INTERACTIONS OF WHITEFLY *BEMISIA TABACI* WITH A *BEGOMOVIRUS* AND ITS HOST PLANT, *SOLANUM LYCOPERSICUM*

by

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(Under the Direction of Rajagopalbabu Srinivasan)

ABSTRACT

Tomato yellow leaf curl virus (TYLCV) is a *Begomovirus* that causes severe symptoms in tomatoes such as curling of the leaves, chlorosis, stunted growth, and yield losses. The vector is *Bemisia tabaci*, which transmits the virus in a persistant and circulative manner. TYLCV arrived in Florida in the mid 1990's and has spread northward into Georgia, South Carolina, and Alabama. TYLCV's overwintering mechanism is currently unknown, so transovarial and sexual transmission in the whitefly were tested for using PCR and plant transmission experiments, as these modes of transmission could indicate TYLCV overwinters in the whitefly. Transovarial and sexual transmission of TYLCV was found, but the insects were not infectious to plants. TYLCV-resistant tomato cultivars are currently the best method to control for yield losses due to the virus. However, in other pathosystems, viruses have broken resistance in their respective crops. TYLCV genomes isolated from resistant and susceptible cultivars were compared to investigate for this threat. There were not any apparent differences between the genomes, indicating that TYLCV-resistant tomatoes can continue to be of use. Also, many studies have examined the phylogeny and introductions of TYLCV into new geographic regions, but a population genetics approach on a world-wide scale has not been conducted. Hundreds of

TYLCV genomes available on GenBank, and from Florida and Georgia, were amassed to test for recombination, polymorphisms, population neutrality, gene flow and genetic differentiation, selection, and phylogeny. The Middle East was confirmed to be the likely origin of TYLCV and showed the highest diversity. In general, the TYLCV species is highly variable and is spreading most rapidly in Southeast Asia. Mixed infections of different *Begomovirus* species have been reported in many different plants. However, few studies have examined mixed infections of different virus isolates from the same species. Mixed TYLCV infections were sought for in tomatoes from the field. A greenhouse experiment was conducted to assess the competitiveness of two different TYLCV isolates. Lastly, tomato genotypes with acylsugar-exuding trichomes were assessed for whitefly resistance. These genotypes showed xenobiosis and antibiosis toward the whitefly, but were still susceptible to TYLCV inoculation by whiteflies.

INDEX WORDS: *Tomato yellow leaf curl virus*, *Bemisia tabaci*, *Solanum lycopersicum*, Transovarial, Phylogenetics, Acylsugars, Vector, Mixed infections, Population genetics, Recombination

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INTRODUCTION

Introduction

Tomato yellow leaf curl virus (TYLCV) is a plant virus that causes severe symptoms and yield loss in tomato crops. The TYLCV pathosystem involves three key biological entities - the virus, the insect vector, and the host plant. The insect vector is comprised of the cryptic species complex, *Bemisia tabaci*, or the sweet potato whitefly. The virus is a non-enveloped DNA virus with a geminate, icosahedral shape. The most well-known host plant for TYLCV is the tomato, *Solanum lycopersicon*, as it is of economic importance. The whitefly feeds on tomatoes and is responsible for the spreading the virus from plant to plant.

The virus

TYLCV is in the family *Geminiviridae*, which has circular, single-stranded DNA components about 2.7 kb in size. All geminiviruses utilize an insect vector to spread themselves to new plants. The genomes of geminiviruses can be either bipartite with two DNA components termed DNA-A and DNA-B, or monopartite with only the DNA-A component. DNA components are encapsidated with coat protein into twinned, icosohedral virions (Bottcher et al. 2004). There are nine genera within the *Geminiviridae* which are *Becurtovirus*, *Begomovirus, Curtovirus, Eragrovirus, Mastrevirus, Topocuvirus, Turncurtovirus, Capulavirus,* and *Grablovirus*. *Capulavirus* and *Grablovirus* were recently described in 2017. The coat protein and replication protein are conserved across the family, while other genes may vary genus to genus

(Varsani et al. 2017). A nonanucleotide sequence, TAATATTAC, is also conserved throughout the *Geminiviridae*.

TYLCV is in the genus *Begomovirus* which is the largest of nine genera in family *Geminiviridae*. Begomoviruses are all transmitted by whiteflies (family Aleyrodidae) and infect dicotyledonous plants. Most begomoviruses have two genomic components, DNA A and DNA B (Navas-Castillo et al. 2011). DNA A contains six genes while DNA B contains two genes. TYLCV, however, has only one genomic component sharing homology with the DNA A of its bipartite congenerics. The genome of TYLCV is approximately 2,800 base-pairs long and contains two genes on the viral strand (V1 and V2) and four genes on the complementary strand (C1, C2, C3, and C4) (Czosnek et al. 2002). V1 codes for the coat protein and is important in virus-vector interactions. V2 codes for a protein that modulates host symptoms and affects virus accumulation (Padidam et al. 1996). C1 codes for the replication-associated protein (Czosnek 2008), C2 codes for the transcriptional activator protein and is involved in suppression of posttranscriptional gene silencing in host plants (van Wezel et al. 2002), C3 codes for the replication enhancer protein, and C4 codes for a protein that determines host symptoms, host range, and systemic virus movement (Tomás et al. 2001, Jupin et al. 1994). The genes cover most of the length of the genome, however, a highly-variable intergenic region exists where no genes are present.

The genome of TYLCV shows high variability. In fact, TYLCV mutates almost as quickly as an RNA virus at a rate of 2.88 × 10-4 substitutions/site/year (Duffy and Holmes 2008). The intergenic region, which contains no genes, mutates at a much higher rate than the rest of the genome (Yang et al. 2014). Besides mutation, recombination is another major factor in the evolution of TYLCV and other begomoviruses (Idris and Brown 2005, Lefeuvre et al. 2007,

Belabess et al. 2016). TYLCV will recombine within its own species and even with begomoviruses of different species such as *Tomato yellow leaf curl Sardinia virus* (Belabess et al. 2016), *Tomato leaf curl Iran virus* (Bananej et al. 2004), and *Tobacco leaf curl virus* (Park et al. 2011). Selection can also affect the evolution of TYLCV. For example, use of TYLCV-resistant tomato cultivars with *Ty*-1 resistance gene allowed TYLCV to outcompete *Tomato yellow leaf curl Sardinia virus* in a tomato-growing region in Spain (García-Andrés et al. 2009). Susceptible tomatoes were more frequently found with *Tomato yellow leaf curl Sardinia virus* while resistant tomatoes were more frequently found with TYLCV. With the increased use of the resistant variety, TYLCV became the prevalent tomato-infecting *Begomovirus* in the region.

The vector

TYLCV is transmitted exclusively by the sweet potato whitefly, *Bemisia tabaci*. This insect belongs to the order Hemiptera which have piercing-sucking mouthparts comprised of two maxillae and two mandibular stylets enveloped by the labium (Borrer et al. 2005). Whiteflies are in the suborder Sternorrhyncha. Insects in this suborder have one- or two-segmented tarsi and their mouthparts arise from between the procoxae. Whiteflies are all in the family Aleyrodidae. Whiteflies are small insects covered with white wax and feed on plant leaves. Adults of the species *Bemisia tabaci* are just under 1 mm long and hold their wings at about a 45° angle which differentiates it from many other whiteflies that hold their wings more flatly over their body (CUES 2013). *B. tabaci* is a cryptic species complex comprised of more than forty sibling species that are morphologically identical and can only be identified using molecular methods (Elfekih et al. 2017, Dinsdale et al. 2010). Two of these sibling species, MED and MEAM1, are highly invasive and misplace native sibling species upon invasion (Muñiz et al. 2011, De Barro and Ahmed 2011). The MEAM1 sibling species (formally called biotype B) is unique from other

sibling species in that it has phytotoxic effects on plants which causes silverleaf and white stem in *Cucurbita* species (Costa and Brown 1991). The MEAM1 sibling species is present in South Georgia where it transmits TYLCV to tomato.

B. tabaci can build up to high numbers quickly. Females lay eggs on the underside of leaves. After hatching from the egg, the insect transitions through four instar stages before reaching the winged adult form (Walker et al. 2009). The first instar nymph is termed the "crawler" and is mobile on the plant on which is what laid upon, usually staying on the same leaf. Second through fourth instar stages are attached on the bottom surface of the leaf. The latter part of the fourth instar stage is called a "pupa", although it is not a true pupa. Winged adults emerge from a T-shaped break in the puparium and are yellowish in color until they begin covering themselves with white wax.

B. tabaci exhibits haplodiploidy sex determination. Males have one set of chromosomes whereas females have two sets of chromosomes. Males occur when eggs are left unfertilized while females occur when eggs are fertilized. A karyotype of *B. tabaci* shows ten unique chromosomes (Blackman and Cahill 1998).

Whiteflies are phloem-feeders. They pierce into plant tissue with their piercing-sucking mouthparts and probe with their stylets between plant cells until they reach phloem tissue. The stylets are comprised of two modified mandibles and two modified maxilla that slide within the labium (Rosell et al. 1995). Two canals are formed by the stylets; the salivary canal and the food canal. The salivary canal delivers saliva into the plant tissue while the food canal intakes fluid from the plant tissue. The mandibular and maxillary stylets can be manipulated separately with the mandibular stylets preceding the maxillary stylets when penetrating plant tissue. During feeding is when whiteflies acquire or inoculate plant viruses.

TYLCV is considered a circulative virus, meaning it must migrate through the whitefly's body to the salivary glands before it can be transmitted to other plants. After a whitefly feeds on the phloem tissue of an infected plant, virions must travel to the filter chamber or midgut and traverse the gut epithelium into the hemolymph. The virions then circulate up to and enter the primary salivary glands with receptor-mediated endocytosis. The virions are retained in the primary salivary glands and are later exuded during feeding which will inoculate a new plant (Cohen and Nitzany 1966, Zeidan and Czosnek 1991, Rosell et al. 1999, Czosnek et al. 2002, Ghanim et al. 2001). This process of circulating through the whitefly body takes at minimum about 8 hours and is called the latent period. Whiteflies are infectious up to weeks after acquisition classifying this virus as persistently-transmitted in contrast to semi-persistently or non-persistently-transmitted viruses which render an insect vector infectious for a shorter amount of time.

The host plant

The cultivated tomato is the best known host plant of TYLCV although there are many other known host plants. This is because tomato is of economic importance and has flamboyant symptoms. New host plants of TYLCV are reported often and span many plant families such as Amaranthaceae, Asteraceae, Caricaceae, Caryophyllaceae, Cucurbitaceae, Euphorbiaceae, Lamiaceae, Leguminosae, Malvaceae, Moraceae, Solanaceae, Urticaceae, and Violaceae (Liang et al. 2013, Shahid and Natsuaki 2014, Li et al. 2014, Sohrab 2016, Al-Ali et al. 2016, Parrella et al. 2015, Zhou et al. 2016, Smith et al. 2015, Kil et al. 2014, Alabi et al. 2017, Kil et al. 2015). TYLCV can cause severe symptoms in tomato plants such as stunted growth, chlorosis, curling of the leaves, and a reduction in fruit yields.

The tomato is in the family Solanaceae which includes many other economically important plants such as peppers, eggplants, tomatillo, potatos, and tobacco (Simpson 2010). These are flowering plants with fruits of berry, drupes, or capsules, and have internal phloem. Many solanaceous plants have trichomes and high levels of alkaloids which can be poisonous, such as plants in the genus *Datura*. The cultivated tomato originates in South America and was brought to Europe by Spanish and Portuguese explorers in the 1500's as an ornamental, as the fruits were thought to be poisonous. In the 1700's, Europeans began eating the tomato fruit and when Europeans began colonizing North America, the cultivated tomato was taken with them (Jones 2007).

Today, both fresh tomatoes and processing tomatoes are grown in the United States. Florida and California produce the vast majority of fresh tomatoes, but the states of Virginia, Ohio, Georgia, and Tennessee also produce fresh tomatoes (USDA ERS 2016). Processing tomatoes are grown almost entirely in California. Indiana, Ohio, and Michigan grow a small amount of processing tomatoes.

TYLCV first became a problem for the United States tomato industry in the mid-1990's when TYLCV's introduction into Florida was detected (Polston et al. 1999). TYLCV has since spread northward into Georgia, South Carolina, and Alabama (Momol et al. 1999, Ling et al. 2006, Akad et al. 2007). For tomato growers, the most effective method of managing TYLCV is to use TYLCV-resistant tomato cultivars (Lapidot et al. 1997, Gilreath et al. 2000). Currently, six different genes have been introgressed from wild tomato species into the cultivated tomato (Scott et al. 2015). These are designated *Ty*-1 through *Ty*-6. *Ty*-1and *Ty*-3 have been identified as RNA-dependent RNA polymerases and are two versions of the same gene (Verlaan et al. 2013). The mechanisms of resistance for the other *Ty* resistance genes are not known. In addition to

virus resistance, lines of tomatoes have been bred to resist whiteflies (Andrade et al. 2017, Dias et al. 2016, Leckie et al. 2012, Resende et al. 2009). These lines have unique trichomes that exude acylsugars which deter herbivore feeding and oviposition. The trichomes and acylsugars have been introgressed from wild tomato relatives into the cultivated tomato. Experiments using an assortment of tomato herbivores, such as the whitefly *B. tabaci*, the two-spotted spider mite *Tetranychus urticae* (Lucini et al. 2015, Rakha et al. 2016), *Tetranychus evansi* (Resende et al. 2008), tobacco thrips *Frankliniella fusca*, western flower thrips *Franklinieallа occidentalis* (Leckie et al. 2016), the tomato leaf miner *Tuta absoluta* (Moreira et al. 2013), the cotton bollworm *Helicoverpa armigera* (Talekar et al. 2006), and the green peach aphid *Myzus persicae* (Silva et al. 2013) have shown the plants exhibit both antixenosis and antibiosis toward herbivores. A next step toward TYLCV control may be to integrate virus resistance with whitefly resistance.

Interactions within the TYLCV pathosystem

My goal was to examine some of the interactions in the TYLCV pathosystem. I examined the interaction of TYLCV with the resistance status of the tomato plant to see if virus-resistant cultivars select for any modifications within the TYLCV genome. I also examined to see if TYLCV is transmitted transovarially or sexually in the whitefly as variable results are currently in the literature and I wanted to see how our local virus isolate interacts with our local whitefly sibling species. Another interaction I examined was between acylsugar-producing tomato genotypes and whiteflies to assess the effectiveness of these genotypes in terms of antibiosis and antixenosis toward the whitefly and in terms of virus transmission. Another phenomenon I focused on was mixed infections of TYLCV isolates within individual tomato plants and assessed if one TYLCV isolate can be more competitive than other. Lastly, I examined hundreds

of TYLCV genomes from around the world on GenBank, along with genomes that I sequenced from Georgia and Florida, to conduct a world-wide population phylogenomic analysis and assessed the various factors that influence its population genetics.

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CHAPTER 2

TOMATO YELLOW LEAF CURL VIRUS: TRANSOVARIAL AND SEXUAL TRANSMISSION IN ITS WHITEFLY VECTOR, *BEMISIA TABACI*[1]

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Abstract

Tomato yellow leaf curl virus (TYLCV) is a *Begomovirus* transmitted by *Bemisia tabaci* in a persistent, circulative manner. Many studies state that TYLCV is either not transmitted to adult offspring transovarially or only at low levels as detected by PCR. Most studies also state that transovarial transmission of TYLCV with subsequent infectivity to plants does not occur. However, studies from Israel report high levels of TYLCV DNA in adult offspring of viruliferous whiteflies and these offspring are infectious to plants. The reason for such discrepancies is unknown. Few studies have investigated sexual transmission of TYLCV. In this study we tested in the MEAM1 whitefly sibling species for transovarial transmission and sexual transmission of a) TYLCV with conventional PCR, b) TYLCV with subsequent infectivity to plants, and c) TYLCV virions with immunocapture PCR. Any whitefly samples that were positive for TYLCV DNA were subjected to real-time PCR to quantify the amount of TYLCV acquired. Lasty, the coat protein of TYLCV from around the world and Israel were compared. We found that TYLCV is transmitted both transovarially (4% fourth instar nymphs, 2% first generation adults, 2% second generation adults) and sexually (4% in mated males, 0% in mated females) using PCR. However, TYLCV virions were not detected with immunocapture PCR. Subsequent infectivity to plants was not transmitted transovarially or sexually. A difference was found between the TYLCV coat protein of the Israel isolate and other isolates that could explain the differences in the virus-vector interactions in these geographic regions.

Introduction

Geminiviruses are insect-transmitted plant pathogens that can cause severe symptoms and yield losses in agricultural crops. Their genomes are comprised of either one or two circular ssDNA components approximately 2.7 kb long. DNA components are encapsidated with coat

protein into twinned, icosahedral-shaped virions (Bottcher et al. 2004). *Begomovirus* is the largest of nine genera in the family *Geminiviridae* (Varsani et al. 2017). All begomoviruses are transmitted by whiteflies (family Aleyrodidae) and infect dicotyledonous plants. *Tomato yellow leaf curl virus* (TYLCV) is a *Begomovirus* of great agricultural importance as it causes large losses in tomato crops around the world. A 100% incidence of TYLCV can occur in unprotected tomato fields (Berlinger et al 1983). Symptoms of infection include stunting, chlorosis, curling of leaves, and yield loss.

The vector of TYLCV is the sweet potato whitefly, *Bemisia tabaci*, which is a cryptic species complex. This complex is composed of tens of cryptic species that are morphologically identical, but can be distinguished based on cytochrome oxidase subunit I sequences (Elfekih et al. 2017, Dinsdale et al. 2010). Several of the sibling species are highly invasive, such as the MEAM1 and MED sibling species, and have invaded new geographic areas and outcompeted many native sibling species (Muñiz et al. 2011, De Barro and Ahmed 2011). The invasive nature of the insect vector has enhanced the spread of the virus around the world (Pan et al. 2012).

TYLCV is a persistent, circulative virus within its whitefly vector. After a whitefly feeds on an infected plant, TYLCV virions cross over from the alimentary canal at the midgut or filter chamber region into the hemolymph of the insect and cross into the salivary glands before it can be transmitted to other plants (Cohen and Nitzany 1966, Zeidan and Czosnek 1991, Rosell et al. 1999, Ghanim et al. 2001, Czosnek et al. 2002). Once in the primary salivary glands, virions are exuded in the saliva during feeding which will inoculate a new plant. This process of circulating through the whitefly body takes at minimum about 8 hours and is called the latent period. Whiteflies are infectious up to weeks after acquisition. Besides acquisition by feeding, the virus is also believed to be transmitted transovarially and sexually within its vector (Ghanim et al.

1998, Wang et al. 2010, Pan et al. 2012, Ghanim and Czosnek 2000). However, transovarial transmission of TYLCV and subsequent infectivity to plants has been a topic of debate, primarily because only a few studies from Israel have suggested that TYLCV can be transmitted transovarially and subsequently infect plants. Their studies revealed that first generation and second generation whiteflies were capable of inoculating tomato plants at percentages of 10% and 8%, respectively (Ghanim et al. 1998). They also report high percentages of TYLCV DNA transmission. For example, eggs, crawlers, and adults were all found to contain TYLCV DNA at percentages of 80.7%, 36.8%, 56.8%, respectively. TYLCV DNA was also detected in eggs, crawlers, and adults of second generation progeny at percentages of 38.2%, 71.4%, and 78.8%, respectively. Other research groups report little to no transfer of TYLCV DNA. For example, one study found that zero percent of MEAM1 adult offspring tested positive for TYLCV DNA, however, 2-3% of the MED adult offspring tested positive for TYLCV DNA (Wang et al. 2010). Pan et al. (2012) found high percentages of TYLCV DNA transovarially transmitted to the eggs (30% in MEAM1 and 50% in MED) and nymphs (11% in MEAM1 and 11% in MED) of viruliferous whiteflies, but not to the adult offspring. Studies by Bosco et al. (2004) and Becker et al. (2015) detected no TYLCV DNA in adult offspring using PCR. Even though many studies have documented transovarial transfer of TYLCV, many studies that have conducted plant transmission studies found that no tomato plants became infected after the adult offspring of viruliferous whiteflies fed on tomato plants (Wang et al. 2010, Ioannou et al. 1985, Becker et al. 2015, Bosco et al. 2004, Cohen and Nitzany 1966).

Less research has been conducted on sexual transmission of TYLCV than on transovarial transmission. A number of studies have examined sexual transmission by testing for TYLCV DNA, but only one study has examined sexual transmission of TYLCV with subsequent

infectivity to plants. A study by Pan et al. (2012) showed that in both the MEAM1 and MED whiteflies, TYLCV DNA can be transmitted to mates. In the MEAM1 sibling species, TYLCV DNA did not move from female to male, but moved from male to female 10% of the time. In the MED sibling species, TYLCV DNA moved from female to male in 50% of the time and from male to female 73.7% of the time (Pan et al. 2012). A study out of China demonstrated TYLCV DNA transmission via mating at a percentage of 2.8% in the MEAM1 sibling species and a percentage of 2.9% in the MED sibling species (Wang et al. 2010). TYLCV DNA moved both male to female and female to male. Another study demonstrating mating transmission of TYLCV DNA showed that female whiteflies that had mated with viruliferous males tested positive for TYLCV DNA at a percentage of 55.6% and for TYLCV coat protein at a percentage of 44.4% (Ghanim and Czosnek 2000). Males that mated with viruliferous females were positive for TYLCV DNA at a percentage of 27.8% and for coat protein at a percentage of 33.3%. When mated whiteflies were clip-caged to tomato plants, a significant number of the plants became infected with TYLCV. For mated males, 34.5% of tomato plants became infected, and for mated females, 23.8% of the plants became infected. This is the only study in the literature that has examined sexual transmission of TYLCV with subsequent infectivity to plants.

In this study, we elucidate whether transovarial transmission and mating transmission of TYLCV occurs with the whitefly sibling species and TYLCV isolate present in the state of Georgia. The TYLCV overwintering mechanism is currently unknown, and transovarial and sexual transmission could indicate that TYLCV may overwinter in the whitefly vector. To explore this possibility, we tested for transovarial and sexual transmission of TYLCV DNA, infectivity toward plants, and transmission of TYLCV virions in whiteflies. The whitefly sibling

species and TYLCV isolate in Georgia was compared to those from Israel, which seem to have unique characteristics in regards to transovarial and mating transmission of TYLCV.

Materials and methods

Transovarial transmission of TYLCV DNA

Whiteflies used throughout this study were of the *B. tabaci* MEAM1 sibling species and were reared on cabbage plants. To obtain viruliferous whiteflies, newly-emerged whiteflies were allowed to feed on infected tomato plants for three days. To begin the transovarial transmission experiment, viruliferous female whiteflies less than one-week old were allowed to lay eggs on cotton, a TYLCV non-host, for one week. After a week, female whiteflies were removed and tested with PCR targeting the C2 gene in TYLCV to verify they were viruliferous. Primers used were C2-1201 (5'- CATGATCCACTGCTCTGATTACA -3') and C2-1800V2 (5'-TCATTGATGACGTAGACCCG-3') which target a 695-nucleotide region of the TYLCV genome encompassing the entire C2 gene. The PCR reactions were run in 10 µl reactions with 5 µl of GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 2 µl of water, 0.5 µl of each primer at 10 µM concentration, and 2 µl of DNA extract. The PCR program had an initial denaturation step at 94° C for 2 minutes followed by 30 cycles of 94° for 30 sec, 52° for 30 sec, 72° for 1 min, and a final extension at 72° for 5 min. Cotton plants with the laid eggs were caged in whitefly-proof cages and placed in a growth chamber to avoid introduction of outside whiteflies. Fourth-instar nymphs were removed from the leaf surface and individually collected into tubes. Individual insects were surface sterilized using the protocol outlined by Lacey and Brooks (1997) with a series of five 500 µL washes in the following order: 70% ethanol, water, 1% bleach, water, water. The final rinsates were collected and tested with PCR to determine there was no external contamination of TYLCV. The surface sterilization process is critical to

obtain accurate results as honeydew from the whitefly's parent could still be on the exterior of the whitefly and give a false positive. After rinsates tested negative, the insect DNA was extracted using Instagene Matrix (BioRad, Hercules, CA) following manufacturer's protocol. The DNA extracts of individual insects were subjected to PCR with the C2-1201 and C2-1800V2 primers to determine if TYLCV DNA was transmitted from mother to offspring. Whiteflies that had acquired TYLCV through feeding were used as positive controls. In addition to collecting fourth-instar nymphs, this experiment was repeated to collect adult offspring and again to collect second generation adult offspring. The experiment was conducted twice for the nymphs, adult offspring, and second generation adult offspring.

Sexual transmission of TYLCV DNA

Individual viruliferous male or female whiteflies were clip-caged with a non-viruliferous whitefly of the opposite sex on cotton for 48-hours to allow for a mating access period. Whiteflies were then collected individually. Whiteflies were surface sterilized and final rinsates were tested with PCR to determine there was no external contamination of TYLCV. Whitefly DNA was then extracted using Instagene Matrix. Originally-viruliferous were tested with PCR to verify they were indeed viruliferous. Originally non-viruliferous whiteflies were tested with PCR to determine if TYLCV DNA was transmitted through mating. Whiteflies that had acquired TYLCV through feeding were used as positive controls. Each experimental replication contained approximately 25 pairs for both positive male/negative female and positive female/negative male combinations. The experiment was conducted twice.

Quantification of TYLCV DNA in positive whitefly samples

Whiteflies that were positive for TYLCV DNA from the transovarial transmission and mating transmission experiments were subjected to real-time PCR to quantify the amount of

TYLCV DNA the whiteflies had acquired. Whiteflies that had acquired TYLCV through feeding were used as positive controls. Primers used to quantify the C2 gene of TYLCV were C2F (5'-GCAGTGATGAGTTCCCCTGT-3') and C2R (5'-CCAATAAGGCGTAAGCGTGT-3'). The real-time PCR reactions were run in 25 µl reactions with 12.5 µl of GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI), 6.5 µl of water, 0.5 µl of each primer at 10 µM concentration, and 5 µl of DNA extract. The PCR program had an initial denaturation at 95° C for 2 min followed by 40 cycles of 95° for 15 sec and 60° for 1 min, followed by a melting curve. Values were normalized with the whitefly β-actin gene which was amplified with the primers whitefly β-actin F (5'–TCTTCCAGCCATCCTTCTTG–3') and whitefly β-actin R (5'–CGGTGATTTCCTTCTGCATT–3') (Sinisterra et al. 2005). The real-time PCR program had an initial 95° denaturation step for 2 m, followed by 40 cycles of 95° for 15 sec and 60° for 1 m, followed by a melting curve. TYLCV C_T values and the whitefly β-actin C_T values were used in the equation developed by Pfaffl (2001) for relative quantification of TYLCV DNA to whitefly β-actin DNA.

Transovarial and sexual transmission of TYLCV and subsequent infectivity to plants

Viruliferous female whiteflies less than one-week old were allowed to lay eggs on cotton, a TYLCV non-host, for one week. After a week, female whiteflies were removed and tested with PCR using the C2-1201 and C2-1800V2 primer set to verify they were viruliferous. After female whiteflies were confirmed positive for TYLCV, the cotton plants with the laid eggs were caged and placed in a growth chamber to avoid introduction of outside whiteflies. As adult offspring emerged from the cotton plants, they were collected, and 50 adult offspring were clip-caged to a tomato plant for a 24-hour inoculation access period. Tomato plants were caged and kept for four weeks inside a growth chamber for possible infection to develop. After four weeks, leaf tissue

was removed and DNA was extracted. The DNA extracts were tested with PCR with the C2-1201 and C2-1800V2 primer set to determine the infection status of the plants. Ten plants were used per experiment and the experiment was conducted twice.

To test for sexual transmission of TYLCV and subsequent infectivity to plants, fifty male or female viruliferous whiteflies less than one-week old were clip-caged on cotton with fifty non-viruliferous whiteflies of the opposite sex aged less than 3-days for 48-hours to allow for a mating access period. Twenty six of the initially non-viruliferous whiteflies were then clip-caged to a tomato plant for a 48-hour inoculation access period. Tomato plants were caged and kept in a growth chamber to avoid outside whiteflies from entering. Tomato plants were maintained for four weeks to allow development of infection. DNA was then extracted from leaf tissue and PCR was performed with the C2-1201 and C2-1800V2 primers to determine the infection status of each plant. Six plants were used per experimental replication and the experiment was conducted twice for both positive male/negative female and positive female/negative male combinations.

Transovarial and sexual transmission of TYLCV virions

Adult offspring from viruliferous whiteflies were produced and collected as described above. Whiteflies were then surface sterilized. Immunocapture PCR was then performed on whitefly homogenates as described by Ghanim et al. (2001), using the antibody against the TYLCV coat protein from Bioreba (Ebringen, Germany) at a dilution rate of 1:1000. The PCR primers and program we used for TYLCV were the C2-1201 and C2-1800V2 primers and the program as described above but with a 5 minute 95° denaturation step before the initiation of PCR program. Whiteflies that had acquired virus through feeding were used as positive controls.

Whitefly mating pairs were set up as described above. Whiteflies were then surface sterilized. Immunocapture PCR was then performed on whitefly homogenates as described above to determine if virions were passed through mating.

TYLCV genome comparisons

TYLCV genomes from around the world were compared to a TYLCV genome from Israel available on GenBank. Genomes and their GenBank accession numbers are included in Table 2.1. Translations and alignments of each of the six reading frames were compared to see if any amino acid differences could explain the differences between the percentages of transovarial and sexual transmission of TYLCV in our pathosystem versus the Israeli pathosystem. This was performed using Geneious Pro v. 8.1.9 (Drummond et al. 2011).

Results

Transovarial transmission of TYLCV DNA

TYLCV DNA was detected in 4% of fourth instar nymphs (n=100). TYLCV DNA was detected in first generation adult offspring at a percentage of 2% (n=102). TYLCV DNA was also detected in the second generation adult offspring at a percentage of 2% (n=103).

Sexual transmission of TYLCV DNA

TYLCV DNA was detected in 4% of initially non-viruliferous males that mated with viruliferous females (n=52). However, none of the initially non-viruliferous females tested positive for TYLCV after mating with viruliferous males (n=53).

Quantification of TYLCV DNA in positive whitefly samples

TYLCV was quantified in whiteflies that tested positive from the transovarial and sexual transmission experiments. Whiteflies that acquired TYLCV DNA from their mothers showed a decreasing trend in TYLCV DNA as they molted or with subsequent generations (Figure 2.1).

First generation nymphs (n=4) had the highest concentration of TYLCV DNA, followed by first generation adult whiteflies (n=2), followed by second generation adult whiteflies (n=2). All of the whiteflies with TYLCV DNA acquired transovarially had lower levels of TYLCV DNA than whiteflies that had acquired TYLCV DNA through feeding. Male whiteflies (n=2) that acquired TYLCV DNA through mating also did so at a level lower than whiteflies that had acquired from feeding.

Transovarial and sexual transmission of TYLCV and subsequent infectivity to plants

No tomato plants became infected after the offspring of viruliferous whiteflies fed for a 48-hour inoculation access period (n=20 plants). This indicates no transovarial transmission of TYLCV plant infectivity. After a 48-hour inoculation access period with whiteflies that had mated with viruliferous whiteflies, none of the tomato plants became infected (n=12 plants for females, n=12 plants for males). This indicates no passage of TYLCV plant infectivity to mates of viruliferous whiteflies.

Transovarial and sexual transmission of TYLCV virions

Virions were not detected using immunocapture PCR in the adult offspring of viruliferous whiteflies (n=99). Virions were also not detected using immunocapture PCR in any of the initially-non-viruliferous whiteflies that mated with viruliferous whiteflies (n=52 for males, n=53 for females).

TYLCV genome comparisons

The amino acid sequences for each of the six TYLCV genes were compared among the Israel TYLCV genome and other genomes from around the world (Table 2.1). The V1 gene, which codes for the coat protein, showed the most notable difference, as the Israeli isolate had a 5 amino acid deviation at position 213-217, which includes a 2 amino acid insertion (Figure 2.2).

High amino acid homology is demonstrated throughout the rest of the V1 gene. The V1 gene is the only gene in which the Israeli TYLCV genome has an amino acid indel.

Discussion

Ours results indicate transovarial transmission of TYLCV at a low percentage as detected by PCR. Four-percent of fourth-instar nymphal offspring, two-percent of first-generation adult offspring, and two-percent of second-generation adult offspring contained TYLCV DNA. Levels of TYLCV DNA in positive offspring were lower than levels found in whiteflies that had acquired TYLCV through feeding. Through molts and subsequent generations, the amount of TYLCV DNA in offspring diminished as shown with real-time PCR. Although TYLCV DNA was transmitted, virions, however, were not detected by immunocapture PCR in adult offspring.

Sexual transmission of TYLCV as detected by PCR was also found in the mating experiments. TYLCV DNA was detected in 4% of males that had mated with viruliferous females. No females that mated with viruliferous males were positive for TYLCV DNA, however. Males that had acquired TYLCV DNA through mating had lower levels of TYLCV DNA than whiteflies that had acquired TYLCV DNA through feeding. Immunocapture PCR did not detect virions in female whiteflies that mated with viruliferous males or male whiteflies that mated with viruliferous females.

Our results demonstrate that transovarial of TYLCV with subsequent infectivity to plants does not occur in our whiteflies. None of the tomato plants from the plant transmission experiments became infected with TYLCV, indicating that the offspring of viruliferous whiteflies are not capable of transmitting the virus the plants. These results corroborate many other studies that indicate no transovarial transmission of TYLCV infectivity to plants (Wang et al. 2010, Ioannou et al. 1985, Becker et al. 2015, Bosco et al. 2004). Many other begomoviruses

also show no transovarial transmission of infectivity to plants, such as *Tomato leaf curl virus* (Butter and Rataul 1977), *Squash leaf curl virus* (Cohen et al. 1983), *Tobacco leaf curl virus* (Aidawati et al. 2002), *Tomato leaf curl Sinoloa virus* (Idris and Brown 1998), *Tomato yellow leaf curl Sardinia virus* (Bosco et al. 2004), *Tomato yellow leaf curl China virus* (Wang et al. 2010), and *African cassava mosaic virus* (Dubern 1994). However, one study states that the age of the female whitefly parent can determine whether or not offspring are infectious to plants (Wei et al. 2017). Adult offspring of viruliferous 1-day-old MED whiteflies infected 0% of plants after a 48-hr inoculation access period using 10 whitefly offspring. However, the adult offspring of 11-day-old whiteflies inoculated 33.3% of plants. This effect even extended into the second generation for both the MEAM1 and MED sibling species. TYLCV DNA was not transmitted to adult offspring of 1-day-old females of the MED sibling species, but was transmitted at a percentage of 67% by 11-day-old females. The authors credit this transmission ability by older whiteflies to the ability of TYLCV's coat protein to bind to the whitefly vitellogenin protein. The TYLCV then hitchhikes with the vitellogenin when endocytosed into oocytes. Our experiments used whiteflies of a younger age and had we used older whiteflies, we may have obtained different results.

Sexual transmission of TYLCV with subsequent infectivity to plants was also not demonstrated by our plant transmission experiments. Only one study is published that examines sexual transmission of TYLCV with subsequent infectivity to plants with plant transmission experiments which uses MEAM1 whiteflies and a TYLCV isolate from Israel (Ghanim and Czosnek 2000). They found that there is sexual transmission of TYLCV infectivity to plants, as tomato plants became infected by the mates of viruliferous whiteflies at a percentage of 34.5% for mated females and 23.8% for mated males. Our results are in disagreement with this study,

but differences in the Israeli TYLCV coat protein and the Georgia TYLCV coat protein may be at play.

The capsid protein of begomoviruses is responsible for virus-vector interactions (Briddon et al. 1990, Höfer et al. 1997). Particular amino acid changes in the TYLCV capsid protein can leave the virus incapable of transmission by whiteflies, but still infectious to plants (Noris et al. 1998). In closely-related *Tomato yellow leaf curl Sardinia virus*, a region between amino acids 129 and 152 in the coat protein was found to be crucial for insect transmissibility (Caciagli et al. 2009). Wei et al. (2017) found that the TYLCV coat protein interacts with vitellogenin in the whitefly. This is how they explain the capability of 11-day old viruliferous females, but not younger females, to transmit TYLCV transovarially to offspring. Younger females would not have had access to TYLCV at the appropriate time during the development of their mature eggs and this explains why they did not transmit TYLCV to offspring. Our comparison of the coat protein from other genomes around the world and the genome from Israel show a notable 5-amino acid difference at amino acids 213-217. This region could alter the way the Israeli TYLCV isolate interacts with its vector and may explain why studies from Israel differ from other regions of the world in their transovarial and mating transmission results. The Israeli studies used the MEAM1 sibling species of whitefly like many other researchers have, so this factor can be ruled out. Further experimentation targeting this 5-amino acid region could help elucidate if it alters the virus-vector interactions.

The importance of surface sterilizing insects prior to PCR testing should be emphasized as we got many positives in preliminary experiments before we adopted the surface sterilization procedure. Honeydew from viruliferous parent whiteflies or mating partners could contaminate

the exterior of the whitefly of interest. By rinsing the outside of the whiteflies, we can be assured that any TYLCV DNA detected was located internally in the whitefly.

The overwintering mechanism of TYLCV in South Georgia still remains undetermined. Although DNA was detected in a small proportion of offspring and mates of viruliferous whiteflies, there was a lack of subsequent transmission to plants by these whiteflies. Therefore, the whitefly vector is unlikely to be the overwintering mechanism of TYLCV. Winter annual *Lamium amplexicaule* is present in South Georgia and has been reported in Korea as a host plant of TYLCV (Kil et al. 2014). This weed and other host plants could be furthered examined as potential TYLCV overwintering reservoirs.

Overall, our results show that TYLCV is transmitted transovarially and sexually as determined by PCR. Immunocapture PCR did not detect virions in these whiteflies, though. Subsequent infectivity to plants is not transmitted among whiteflies transovarially or sexually. TYLCV DNA transmitted both transovarially and sexually occurred at a lower levels than TYLCV DNA acquired by feeding. Our overall results are similar to those of many other studies, but differ from those found in Israel. The Israeli TYLCV may differ from other TYLCV isolates around the world due to amino acid differences in the coat protein. Further work is warranted to determine if the amino acid differences in the coat protein contribute to the differences seen in virus-vector interactions in Israel. Also, the overwintering mechanism of TYLCV in South Georgia remains unknown and whiteflies seem unlikely to be the culprit. Plants present in the winter, such as *Lamium amplexicaule*, should be further examined as possible overwintering reservoirs.

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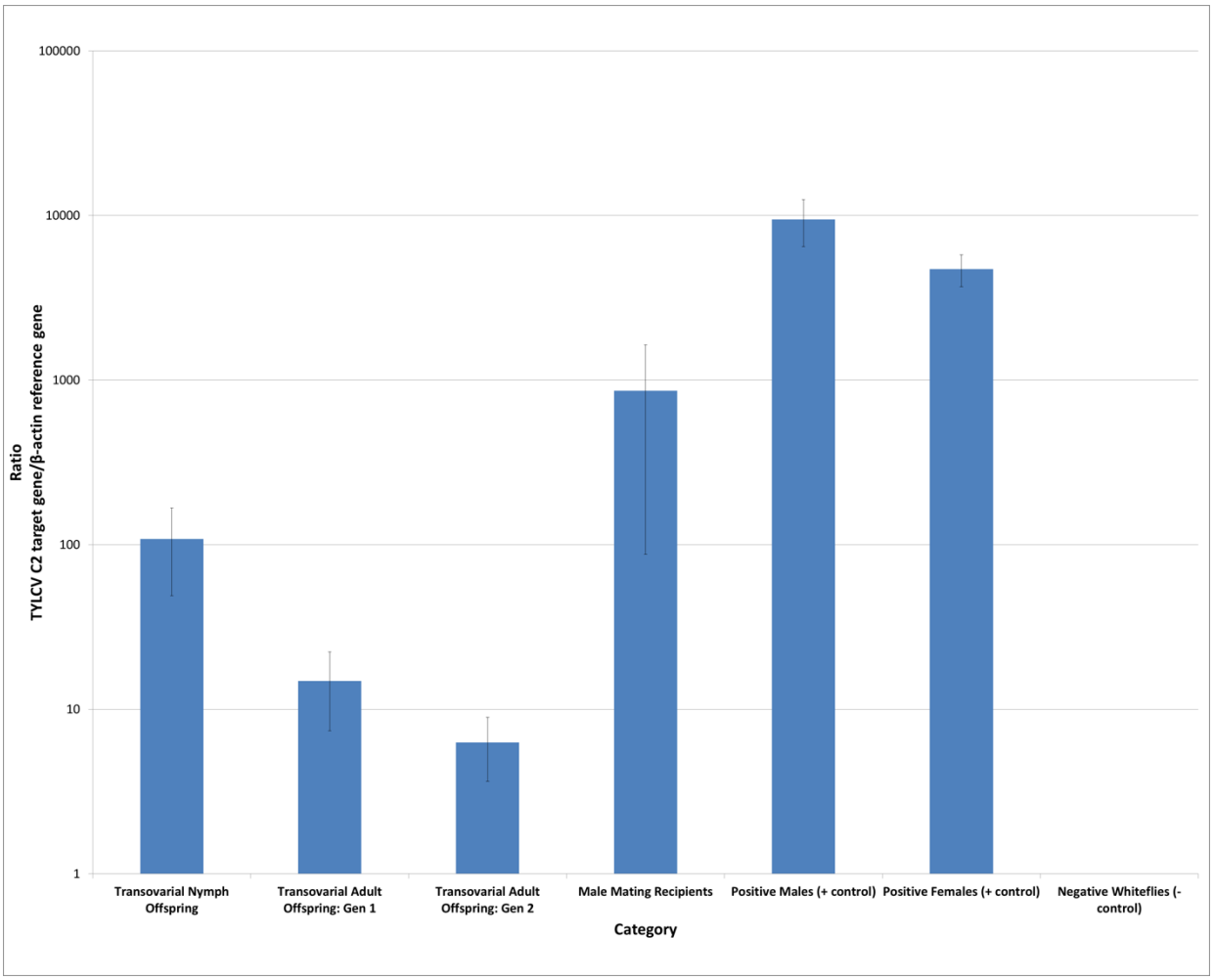

Figure 2.1. Amount of TYLCV DNA in whiteflies that acquired TYLCV DNA transovarially or sexually. Error bars are standard errors.

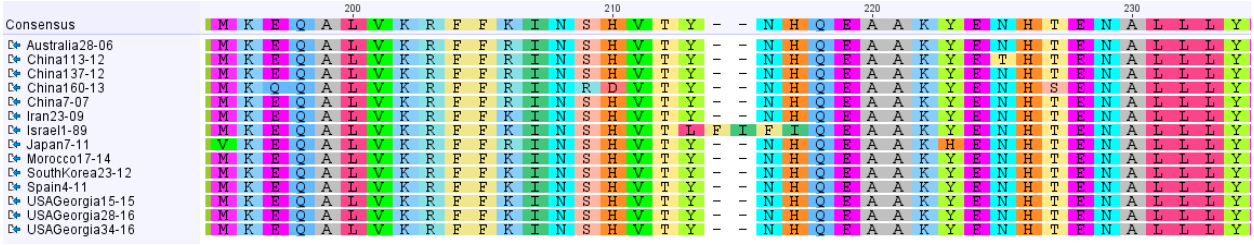

Figure 2.2. Alignment of amino acid sequence from the V1 gene demonstrating the 5 amino acid deviation between the Israel TYLCV isolate and other TYLCV isolates from around the world.

Table 2.1. Genomes from around the world and their GenBank accession numbers used in amino acid sequence sequence comparisons.

Genome	GenBank Accession Number
Australia28-06	KX347120
China113-12	KC312656
China137-12	KC999844
China160-13	KM435325
China7-07	FN252890
Iran23-09	KX347162
Israel1-89	X15656
Japan7-11	KJ466047
Morocco17-14	LN846615
SouthKorea23-12	JX961666
Spain4-11	KT099157
USAGeorgia15-15	KY971369
USAGeorgia28-16	KY971342
USAGeorgia34-16	KY971340

CHAPTER 3

COMPARISION OF TOMATO YELLOW LEAF CURL VIRUS GENOMES ISOLATED FROM RESISTANT AND SUSCEPTIBLE TOMATO CULTIVARS IN FLORIDA AND GEORGIA[1]

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Abstract

Tomato yellow leaf curl virus (TYLCV) causes devastating symptoms in tomato crops that can result in total yield loss. TYLCV-resistant cultivars have been developed to overcome this barrier. Viruses in other pathosystems have overcome resistance in their respective crops either by simple mutation or recombination. To determine if this is an eminent threat for TYLCV-resistant tomatoes, TYLCV were propagated through ten generations of a TYLCV-resistant and a TYLCV-susceptible tomato cultivar. TYLCV genomes were analyzed for genetic differentiation and selection to determine if they differed. Field-collected samples from Georgia and Florida from susceptible and resistant cultivars were also sampled, sequenced, and analyzed for differences. Lastly, phylogenetic and population genetic analyses were performed on the Georgia and Florida sequences to examine differences based on geography. TYLCV genomes propagated through the resistant and susceptible cultivars began differentiating with increasing generation number, but no specific mutations were repeatedly observed and no selection was detected. TYLCV genomes from field-collected resistant and susceptible samples showed some differentiation with some of the population genetic statistics, however, the Fst value was low indicating a lack of genetic structure. No codons were determined to be under selection. The TYLCV populations from Georgia and Florida were highly distinct with all statistics and a phylogenetic tree in agreement. Currently, TYLCV-resistant tomatoes are still of use and there is little evidence of selection at this point in time. For now, geography seems to be playing a bigger role in the differentiation of TYLCV genomes.

Introduction

Tomato yellow leaf curl virus (TYLCV) is a pathogen of tomatoes that can cause tremendous yield losses in tomato crops. Symptoms of infection in tomato plants include stunted growth, chlorosis, curling of the leaves, and reduced fruit yield. TYLCV is in the family *Geminiviridae* and genus *Begomovirus*. The monopartite, circular genome contains six genes with two genes on the viral strand (V1 - V2), and four genes on the complementary strand (C1 – C4). The virus is transmitted exclusively by the silverleaf whitefly, *Bemisia tabaci*, in a persistent and circulative manner.

TYLCV has been problematic for tomato growers in the Southeastern US since its introduction into Florida in the mid-1990's (Polston et al. 1999). Tomato cultivars resistant to TYLCV have been developed to help manage the virus. These cultivars have proven to be a highly effective tool for managing the virus (Lapidot et al. 1997, Gilreath et al. 2000). For example, a field trial in Florida using susceptible and resistant tomatoes cultivars showed the standard susceptible variety, FL47, produced only 10.7 tons/acre of tomatoes while resistant varieties produced up to 25.9 tons/acre (Ozores-Hampton et al. 2013).

TYLCV-resistant tomato cultivars do not completely stop replication of TYLCV. TYLCV-resistant cultivars exhibit milder symptoms and less yield loss than susceptible cultivars. Resistant cultivars also accumulate lower levels of viral DNA compared to susceptible varieties (Legarrea et al. 2015). Six different genes, designated *Ty*-1 through *Ty*-6, have been identified and introgressed from wild tomato species into tomato that give the plants resistance to the virus (Scott et al. 2015). The mechanism of resistance is not known for all of the resistance genes, but *Ty*-1and *Ty*-3 have been identified as RNA-dependent RNA polymerases (Verlaan et al. 2013).

Continual use of resistant cultivars in the field could potentially be placing positive selection pressure on the virus and could give rise to resistance-breaking TYLCV strains. Although a DNA virus, TYLCV mutates at a rate approaching RNA viruses. The rate of mutation for the full-length genome is 2.88×10^{-4} substitutions/site/year (Duffy and Holmes 2008). A high mutation rate with selection pressure from continual use of resistant cultivars could lead to the emergence of a resistance-breaking virus isolate.

Numerous examples of resistance-breaking virus isolates have been documented in other pathosystems. *Beet necrotic yellow vein virus*, an RNA virus in genus *Benyviris*, has overcome the *Rz1*-resistance in sugar beet. The resistance-breaking virus strains have been linked to mutations within a four amino acid region in the P25 gene of the virus. Resistance-breaking events due to mutations in this region have occurred multiple times resulting in numerous resistance-breaking amino acid motifs within the four amino acid region (Bornemann et al. 2015).

Tomato spotted wilt virus, an RNA virus in family Bunyaviridae, has overcome the resistance gene *Tsw* in pepper. The virus has broken *Tsw* resistance in Argentina, Australia, China, Italy, Hungary, Spain, and Turkey (Ferrand et al. 2015, Sharman and Persley 2006, Jiang et al. 2016, Roggero et al. 2002, Almasi et al. 2015, Debreczeni et al. 2015, Deligoz et al. 2014). Another example is *Cotton leafroll dwarf virus* (CLRDV), an RNA virus in family *Luteoviridae*. In Brazil, two isolates of CLRDV were discovered that overcame previously-resistant cotton accessions (da Silva et al. 2015).

No instances of TYLCV overcoming TYLCV-resistance in the field have been documented. However, an experiment in the lab has led to a resistance-breaking strain of TYLCV. The tomato cultivar H24 is homozygous for the *Ty*-2 TYLCV-resistance gene and is

resistant to the IL form of TYLCV, but not the Mld strain. However, a virus chimera created in lab with the C4 and C1 (Rep) genes from the Mld isolate and the remainder of the genome from the IL isolate was able to cause disease in the H24 tomato line (Ohnishi et al. 2016). This indicates that the C4 and/or C1 gene from the Mld strain is involved in the strain's ability to break the *Ty*-2 TYLCV resistance in the H24 tomato line. If a natural recombination event occurred with these regions, a resistance-breaking TYLCV isolate could emerge in the field.

Use of TYLCV-resistant cultivars can also displace certain begomoviruses in favor of others. A study conducted in Spain surveying numerous tomato fields found that susceptible tomato fields were more often infected with *Tomato yellow leaf curl Sardinia virus* (TYLCSV) while resistant tomato fields with the *Ty*-1 gene were more often infected with TYLCV (García-Andrés et al. 2009). The *Ty*-1 plants were permitting TYLCV to outcompete TYLCSV. Use of resistant *Ty*-1 tomato varieties and decreased use of susceptible varieties in this region of Spain selected for TYLCV over TYLCSV. Mixed infections and recombinants comprised of TYLCV and TYLSCV were also found. Recombination is a rapid mechanism for creating genetic variation. Rapidly-emerging genetic variation within the begomoviruses could lead to a resistance-breaking event. This has been witnessed in southern Morocco with the recombinant TYLCV-IS76 that outcompetes its parents TYLCV-IL and TYLCSV-ES in tomato cultivars with the *Ty*-1 resistance gene (Belabess et al. 2016). The recombinant is now the prevalent TYLCV isolate present in the region.

We would like to examine if there is any evidence of TYLCV becoming capable of overcoming TYLCV-resistant cultivars in the states of Florida and Georgia. By examining the full-length genomes of TYLCV isolated from TYLCV-resistant and susceptible tomato cultivars, we can determine if there is an eminent threat to resistance-breaking. We will also perform a

greenhouse study in which we propagate TYLCV through resistant and susceptible tomato cultivars to determine if repeated use of resistant cultivars over multiple generations will select for particular TYLCV isolates. In addition to comparing resistant and susceptible populations to one another, we will compare the Florida and Georgia TYLCV populations to one another to see if the TYLCV genome varies over geography.

Materials and methods

Propagation of TYLCV through multiple generations of a TYLCV-resistant and TYLCV-susceptible cultivar

The TYLCV isolate used for inoculation was isolated from a tomato field in Tifton, Georgia in 2015 and maintained in the greenhouse. Five plants at the ten-leaf stage of either the TYLCV-susceptible cultivar, Lanai, or the TYLCV-resistant cultivar, Inbar (Hazera Genetics, Israel), were individually caged and inoculated by clip-caging twenty viruliferous whiteflies to their upper leaves. After a 48-hour inoculation access period, the whiteflies were removed. The tomato plants were allowed to develop infection for three weeks. Twenty non-viruliferous whiteflies were then clip-caged to the upper leaves of the plants and given a 48-hour acquisition access period. These whiteflies were transferred to a new, non-infected plant of the same cultivar for a 48-hour inoculation access period. The new plants were maintained for three weeks and the TYLCV was again passed on to new plants. This process was repeated until ten generations of tomato plants had been infected. Samples of plant tissue were taken from each of the plants at generations one, five, and ten for DNA extraction in order to sequence three full-length TYLCV genomes from each of the five replicates for the resistant and susceptible cultivars. The full-length genomes were deposited in GenBank with accession numbers KY965834 - KY965923.

Isolation of TYLCV from field-collected TYLCV-resistant and TYLCV-susceptible tomato cultivars

Leaf tissue was collected from symptomatic tomato plants in agricultural fields from both TYLCV-resistant and TYLCV-susceptible cultivars located in Tifton, Georgia during 2015 and 2016 and in Immokalee, Florida during 2015. Sample information is included in Table 3.1. Sequences were deposited in GenBank with accession numbers KY971320 - KY971372. For analyses comparing Florida and Georgia populations (but not resistant and susceptible populations), the TYLCV sequence available from GenBank, accession AY530931 from Florida, was added to the data set.

Cloning of TYLCV genomes

DNA from leaf tissue was extracted using GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA). TYLCV DNA from susceptible tomato cultivars was amplified with rolling circle amplification. TYLCV DNA from resistant cultivars did not amplify well with rolling circle amplification, as resistant cultivars accumulate much lower levels of viral DNA, and a PCR-based cloning method was employed. TYLCV DNA from susceptible cultivars was amplified using TempliPhi (GE Healthcare, Chicago, IL) with the protocol outlined by Inoue-Nagata et al. (2004). Amplified DNA was digested with SacI (Fisher BioReagents, Pittsburgh, Pennsylvania). To purify the DNA, a gel extraction was performed on the SacI-digested DNA using crystal violet (Fisher Chemical, Fair Lawn, NJ) as the DNA-visualizing agent. The DNA was then ligated into the vector pGEM-3Z (Promega Corporation, Madison, WI) and a transformation was performed into One Shot TOP10 Chemically Competant *E. coli* (Invitrogen, Carlsbad, CA). Colonies were screened using colony PCR with primers T7F (5'-TAATACGACTCACTATAGGG-3') and and M13R (5'- CAGGAAACAGCTATGACC-3'),

and purified plasmids were sent for Sanger sequencing (Eurofins Genomics, Louisville, KY) using the following primers: 5370F (5'–TTCGCTATTACGCCAGCT–3'), 2941R (5'–CCCAGGCTTTACACTTTATGCTTCC–3'), 710F (5'–TCTTATATCTGTTGTAAGGGCCCGT–3'), and 1400F (5'–ACGAGAACCATACTGAAAACGCCTT–3').

TYLCV DNA from resistant cultivars was amplified using PCR with three different primer sets to cover the full-length of the TYLCV genome. The first segment amplified with primers 1470R (5'-TGCATACACTGGATTAGAGGCATG-3') and 2243F (5'-GAAACATAAACTTCTAAAGGAGGAC-3') and a PCR program with an initial 95° C denaturation step for 3 min followed by 35 cycles of 95° for 30 s, 49° for 30 s, and 72° for 1 min 50 s, with a final extension step of 72° for 5 min. The PCR reactions were run in 10 µl reactions with 5 µl of GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 2 µl of water, 0.5 µl of each primer at 10 µM concentration, and 2 µl of DNA extract. The second segment amplified with primers C2R (5'-CCAATAAGGCGTAAGCGTGT-3') and 1371F (5'-AACTTATAATCATCAGGAGGCAGCC-3') and the third segment with C2F (5'-GCAGTGATGAGTTCCCCTGT-3') and 2326R (5'-GAGGCCCTCAATATATTAAAAGA-3'). Both the second and third segments amplified with a PCR program starting with an initial denaturation step of 95° C for 3 min followed by 35 cycles of 95° for 30 s, 47° for 30 s, and 72° for 50 s, with a final extension step of 72° for 5 min. The three segments were cloned using CloneJET PCR Cloning Kit (Thermo Scientific, Waltham, MA). Ligated vectors were transformed into One Shot TOP10 Chemically Competant *E. coli* (Invitrogen, Carlsbad, CA). Colonies were screened using colony PCR with and purified plasmids were sent for Sanger sequencing (Eurofins Genomics, Louisville, KY) using primers pJET1.2F (5'-

CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2R (5'-AAGAACATCGATTTTCCATGGCAG-3'). Vector sequences were manually excised from sequences to leave only TYLCV sequence intact. Reads were then assembled into full-length genomes using Geneious Pro v. 8.1.9 (Drummond et al. 2011).

Gene flow and genetic differentiation

To determine if the resistant and susceptible populations and Florida and Georgia populations were differentiated from one another, nucleotide sequence-based Ks, Kst, Snn, Z, and Fst (Hudson et al. 1992b) statistics were calculated using DnaSP v5.10.01 (Librado and Rozas 2009) using the Gene Flow and Genetic Differentiation tool. To test for level of significance, a permutation test with 1000 replications was performed during the test. Values were considered significant if p-values were less than 0.05.

Test of positive selection

All six genes of the TYLCV genome were analyzed for positive selection using the HyPhy tool (Pond and Muse 2005) in MEGA 7.0.21 (Kumar et al. 2016). The HyPhy tool determines nonsynonymous (dN) and synonymous (dS) nucleotide substitutions for each codon. The Tamura-Nei model was selected as the substitution model (Tamura and Nei 1993). Codons with a dN greater than dS and a p-value less than 0.05 were considered to be under positive selection.

Tests of population neutrality

To test for neutrality of the TYLCV populations, Tajima's D (Tajima 1989) was computed in DnaSP using the Tajima's Test tool. Tajima's D is determined by the average number of nucleotide pair-wise differences and the number of segregating sites among all sequences.

Fu and Li's D and F statistics (Fu and Li 1993) were also calculated using DnaSP. The D statistic is calculated based on the number of mutations appearing just once and the total number of mutations. The F statistic is calculated based the number of mutations appearing just once and the average pairwise differences between sequences.

Recombination detection

All TYLCV genomes acquired from field-collected samples were analyzed in Recombination Detection Program v.4.80 (RDP4) (Martin et al. 2015) to determine if any recombinants could be detected within the data set. RDP4 uses seven different detection tests to screen for recombination. A threshold of three positive tests and a phylogenetic confirmation were the criteria used for a positive detection of recombination.

Phylogenetic analysis

A maximum-likelihood phylogenetic tree was constructed in MEGA 7.0.21. One thousand bootstrap replications were performed and the Tamura Nei substitution model was used. *Tomato yellow leaf curl China virus* (NC_004044) and *Tomato yellow leaf curl Sardinia virus* (GU951759) were used as outgroups.

Results

TYLCV propagation experiment

Generations 1, 5, and 10 were each assessed to determine if TYLCV populations from the susceptible and resistant plants became differentiated from one another. The nucleotide sequence-based genetic differentiation statistics Ks, Kst, Snn, and Z show a statistically-significant differentiation occurring at generations 5 and 10, but not 1 (Table 3.2). The Fst value increases with generation number, indicating an increasing differentiation between the populations with time.

All six of the genes were tested for positive selection by determining the nonsynonymous to synonymous substitution ratio (dN/dS) for each codon. Resistant and susceptible populations were tested both separately and together at generations 1, 5, and 10. No positive selection of any of the codons was detected with a statistical significance of a p-value < 0.05.

Fu and Li's F and D statistics and Tajima's D statistic were calculated for Generations 1, 5, and 10 for susceptible and resistant populations. Fu and Li's F and D statistic and Tajima's D statistic were only significant for the resistant population at generation 1 (Table 3.3). This is possibly due to the man-made bottleneck event occurring since all plant replicates were inoculated from the same original plant.

An alignment containing the TYLCV genomes from all field-collected was loaded into RDP4. No recombination events were detected in the resistant, susceptible, or combined populations.

Resistant vs susceptible field samples

A population of twenty seven resistant genomes and a population of twenty six susceptible genomes were compared with one another. The nucleotide-based genetic differentitiation statistics Ks, Kst, Snn, and Z values calculated were determined by the permutation test to be significantly different, indicating that the resistant and susceptible populations are detectably different (Table 3.4). The Fst value is low indicating a low level of genetic structure between the resistant and susceptible populations.

To test for positive selection on genes within the TYLCV genomes, the HyPhy codon selection test was performed on all six genes for the resistant, susceptible, and combined populations. No codons were determined to be under positive selection at a statistically significant level.

Population neutrality statistics Fu and Li's D and F statistics and Tajima's D were calculated for the resistant, susceptible, and combined populations. These statistics examine the frequency of segregating sites across the population(s) examined. The resistant and susceptible populations had negative values for all three of these values, however, they were not considered statistically significant (Table 3.5). The combined population did have statistically significant results for Fu and Li's F and D statistics. Negative values for Fu and Li's D and F values indicate either a recent population expansion or purifying selection.

An alignment with the TYLCV genomes from resistant and susceptible cultivars were loaded into RDP4 and analyzed. No recombination events were detected in the field-collected samples.

Florida vs Georgia field samples

The TYLCV genomes were divided into populations based on the state they were collected from (Florida or Georgia). Additionally, an extra genome available on GenBank (AY530931) was added to the Florida population. The nucleotide-based genetic differentiation statistics Ks, Kst, Snn, and Z statistics with their corresponding permutation tests determined the two populations to be differentiated (Table 3.6).

All six genes from the Florida, Georgia, and combined populations were tested with the HyPhy codon selection test. No codons were under positive selection at a statistically significant level.

The Florida, Georgia, and combined populations were tested for neutrality with Fu and Li's D and F and Tajima's D. The Florida and Georgia populations both have negative values for all three statistics, however, not at a statistically significant level (Table 3.7). The combined population did have statistically significant values for Fu and Li's D and F statistics, but not for

Tajima's D. The negative values of the Fu and Li's D and F statistics indicate either population expansion or purifying selection.

The maximum likelihood tree shows the Florida and Georgia samples clearly parsed from one another (Figure 3.1). The Georgia clade appears to emerge from the Florida population. This could indicate that the Georgia TYLCV population arose from an introduction from Florida. The TYLCV samples from resistant and susceptible cultivars do not parse with one another. There does not appear to be any phylogenetic relationship between TYLCV genomes and the resistance status of the cultivar it was collected from.

Discussion

Overall, there is little evidence from our data to indicate TYLCV is currently undergoing significant positive selection from the TYLCV-resistant cultivars. The results from the TYLCV propagation experiment did not indicate that particular TYLCV isolates were being selected for by the TYLCV-resistant tomato cultivar, Inbar. The nucleotide sequence-based population statistics, Ks, Kst, and Snn, did detect increasing differences between the susceptible and resistant populations as the generation number increases. The Fst value also increases with generation number. However, the mutations accumulated with increasing generation appear to be random between plant replications. Very few consistently-occurring mutations were found between replications. The very few consistently-occurring mutations seen in alignment did not result in an amino acid change. This is shown with the dN/dS ratios calculated by the HyPhy codon selection test which did not detect any positive selection on any of the codons in the six different TYLCV genes. Overall, the propagation through ten generations of a resistant cultivar did not yield any detectable positive selection. The experiment lasted a total of 30 weeks. If the

experiment ran longer, the resistant and susceptible populations may have become further differentiated and positive selection may have eventually occurred.

The analyses for the field-collected samples show mixed results. The nucleotide sequence-based population statistics did detect statistically-significant differences for Ks, Kst, and Snn. However, the Fst value is quite low indicating that these resistant and susceptible populations have a low level of genetic differentiation. In addition to the low Fst value, the phylogenetic tree shows TYLCV genomes from resistant cultivars intermixed with TYLCV genomes from susceptible cultivars. The genomes from resistant cultivars do not parse out separately from the susceptible cultivars. The lack of significant results from the HyPhy tool indicates there may not be significant positive selection currently acting on these populations. The majority of our evidence indicates the resistant and susceptible populations are not distinctive from one another.

Recombination can play a major role in the evolution of begomoviruses (Navas-Castillo et al. 2000; Belabess et al. 2016). Recombinants can outcompete parental virus strains in resistant cultivars as has been seen in southern Morocco (Belabess et al. 2016). We did not detect any recombination events in our data set. Recombination could be occurring, but is not being detected since the TYLCV isolates are so similar to one another within the two geographic regions we examined. Another tomato-infecting *Begomovirus, Tomato mottle virus* (ToMoV), is present in Florida. ToMoV is bipartite while TYLCV is monopartite. TYLCV and ToMoV have been documented to co-infected individual tomato plants (Akad et al. 2007), but there is no documentation that these two viruses recombine. Introduction of a new *Begomovirus*, specifically a monopartite species, could offer an opportunity for recombination with TYLCV. TYLCV has been documented to recombine with the monopartite begomoviruses *Tomato yellow*

leaf curl Sardinia virus (Belabess et al. 2016), *Tomato leaf curl virus* (Navas-Castillo et al. 2000), *Tomato leaf curl Comoros virus* (Urbino et al. 2013), *Tomato leaf curl Sudan virus* (Idris and Brown 2004), *Tomato leaf curl Iran virus* (Bananej et al. 2004), and *Tobacco leaf curl virus* (Park et al. 2011). TYLCV has also recombined with *Tomato yellow leaf curl Thailand virus* (Kim et al. 2011), which typically has two genomic components, but DNA-A alone has been demonstrated to be sufficient for plant infection (Guo et al. 2009). Preventing the introduction of new begomoviruses is crucial in order to prevent recombination opportunities with TYLCV.

The geographic region in which the TYLCV genomes were collected from seems to play a key role in the diversification of TYLCV. The Florida and Georgia populations were determined to be differentiated at a statistically-significant level by the nucleotide sequence-based statistics, Ks, Kst, and Snn. These values indicated a greater degree of differentiation for the Florida and Georgia populations compared to the resistant and susceptible populations. Additionally, the Fst value between Florida and Georgia populations was considerably higher than the Fst value between resistant vs susceptible populations. For our data set, geography played a much greater role in the genetic makeup of TYLCV genomes than the resistance status of the plant. The phylogenetic tree demonstrates that the Florida and Georgia samples parse separately. The phylogenetic tree hints that the Georgia TYLCV population may be derived from an introduction from the Florida population.

Currently, resistant cultivars are still effective for the management of TYLCV in the regions of Florida and Georgia. Based on our data, the use of TYLCV-resistant tomato cultivars does not currently face the danger of a resistance-breaking strain. However, novel mutations or a recombination event could change this in the future. Certain cropping strategies can be employed to reduce the risk of resistance-breaking strains emerging (Fabre et al. 2012). One strategy is to

plant a mixture of resistant and susceptible varieties of crop in order to reduce the overall selection pressure on the virus from the resistant crop. The other strategy is to plant only resistant varieties on a landscape level if the virus requires two or more mutations in order to overcome resistance. This strategy will eventually deplete surrounding reservoir plants of virus over time. Resistant cultivars are an invaluable tool for growing tomatoes in TYLCV-affected areas and measures should be taken to preserve their usefulness.

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Figure 3.1. Maximum-likelihood phylogenetic tree constructed with field-collected TYLCV genomes from Florida and Georgia. Samples with an ‘R’ at the end of their name were isolated from a resistant cultivar. All other TYLCV genomes were isolated from a susceptible cultivar, except ‘Florida AY530931’ which comes from an unknown cultivar.

Table 3.1. Sample name, collection date, location, host plant, and TYLCV susceptibility of host plant of TYLCV genomes isolated from field-collected susceptible and resistant tomato cultivars.

Sample name	Collection date	Location	Host plant	TYLCV-susceptibility of host plant
Florida_1.2	Mar-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Florida_11.1_R	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Skyway 687 (Enza Zaden)	resistant
Florida_11.2_R	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Skyway 687 (Enza Zaden)	resistant
Florida_11.3_R	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Skyway 687 (Enza Zaden)	resistant
Florida_17.1	Jun-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Suddath's Strain (Nature and Nuture Seeds)	susceptible
Florida_18.1	Jun-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Orange Strawberry (Baker Creek Heirloom Seeds)	susceptible
Florida_19.1	Jun-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Legend (Tomato Growers)	susceptible
Florida_2.1	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Plum Regal (Bejo Seeds)	susceptible
Florida_3.1	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. BHN 685 (Siegers Seed Company)	susceptible
Florida_4.2_R	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. HM 8845 (Harris Moran Seed Company)	resistant
Florida_4.4_R	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. HM 8845 (Harris Moran Seed Company)	resistant
Florida_5.1	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Juliet (Johnny's Selected Seeds)	susceptible
Florida_6.5	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Gator (Bejo Seeds)	susceptible
Florida_7.1	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. yellow pear heirloom (Tomato Growers)	susceptible
Florida_7.2	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. yellow pear heirloom (Tomato Growers)	susceptible
Florida_7.3	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. yellow pear heirloom (Tomato Growers)	susceptible
Florida_7.5	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. yellow pear heirloom (Tomato Growers)	susceptible
Florida_8.4	Apr-2015	USA: Immokalee, Florida	Solanum lycopersicum cv. Brickyard (Syngenta)	susceptible
Georgia_107.2	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Lanai (lab cultivar)	susceptible
Georgia_108.1	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_108.2	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_112.1	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_118.1_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Security (Harris Seeds Company)	resistant
Georgia_122.2_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Security (Harris Seeds Company)	resistant
Georgia_124.3_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Inbar (Hazera Genetics)	resistant
Georgia_127.1_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Inbar (Hazera Genetics)	resistant
Georgia_130.1_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Shanty (Hazera Genetics)	resistant
Georgia_130.2_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Shanty (Hazera Genetics)	resistant
Georgia_132.1_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Shanty (Hazera Genetics)	resistant
Georgia_133.1_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Tygress (Seminis)	resistant
Georgia_133.2_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Tygress (Seminis)	resistant
Georgia_135.1_R	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Tygress (Seminis)	resistant
Georgia_135.2	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Tygress (Seminis)	resistant

_R				
Georgia_25.1_R	Sep-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Security (Harris Seeds Company)	resistant
Georgia_30.1_R	Sep-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Shanty (Hazera Genetics)	resistant
Georgia_35.1	Sep-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_40.2_R	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Shanty (Hazera Genetics)	resistant
Georgia_47.1_R	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Tygress (Seminis)	resistant
Georgia_50.1_R	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Security (Harris Seeds Company)	resistant
Georgia_57.1	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_58.1	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Red Bounty (Harris Seeds Company)	susceptible
Georgia_58.2	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Red Bounty (Harris Seeds Company)	susceptible
Georgia_59.1	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_59.3	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_63.1_R	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Tygress (Seminis)	resistant
Georgia_67.2_R	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Tygress (Seminis)	resistant
Georgia_72.1_R	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Shanty (Hazera Genetics)	resistant
Georgia_76.2_R	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Security (Harris Seeds Company)	resistant
Georgia_81.3_R	Oct-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Security (Harris Seeds Company)	resistant
Georgia_9.10	Jan-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_9.9	Jan-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. FL47 (Seminis)	susceptible
Georgia_92.1_R	Nov-2015	USA: Tifton, Georgia	Solanum lycopersicum cv. Security (Harris Seeds Company)	resistant
Georgia_99.1	Sep-2016	USA: Tifton, Georgia	Solanum lycopersicum cv. Lanai (lab cultivar)	susceptible

Table 3.2. Genetic differentiation statistics between resistant and susceptible TYLCV populations of different generations.
[x]Kt is the average number of pairwise nucleotide differences across genomes in both populations.
[y]Ks, Kst, and Snn, Z are nucleotide sequence-based genetic differentiation statistics.
[z]Fst is a genetic differentiation statistic. Values range from 0 to 1. Low Fst values indicate a high level of similarity between populations while high Fst values indicate genetically distinct groups.

Populations Analyzed	Kt[x]	Ks[y]	Kst[y]	p-value of Ks and Kst	Snn[y]	p-value of Snn	Z[y]	p-value of Z	Fst[z]
Generation 1: Resistant vs. Susceptible	11.82069	11.79048	0.00256	0.3310	0.46667	0.6400	214.12143	0.1610	0.00493
Generation 5: Resistant vs. Susceptible	12.07126	11.40000	0.05561	**0.0010**	0.93333	**0.0000**	202.68333	**0.0020**	0.10221
Generation 10: Resistant vs. Susceptible	12.42989	11.38095	0.08439	**0.0000**	0.93333	**0.0000**	188.65238	**0.0000**	0.15124

Table 3.3. Tests of neutrality for resistant and susceptible TYLCV populations of different generations. Negative Fu and Li's D and F values and Tajima's D values indicate population expansion or purifying selection.

Population Analyzed	Fu and Li's D	p-value	Fu and Li's F	p-value	Tajima's D	p-value
Generation 1 - Resistant	-2.39208	**P < 0.05**	-2.59396	**P < 0.05**	-1.88472	**P < 0.05**
Generation 5 - Resistant	-0.63476	P > 0.10	-0.78389	P > 0.10	-0.79782	P > 0.10
Generation 10 - Resistant	-0.91053	P > 0.10	-1.12974	P > 0.10	-1.15968	P > 0.10
Generation 1 - Susceptible	-1.22645	P > 0.10	-1.45762	P > 0.10	-1.36701	P > 0.10
Generation 5 – Susceptible	-1.10155	P > 0.10	-1.28177	P > 0.10	-1.14230	P > 0.10
Generation 10 - Susceptible	-1.06583	P > 0.10	-1.20065	P > 0.10	-0.98299	P > 0.10

Table 3.4. Genetic differentiation statistics between field-collected resistant and susceptible TYLCV samples.
[x]Kt is the average number of pairwise nucleotide differences across genomes in both populations.
[y]Ks, Kst, and Snn, Z are nucleotide sequence-based genetic differentiation statistics.
[z]Fst is a genetic differentiation statistic. Values range from 0 to 1. Low Fst values indicate a high level of similarity between populations while high Fst values indicate genetically distinct groups.

Populations Analyzed	Kt[x]	Ks[y]	Kst[y]	p-value of Ks and Kst	Snn[y]	p-value of Snn	Z[y]	p-value of Z	Fst[z]
Field Samples: Resistant vs. Susceptible	24.61974	23.86067	0.03083	**0.0170**	0.67977	**0.0040**	670.42164	**0.0340**	0.05857

Table 3.5. Tests of neutrality for field-collected resistant and susceptible TYLCV samples.
Negative Fu and Li's D and F values and Tajima's D values indicate population expansion or purifying selection.

Population Analyzed	Fu and Li's D	p-value	Fu and Li's F	p-value	Tajima's D	p-value
Resistant	-1.18636	P > 0.10	-1.32671	P > 0.10	-0.99818	P > 0.10
Susceptible	-2.15866	0.10 > P > 0.05	-2.24789	0.10 > P > 0.05	-1.38280	P > 0.10
Combined	-3.21125	**P < 0.05**	-3.12489	**P < 0.05**	-1.64132	0.10 > P > 0.05

Table 3.6. Genetic differentiation statistics between field-collected Florida and Georgia TYLCV samples.
[x]Kt is the average number of pairwise nucleotide differences across genomes in both populations.
[y]Ks, Kst, and Snn, Z are nucleotide sequence-based genetic differentiation statistics.
[z]Fst is a genetic differentiation statistic. Values range from 0 to 1. Low Fst values indicate a high level of similarity between populations while high Fst values indicate genetically distinct groups.

Populations Analyzed	Kt[x]	Ks[y]	Kst[y]	p-value of Ks and Kst	Snn[y]	p-value of Snn	Z[y]	p-value of Z	Fst[z]
Field Samples: FL vs GA	24.86932	16.34665	0.34270	**0.0000**	0.98148	**0.0000**	455.61036	**0.0000**	0.49763

Table 3.7. Tests of neutrality for field-collected Florida and Georgia TYLCV samples.
Negative Fu and Li's D and F values and Tajima's D values indicate population expansion or purifying selection.

Population Analyzed	Fu and Li's D	p-value	Fu and Li's F	p-value	Tajima's D	p-value
FL	-1.92670	P > 0.10	-2.18758	0.10 > P > 0.05	-1.76221	0.10 > P > 0.05
GA	-2.14999	0.10 > P > 0.05	-2.12725	0.10 > P > 0.05	-1.10892	P > 0.10
Combined	-3.23971	**P < 0.05**	-3.15494	**P < 0.05**	-1.66634	0.10 > P > 0.05

CHAPTER 4

PHYLOGENETIC AND POPULATION GENETIC ANALYSES OF *TOMATO YELLOW LEAF CURL VIRUS* ON A WORLD-WIDE SCALE[1]

[1]Marchant, W.G., Brown, J.K., and Srinivasan, R. To be submitted to *BMC Genomics*.

Abstract

Begomoviruses are whitefly-transmitted plant viruses with circular, single-stranded DNA genomes. *Tomato yellow leaf curl virus* (TYLCV) is a well-studied monopartite *Begomovirus* with a genome of approxiamately 2,800 base pairs that can easily be sequenced in entirety, making it an ideal model organism for studying evolutionary relationships. Many studies have examined the phylogenetic history and spread of TYLCV, however, the genetic factors shaping TYLCV populations worldwide are not clear. All available full-length TYLCV genomes were downloaded from GenBank, along with some of our own genomes from Georgia and Florida, and parsed into four geographical regions which were Africa-Europe-Middle East, Southeast Asia, Americas, and Ocenia. Population genetics influencing factors such as recombination, polymorphisms, gene flow and genetic differentiation, population neutrality, positive selection, and Bayesian evolutionary analyses were conducted for each geographical group. Recombination and neutrality were key players in shaping the populations. The Africa-Europe-Middle East region showed the highest percentage of recombinants and average number of nucleotide differences, proving it is the most diverse region of the four. This region is also most basal on the phylogenetic tree and has the oldest most common recent ancestor, supporting other studies stating the Middle East is the likely origin of TYLCV. Oceania had the lowest average number of nucleotide differences and number of recombinants, and negative population neutrality values which all corroborate that TYLCV is newly-introduced and rapidly spreading in Oceania. TYLCV is also rapidly spreading thoughout Southeast Asia as the population neutrality statistics indicate. The Americas show intermediate values for average number of nucleotide differences and have about six separate introduction events from several different places but may not be

spreading as rapidly as in Southeast Asia, perhaps due to the introduction and spread of the MED whitefly sibling species.

Introduction

Viruses in the family *Geminiviridae* are plant pathogens that are transmitted by Hemipteran insects. These viruses are very important in agriculture as they create yield losses in a number of crops (Varma and Malathi 2003, Thottappilly 1992, Legg 1999, Briddon 2003, Picó et al. 1996). The genomes of geminiviruses are comprised of circular, single-stranded DNA components of about 2.6 kb in size. Genomes can be either bipartite with two DNA components termed DNA-A and DNA-B, or monopartite with only the DNA-A component. DNA components are encapsidated with coat protein into twinned, icosohedral virions (Bottcher et al. 2004).

Begomovirus is one of the nine genera in the family *Geminiviridae* (Varsani et al. 2017) and is the largest genus in the family. These viruses are all transmitted by whiteflies (family Aleyrodidae) and infect dicotyledonous plants. *Tomato yellow leaf curl virus* (TYLCV) is an extensively studied *Begomovirus* species that serves as an important model organism for studying evolutionary relationships. This virus has only one genetic component (DNA-A) which simplifies genetic analyses by eliminating the complications of pseudorecombination when there are multiple DNA components. The genome is small and can easily be amplified, cloned, and sequenced with Sanger sequencing. TYLCV is now present in most parts of the world and many full-length genomes are available on GenBank and can be analyzed as separate populations.

The genome of TYLCV is approximately 2,800 base-pairs long and contains a total of six genes. Two genes, designated V1 and V2, are on the viral strand of the genome. V1 codes for the coat protein and V2 codes for a protein that modulates host symptoms and affects virus

accumulation (Padidam et al. 1996). Four other genes, designated C1-C4, are on the complementary strand of the genome (Czosnek 2008). C1 codes for the replication-associated protein, C2 codes for the transcriptional activator protein and is involved in suppression of posttranscriptional gene silencing in host plants (van Wezel et al. 2002), C3 codes for the replication enhancer protein, and C4 codes for a protein that determines host symptoms, host range, and systemic virus movement (Tomás et al. 2001, Jupin et al. 1994). The genes cover most of the length of the genome, however, a highly-variable intergenic region exists where no genes are present.

The virus was first reported in Israel in the 1930's (Cohen and Antignus 1994), and within the last half century has swiftly spread to many parts of the world. The virus is now present on most continents, including Asia (Wu et al. 2006, Kimihiko et al. 1998), Africa (Peterschmitt et al. 1999a), North America (Polston et al. 1999, Brown and Idris 2006, Ascencio-Ibáñez et al. 1999), Australia (Van Brunschot et al. 2010), Europe (Botermans et al. 2009, Louro et al. 1996, Moriones et al. 1993, Accotto et al. 2003, Avgelis et al. 2001), South America (Zambrano et al. 2007), and even on small islands such as Reunion Island and Puerto Rico (Peterschmitt et al. 1999b, Bird et al. 2001).

One possible reason for the rapid spread of TYLCV is the introduction of invasive whitefly sibling species from the *Bemisia tabaci* species complex to new geographic areas. The *B. tabaci* species complex is the only vector of TYLCV. The sibling species of the *B. tabaci* complex are morphologically indistinguishable, but can be differentiated based on the sequence of the cytochrome oxidase subunit I gene (Elfekih et al. 2017, Dinsdale et al. 2010). The MEAM1 (formerly biotype B) and MED (formerly biotype Q) sibling species are the most notoriously invasive sibling species of *B. tabaci* (Muñiz et al. 2011, De Barro and Ahmed 2011)

and both can transmit TYLCV (Pan et al. 2012, Li et al. 2010). In fact, in China, the MEAM1 and MED sibling species transmit TYLCV more effectively than the native ZHJ2 sibling species (Li et al. 2010). The MED sibling species is credited to have spread TYLCV through China during its invasion starting in 2003 (Pan et al. 2012).

Despite being a DNA virus, TYLCV mutates almost as quickly as an RNA virus at a rate of 2.88 × 10-4 substitutions/site/year (Duffy and Holmes 2008). The intergenic region mutates at a much higher rate than the rest of the genome (Yang et al. 2014). The rapid mutation rate of TYLCV has led researchers to refer to TYLCV as a "quasispecies" (Seal et al. 2006, Roossinck 1997), a term usually associated with RNA viruses. A quasispecies is a virus population that contains a wide distribution of mutants, rather than a population of completely homogenous genomes (Domingo et al. 2012). TYLCV has many features of a quasispecies such as a high rate of mutation and the presence of many isolates.

Phylogenetic analyses and reconstruction of TYLCV introduction events have been published by numerous groups (Duffy and Holmes 2007, Mabvakure et al. 2016, Lefeuvre et al. 2010, Yang et al. 2014, Lee et al. 2010, Hosseinzadeh et al. 2014, Romay et al. 2013, Zhang et al. 2014). It is also well known that mutation and especially recombination are powerful forces in *Begomovirus* evolution. However, other factors have been less examined such as population genetic factors like gene flow and genetic differentiation, neutrality, or positive selection. We would like to examine all the TYLCV genomes available on GenBank as well as some of our local TYLCV genomes from Georgia and Florida to perform a world-wide analysis on the population structures of TYLCV in different geographic regions of the world. Including as many genomes as possible in our analysis will increase the odds of detecting differences between populations and factors shaping these populations. We will examine data sets both with and

without recombinant genomes, as recombinant genomes have different portions of their genomes with different phylogenetic histories.

Materials and methods

Acquisition of TYLCV genomes

To collect TYLCV genomes from GenBank, several representative TYLCV genomes were queried with BLAST on NCBI. These were accessions FJ956705 from Oman, FN256259 from China, LN846599 from Morocco, and X15656 from Israel. Resulting genomes that shared 85% identity or higher with the queried genomes were collected. Additionally, sequences from Florida and Georgia that had not yet been added to GenBank were added to the data set (KY971320-KY971372). A total of 666 genomes comprised the total data set (Appendix A). Genomes were renamed to their country of origin and a number if there were multiple genomes from the same country. The last two digits of the collection year, if available, were added after a dash.

Grouping of TYLCV genomes into geographic regions

TYLCV genomes were grouped into one of four geographic regions for population genetics and BEAST analyses. These four groups were Africa-Europe-Middle East with 226 genomes, Oceania with 69 genomes, Southeast Asia with 296 genomes, and Americas with 75 genomes.

Recombination detection

The TYLCV data set was uploaded and tested for recombination events in Recombination Detection Program v.4.80 (RDP4) (Martin et al. 2015). Genomes that tested positive for recombination with three or more of the seven testing methods present in RDP4 (RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiSscan, and 3Seq) and were

phylogenetically supported were considered recombinants (Appendix B). Recombinant genomes were removed from the data set resulting in a set of 493 genomes referred to as the "without-recombinants" data set (Appendix C). The original data set of 666 genomes which includes recombinants is referred to as the "with-recombinants" data set.

Polymorphism analysis

Both with-recombinants and without-recombinants data sets were analyzed for polymorphisms in DnaSP v5.10.01 (Librado and Rozas 2009) using the DNA Polymorphism tool. The data sets were subdivided into their geographic regions to compare polymorphisms between geographic regions. Polymorphic traits examined were number of polymorphic sites, number of haplotypes, haplotype diversity, and average number of nucleotide differences (k).

Gene flow and genetic differentiation

The different geographic regions of the with-recombinants and without-recombinants data sets were analyzed to determine if the TYLCV populations were differentiated from one another. Nucleotide sequence-based Ks, Kst, Snn, Z, and Fst statistics (Hudson et al. 1992a, Hudson et al. 1992b, Hudson 2000) were calculated using DnaSP with the Gene Flow and Genetic Differentiation tool. A permutation test with 1000 replications was performed to test for levels of significance. Values were considered significant if p-values were less than 0.05.

Tests of population neutrality

Tajima's D (Tajima 1989) was calculated for each geographic region for both the with-recombinants and without-recombinants data sets. The values were calculated in DnaSP with the Tajima's Test tool which uses the average number of nucleotide pair-wise differences along with the number of segregating sites among all sequences to calculate a value for each TYLCV population group.

Fu and Li's D and F statistics (Fu and Li 1993) were also calculated for each geographic region in both data sets using the Fu and Li's Tests tool in DnaSP. The D statistic is determined by the number of mutations appearing just once and the total number of mutations. The F statistic is determined by number of mutations appearing just once and the average pairwise differences between sequences.

Test of positive selection

The six genes of the TYLCV genome were analyzed for positive selection in each of the geographic groups for both data sets. The HyPhy tool (Pond and Muse 2005) in MEGA 7.0.21 (Kumar et al. 2016) was used to identify positively-selected codons by examining the number of nonsynonymous (dN) and synonymous (dS) nucleotide substitutions for each codon. The substitution model selected was the Tamura-Nei model (Tamura and Nei 1993). Codons with a dN greater than dS and a p-value less than 0.05 were considered to be under positive selection.

Bayesian evolutionary analysis

Evolutionary analysis for both the with- and without-recombinants data sets was conducted using Bayesian Evolutionary Analyses Sampling Trees (BEAST v2.4.7, Drummond and Bouckaert (2015)). The best suitable nucleotide substitution model was identified for each data set using jModelTest 2.1.10 v20160303 (Darriba et al. 2012). Among the 88 models, the best fit models based on lowest Bayesian Information Criterion (BIC) Scores were selected. The best models turned out to be Generalized Time Reversible (GTR models) for both data sets. The data sets along with nucleotide substitution model details were then exported in BEAUti2:Standard. In addition, a gamma site heterogeneity model was selected to allow variation between sites within the alignment. Each data set was then partitioned in to four taxon sets based on location proximities (details included above). A relaxed log normal clock model

was chosen for this analysis. The coalescent constant population model was set as the tree prior, and taxon sets were assumed to be distributed uniformly. The calibration time limits for estimation of Most Recent Common Ancestor (MRCA) for each taxon set were set at 290 and 40211 years ago based on previously published results (Duffy and Holmes 2007). Monte Carlo Markovian Chain analyses was conducted for a length for 75,000,000 with trace log generated for every 1000 trees. An Xml file was generated and was used as a BEAST input file. The run was repeated once more and a combined tracer (log) file was generated using LogCombiner. The MCMC output file was then visualized using Tracer v1.6.0 (Rambaut et al. 2015). TREEANNOTATOR was then used to develop a target tree from a sample of trees generated by BEAST. A maximum clade credibility tree was then generated with posterior probability values. The tree was visualized using FigTree V1.4.3 (Rambaut and Drummond 2009).

Results

Recombination detection

A total of 44 recombination events affecting 172 of the genomes from our total data set were detected with the RDP4 software. To be considered a true recombination event, each event had to test positive for at least three out of the seven test methods in RDP4 and be phylogenetically supported by showing a shift in tree position depending on recombinant region of the genome examined. The Africa-Europe-Middle East region contained the large majority of the recombinant genomes with 157 genomes out of the 172 total recombinant genomes detected. The Americas, Southeast Asia, and Oceania regions had comparatively fewer recombinant genomes compared to Africa-Europe-Middle East region (Table 4.1). The Africa-Europe-Middle East region was further broken down geographically to examine more specifically where the

recombinants are from (Table 4.2). The Middle East has the highest proportion of recombinant genomes, however, Africa, and Europe, also have a large proportion of recombinant genomes. An example of a recombinant genome is Iran7-06 which is comprised of parental genomes Oman52-13 and SaudiArabia1-12. Iran7-06 shares a high nucleotide identity with Oman52-13 for a portion of its genome, and shares a high identity with SaudiArabia1-12 for a different portion of its genome (Figure 4.1). This recombination event is phylogenetically-supported as Iran7-06 parses with Oman52-13 in one tree and parses with SaudiArabia1-12 in the other tree (Figure 4.2). Another example is Oman9-11 which is comprised of parental genomes Oman16-11 and Oman36-13. Oman9-11 shares a high identity with Oman36-13 for a portion of its genome and a high identity with Oman16-11 for another portion of its genome (Figure 4.3). This recombination event is phylogenetically-supported as Oman9-11 parses with Oman16-11 in one tree and parses with Oman36-13 in another tree (Figure 4.4).

Polymorphism analysis

Polymorphisms were calculated for each of the four geographic regions for both the with-recombinants and without-recombinants data sets (Table 4.3 and 4.4). All geographic regions exhibit a higher average number of nucleotide differences (k) between genomes in the with-recombinants data set than the without-recombinants data set indicating that recombination is a substantial source of genetic diversity. This is especially true of the Africa-Europe-Middle East region. The Africa-Europe-Middle East region also shows the highest average number of nucleotide differences (k) between genomes compared to all other geographic regions for both the with- and without-recombinants data sets. Oceania exhibits the lowest value of all geographic regions in both data sets. All regions have a high haplotype diversity indicating that is uncommon to find completely identical virus genomes, even within the same region.

Gene flow and genetic differentiation

Nucleotide-based statistics Ks, Kst, Snn, Z, and Fst were calculated for both the with- and without-recombinants data sets. Each geographic region was compared with one another to determine the degree of differentiation between each population. p-values for Ks, Kst, Snn, and Z indicate that all geographic regions are significantly differentiated from one another (Table 4.5 and 4.6). Fst values are greatest for the Oceania and Americas comparison for both with- and without-recombinants data sets indicating a high degree of genetic differentiation. Oceania and SE Asia exhibit the lowest Fst value in both data sets indicating a high level of gene flow or low level of genetic differentiation between the two regions.

Tests of population neutrality

Fu and Li's D and F statistics and Tajima's D statistic were calculated for each geographic region for both with- and without-recombinants data sets (Table 4.7 and 4.8). Negative values for all three of these statistics indicate purifying selection or a population expansion. Negative values result from a high number of mutations that occur rarely or only once within the population. For the without-recombinants data set, all four geographic regions exhibit negative Fu and Li's D and F statistics with statistical significance. Oceania and Southeast Asia have negative values for Tajima's D with statistical significance while Africa-Europe-Middle East and Americas do not have statistical significance.

The with-recombinants data set shows Southeast Asia and Oceania, again, as negative values with statistical significance for all three statistics. Africa-Europe-Middle East is only negative and statistically significant for the Fu and Li's D statistic and the Americas is only negative and statistically significant for Tajima's D statistic. Inclusion of recombinants for the

Africa-Europe-Middle East and Americas regions weakens the signal for population expansion or purifying selection.

Test of positive selection

Positively-selected codons were screened for in the six different genes of the TYLCV genome. Each geographic region was examined in the with- and without-recombinants data sets. The without-recombinants data set showed no positively-selected codons while the with-recombinants data set had fourteen codons under positive selection in the Africa-Europe-Middle East region and one codon under positive selection in the Southeast Asia region (Table 4.9). Codon positions under positive selection have amino acid polymorphisms within the population that are co-existing. Codon positions without positive selection detected often have single amino acid or there are just a small number of genomes with a different amino acid. An example of a codon under positive selection is in the C2 gene from the Africa-Europe-Middle East region in the with-recombinants data set. At codon position 83, 23.3% of the population has a proline and while the other 75.8% of the genomes have a threonine. Another example is codon 109 in which 15.1% of the genomes have an isoleucine, 36.5% have asparagine, 33.8% have a threonine, 13.2% have a valine, 0.5% have an alanine, and 0.5% have a tyrosine. The vast majority of codons in the C2 are not under positive selection. An example is with codon position 95 in which 98.6% of the genomes have a histidine and just 1.4% have a threonine. Another example is a position 60 in which 100% of genomes have a cysteine.

Bayesian evolutionary analysis

Bayesian trees were constructed for both the with- and without-recombinants data sets. Individual genomes were color-coded by the region they belonged to (Figure 4.5 and 4.6, and Appendix D and E). Genomes from the Africa-Europe-Middle East region are the most basal on

the tree, indicating they are more ancestral than other genomes. Introduction events are also apparent as a few genomes from one region will be included within a clade mostly comprised of another region. For example, six genomes from New Caledonia are clustered within a clade comprised of Africa-Europe-Middle East genomes indicating the genomes in New Caledonia originate from the Africa-Europe-Middle East region. It is also apparent that genomes from Australia are likely from an introduction from China as Australian genomes emerge from clades largely comprised of Chinese samples. Mexico seems to have had two introduction events; one from the Middle East and one from China as Mexican isolates are embedded within clades of either Middle East or Chinese origins.

The time of the most recent common ancestor (MRCA) was calculated for each of the geographic regions for both with- and without-recombinants data sets (Figure 4.7 and 4.8). The Africa-Europe-Middle East region has the oldest MRCA for both data sets, which is especially pronounced in the without-recombinants data set. In the without-recombinants data set, the Americas, Southeast Asia, and Oceania have similar values for the MRCA which are all well below the MRCA value for the Africa-Europe-Middle East region. In the with-recombinants data set, Africa-Europe-Middle East region has the oldest MRCA, followed by both the Americas and Southeast Asia which have similar MRCA values. Oceania has the newest MRCA. The mean substitution rate for the without-recombinants data set is 3.906×10^{-3} nucleotides/year. The mean substitution rate for the with-recombinants data set is 1.448×10^{-2} nucleotides/year.

Discussion

The Middle East is thought to be the origin of TYLCV (Lefeuvre et al. 2010) and our analyses seem to corroborate this. Israel is where TYLCV was first observed infecting tomato crops in the 1930's (Cohen and Antignus 1994). Mabvakure et al. (2016) state from their

analyses that either the Eastern Mediterranean or the Middle East is the likely origin of the genomes they analyzed. Genomes from the Middle East are most basal in both their phylogenetic tree and our phylogenetic tree. The Africa-Europe-Middle East region has the oldest MRCA estimate of the four regions we analyzed for both the with- and without-recombinants data sets which is indicative of the virus species originating there as geographical centers of origin often have higher levels of genetic diversity than other regions. The Africa-Europe-Middle East region has the highest average number of nucleotide differences (k) across its set of genomes indicating the highest diversity of all the geographic regions analyzed. The Africa-Europe-Middle East region also has the highest proportion of recombinant genomes, which suggests a greater amount of diversity to create recombinants and a greater amount of time for recombination events to have occurred. When the Africa-Europe-Middle East region is further broken down into more specific geographic regions, we see that the Middle East has the highest proportion of recombinant genomes. Hosseinzadeh et al. (2014) reported that the Middle Eastern country of Iran is a highly active area for recombination of TYLCV-like viruses, which supports our findings. In fact, we found 44 out of the 55 genomes from Iran to be recombinants. The Africa-Europe-Middle East region also exhibits the highest number of codons under positive selection, indicating that certain polymorphisms are being permitted to emerge and co-exist in this population which increases the diversity of the population.

While the Africa-Europe-Middle East region appears to have the greatest diversity of TYLCV genomes, Oceania has the least diversity of TYLCV genomes based on the average number of nucleotide differences (k). This is likely because Oceania is one of the last regions to be invaded by TYLCV and introductions into Oceania have come from a limited geographic area. For example, Australia did not have reports of TYLCV until 2006 (Van Brunschot et al.

2010) and based on our phylogenetic tree, has only had introductions from Southeast Asia. New Caledonia has only had an introduction from the Middle East. The Americas, in contrast, has had introductions from multiple regions of the world starting a decade earlier in the 1990's (Polston et al. 1999, and Bird et al. 2001).

The Southeast Asia region exhibits the most extreme values for the population neutrality statistics. Negative values for Fu and Li's F and D statistics and Tajima's D statistic indicate two possibilities: population expansion or purifying selection. Evidence suggests that population expansion is likely the most significant of these two as TYLCV was only first reported in China in 2006 and by 2009, had already spread to 11 provinces (Pan et al. 2012). By 2014, TYLCV was reported in 13 provinces or autonomous regions in China (Yang et al. 2014). Our data set from GenBank downloaded at the end of 2016 contained genomes from 13 provinces (Anhui, Fujian, Guangdong, Hebei, Henan, Hubei, Jiangsu, Jilin, Liaoning, Shandong, Shanxi, Yunnan, and Zhejiang), 3 municipalities (Beijing, Shanghai, and Tianjin), and 1 autonomous region (Xinjiang Uyghur) in China. The rapid spread of the MED sibling species (formally biotype Q) of *Bemisia tabaci* throughout China during this time may have aided in the rapid spread of TYLCV (Zhang et al. 2014, and Pan et al. 2012). The MED sibling species has been reported to be a more effective vector of TYLCV than the MEAM1 sibling species which it is outcompeting (Zhang et al. 2014, and Pan et al. 2012). The swift sweep of the virus throughout Southeast Asia gives a signature in the population's sequences that is detectable by these neutrality tests.

Our study also demonstrates the large influence of recombination on the TYLCV population. The total number of TYLCV genomes in our data set affected by recombination was 172 out of 666, or 25.83%. The differences between the population genetics results from our with- and without-recombinants data sets were noticeable. For example, the Africa-Europe-

Middle East region in the with-recombinants data had a value for the average number of nucleotide differences (k) that was 73 nucleotides higher than the Africa-Europe-Middle East region in the without-recombinants data set. This demonstrates that recombinants added an additional 73 nucleotides of difference between the genomes, adding to the genetic diversity of the population. Inclusion of recombinants also contributed to the detection of codons under positive selection in the Africa-Europe-Middle East region. The Africa-Europe-Middle East region exhibited no codons under positive selection in the without-recombinants data set, but exhibited fourteen codons under positive selection in the with-recombinants data set. Again, inclusion of recombinants is adding genetic diversity to the population. Part of the reason that recombination adds so much diversity to the TYLCV population is because TYLCV often undergoes recombination with other begomoviruses. For example, Lefeuvre et al. (2010) determined that out of 18 recombination events they detected from their data set from the Middle East and Western Mediterranean, 16 events involved two virus species. They reported TYLCV to recombine with *Tomato leaf curl Iran virus*, *Tomato leaf curl Sardinia virus*, *Tomato leaf curl Karnataka virus*, and *Cotton leaf curl Gezira virus*. Other researchers have reported TYLCV to recombine with *Tomato yellow leaf curl Sardinia virus* (Belabess et al. 2016), *Tomato leaf curl virus* (Navas-Castillo et al. 2000), *Tomato leaf curl Comoros virus* (Urbino et al. 2013), *Tomato leaf curl Sudan virus* (Idris and Brown 2005), *Tomato leaf curl Iran virus* (Bananej et al. 2004), *Tobacco leaf curl virus* (Park et al. 2011), and *Tomato yellow leaf curl Thailand virus* (Kim et al. 2011). Recombinants can exhibit selective advantages over their parental genomes such as an increased host range or a modification in host symptom severity (Stenger et al. 1994, Martin et al. 2001, Zhou et al. 1997). For example, a recombinant resulting from TYLCV and *Tomato*

yellow leaf curl Sardinia virus exhibited an increased host range and a reduction in host plant symptom severity (Monci et al. 2002).

TYLCV's rapid mutation rate of 2.88 × 10-4 substitutions/site/year (Duffy and Holmes 2008) also contributes to the diversity and evolution of TYLCV. All the regions analyzed had high percentages of haplotype diversity (Table 4.3 and 4.4), even for the without-recombinants data set. Few genomes share 100% identity with one another, even when sampled from the same region at the same time. The rapid mutation rate allows for the emergence of new amino acid sequences that may be more adaptive than previously-prevailing isolates in the ever-changing environmental conditions this virus is exposed to.

Our phylogenetic tree demonstrates the extent to which TYLCV has been introduced to new regions all over the world. For example, the tree shows that isolates in the Americas are not from a single introduction, but likely from about six different introductions. Duffy and Holmes reported back in 2007 that two introduction events, one from Asia and one from the Middle East, had occurred into North America. Lefeuvre et al. (2010) also reported two separate introduction events from the same regions into North America. Our results corroborate the findings of both of these studies as we see one group of Mexican isolates which clade with Chinese isolates and another large group of Caribbean, Mexican, and United States isolates that emerge from a Middle Eastern clade. We also see two Dominican Republic isolates and a Venezuelan isolate emerging from a clade of Middle Eastern and Mediterranean isolates. The larger size of our data set and the addition of more recent TYLCV genomes reveal three additional introductions into mainland North America. Our tree shows another event from Australia into California and a fourth event from China into Costa Rica. A single genome from Florida (USAFlorida12-15) appears to have possibly come from Southeast Asia. Overall, we show five introduction events

into mainland North America and two introduction events into the Caribbean (the mainland and the Caribbean share one introduction of the same origin).

Mabvakure et al. (2016) reported three introductions into mainland North America, and three introductions into the Caribbean. They list introduction events into the mainland North America from Australia, East Asia, and the Caribbean, with the Caribbean isolates being of Mediterranean origin. They also state that the Caribbean experienced introductions from the Eastern Mediterranean, Western Mediterranean, and East Asia. We see two introductions into the Caribbean islands in our present study – one from China and two from the Middle East. The researchers grouped geographic regions differently than we did, so some countries we placed into the Middle East, they had placed into Eastern Mediterranean. Regardless, all the published studies and our own data report that American isolates originate from both the Middle East (Eastern Mediterranean), and Asia.

Asia also seems to have had multiple introduction events from the Middle East as large clusters of Asian isolates are embedded in clades with a basal Middle Eastern isolate. Looking at our tree, there are four large clusters of genomes from Asia, three which originate from the Middle East and one which originates from Australia. Wan et al. 2015 noted that Chinese isolates do not clade together, and instead are members of three different clades which is possible evidence of three separate introductions. Our tree, instead, shows two large clades comprised of Chinese isolates. South Korea appears to have had three introductions based on our phylogenetic tree. Back in 2010, Lee et al. determined that TYLCV isolates in Korea form two separate clades and that each clade is derived from isolates in Japan. Based on our tree, this is feasible as we see two clusters of South Korean isolates clustered with Japan, but we see an additional group of Korean isolates that seem to have come from China.

Oceania has also had multiple introductions from Asia. Isolates from Australia form three clusters, all within Asian clades. Mabvakure et al. (2016) also describes Australia as having three introduction events from East Asia. Our tree shows that isolates in New Caledonia, however, appear to originate from Europe as they clade most closely with Spanish isolates. Mabvakure et al. (2016) also notes that New Caledonian isolates originate from the Western Mediterranean.

The geographic distribution of TYLCV has changed dramatically over that last half century as international trade has inadvertently spread invasive whiteflies and infected plant material around the world. Introduction events, recombination, selection pressure, and baseline mutations are shaping the genetics of the TYLCV populations. This study provides a snap shot of what the TYLCV populations are like now, but they are likely to change in the future.

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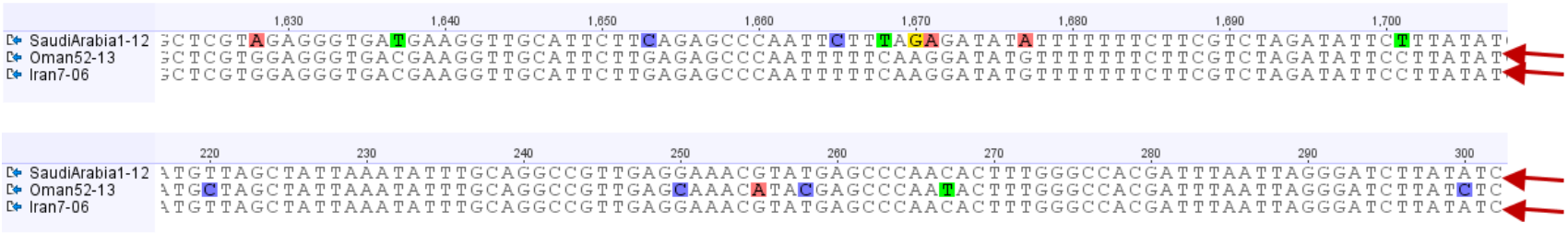

Figure 4.1. Nucleotide alignments of recombinant Iran7-06 and parental genomes Oman52-13 and SaudiArabia1-12. The top alignment shows a portion of Iran7-06's genome that shares a high identity with Oman52-13. The bottom alignment shows a portion of Iran7-06's genome that shares a high identity with SaudiArabia1-12.

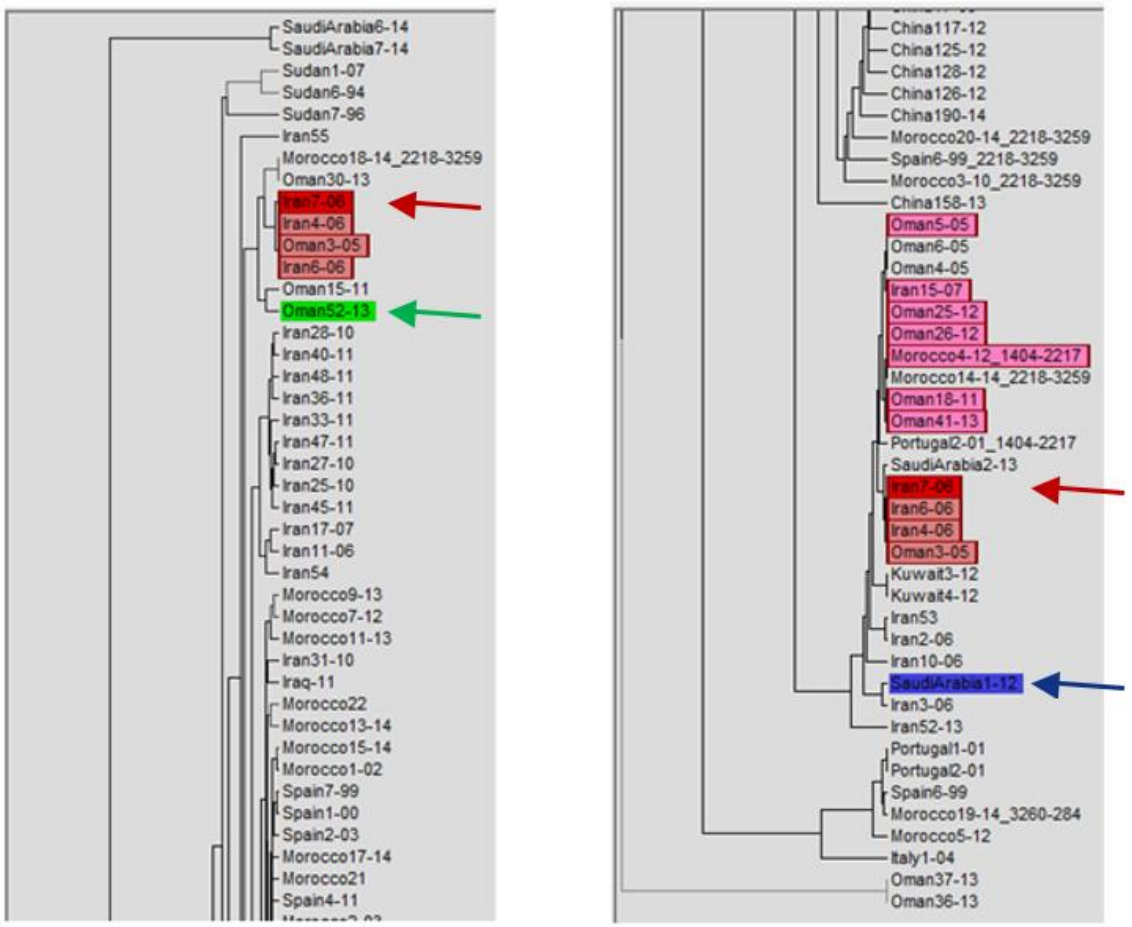

Figure 4.2. Trees created by RDP4 showing how one portion of Iran7-06's genome phylogenetically parses with Oman52-13 and another portion of Iran7-10's genome phylogenetically parses with SaudiArabia1-12.

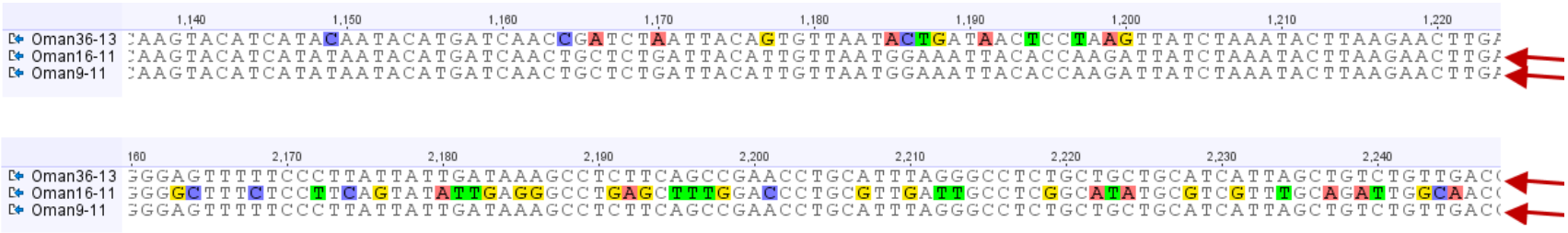

Figure 4.3. Nucleotide alignments of recombinant Oman9-11 and parental genomes Oman16-11 and Oman36-13. The top alignment shows a portion of Oman9-11's genome that shares a high identity with Oman16-11. The bottom alignment shows a portion of Oman9-11's genome that shares a high identity with Oman36-13.

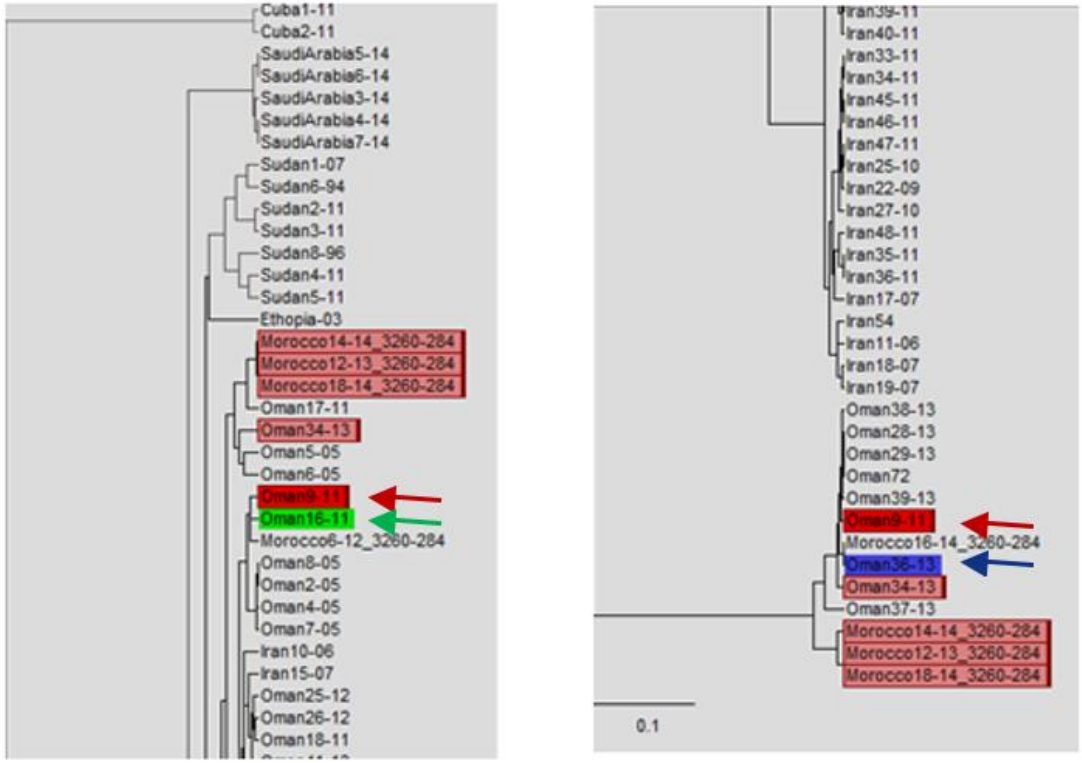

Figure 4.4. Trees created by RDP4 showing how one portion of Oman9-11's genome phylogenetically parses with Oman16-11 and another portion of Oman9-11's genome phylogenetically parses with Oman36-13.

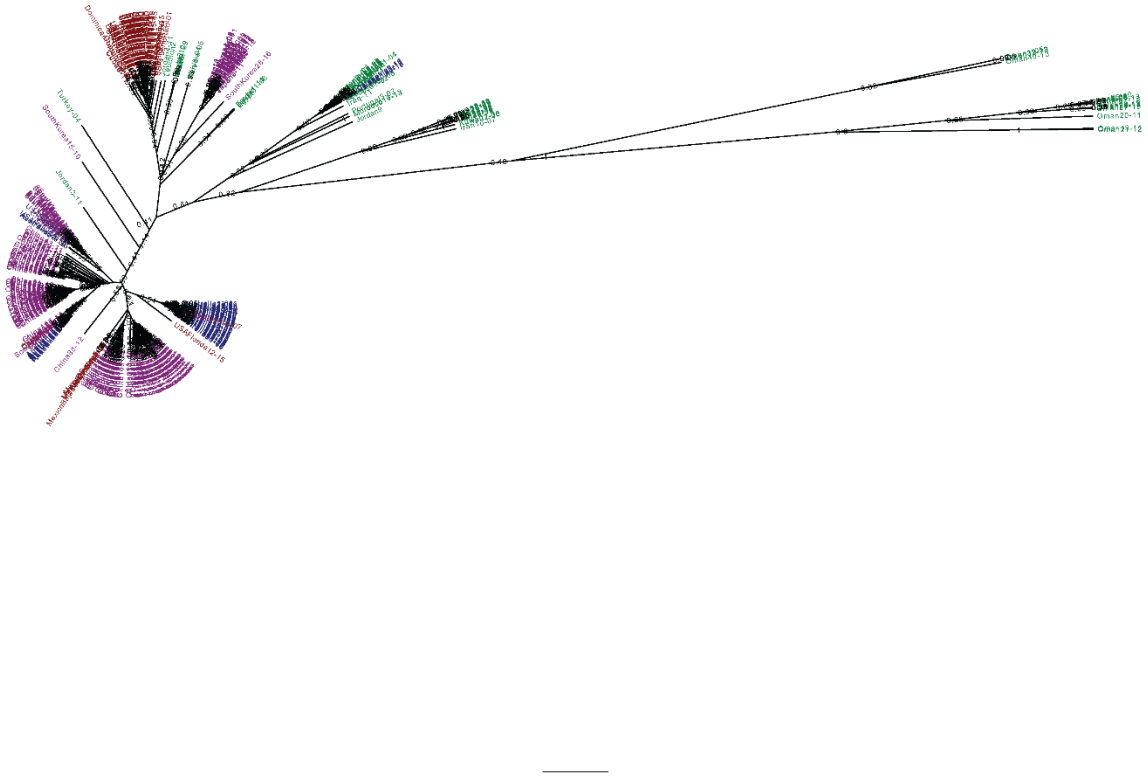

Figure 4.5. Bayesian tree constructed with the without-recombinants data set. Genomes are color-coded by region: green is Africa-Europe-Middle East, blue is Oceania, pink is Southeast Asia, and red is Americas.

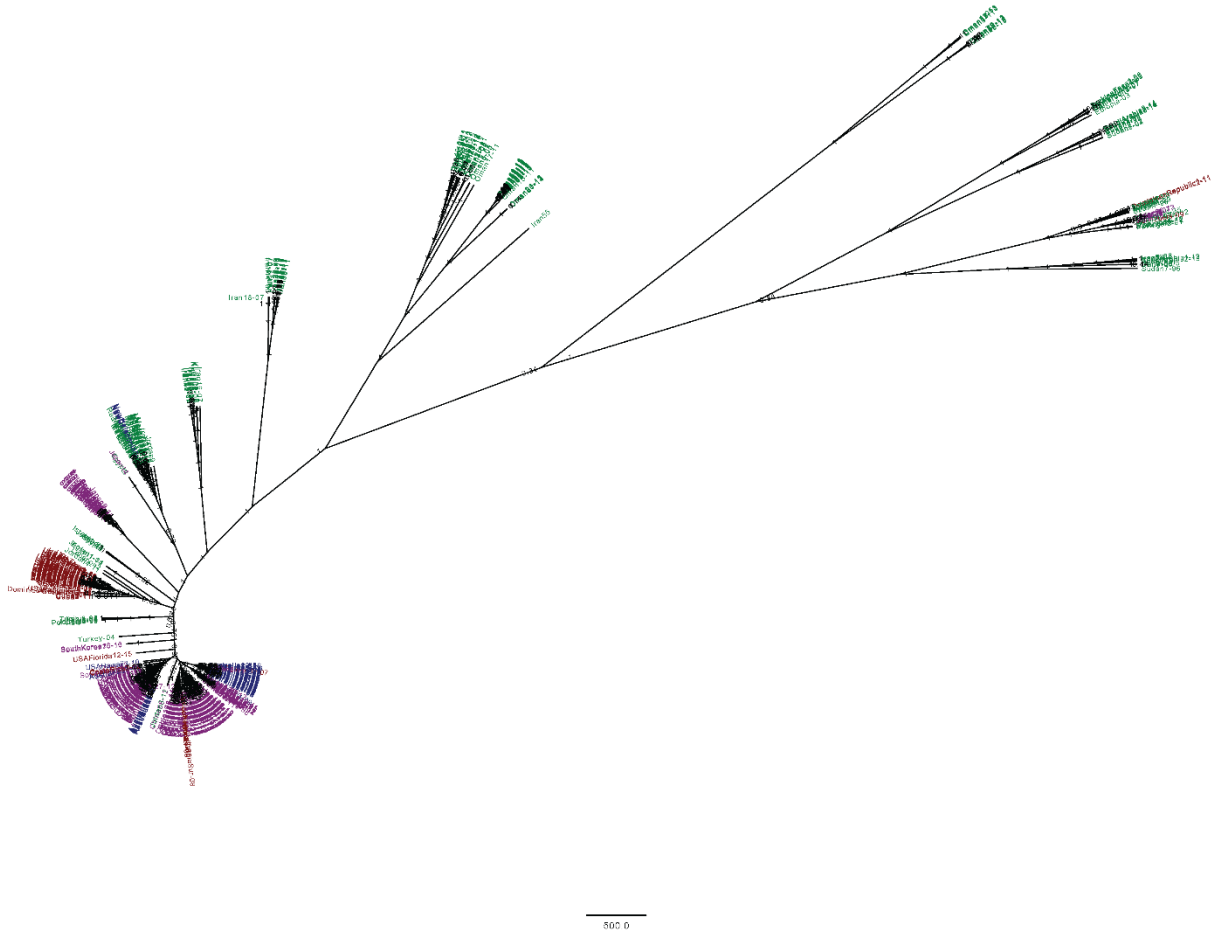

Figure 4.6. Bayesian tree constructed with the with-recombinants data set. Genomes are color-coded by region: green is Africa-Europe-Middle East, blue is Oceania, pink is Southeast Asia, and red is Americas.

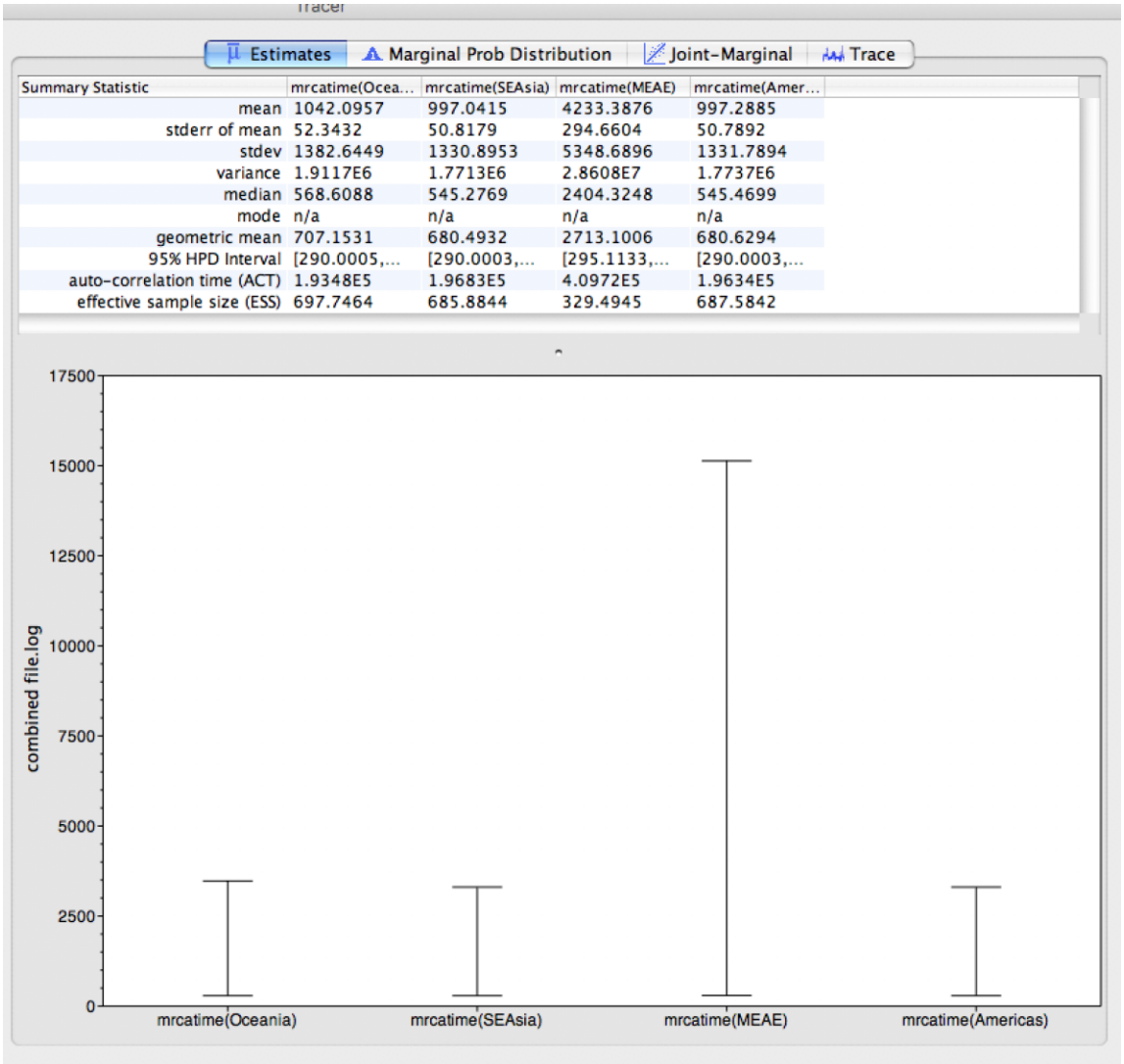

Figure 4.7. Age of the most recent common ancestor (MRCA) is calculated for each of the four geographic regions for the without-recombinants data set.

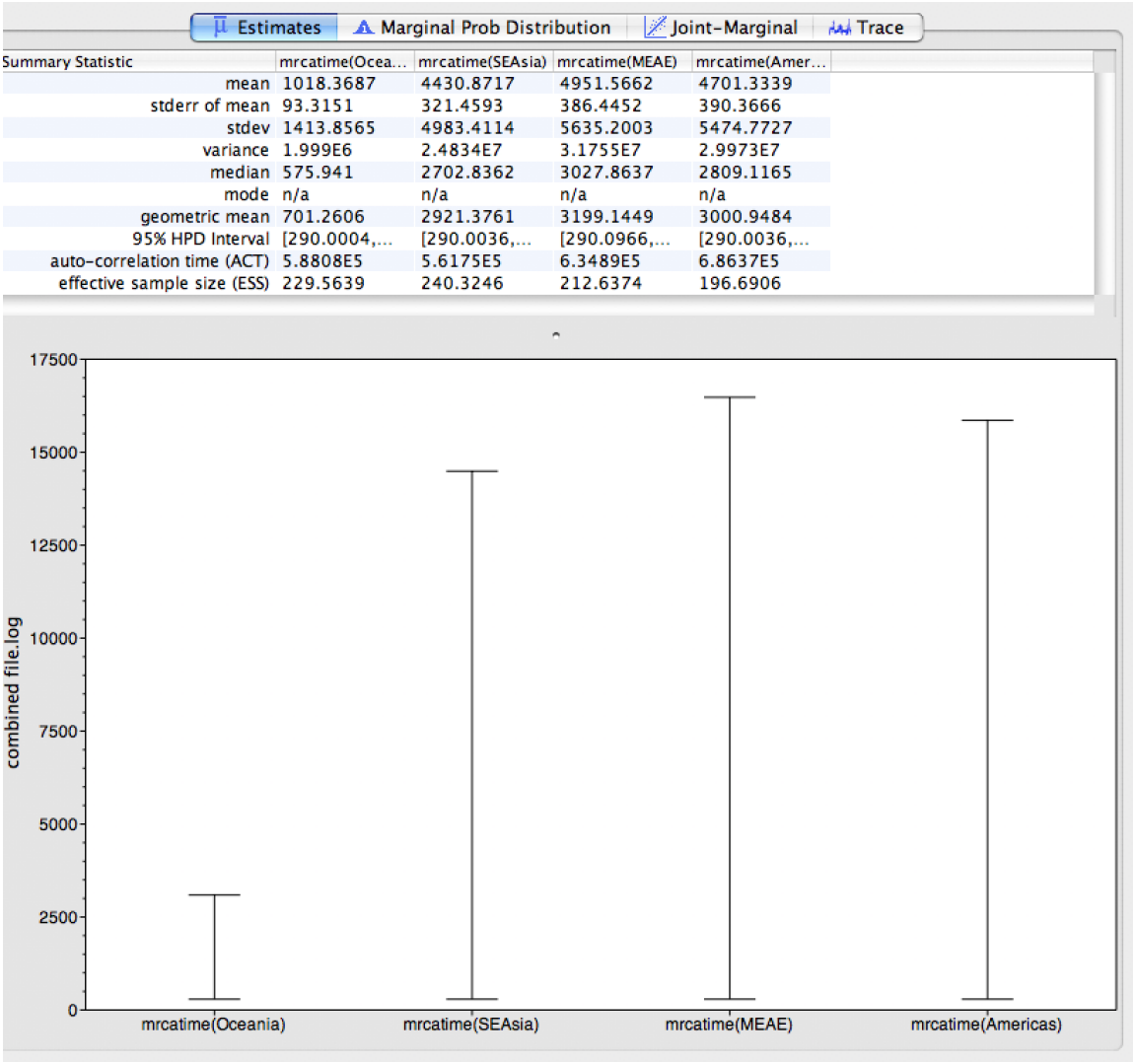

Figure 4.8. Age of the most recent common ancestor (MRCA) is calculated for each of the four geographic regions for the with-recombinants data set.

Table 4.1. Proportion of TYLCV genomes that are recombinants in each geographic region.

	Africa-Europe-Middle East	Americas	Oceania	Southeast Asia
Number of Recombinant Genomes/Total Number of Genomes (as a percentage)	157/226 (69.5%)	5/75 (6.7%)	1/69 (1.4%)	10/296 (3.4%)

Table 4.2. Proportion of TYLCV genomes that are recombinants in each geographic region.

	Africa	Europe	Middle East
Number of Recombinant Genomes/Total Number of Genomes (as a percentage)	36/54 (66.7%)	9/18 (50.0%)	112/154 (72.7%)

Table 4.3. Polymorphism analysis statistics calculated for the without-recombinants data set.

	Africa-Europe-Middle East	Americas	Oceania	Southeast Asia
Number of sequences	69	70	68	286
Number of Polymorphic Sites	976	287	257	1498
Number of Haplotypes	64	59	66	278
Haplotype Diversity + standard deviation	0.997 + 0.003	0.989 + 0.007	0.999 + 0.003	0.9998 + 0.0003
Average Number of Nucleotide Differences (k)	151.066	30.578	22.907	42.936

Table 4.4. Polymorphism analysis statistics calculated for the with-recombinants data set.

	Africa-Europe-Middle East	Americas	Oceania	Southeast Asia
Number of sequences	226	75	69	296
Number of Polymorphic Sites	1335	732	302	1551
Number of Haplotypes	194	64	67	288
Haplotype Diversity + standard deviation	0.9945 + 0.0023	0.991+ 0.006	0.999 + 0.003	0.9998+ 0.0003
Average Number of Nucleotide Differences (k)	224.548	63.992	25.173	49.977

Table 4.5. Nucleotide-based genetic differentiation statistics calculated for the without-recombinants data set.
Ks, Kst, and Z are nucleotide-based genetic differentiation developed by Hudson et. al (1992a).
Snn is a nucleotide-based genetic differentiation statistic developed by Hudson (2000). Snn values approaching 1 indicate differentiation.
Fst values range from 0 to 1. Low Fst values indicate a high level of mixing between populations while high Fst values indicate genetically distinct groups. Developed by Hudson et. al (1992b).
p-values are determined by a permutation test with 1000 replications.

Population 1	Population 2	Ks	Kst	p-value of Ks and Kst	Snn	p-value of Snn	Z	p-value of Z	Fst
Oceania	SE Asia	38.67217	0.02390	0.0000	0.98493	0.0000	30245.62672	0.0000	0.08717
Oceania	Americas	25.65493	0.34418	0.0000	0.98551	0.0000	3043.45580	0.0000	0.51100
Oceania	Africa-Europe-Middle East	85.42058	0.12447	0.0000	0.98175	0.0000	3913.73345	0.0000	0.22111
SE Asia	Americas	40.50617	0.16976	0.0000	0.99579	0.0000	25177.96297	0.0000	0.41561
SE Asia	Africa-Europe-Middle East	61.16734	0.10790	0.0000	0.98873	0.0000	28091.14506	0.0000	0.19840
Americas	Africa-Europe-Middle East	89.43963	0.13550	0.0000	0.97122	0.0000	3947.02966	0.0000	0.23648

Table 4.6. Nucleotide-based genetic differentiation statistics calculated for the with-recombinants data set.

Population 1	Population 2	Ks	Kst	p-value of Ks and Kst	Snn	p-value of Snn	Z	p-value of Z	Fst
Oceania	SE Asia	44.70786	0.01972	0.0000	0.98539	0.0000	32229.32874	0.0000	0.07493
Oceania	Americas	43.83417	0.22660	0.0000	0.98611	0.0000	3573.59541	0.0000	0.37288
Oceania	Africa-Europe-Middle East	176.71355	0.09221	0.0000	0.99322	0.0000	19695.69092	0.0000	0.28960
SE Asia	Americas	51.88812	0.13199	0.0000	0.99259	0.0000	28367.53478	0.0000	0.30355
SE Asia	Africa-Europe-Middle East	119.98679	0.15403	0.0000	0.98691	0.0000	57243.73834	0.0000	0.25213
Americas	Africa-Europe-Middle East	180.26972	0.09107	0.0000	0.98000	0.0000	20289.57946	0.0000	0.25643

Table 4.7. Population neutrality statistics calculated for the without-recombinants data set.
Fu and Li's D and F Statistics. Negative values indicate population expansion or purifying selection. Bold values are statistically significant.
Tajima's D Statistic. Negative values indicate population expansion or purifying selection. Bold values are statistically significant.

Geographic Region	Fu and Li's D Statistic	Fu and Li's F Statistic	Tajima's D Statistic
Africa-Europe-Middle East	-3.11590 $p < 0.05$	-2.64949 $p < 0.05$	-0.90490 $p > 0.10$
Americas	-2.85203 $p < 0.05$	-2.85861 $p < 0.05$	-1.69989 $0.10 > p > 0.05$
Oceania	-4.35191 $p < 0.02$	-4.08170 $p < 0.02$	-2.00530 $p < 0.05$
Southeast Asia	-12.89224 $p < 0.02$	-8.70732 $p < 0.02$	-2.58601 $p < 0.001$

Table 4.8. Population neutrality statistics calculated for the with-recombinants data set.
Bold values are statistically significant ($p<0.05$).

Geographic Region	Fu and Li's D Statistic	Fu and Li's F Statistic	Tajima's D Statistic
Africa-Europe-Middle East	-3.23198 P < 0.05	-1.84413 P > 0.10	0.02702 P > 0.10
Americas	0.02936 P > 0.10	-0.98318 P > 0.10	-2.00149 P < 0.05
Oceania	-5.05737 P < 0.02	-4.62386 P < 0.02	-2.09887 P < 0.05
Southeast Asia	-12.34404 P < 0.02	-8.31622 P < 0.02	-2.50741 P < 0.001

Table 4.9. Codons under positive selection by gene and region for the with-recombinants data set.

Geographic Region	Gene					
	V1	V2	C1	C2	C3	C4
Africa-Europe-Middle East	33	-	36 79 331	83 98 105 109	12 81 91	14 21 30
Americas	-	-	-	-	-	-
Oceania	-	-	-	-	-	-
Southeast Asia	-	-	-	-	-	14

CHAPTER 5

CO-INFECTION OF MULTIPLE *TOMATO YELLOW LEAF CURL VIRUS* ISOLATES IN INDIVIDUAL HOST PLANTS[1]

[1]Marchant, W.G., and Srinivasan, R. To be submitted to *Virology*.

Abstract

Begomoviruses are whitefly-transmitted DNA viruses that affect many agricultural crops. There are many reports of individual host plants harboring two or more begomoviruses. These mixed infections allow recombination events to occur within and among *Begomovirus* species. Most studies have examined co-infections of different virus species, while a few have examined co-infections of different strains within in the same species. The frequency of mixed infections of *Tomato yellow leaf curl virus* (TYLCV) in tomatoes in the field was assessed. Infected tomato samples from the field showed a high percentage of mixed TYLCV infections at 45%. There were many isolates that varied by just a few nucleotides, making TYLCV very characteristic of a quasispecies. We found up to two different TYLCV isolates in individual plants. We further examined co-infection with two isolates of TYLCV, called "isolate #2" and "isolate #4", which share 99.5% nucleotide identity and differ by just several amino acids in the greenhouse. Individual performance, competition, and whitefly acquisition of the two isolates were assessed. Results indicated that recipient plants were inoculated via whiteflies at similar frequencies by isolate #2 and isolate #4, however isolate #4 accumulated to higher levels in the plants. Whiteflies acquired isolate #2 and isolate #4 at similar percentages. Whiteflies acquired both isolates at higher amounts from individually-infected plants than from mixed infected plants.

Introduction

Geminiviridae is a family of insect-transmitted viruses that infect many important agricultural crops. *Begomovirus* is the largest of nine genera in the family *Geminiviridae* (Varsani et al. 2017) and infects dicotyledonous plants. Begomoviruses are all transmitted by the whitefly species complex *Bemisia tabaci* and affect many important crops such as cotton, tomato, cassava, beans, and squash (Varma and Malathi 2003, Thottappilly 1992, Legg 1999,

Briddon 2003, Picó et al. 1996). These viruses are either monopartite or bipartite with circular ssDNA components approximately 2.6 kb long. The DNA components are termed DNA-A and DNA-B for bipartite genomes or just DNA-A for monopartite genomes. The DNA components are encapsidated into icosahedral, geminate particles (Bottcher et al. 2004).

A *Begomovirus* of great agricultural importance is *Tomato yellow leaf curl virus* (TYLCV). This virus causes serious disease in tomato plants with symptoms such as curling of leaves, stunted growth, chlorosis, and reduced fruit yield. The whitefly species complex, *Bemisia tabaci* (Genn.), transmits the virus in a persistent and circulative manner. TYLCV is monopartite with a genome size of approximately 2.8 kb (Czosnek 2008). The genome contains six genes; two on the viral strand designated V1 and V2 and four on the complementary strand designated C1, C2, C3, and C4. TYLCV is just one of many begomoviruses that infects tomatoes causing symptoms as described above. In fact, multiple begomoviruses can be found in co-infections within individual plants. For example, mixed infections of TYLCV and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) have been found in individual tomato plants and black nightshade plants, *Solanum nigrum* (García-Andrés et al. 2006, García-Andrés et al. 2009). In an inoculation experiment, researchers were able to co-infect plants with TYLCV and TYLCSV and even found recombinants of the two viruses (García-Andrés et al. 2009). Another research group found that in tomato plants co-infected with TYLCV and TYLCSV, one-fifth of infected nuclei contained both viruses (Morilla et al. 2004).

Other species of begomoviruses have also been found in mixed infections in individual plants (Harrison et al. 1997, Fondong et al. 2000). *Malvastrum leaf curl Guangdong virus* and *Ageratum yellow vein virus* were found to co-infect *Malvastrum coromandelianum* plants in China (Yang et al. 2008). Mixed *Begomovirus* infections containing up to three *Begomoviruses*

were also detected in tomato plants from Nicaragua. Individual plants were found to contain *Tomato severe leaf curl virus*–Nicaragua along with *Tomato leaf curl Sinaloa virus* and/or *Pepper golden mosaic virus*. A cushaw plant from the same study harbored both *Squash yellow mottle virus* and *Pepper golden mosaic virus* (Ala-Poikela et al. 2015). *Tomato yellow leaf curl virus*–Oman and *Chili leaf curl virus*–Oman were both detected in radishes from crops in Oman (Al-Shihi et al. 2017).

Viruses co-infecting the same host can affect one another. Experiments conducted on *Nicotiana benthamiana* with the two begomoviruses *Tomato yellow leaf curl virus*–Oman and *Chili leaf curl virus*–Oman showed synergistic effects of co-infection on host symptoms and increased virus accumulation of both viruses compared to singly-infected plants (Al-Shihi et al. 2017). In contrast, another experiment inoculating tomato plants with *Tomato yellow spot virus* (ToYSV) and *Tomato rugose mosaic virus* (ToRMV) showed lower virus accumulations of both viruses in dual-inoculated plants than in singly-infected tomato plants, indicating that these viruses interfere with one another (Alve-Júnior et al. 2009). Another study examined competition between TYLCV-IL, *Tomato yellow leaf curl Sardinia virus*-ES, and their recombinant, TYLCV-IS76 in tomato plants with the *Ty*-1 TYLCV-resistance gene (Belabess et al. 2016). The recombinant TYLCV-IS76 accumulated to much higher levels in tomato plants than both parental viruses in single-, dual-, and triple-infected plants. Co-infection with recombinant TYLCV-IS76 also had a deleterious effect on the accumulation of parental virus TYLCV-IL.

In addition to plants, whiteflies can also harbor multiple viruses simultaneously. For example, whiteflies can acquire and transmit two different strains of TYLCV (Ohnishi et al. 2011). These two strains are the Israeli and Mild strain of TYLCV, which share a 91.5% nucleotide identity. After given an acquisition access period on tomato plants infected with the

Israeli strain and then tomatoes with the Mild strain, or vice versa, most whiteflies acquired both viruses. Plants that became infected after an inoculation access period by these whiteflies most often became co-infected with both strains. There were instances, however, in which plants become infected with just one of the two virus strains. Successive inoculation access periods on different tomato plants using the same whitefly showed that some tomato plants became co-infected, while others became infected with just one of the two virus strains. The researchers also stated that the two strains did not appear to compete or interfere with one another's circulation in the whitefly.

Further evidence for co-infection in single plants is the existence of recombinant viruses. Co-infection is a pre-requisite for recombination as this provides a physical setting for a recombination event to occur. TYLCV recombines within its own species, and has also been documented to recombine with *Tomato leaf curl Iran virus, Tomato leaf curl Sardinia virus, Tomato leaf curl Karnataka virus, Cotton leaf curl Gezira virus* (Lefeuvre et al. 2010), *Tomato yellow leaf curl Sardinia virus* (Belabess et al. 2016), *Tomato leaf curl virus* (Navas-Castillo et al. 2000), *Tomato leaf curl Comoros virus* (Urbino et al. 2013), *Tomato leaf curl Sudan virus* (Idris and Brown 2005), *Tomato leaf curl Iran virus* (Bananej et al. 2004), *Tobacco leaf curl virus* (Park et al. 2011), and *Tomato yellow leaf curl Thailand virus* (Kim et al. 2011). In order for these recombinants to be created, TYLCV and the other viruses must have to occupy the same cell within a single organism, indicating a co-infection.

Studies have examined mixed-infections with congeneric virus species, such as those in genus *Begomovirus* (Yang et al. 2008, Ala-Poikela et al. 2015) and even viruses within the same species such as TYLCV-IL and TYLCV-Mld which share a 91.5% nucleotide identity (Ohnishi et al. 2011) and TYLCV-IL and its recombinant TYLCV-IS76 which share a 97.8% nucleotide

identity (Belabess et al. 2016). Mixed infections of begomoviruses with a higher level of similarity have not been examined. We would like to examine mixed infections of TYLCV that share 99.5% nucleotide identity and differ by just several amino acids. Individual performance in singly-inoculated plants and competition in dual-inoculated plants will be examined in greenhouse experiments. We would like to see if there is a difference in the performance of the isolates and to see if there are competitive forces in dually-inoculated plants. We would also like to conduct a survey of TYLCV field isolates to examine the frequency of mixed infections in tomato cropping systems.

Materials and methods

Frequency of mixed infections in field tomatoes

Tissue from symptomatic tomato plants was collected during the fall of 2016 from three tomato fields in Tifton, Georgia. Ten samples of leaf tissue were taken from each of four different tomato cultivars which were Brandywine (Johnny's Selected Seeds), FL47 (Seminis), Lanai (lab cultivar), and Red Bounty (Harris Seeds Company). DNA was extracted from the tissue samples and subjected to PCR with to verify TYLCV infection status. Primers were C2-1201 (5'- CATGATCCACTGCTCTGATTACA -3') and C2-1800V2 (5'-TCATTGATGACGTAGACCCG-3'), which target a 695 nucleotide region of the TYLCV genome that encompasses the entire C2 gene. The PCR reactions were run in 10 µl reactions with 5 µl of GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 2 µl of water, 0.5 µl of each primer at 10 µM concentration, and 2 µl of DNA extract. The PCR program had an initial denaturation step at 94° C for 2 minutes followed by 30 cycles of 94° for 30 sec, 52° for 30 sec, 72° for 1 min, and a final extension at 72° for 5 min. Samples testing positive were amplified with rolling circle amplification using TempliPhi (GE Healthcare, Chicago, IL) with the protocol

outlined by Inoue-Nagata et al. (2004). Amplified DNA was digested with SacI (Fisher BioReagents, Pittsburgh, Pennsylvania). The digested DNA was then gel-extracted using crystal violet (Fisher Chemical, Fair Lawn, NJ) as the DNA-visualizing agent to avoid use of UV light which would degrade the DNA. The DNA was ligated into vector pGEM-3Z (Promega Corporation, Madison, WI) and a transformation was performed into One Shot TOP10 Chemically Competant *E. coli* (Invitrogen, Carlsbad, CA). Colonies were tested with colony PCR using primers T7F (5'-TAATACGACTCACTATAGGG-3') and M13R (5'-CAGGAAACAGCTATGACC-3') to determine if plasmids had the appropriately sized inserts for a TYLCV genome. Five colonies from each plant sample with the appropriate length insert were cultured and the plasmids purified. Plasmids were sent for Sanger sequencing (Eurofins Genomics, Louisville, KY) using the following primers: 5370F (5'–TTCGCTATTACGCCAGCT–3'), 2941R (5'–CCCAGGCTTTACACTTTATGCTTCC–3'), 710F (5'–TCTTATATCTGTTGTAAGGGCCCGT–3'), and 1400F (5'–ACGAGAACCATACTGAAAACGCCTT–3'). Full-length TYLCV genomes were assembled in Geneious Pro v. 8.1.9 (Drummond et al. 2011). All six TYLCV genes from the five genomes were translated and aligned to examine for differences. Recombination events among the genomes were tested for in Recombination Detection Program v.4.80 (RDP4) (Martin et al. 2015). RDP4 uses seven different detection tests (RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiSscan, and 3Seq) to screen for recombination. A threshold of three positive tests and a phylogenetic confirmation were the criteria used for a positive detection of recombination.

TYLCV isolate acquisition and maintenance

Whiteflies collected in the field in 2015 in Tifton, Georgia were placed on non-infected tomato plants and allowed to inoculate the plants. Samples from resulting infected plants were

taken and clones of full-length genome of the TYLCV were sequenced from individual plants. Isolates designated #2 (GenBank accession MF687351) and #4 (accession MF687350) were selected for this experiment. The isolates share 99.5% nucleotide identity and differ by 1 amino acid in the V1 gene, 1 amino acid in the C2 gene, and most notably, by a 7 amino acid truncation of the C1 gene in the #2 isolate while the #4 isolate has a full-length C1 gene. Plants with these isolates were maintained in cages in a greenhouse in separate insect-proof cages.

TYLCV inoculation of different isolates into tomato plants

Whiteflies were given an acquisition access period of 48-hours on either an infected tomato plant with the #2 or #4 TYLCV isolate. Whiteflies were moved to non-infected tomato plants for an inoculation access period of 48-hours. Three treatments with six plants each were used. The three treatments were plants individually-inoculated with isolate #2, plants individually-inoculated with isolate #4, and plants dual-inoculated with isolate #2 and #4. Two clip-cages with twenty whiteflies each from either the #2 or #4 were clipped to non-infected tomato plants to infect the individually-inoculated plants. One clip-cage with twenty whiteflies from #2 isolate and one clip-cage with whiteflies from #4 were placed non-infected tomato plants to infect the dual-inoculated plants. Infection was allowed to develop for three weeks. The experiment was conducted twice. The inoculation data was analyzed using GLIMMIX PROCEDURE in SAS with a binomial distribution.

Three weeks after inoculation, leaf tissue from tomato plants was collected and DNA was extracted. Samples were tested with PCR for both isolates of TYLCV. Specialized primers for SNP detection to distinguish the TYLCV isolates were designed using recommendations by Liu et al. (2012). Isolate #2 was detected with primers #2F G mismatch (5'–GCCTTATTGGTTTCTTCGTG–3') and 2260R (5'–CCGCATTATTTAAAGCACTTCAAAG–

3') with the following PCR program: 95° denaturation step for 2 min followed by 30 cycles of 95° for 1 min, 55° for 30 sec and 72° for 30 sec, with a final extension of 72° for 2 min. Isolate #4 was detected with primers #4F G mismatch (5'-GCCTTATTGTTTTCTTCGTA-3') and 2260R with the following PCR program: 95° denaturation step for 2 min followed by 30 cycles of 95° for 1 min, 52° for 30 sec and 72° for 30 sec, with a final extension of 72° for 2 min.

TYLCV accumulation of different isolates in individual tomato plants

Plant samples testing positive with either of the above primer sets were subjected to TYLCV quantification using Custom TaqMan® SNP Genotyping Assay (Life Technologies, Carlsbad, CA). This assay uses a primer set to amplify the region of interest and FAM and VIC probes to detect the SNP's on the amplicons by binding preferentially to one TYLCV isolate or the other. The primer set used to amplify the region of interest was 5'-GTCTACACGCTTACGCCTTATTG-3' and 5'-ACTGTTCGCAAGTATCAATCAAGGT-3' and amplified a 74 bp region of the TYLCV genome containing the SNPs of interest. The reporter sequences were 5'-CACAAGATAGCCAAGAAG-3' linked with VIC reporter dye which detects TYLCV isolate #2 and 5'-ACACAAGATAGCTAAGAAG-3' linked with FAM reporter dye which detects TYLCV isolate #4. The PCR reactions were run in 25 µl reactions with 12.5 µl of TaqMan® Genotyping Master Mix (Applied Biosystems, Foster City, California), 1.25 µl of 20X Custom TaqMan® SNP Genotyping Assay working stock, and 11.25 µl of DNA extract. The real-time PCR program started with a 95° denaturation step for 10 min followed by 40 cycles of 95° for 15 s and 60° for 1 min. To normalize TYLCV DNA quantification in the plant samples, the samples were also run with tomato 25S rRNA primers Tomato 25S rRNA F (5′-ATAACCGCATCAGGTCTCCA-3′) and Tomato 25S rRNA R (5′-CCGAAGTTACGGATCCATTT-3′) from Mason et al. (2008) with a PCR program with an

initial denaturation of 95° for 2 min followed by 40 cycles of 95° for 15 sec and 53° for 1 min followed by a melting curve. The real-time PCR reactions were run in 25 µl reactions with 12.5 µl of GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI), 6.5 µl of water, 0.5 µl of each primer at 10 µM concentration, and 5 µl of DNA extract. C_T values from both the SNP assay and Tomato 25S rRNA were used in the equation developed by Pfaffl (2001) for relative quantification of TYLCV DNA to tomato 25S rRNA DNA. A low level of cross reaction with the FAM (#4) probe occurred and a uniform level was subtracted from all samples to ensure values indicate only the TYLCV isolate of interest. Values were analyzed for statistical significance using ANOVA with randomized block design with two experimental replications as the blocks in R version 3.4.0. A Tukey's Honestly Significant Differences was performed to determine significant pairwise-comparisons.

To verify the TYLCV isolate quantification with the TaqMan assay, cloning was performed. First, a 215 bp region of TYLCV DNA encompassing the isolates SNPs was amplified with PCR using 1600F (5'–AGTTCCCCTGTGCGTGAATCC–3') and 1814R (5'–AGACGAAGAAAAAAACATATC–3'). The PCR program was 95° for 2 min followed by 26 cycles of 95° for 30 s, 45° for 30 s, 72° for 30 s, followed by a final extension of 72° for 2 min. The resulting amplicons were blunted and ligated into pJET1.2 vector with CloneJET PCR Cloning Kit (Thermo Scientific) and One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) were transformed with the resulting plasmids. Five colonies from each sample were sequenced using Sanger sequencing (Eurofins Genomics, Louisville, KY) using primer pJET1.2R (5'-AAGAACATCGATTTTCCATGGCAG-3').

Whitefly acquisition of different isolates

Twenty newly-emerged whiteflies were clip-caged to a tomato plant infected with either isolate #2, isolate #4, or mixed-infected for a 48-hour acquisition access period. Whiteflies were then removed. The experiment was conducted twice. Whiteflies were surface-sterilized using the protocol from Lacey and Brooks (1997) with a series of washes in the following order: 70% ethanol, water, 1% bleach, water, water. Six whiteflies from each plant underwent a DNA extraction using Instagene Matrix (BioRad, Hercules, CA). PCR was performed on the DNA extracts with primers #2F G mismatch and 2260R with its appropriate PCR program and with #4F G mismatch and 2260R with its appropriate PCR program to determine the presence or absence of TYLCV infection #2 or #4 in each of the samples. The acquisition frequencies were analyzed using the GLIMMIX PROCEDURE in SAS with a binomial distribution. Samples that were positive for either of the isolates were subjected to the TaqMan assay to quantify the amount of each of the TYLCV isolates. Values were normalized with the whitefly β-actin gene, which was amplified with the primers whitefly β-actin F (5'–TCTTCCAGCCATCCTTCTTG–3') and whitefly β-actin R (5'–CGGTGATTTCCTTCTGCATT–3') (Sinisterra et al. 2005). The real-time PCR program had an initial 95° denaturation step for 2 m, followed by 40 cycles of 95° for 15 sec and 60° for 1 m, followed by a melting curve. Values from the Taqman assay and the whitefly β-actin assay were used in the equation developed by Pfaffl (2001) for relative quantification of TYLCV DNA to whitefly β-actin DNA. Values were analyzed for statistical significance using ANOVA with randomized block design with two experimental replications as the blocks in R version 3.4.0. A Tukey's Honestly Significant Differences was performed to determine significant pairwise-comparisons.

Results

Frequency of mixed infections in field tomatoes

Of the twenty samples cloned and sequenced, mixed infections were detected in nine of the samples (45%). Here, we consider different isolates to be those that vary by at least one amino acid. Genomes with a silent nucleotide mutation without amino acid differences were not considered different isolates. The genomes were archived in GenBank (accessions MF669088-MF669119). Most amino acid polymorphisms in mixed infections occurred in the C1 and C3 genes (Table 5.1). No recombination events were detected by RDP4 in our data set of 32 genomes.

TYLCV inoculation of different isolates into tomato plants

The first experimental replication of the plant inoculation test yielded all plants developing infection with the isolates they were inoculated with (Table 5.2). In the second experimental replication, all the plants inoculated with isolate #2 became infected, only two plants inoculated with isolate #4 became infected, two mixed-inoculated plants became infected with both isolates, and two mixed-inoculated plants became infected with just isolate #2 (Figure 5.1 and 5.2). Neither isolate #2 or isolate #4 inoculated plants at a frequency that was statistically different from the other ($F(1,3)=5.09$, $p=0.1093$).

TYLCV accumulation of different isolates in individual tomato plants

Plant samples that were positive from the inoculation test were subjected to the TaqMan assay to quantify the amount of viral DNA each TYLCV isolate accumulated (Figure 5.3). Treatment groups analyzed with ANOVA were isolate #2 and isolate #4 from individually-inoculated plants, and isolate #2 and isolate #4 from mixed-inoculated plants. Statistically significant results were found ($F(3,33)= 4.210$, $p=0.0126$). Isolate #4 in individually-inoculated

plants accumulated to higher levels than isolate #2 in both individually-inoculated plants and mixed-inoculated plants (Tukey HSD: p=0.0215, and p= 0.0144, respectively). The effect of experimental replications (blocking) did have statistically significant effect (F(1,33)=5.359, p=0.0270) on the results, indicating that the two experimental replications had different results in the performance of each TYLCV isolate. An ANOVA was performed on total TYLCV accumulation for treatment groups isolate #2, isolate#4, and mixed (by adding isolate #2 and isolate #4 accumulation together)(Figure 5.4). These results were statistically significant (F(2,26)=4.186, p=0.265) and suggest that isolate #4 in individually-inoculated plants accumulated to higher levels than isolate #2 in individually-inoculated plants (Tukey HSD: p=0.0210). However, the mixed-infected plants did not differ in accumulation compared to individually-infected plants (Tukey HSD: mixed vs isolate #2 p=0.6229, and mixed vs isolate #4 p=0.1526).

The colonies sequenced from cloning from the individually-inoculated plants were consistently the single isolate the plants were inoculated with (Table 5.3). The colonies from mixed-infected plants Mixed A and Mixed C showed a mixture of isolate #2 and isolate #4. Colonies from mixed-infected plants Mixed B, Mixed D, Mixed E, and Mixed F showed exclusively one isolate or another, and is consistently the isolate with the higher concentration in each plant as seen with the Taqman assay.

Whitefly acquisition of different isolates

Whiteflies did not acquire isolate #2 or isolate #4 more frequently than the another (F(1,3)=1.14, p= 0.3633) (Table 5.4). Whitefly samples that were positive for either TYLCV isolate were subjected to the TaqMan assay to quantify the amount of viral DNA each whitefly acquired (Figure 5.5). Statistically significant results were obtained (F(3,31)=25.1, p=1.96e-08).

Total TYLCV accumulations analyzed with ANOVA and Tukey HSD determined that whiteflies acquired more TYLCV virus, regardless of isolate, from individually-inoculated plants than mixed-inoculated plants ($F(2,23)=14.19$, $p=9.67e-05$) (Figure 5.7).

Cloning results showed all colonies from whiteflies on individually-inoculated plants to have completely all of the same isolate from the plant they acquired from (Table 5.5). Two whitefly samples (Mixed G and Mixed K) from the mixed-infected group showed a mixture of isolates while three whitefly samples (Mixed H, Mixed I, and Mixed L) showed only colonies with the #2 isolate. This corroborates the fact that the #2 isolate was higher than the #4 isolate in whiteflies Mixed I and Mixed L and isolate #2 was acquired alone in Mixed H.

Discussion

Mixed infections of TYLCV isolates are very common in the field as we found 45% of tomato plant samples to contain multiple isolates of TYLCV. No more than two isolates were ever detected in a single plant in our data set, however, this does not rule out the possibility that three or more isolates could be found in individual plants if more sampling occurred. Many others have reported the frequent nature of begomoviruses co-infecting plants in agricultural systems (García-Andrés 2006, García-Andrés et al. 2009, Morilla et al. 2004, Harrison et al. 1997, Fondong et al. 2000, Al-Shihi et al. 2017, Ala-Poikela et al. 2015, Yang et al. 2008). Perhaps this is because *B. tabaci* is a vector of many different viruses and can acquire and transmit multiple viruses simultaneously (Ohnishi et al. 2011, Alabi et al. 2017). This potentially explains why there are so many cases of mixed infections with whitefly-transmitted viruses.

Mixed infections allow for recombination events to occur. When researchers inoculated plants with both TYLCV and TYLCSV, recombinants of these two species were found (García-Andrés et al. 2009). In another study, TYLCV-Mld and *Tomato leaf curl Comoros virus* were

dual-inoculated into tomato plants and infection was allowed to develop and establish for four months. Twenty-nine percent of recovered genomes sampled from these plants were recombinants of TYLCV-Mld and *Tomato leaf curl Comoros virus* (Martin et al. 2011). This demonstrates the common occurrence of recombination in begomoviruses. We did not detect any recombinants in our data set, although this could be due to the high similarity of our genomes such that a recombination event would not be detected because the recombinant genome would not differ much from either of the parental genomes. Nonetheless, recombination is an important driver of *Begomovirus* evolution and mixed infections are opportunities for these events to occur (García-Andrés et al. 2009, Morilla et al. 2004).

Mutation is another mechanism that can create multiple TYLCV isolates for plants to become co-infected with. TYLCV possesses a rapid mutation rate of 2.88 × 10-4 substitutions/site/year (Duffy and Holmes 2008) which generates many different co-existing isolates (Figure 5.7). The presence of many different TYLCV isolates and the frequent nature of mixed infections permits TYLCV to be described as a quasispecies. Other researchers have also commented on the quasispecies quality of TYLCV and other *Begomoviruses* (Seal et al. 2006, Roossinck 1997). A quasispecies refers to a virus population that is a distribution of mutants, rather than a population of completely homogenous genomes (Domingo et al. 2012). The concept of quasispecies is usually applied to RNA viruses, however, TYLCV seems to have characteristics of a quasispecies such as a rapid mutation rate and existence of many different isolates, even in a small geographic area during a short period of time. Presence of many isolates can allow a virus species to rapidly adapt to new environmental conditions as many mutants are available that may outperform previously-prevailing isolates.

For our greenhouse experiment, our results show that isolate #2 and isolate #4 inoculated plants at statistically similar frequencies. However, isolate #4 accumlated to higher levels in plants than isolate #2. When comparing individually-infected to mixed-infected plants, total TYLCV accumulation did not differ statistically between individually-inoculated plants and mixed-inoculated plants. This indicates there were no synergistic or interfering effects observed in dual-inoculated tomato plants. These results differ from Al-Shihi et al.'s (2017) study that showed a synergizing effect on virus accumulation in plants with a mixed infection of *Tomato yellow leaf curl virus*–Oman and *Chili leaf curl virus*. Conversely, Alve-Júnior et al.'s (2009) study showed an interfering effect in which a mixed infection of *Tomato yellow spot virus* and *Tomato rugose mosaic virus* caused a decrease in virus accumulation for both viruses in mixed-infected plants. Our experiments with mixed infections of TYLCV isolates #2 and #4 did not demonstrate a synergizing or interfering effect in virus accumulations. However, the other researchers used viruses of species while we used different isolates of the same species.

There seems to be a stochastic nature to the inoculation and establishment of the different TYLCV isolates in plants as isolate #2 accumulated to higher levels in three of the mixed-infected plants while isolate #4 accumulated to higher levels in five of the mixed-infected. We also had two dual-inoculated plants that become infected with isolate #2 alone. Ohnishi et al. (2011) also seemed to find similar results as plants inoculated with whiteflies harboring both TYLCV-IL and TYLCV-Mld did not always become infected with both strains. Plants became infected by zero, just one, or both of the TYLCV strains without predictability.

Our results show that whiteflies did not preferentially acquire one isolate over another in the acquisition test. Whiteflies were able to acquire the separate virus isolates from individually-inoculated tomato plants at statistically similar levels. Whiteflies also acquired both isolates from

the mixed-infected plants at similar levels. We did, however, have two whiteflies that acquired only isolate #2 from the mixed-infected plants. The polymorphisms between isolate #2 and #4 did not seem to affect the ability of the whiteflies to acquire each of the viruses.

Mixed infections with TYLCV and relatives can easily be established in plants for greenhouse experiments. We have demonstrated here the co-inoculation and establishment of isolates #2 and #4 into individual tomato plants. García-Andrés et al. (2009) co-inoculated plants with both TYLCV and TYLCSCV and Ohnishi et al. (2011) co-infected individual plants with both the Mild and Israeli strains of TYLCV. There are at least three different ways whiteflies can inoculate an individual plant with multiple virus isolates. Whiteflies can acquire a mixture of TYLCV isolates from a single mixed-infected plant as this study shows and go on to infect a new plant by feeding. Secondly, a whitefly can acquire a mixture of TYLCV isolates from a succession of feeding on separate single-infected plants (Ohnishi et al. 2011). Another method in which tomato plants could become co-infected is by allowing separate whiteflies that have each acquired a different isolate from separate plants to feed on the same plant at around the same time as we did in this study.

Overall, mixed TYLCV infections are very common in field samples and are potentially drivers of *Begomovirus* evolution as they provide opportunities for recombination. The rapid mutation rate of TYLCV generates many new isolates that can co-infect individual plants. The heterogeneous nature of TYLCV seems to warrant its characterization as a quasispecies.The two TYLCV isolates were inoculated by whiteflies into tomato plants at statistically similar frequencies, however isolate #4 accumulated to higher levels. In mixed-infected plants, the isolates did not outcompete one another or cause synergizing or interfering effects on one

another in co-inoculated plants. Whiteflies did not preferentially acquire one virus isolate over another.

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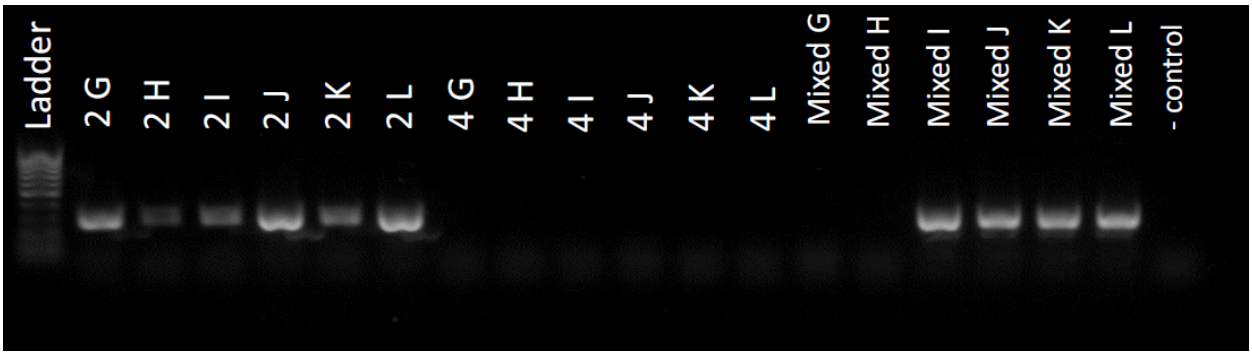

Figure 5.1. Plant samples from experimental replication 2 tested with primer set #2F G mismatch and 2260R which detects the #2 isolate of TYLCV. Lane 1 is ladder, lanes labelled "2G-2L" are individual plant samples inoculated with isolate #2, lanes labelled "4G-4L" are individual plant samples inoculated with isolate #4, and lanes labelled "Mixed G-MixedL" are individual plant samples inoculated with both isolates #2 and #4.

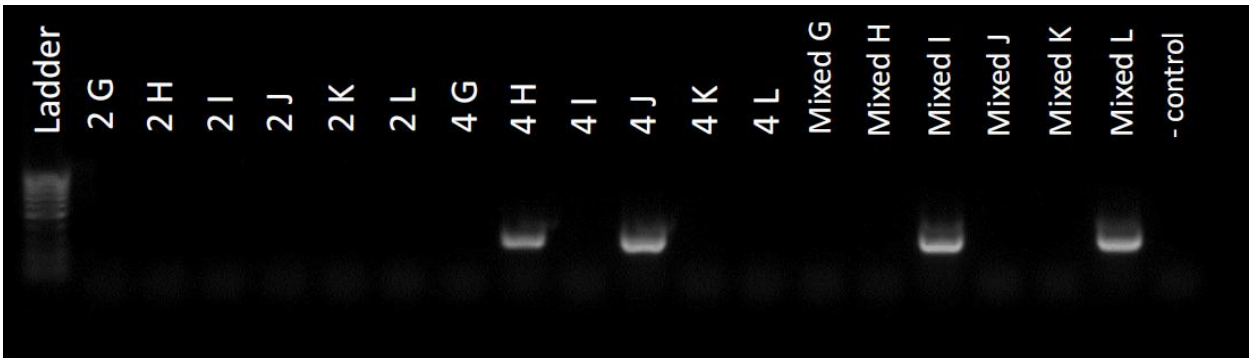

Figure 5.2. Plant samples from experimental replication 2 tested with primer set #4F G mismatch and 2260R which detects the #4 isolate of TYLCV. Lane 1 is ladder, lanes labelled "2G-2L" are individual plant samples inoculated with isolate #2, lanes labelled "4G-4L" are individual plant samples inoculated with isolate #4, and lanes labelled "Mixed G-MixedL" are individual plant samples inoculated with both isolates #2 and #4.

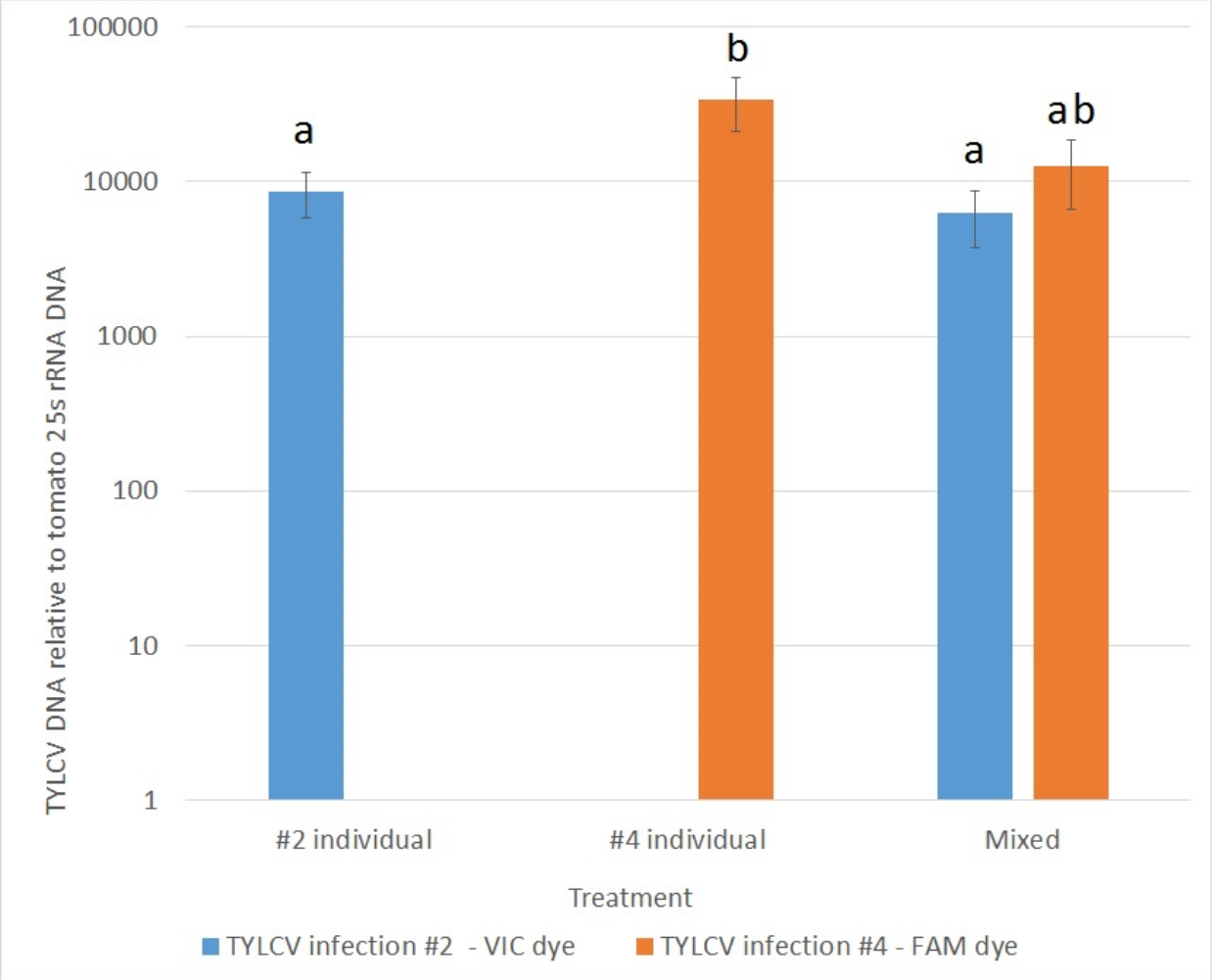

Figure 5.3. TYLCV accumulation of each of the TYLCV isolates in the plant inoculation experiment.

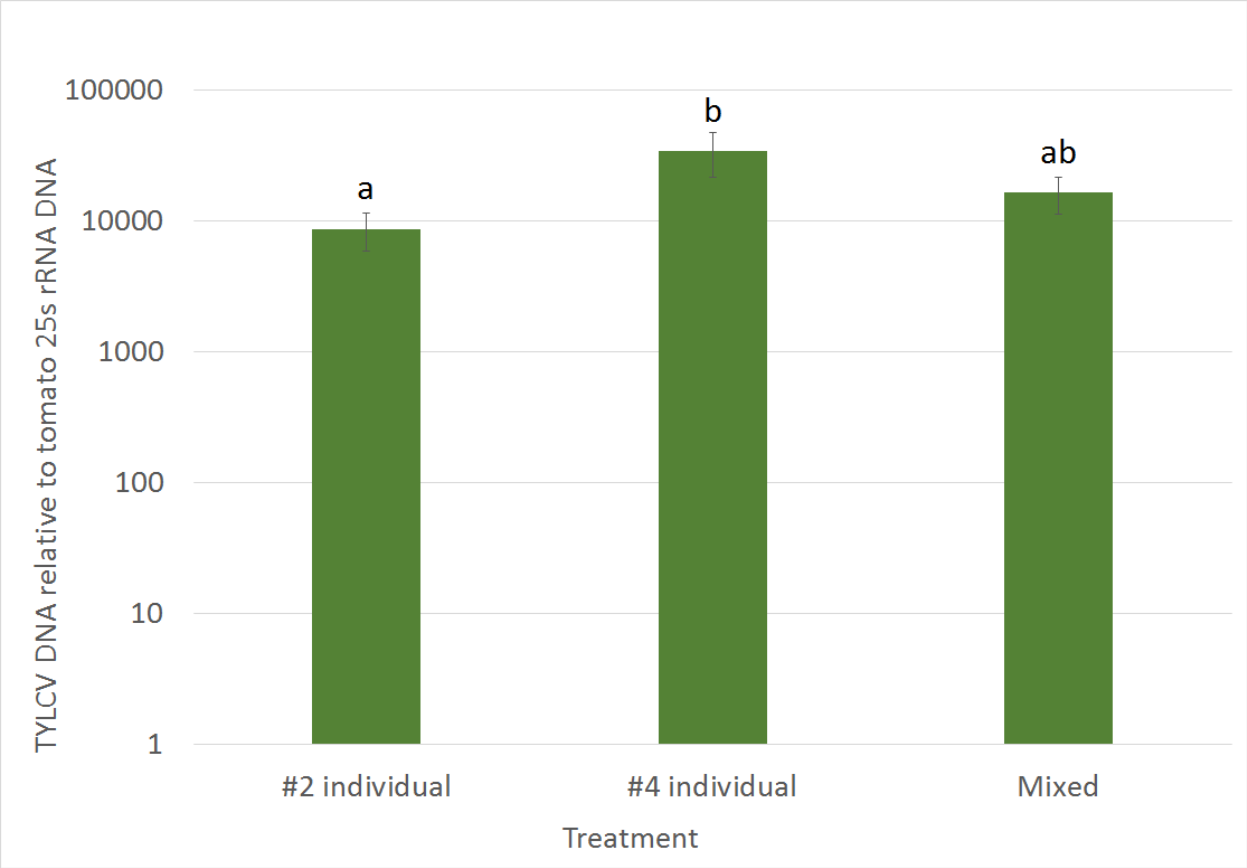

Figure 5.4. Total TYLCV accumulation, regardless of isolate, in the plant inoculation experiment.

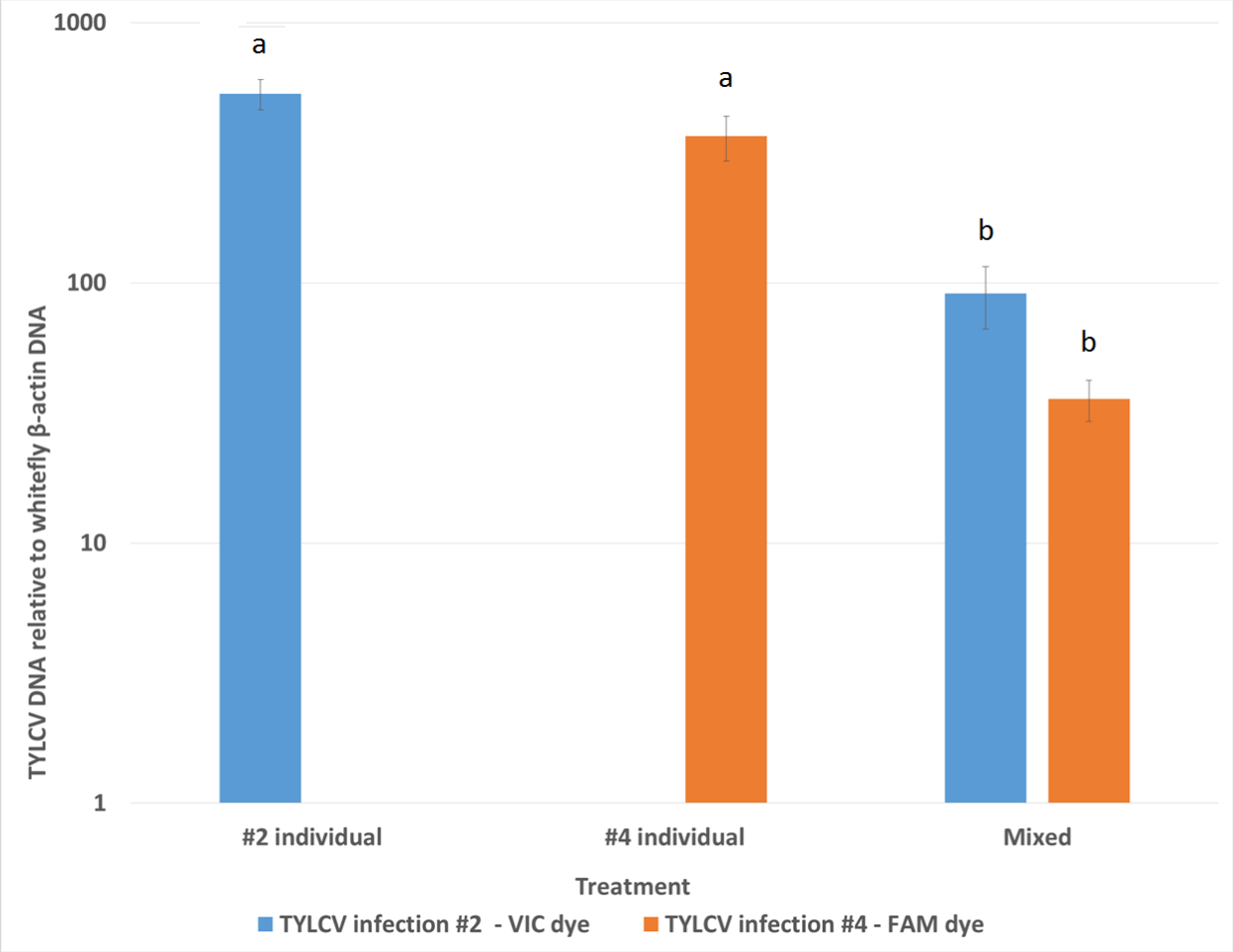

Figure 5.5. Whitefly acquisition of each of the TYLCV isolates.

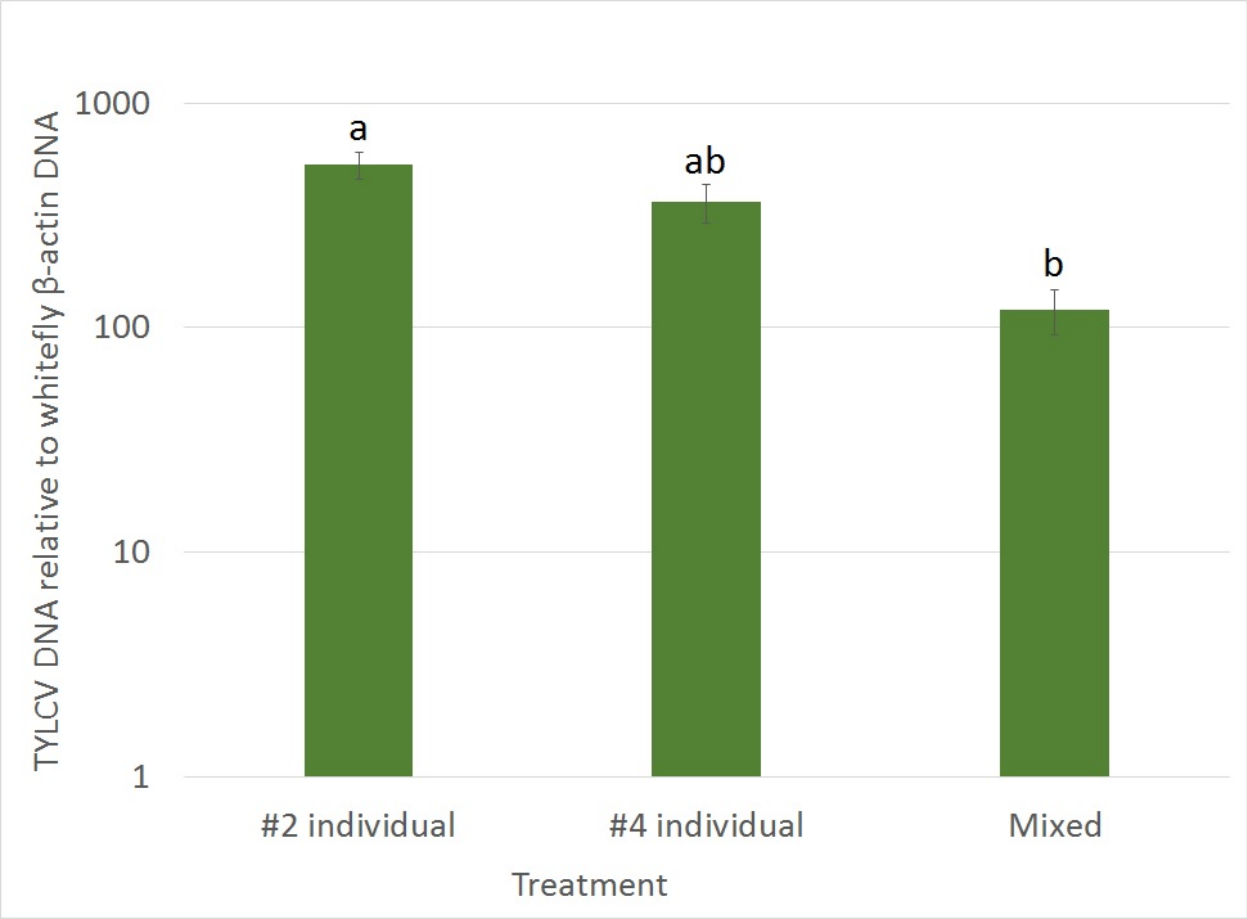

Figure 5.6. Whitefly acquisition of total TYLCV, regardless of isolate.

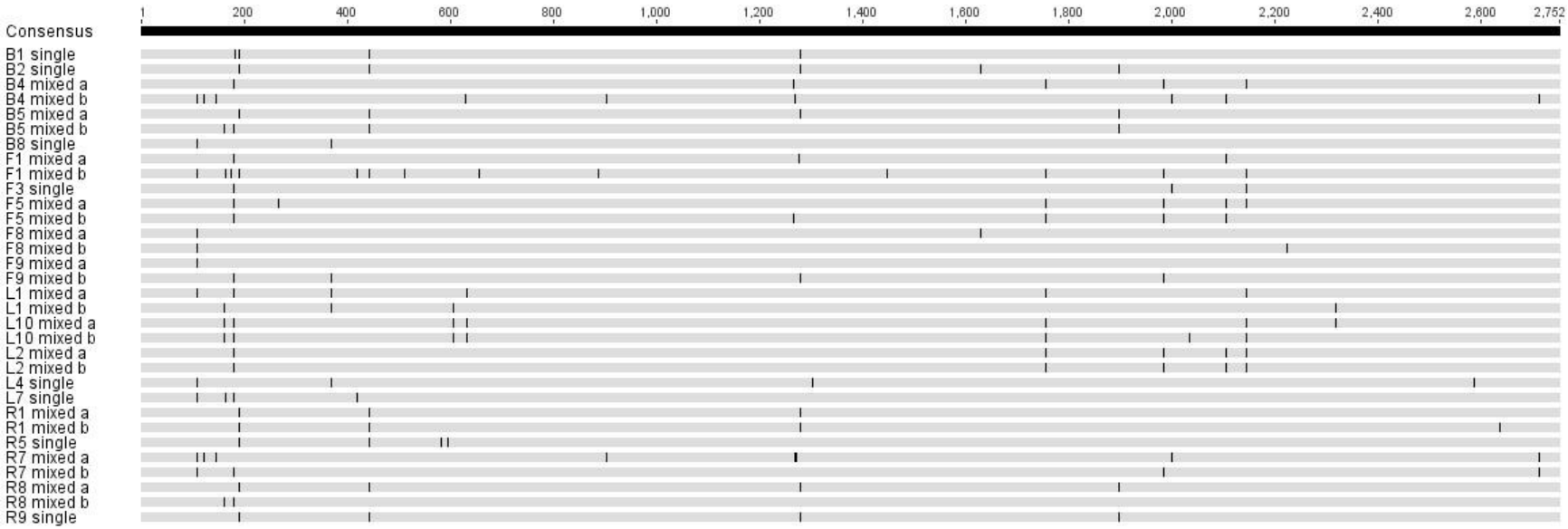

Figure 5.7. Alignment of field-collected samples that shows the high variability of the TYLCV genome. Tick marks indicate nucleotides that depart from the consensus sequences.

Table 5.1. Table of tomato plant samples detailing whether each was a mixed infection and the polymorphisms between the two isolates if a mixed infection.

Sample Name	Mixed TYLCV Infection?	Number of Polymorphic Amino Acid Sites Between TYLCV Isolates					
		V1 protein	V2 protein	C1 protein	C2 protein	C3 protein	C4 protein
Brandywine 1	No						
Brandywine 2	No						
Brandywine 4	Yes		1	4		1	
Brandywine 5	Yes					1	
Brandywine 8	No						
FL47 1	Yes		Full-length protein versus 40-aa shortened protein	3	1	2	
FL47 3	No						
FL47 5	Yes			1		1	
FL47 8	No						
FL47 9	Yes			1		1	
Lanai 1	Yes	2		3			
Lanai 10	Yes			1			
Lanai 2	No						
Lanai 4	No						
Lanai 7	No						
Red Bounty 1	No						
Red Bounty 5	No						
Red Bounty 7	Yes			1		2	
Red Bounty 8	Yes					1	
Red Bounty 9	No						

Table 5.2. Presence or absence of TYLCV isolates #2 and #4 in the plant inoculation experiment based on PCR results.

	Inoculated with #2												Inoculated with #4												Inoculated with #2 and #4											
Sample Name	2 A	2 B	2 C	2 D	2 E	2 F	2 G	2 H	2 I	2 J	2 K	2 L	4 A	4 B	4 C	4 D	4 E	4 F	4 G	4 H	4 I	4 J	4 K	4 L	Mixed A	Mixed B	Mixed C	Mixed D	Mixed E	Mixed F	Mixed G	Mixed H	Mixed I	Mixed J	Mixed K	Mixed L
Isolate #2	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
Isolate #4	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	-	-	+	-	-	+

Table 5.3. Cloning results from plant inoculation experimental replication 1. Values indicate the number of colonies representing each TYLCV isolate.

	Inoculated with #2						Inoculated with #4						Inoculated with #2 and #4					
Sample Name	2 A	2 B	2 C	2 D	2 E	2 F	4 A	4 B	4 C	4 D	4 E	4 F	Mixed A	Mixed B	Mixed C	Mixed D	Mixed E	Mixed F
Number of Isolate #2 Colonies	5	5	5	5	5	5	0	0	0	0	0	0	4	5	2	5	0	5
Number of Isolate #4 Colonies	0	0	0	0	0	0	5	5	5	5	5	5	1	0	3	0	5	0

Table 5.4. Whitefly acquisition of TYLCV isolates. A "+" sign indicates a positive PCR result.

	Acquired from #2 plant												Acquired from #4 plant												Acquired from #2 and #4 dual-infected plant											
Sample Name	2 A	2 B	2 C	2 D	2 E	2 F	2 G	2 H	2 I	2 J	2 K	2 L	4 A	4 B	4 C	4 D	4 E	4 F	4 G	4 H	4 I	4 J	4 K	4 L	Mixed A	Mixed B	Mixed C	Mixed D	Mixed E	Mixed F	Mixed G	Mixed H	Mixed I	Mixed J	Mixed K	Mixed L
Isolate #2	+	-	-	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+	+
Isolate #4	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	-	+	+

Table 5.5. Cloning results from whitefly acquisition experimental replication 2. Values indicate the number of colonies representing each TYLCV isolate.

	Acquired from #2 plant						Acquired from #4 plant						Acquired from #2 and #4 dual-infected plant					
Sample Name	2 G	2 H	2 I	2 J	2 K	2 L	4 G	4 H	4 I	4 J	4 K	4 L	Mixed G	Mixed H	Mixed I	Mixed J	Mixed K	Mixed L
Number of Isolate #2 Colonies	5	5	5	5	5	5	0	0	0	0	0	0	4	5	5	No TYLCV infection	4	5
Number of Isolate #4 Colonies	0	0	0	0	0	0	5	5	5	5	5	5	1	0	0	No TYLCV infection	1	0

CHAPTER 6

EFFECTS OF ACYLSUGAR-PRODUCING TOMATO GENOTYPES ON WHITEFLY *BEMISIA TABACI* AND *TOMATO YELLOW LEAF CURL VIRUS*[1]

[1]Marchant, W.G., Smeda, J.R., Mutschler-Chu, M.A., and Srinivasan, R. To be submitted to *Journal of Economic Entomology*.

Abstract

Bemisia tabaci, the sweet potato whitefly, is a major pest in agricultural crops as it has phytotoxic effects on crops, transmits yield-reducing viruses, and its honeydew promotes sooty mold growth. Several wild tomato species exhibit resistance to herbivores as a result of acylsugars that are exuded by type IV trichomes. These trichomes and acylsugars have been introgressed into the cultivated tomato to help control the whitefly. We tested tomato genotypes with various quantitative trait loci from *S. pennellii* which included Cornell University's benchmark acylsugar-producing genotype, CU071026, and subsequent crosses of CU071026 with *S. pennellii* which were FA2/AS, FA7/AS, FA2/FA7/AS, QTL6/AS. Cultivar FL47 was used as a control. Whitefly settling preference, survival percentage, and developmental time were measured on the genotypes. The inoculation percentage and accumulation of whitefly-transmitted *Tomato yellow leaf curl virus* (TYLCV) were also tabulated for each genotype along with whitefly acquisition of TYLCV. We found that whiteflies preferred to settle on control cultivar FL47 over acylsugar-producing genotypes. Lower survival of whiteflies was observed on the acylsugar-producing genotypes. However, whitefly developmental time was shorter on acylsugar-producing genotypes than on FL47. TYLCV was transmitted to acylsugar-producing genotypes at lower percentages than that of FL47. However, accumulation of TYLCV was similar across tomato lines. In general, the acylsugar-producing genotypes exhibited both antibiosis and antixenosis toward the whiteflies. Limited, but not complete, control of TYLCV was observed. These genotypes could be improved for controlling TYLCV by introgressing TYLCV-resistance genes into them.

Introduction

Bemisia tabaci, the sweet potato whitefly, is an insect pest of enormous economic importance for many agricultural crops. *B. tabaci* is a cryptic species complex comprised of morphologically-identical sibling species that can only be identified using molecular methods (Elfekih et al. 2017, Dinsdale et al. 2010). Two of the sibling species, MEAM1 and MED, are highly invasive and have invaded many areas of the world misplacing native *B. tabaci* sibling species (Muñiz et al. 2011, De Barro and Ahmed 2011). The MEAM1 sibling species (formally called biotype B) is unique from other sibling species in that it has phytotoxic effects on plants which causes silverleaf and white stem in *Cucurbita* species (Costa and Brown 1991). Honeydew produced by whiteflies can cause sooty mold to grow on crop plants which can reduce photosynthetic potential of the leaves and discolor fiber (Invasive Species Compendium 2017, Perkins 1983). Even more damaging is the ability of *B. tabaci* to transmit viral diseases to plants. *B. tabaci* can transmit plant viruses from the families of *Closteroviridae*, *Potyviridae*, *Secoviridae*, *Betaflexiviridae*, and most importantly, *Geminiviridae* (Dombrovsky et al. 2013, Caciagli 2001, Invasive Species Compendium 2017). *B. tabaci* is an extremely important pest in tomatoes as it transmits *Begomovirus Tomato yellow leaf curl virus* (TYLCV) which can cause tremendous yield losses.

TYLCV originated in the Middle East (Lefeuvre et al. 2010) and has rapidly spread around the world within the last half century, including to the United States (Mabvakure et al. 2016). Symptoms of TYLCV are curling of the leaves, chlorosis, stunted growth, and reduced fruit yield. Unprotected fields can have an incidence of 100% (Berlinger et al 1983). The best method for controlling yield losses is the use of TYLCV-resistant tomato cultivars (Lapidot et al. 1997, Dagnoko et al. 2011). TYLCV is able to replicate within these plants, but at much lower

levels than in susceptible cultivars and symptoms are greatly reduced (Srinivasan et al. 2012, Legarrea et al. 2015). Insecticides to control the whitefly vector are another method that has been used to hinder the spread of TYLCV, but they have less longevity than TYLCV-resistant cultivars because whiteflies often develop insecticide resistance (Panini et al. 2017, Ahmad and Khan 2017, Wang et al. 2017). To best control for TYLCV, cultivars should ideally have resistance to both the whitefly vector and the virus. Whitefly-resistant tomato genotypes with acylsugars introgressed from wild tomato species are one of the possible options available for whitefly resistance.

Acylsugars are herbivore-resisting substances that have been identified in several genera of the Solanaceae, including *Solanum*, which includes the cultivated tomato and its wild relatives. Acylsugars are exuded by a specific type of glandular trichome. There are eight different types of trichomes that have been identified in tomato (Luckwill 1943, Channarayappa et al. 1992). Type IV trichomes are the type that exude acylsugars. They have a glandular cell on the tip that ruptures open when mechanically disrupted and exudes the acylsugars (Glas et al. 2012). Released acylsugars can entrap insects as they are sticky and are thought to be potentially toxic. The chemistry of these compounds includes a sugar, such as sucrose or glucose, esterified at the hydroxyl group with fatty acids of varying numbers, lengths, and chemistries.

The cultivated tomato does not produce acylsugars. Genes for acylsugar production have been introgressed from wild relatives such as *Solanum pimpinellifolium, Solanum pennellii, Solanum galapagense* into the cultivated tomato for testing against herbivore pests (Silva et al. 2014, Andrade et al. 2017, Leckie et al. 2013). Resulting genotypes that exhibit acylsugar-producing properties have been tested against a number of different tomato herbivores such as the whitefly *B. tabaci*, two-spotted spider mite *Tetranychus urticae* (Lucini et al. 2015, Rakha et

al. 2016), *Tetranychus evansi* (Resende et al. 2008), tobacco thrips *Frankliniella fusca*, western flower thrips *Franklinialla occidentalis* (Leckie et al. 2016), the tomato leaf miner *Tuta absoluta* (Moriera et al. 2013), the cotton bollworm *Helicoverpa armigera* (Talekar et al. 2006), and the green peach aphid *Myzus persicae* (Silva et al. 2013). In general, the acylsugar-producing genotypes exhibit antibiosis and antixenosis characteristics towards herbivores. For example, in a choice test between leaf discs with or without acylsugars, the two-spotted mite showed a preference toward leaf discs without acylsugars. Mites also exhibited a higher mortality, a decrease in oviposition, a decrease in egg viability, and a longer egg incubation time on leaf tissue with higher levels of acylsugars (Lucini et al. 2015). The tomato pinworm exhibited a non-preference toward ovipositing on plants of acylsugar-producing genotypes which led to fewer larvae and less damage on the plant compared to non-acylsugar-producing genotypes (Dias et al. 2013).

Many studies examining herbivore resistance with acylsugar-producing genotypes have focused on *B. tabaci*. The number of eggs and the number of nymphs are found to be lower on leaves of acylsugar-producing genotypes (Andrade et al. 2017, Resende et al. 2009, Leckie et al. 2012, Dias et al. 2016). Whiteflies also land with less frequency on an acylsugar-producing genotype (ABL 14-8) compared to the cultivated tomato cultivar Moneymaker in no-choice tests, but this effect was only seen at the 10-leaf stage and not the 4-leaf stage as tomato plants do not produce ample acylsugars at early stages of growth (Rodríguez-López et al. 2011). EPG studies demonstrated that whiteflies on the acylsugar-producing genotype had longer times before first probing and a fewer number of probes. However, once probing was initiated, the duration time of ingestion was not different between the acylsugar-producing genotype and traditional cultivar. While whiteflies showed a preference to settle on the abaxial side of the leaf in Moneymaker, the

whiteflies settled without preference on either the abaxial or adaxial side of the leaf in ABL 14-8 (Rodríguez-López et a. 2012). Whiteflies also fed for a longer duration on the adaxial side of the leaf on ABL 14-8 than on the abaxial side. Additionally, whiteflies that fed on the abaxial side of the leaf were unable to reach the phloem sieve elements on ABL 14-8, but could from the adaxial side of the leaf.

One study has examined the effects of acylsugars on the spread of *Tomato yellow leaf curl virus* (Rodríguez-López et al. 2011). The genotype with acylsugars, ABL14-8, experienced a lower percentage of TYLCV infection compared to the cultivar without acylsugars, however, acylsugars did not completely hinder TYLCV inoculation by whiteflies. Secondary spread of the virus was also reduced, but not eliminated, in the acylsugar-producing genotype. We would also like to examine the effects of acylsugars on the TYLCV transmission by whiteflies. Genotype CU071026 is a tomato line resulting from the cross of the cultivated tomato and acylsugar-producing *S. pennellii* accession LA716. CU071026 produces acylsugars and is the benchmark acylsugar line from Cornell University (Smeda et al. 2017). CU071026 has been further crossed with *S. pennellii* to create a number of new genotypes with quantitative trait loci from *S. pennelii* that create variation in the quantity and chemistry of the fatty acids in the acylsugars. These lines are FA2/AS, FA7/AS, FA2/FA7/AS, and QTL6/AS. We would like to test these genotypes, along with control cultivar FL47, which does not produce acylsugars, for resistance to whiteflies and resistance to TYLCV inoculation by whiteflies to determine if acylsugars can provide a practical application for controlling the spread of TYLCV.

Materials and methods

Tomato genotypes

Tomato lines used were acylsugar-producing genotypes and include FA2/AS, FA7/AS, FA2/FA7/AS, QTL6/AS, and Cornell acylsugar-producing benchmark genotype CU071026. FL47 was used as a non-acylsugar-producing control cultivar. FA2/AS, FA7/AS, and QTL6/AS are crosses of CU071026 with *S. pennellii* and have introgressed quantitative trait loci from *S. pennelli* that affect either the quantity of or chemistry of the acylsugars. FA2/FA7/AS is the resulting cross of FA2/AS and FA7/AS. Genotypes FA2/AS, FA7/AS, and FA2/FA7/AS have, respectively, 117.8%, 102.0%, and 141.0% the acylsugar levels of CU071026 (Smeda et al. 2017), along with different fatty acid profiles. QTL6/AS has a similar fatty acid profile as CU071026, but an increase in the density of Type IV trichomes and acylsugar levels.

Inoculation and accumulation of TYLCV in tomato lines

All tomato lines were evaluated to determine the percentage of plants that are inoculated with TYLCV using whiteflies. Plants were grown in the greenhouse in whitefly-proof cages to the ten true-leaf stage. Twenty viruliferous whiteflies were clip-caged to a leaflet of the eighth true-leaf eight for 24-hours. Whiteflies were removed and plants were sprayed with insecticidal soap (Garden Safe, Bridgeton, MO) to kill remaining adults or eggs laid on the plants. Six plants of each genotype were inoculated per experiment. The experiment was conducted four times. Plants were maintained for three weeks to allow development of infection. Leaf tissue samples from the newest true-leaf were collected and washed to remove external contamination. Tissue was ground in tube with a pestle and underwent a DNA extraction with GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific). PCR was conducted to determine TYLCV infection status. Primers used were C2-1201 (5’-CATGATCCACTGCTCTGATTACA-3’) and C2-

1800V2 (5’-TCATTGATGACGTAGACCCG-3’), which target 695 nucleotides of the TYLCV genome and encompasses the entire C2 gene. The PCR reactions were run in 10 µl reactions with 5 µl of GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 2 µl of water, 0.5 µl of each primer at 10 µM concentration, and 2 µl of DNA extract. The PCR program had an initial denaturation step at 94° C for 2 minutes followed by 30 cycles of 94° for 30 sec, 52° for 30 sec, 72° for 1 min, and a final extension at 72° for 5 min. Inoculation data was analyzed in SAS 9.4 (SAS Institute Inc., Cary, NC) with the GLIMMIX PROCEDURE using a binomial distribution. A least squares means separation was performed for pair-wise comparisons.

Samples testing positive were then subjected to real-time PCR to quantify TYLCV DNA concentrations relative to the tomato 25S rRNA gene using the mathematical formula from Pfaffl (2001). Real-times primers for TYLCV DNA were TYLC-C2-For (5’-GCAGTGATGAGTTCCCCTGT-3’) and TYLC-C2-Rev (5’-CCAATAAGGCGTAAGCGTGT-3’), which cover a 102 nucleotide region over the TYLCV C2 gene. The real-time PCR reactions were run in 25 µl reactions with 12.5 µl of GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI), 6.5 µl of water, 0.5 µl of each primer at 10 µM concentration, and 5 µl of DNA extract. The real-time PCR program for the C2 gene had an initial denaturation step at 95° C for 2 min followed by 40 cycles of 95° for 15 sec and 60° for 1 min followed by a melting curve. Primers for the tomato 25S rRNA gene were Tomato 25S rRNA F (5′-ATAACCGCATCAGGTCTCCA-3′) and Tomato 25S rRNA R (5′-CCGAAGTTACGGATCCATTT-3′) (Noris and Miozzi 2015). The real-time PCR program for the tomato 25S rRNA gene had an initial denaturation step at 95° C for 2 min followed by 40 cycles of 95° for 15 sec and 53° for 1 min followed by a melting curve. Accumulation values were analyzed using ANOVA in R version 3.4.0.

Whitefly acquisition of TYLCV from infected tomato lines

Twenty adult whiteflies were clip-caged to a leaflet of the tenth true-leaf of an infected tomato plant that had been inoculated with TYLCV four weeks prior. Whiteflies were clip-caged for 72-hours and removed. The experiment was conducted four times. Six whiteflies were sampled per clip-cage. A DNA extraction was performed on individual whiteflies using Instagene Matrix (BioRad, Hercules, CA). Samples were tested for TYLCV using primers C2-1201 and C2-1800V2. Acquisition data was analyzed in SAS with the GLIMMIX PROCEDURE using a binomial distribution. A least square means separation was performed for pair-wise comparisons.

Whitefly samples that tested positive for TYLCV with PCR were subjected to real-time PCR with the TYLC-C2-For and TYLC-C2-Rev primer set. Values were normalized with the whitefly β-actin gene, which was amplified with the primers whitefly β-actin F (5'–TCTTCCAGCCATCCTTCTTG–3') and whitefly β-actin R (5'–CGGTGATTTCCTTCTGCATT–3') (Sinisterra et al. 2005). The real-time PCR program had an initial 95° denaturation step for 2 m, followed by 40 cycles of 95° for 15 sec and 60° for 1 m, followed by a melting curve. Values for TYLCV and the whitefly β-actin were used in the equation developed by Pfaffl (2001) for relative quantification of TYLCV DNA to whitefly β-actin DNA. TYLCV acquisition quantities were analyzed using ANOVA in R.

Whitefly settling preference

Genotypes CU071026, FA2/AS, FA7/AS, QTL6/AS, and FA2/FA7/AS were each paired with FL47 in a settling arena. In another experiment, lines FA2/AS, FA7/AS, QTL6-AS, and FA2/FA7-AS were each paired with CU071026. Each experiment was conducted twice using six plants per line. One leaf from each ten true-leaf stage tomato plant was inserted into a settling

arena. A vial containing one hundred whiteflies was placed at the bottom of the arena. After 24-hours, the number of whiteflies settled on both the abaxial and adaxial side of each leaf was tabulated. Preference data was analyzed with a two-way ANOVA in SAS using split-plot design. Comparisons were blocked by replication with genotype as the main effect and side of leaf as subplot effect. A Tukey's Honestly Significant Differences (HSD) was performed to determine significant pairwise-comparisons.

Whitefly survival from egg to nymph

Lines CU071026, QTL6/AS, FA2/FA7/AS, and FL47 were used to conduct a two-week survival test on insects from the egg stage to the nymphal stage. Female whiteflies were clip-caged to the eighth true-leaf of a tomato plant and allowed to lay eggs for two days. Six plants were used per genotype and the experiment was conducted twice. Female whiteflies were then removed and the number of eggs was counted. Plants were maintained for two weeks. The number of nymphs was then counted to determine the percentage of survival on each tomato line.

Whitefly developmental time from egg to adult eclosion

Lines CU071026, QTL6/AS, FA2/FA7/AS, and FL47 were used to determine the length of whitefly developmental time from egg to adult eclosion. Female whiteflies were clip-caged to the eighth true-leaf of a tomato plant and allowed to lay eggs for two days. Six plants were used per line and the experiment was conducted twice. Individual eggs were monitored bi-daily as they developed through nymphal stages and finally to adult eclosion. The time from egg to adult eclosion was recorded. A Kruskal-Wallis rank sum test was conducted in R followed by a Dunn post-hoc multiple comparisons to determine significant pairwise-comparisons.

Results

Inoculation and accumulation of TYLCV in tomato lines

The different tomato lines were inoculated by whiteflies at percentages that were statistically different ($F(5,13)=3.23$, $p=0.0412$). The least squares means comparisons revealed that FL47 was inoculated more frequently than the acylsugar-producing tomato lines, with the exception of QTL6/AS (Table 6.1).

Accumulation of TYLCV DNA in the different tomato lines did not differ significantly based on the ANOVA results ($F(5,61)=0.5522$, $p=0.7360$). TYLCV DNA accumulated to similar amounts in all tomato lines (Figure 6.1).

Whitefly acquisition of TYLCV from infected tomato lines

The number of whiteflies that acquired TYLCV from the different tomato lines did not differ at a statistically significant level ($F(5,13)=2.62$, $p=0.0755$)(Table 6.2). Additionally, the quantity of TYLCV DNA acquired by positive whiteflies, as determined by real-time PCR, did not differ between lines at a statistically significant level based on the ANOVA ($F(5,45)=1.4038$, $p=0.2411$).

Whitefly settling preference

The two-way ANOVA detected significant differences for both tomato genotype ($F(1,11)=10.21$, $p=0.0085$) and side of the leaf ($F(1,22)=38.15$, $p<0.0001$) whiteflies settled on, as well as a statistically-significant interacting effect ($F(1,22)=123.77$, $p<0.0001$). Whiteflies preferred to settle on control cultivar FL47 over three (CU071026, FA7/AS, and QTL6/AS) of the five acylsugar-producing genotypes at statistically-significant levels (Figure 6.2). Whiteflies did not exhibit a preference or deterrence for CU071026 when paired with the other four acylsugar-producing lines.

The surface of the leaflet that whiteflies settled on was also tabulated, as key differences became quickly apparent between control cultivar FL47 and acylsugar-producing genotypes (Figure 6.3). Whiteflies had a strong preference for the abaxial side of the leaf when settled on FL47. However, when settled on acylsugar-producing genotypes, whiteflies exhibited either no preference for the abaxial or adaxial side of the leaflet, or exhibited a preference for the adaxial side of the leaflet.

Whitefly survival from egg to nymph

The percentage of insects that survived from the egg stage into the third or fourth instar stage two weeks later was higher on control cultivar FL47 compared to the acylsugar-producing lines CU071026, FA2/FA7/AS, and QTL6/AS (Table 6.3).

Whitefly developmental time from egg to adult eclosion

The number of days individual insects took to develop from egg to adult eclosion differed at a statistically significant level according to the Kruskal-Wallis test ($H=27.966$, $df=3$, $p<0.0001$). The post-hoc Dunn test determined that FL47 differed from the three acylsugar-producing lines CU071026, FA2/FA7/AS, and QTL6/AS. Whiteflies took approximately two days longer to develop on FL47 than on the acylsugar-producing lines (Table 6.4).

Discussion

The acylsugar-producing genotypes exhibited both antibiosis and antixenosis effects on the whitefly *B. tabaci.* Whiteflies showed a preference to settle on control cultivar FL47 over the acylsugar-producing genotypes. Survival percentages were also lower for developing whiteflies in the egg to 3rd or 4th instar nymphal stage on the acylsugar genotypes compared to FL47. Interestingly, whiteflies took longer to develop on control cultivar FL47 than the acylsugar-producing genotypes. Acylsugars exhibit antifungal and antibacterial properties (Luu et al. 2017,

Chortyk et al. 1993) and may have reduced the pressure of fungal and/or bacterial pathogens on developing whiteflies.

Another main difference between the acylsugar-producing genotypes and FL47 was the side of the leaf in which whiteflies chose to settle on. Whiteflies exhibited a clear preference to settle on the abaxial side of the leaf when settling on FL47. However, on the acylsugar-producing genotypes, whiteflies either showed no preference for either the abaxial or adaxial side, or showed a preference for the adaxial side of the leaf. This effect was also noted by Rodríguez-López et al. (2012) as whiteflies preferred the abaxial leaf surfaces in their control cultivar, Moneymaker, but showed no preference toward abaxial or adaxial leaf surfaces in their acylsugar-producing line, ABL14-8. Electrical Penetration Graph demonstrated that whiteflies most often fed on the abaxial leaf surface in control cultivar Moneymaker, but with the acylsugar-producing line, whiteflies did not feed on the abaxial leaf surface and only did so on the adaxial leaf surface. In a study examining the effects of acylsugars on the two-spotted spider mite, Rakha et al. (2017) mentioned that in *S. pimpinellifolium* accession VI030462, type IV trichomes were found in higher abundance on the abaxial leaf surface than the adaxial leaf surface. The two-spotted spider mites preferred to lay their eggs at significantly higher levels on the adaxial leaf surface compared to the abaxial leaf surface of this accession. A next step for our acylsugar-producing genotypes could be to examine the density of type IV trichomes on both the abaxial and adaxial leaf surfaces to determine if this is the mechanism behind the whiteflies' settling behavior.

Acylsugar-producing genotypes were inoculated with TYLCV by whiteflies at lower percentages than control cultivar FL47. This demonstrates that the acylsugars could potentially be effective in restricting the spread of TYLCV. However, whiteflies were able to acquire

TYLCV from both the acylsugar-producing genotypes and FL47, and at percentages that did not differ significantly. The amount of virus whiteflies acquired from each line also did not vary at a statistically-significant level. EPG study by Rodríguez-López et al. (2012) noted that although whiteflies had longer times before first probing and a fewer number of probes on their acylsugar-producing line ABL 14-8, the duration time of ingestion was not different between the acylsugar-producing line and control cultivar Moneymaker once feeding was initiated. This corroborates our results showing that whiteflies did not acquire TYLCV at lower quantities on acylsugar-producing genotypes compared to FL47.

Accumulation of TYLCV in plant tissue did not differ between the lines as the acylsugar-producing lines do not have TYLCV resistance genes. Currently, the most effective tool for managing TYLCV is use of TYLCV-resistant tomato cultivars (Lapidot et al. 1997, Gilreath et al. 2000). As an example of their effectiveness, a field trial in Florida using susceptible and resistant tomatoes cultivars in an area where TYLCV was established showed the standard susceptible variety, FL47, produced only 10.7 tons/acre of tomatoes while resistant varieties produced up to 25.9 tons/acre (Ozores-Hampton et al. 2013). Ideally, resistance genes to both the whitefly vector and the virus integrated into a single line could provide the best control of TYLCV.

Based on our results, the acylsugar-producing lines could provide limited control of TYLCV. The inoculation percentages of the acylsugar-producing lines are lower than that of control cultivar FL47. However, because the acylsugars do not completely deter whitefly settling and feeding, a portion of the whiteflies will still feed on and inoculate some plants. Only one whitefly feeding for 15-30 minutes is needed to inoculate a plant or acquire sufficient virus to inoculate a new plant (Czosnek et al. 2002). Only with complete deterrence of whitefly feeding

can there be complete control in the spread of TYLCV. A field experiment in an area with established TYLCV could better assess the efficacy of acylsugar-producing genotypes on preventing the spread of TYLCV.

Overall, we see that the acylsugar-producing tomato lines exhibit both antixenosis and antibiosis effects on the whitefly *B. tabaci*. In general, whiteflies preferred to settle on control cultivar FL47 over acylsugar-producing lines. Whiteflies also showed a shift in settling toward the adaxial side of the leaf in acylsugar-producing lines compared to FL47. Survival from the egg to third or fourth instar stage was lower in the acylsugar-producing lines compared to FL47. However, whiteflies developed faster on the acylsugar-producing lines compared to FL47, which could be due to the antibacterial or antifungal properties of acylsugars. The percentages of TYLCV inoculation were lower in the acylsugar-producing genotypes compared to control cultivar FL47, indicating these lines may provide limited control of TYLCV. Whiteflies were, however, capable of acquiring TYLCV from the acylsugar-producing cultivars and acquired amounts of virus that were similar to FL47. TYLCV also accumulated within the plants to similar levels across the tomato lines. Introgression of a TYLCV-resistance gene into these acylsugar-producing genotypes could lead to a very promising method of controlling TYLCV.

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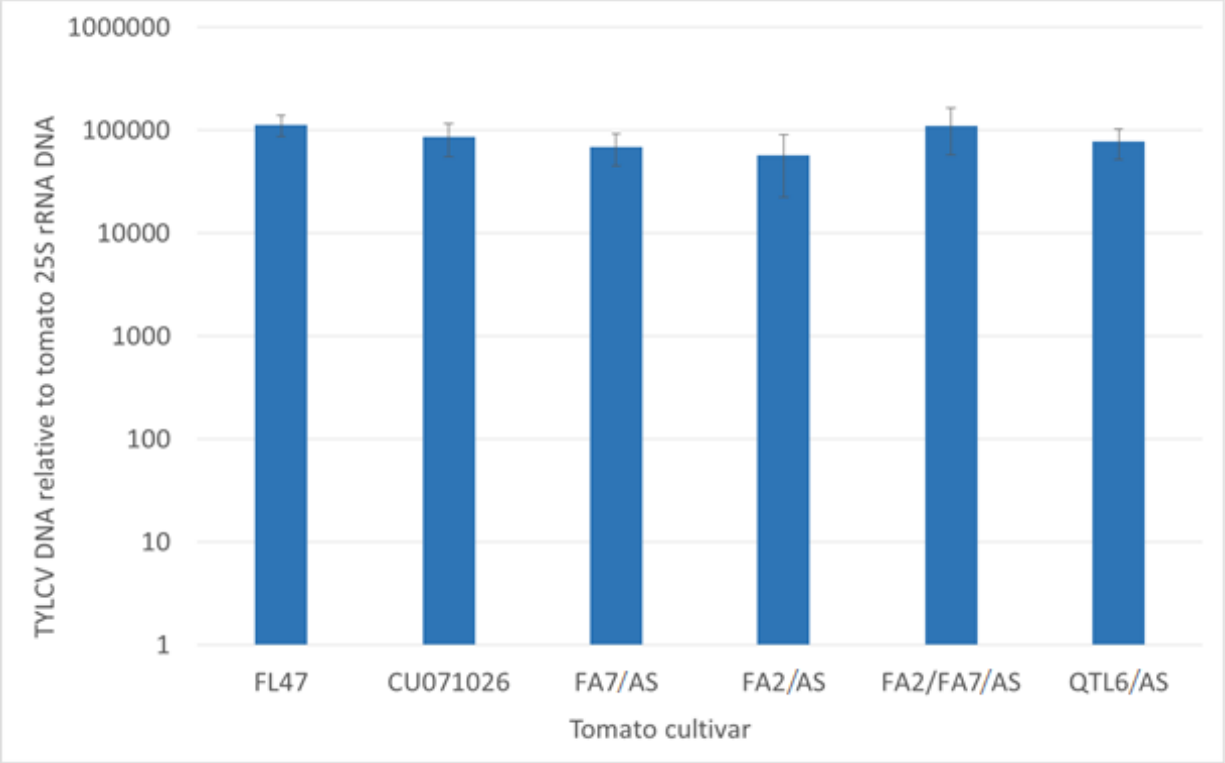

Figure 6.1. Accumulation of TYLCV in the different tomato lines.

Error bars are standard errors.

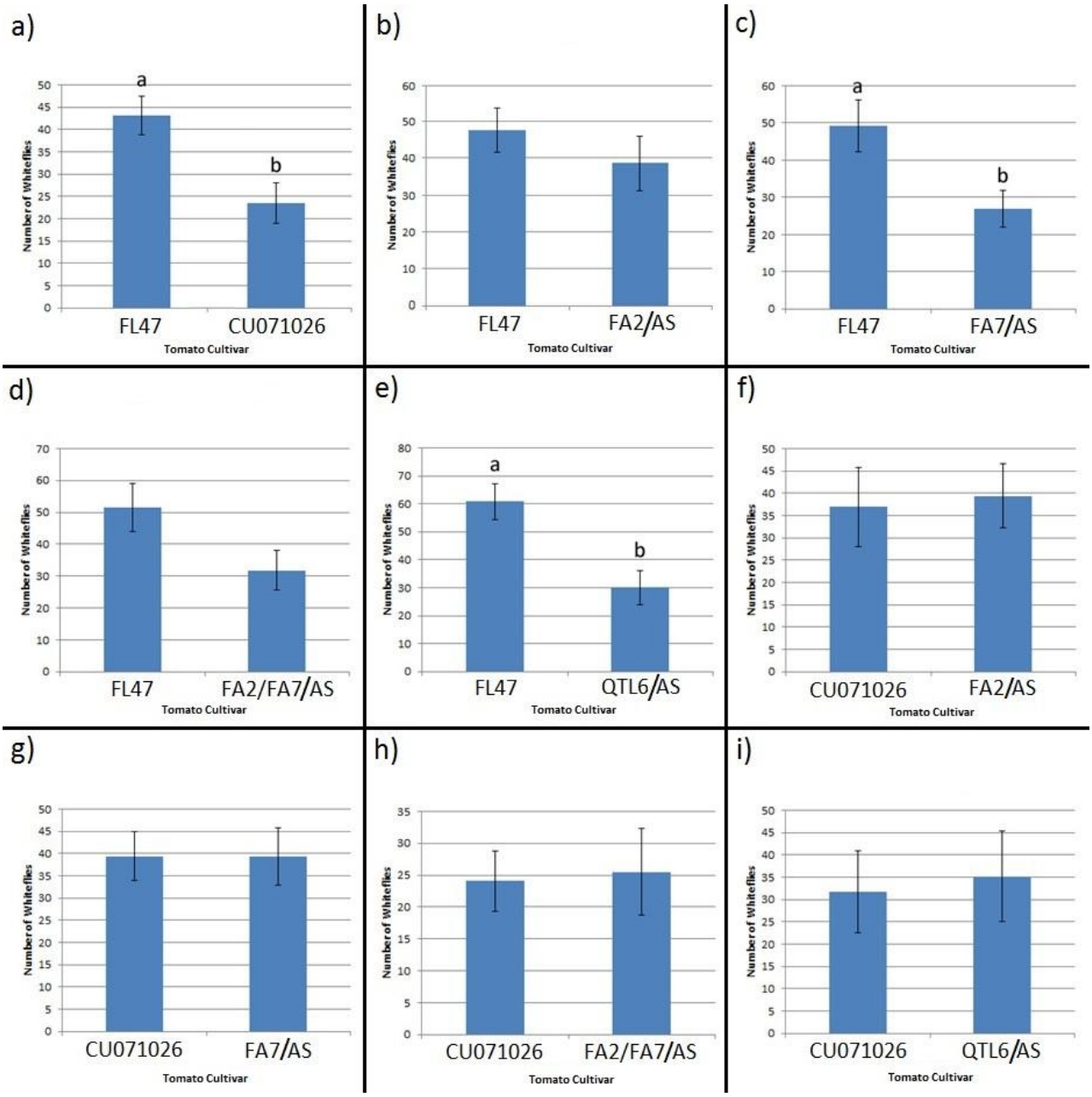

Figure 6.2. Whitefly settling preferences after 24-hours for choice test experiments pairing control cultivar FL47 versus acylsugar-producing genotypes, and CU071026 versus other acylsugar-producing genotypes. a) FL47 versus CU071026, b) FL47 versus FA2/AS, c) FL47 versus FA7/AS, d) FL47 versus FA2/FA7/AS, e) FL47 versus QTL6/AS, f) CU071026 versus FA2/AS, g) CU071026 versus FA7/AS, h) CU071026 versus FA2/FA7/AS, and i) CU071026 versus QTL6/AS. Error bars are standard errors.

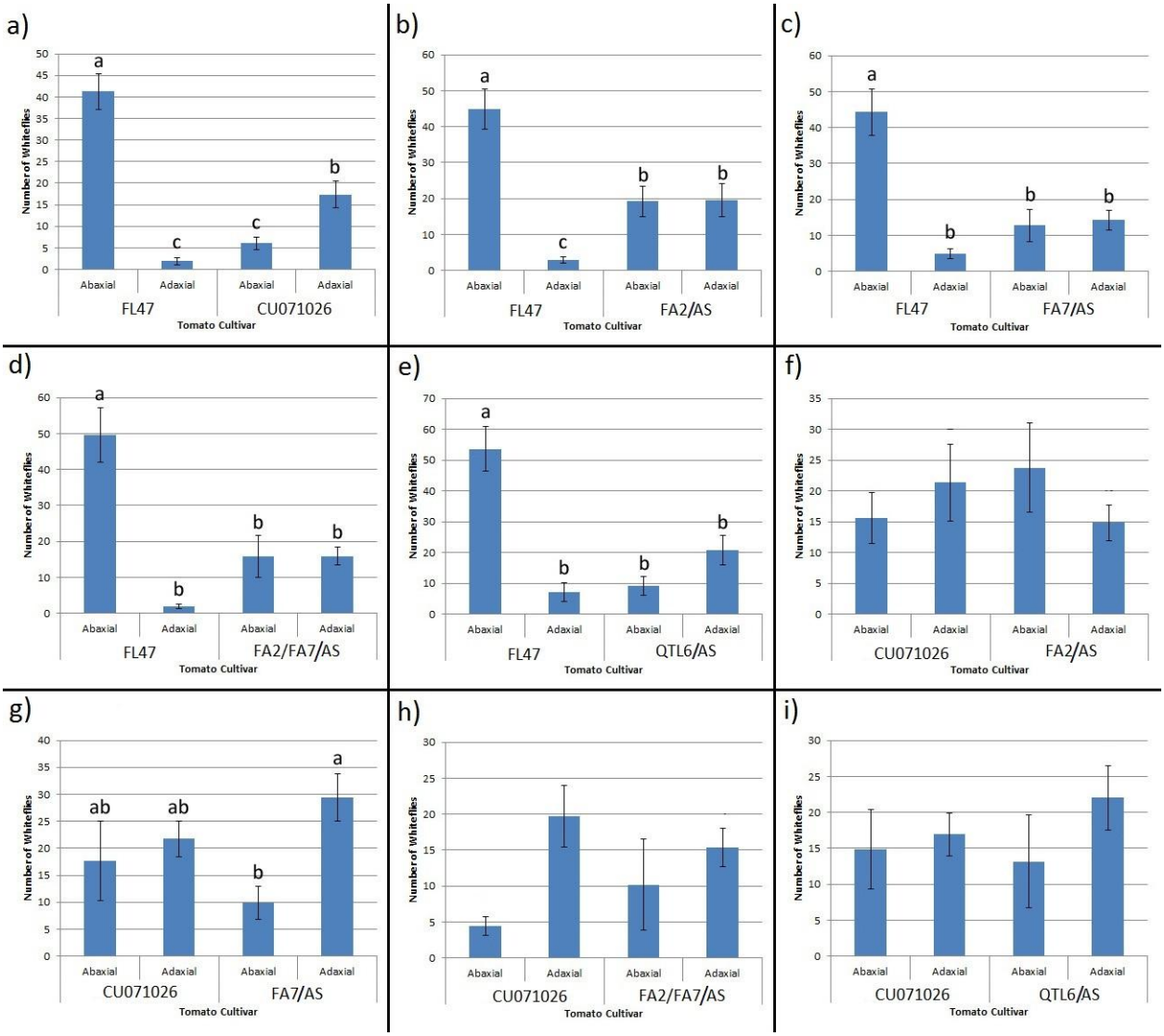

Figure 6.3. Whitefly settling preferences by both tomato line and leaf surface orientation after 24-hours. Choice tests were performed with control cultivar FL47 versus acylsugar-producing genotypes, and CU071026 versus other acylsugar-producing genotypes. a) FL47 versus CU071026, b) FL47 versus FA2/AS, c) FL47 versus FA7/AS, d) FL47 versus FA2/FA7/AS, e) FL47 versus QTL6/AS, f) CU071026 versus FA2/AS, g) CU071026 versus FA7/AS, h) CU071026 versus FA2/FA7/AS, and i) CU071026 versus QTL6/AS. Error bars are standard errors.

Table 6.1. Average percentages of plants that were successfully inoculated with TYLCV for each tomato line.

Tomato line	Average percentage of plants inoculated ± standard error	Least squares means separations
FL47	82.6 ± 11.85%	A
CU071026	34.8 ± 1.675%	B
FA2/AS	41.7 ± 19.85%	B
FA7/AS	54.2 ± 20.85%	B
FA2/FA7/AS	29.2 ± 14.2%	B
QTL6/AS	58.3 ± 17.35%	AB

Table 6.2. Average percentages of whiteflies that acquired TYLCV on each tomato line.

Tomato line	Average percentage of whiteflies that acquired virus ± standard error
FL47	54.2 ± 18.45%
CU071026	25 ± 15.95%
FA2/AS	16.7 ± 11.8%
FA7/AS	20.8 ± 15.75%
FA2/FA7/AS	50 ± 20.4%
QTL6/AS	29.2 ± 14.2%

Table 6.3. Average percentages of individual whiteflies surviving from egg to either the third or fourth instar stage two weeks later.

Tomato line	Average percentage of whiteflies surviving ± standard error
FL47	90.7 ± 4.86%
CU071026	47.6 ± 2.64%
FA2/FA7/AS	49.1 ± 11.79%
QTL6/AS	61.5 ± 11.93%

Table 6.4. Median number of days for whiteflies to develop from egg to adult eclosion on four of the tomato lines.

Tomato line	Median number of days to develop (range in parentheses)	Dunn test separation
FL47	27 (22-35)	A
CU071026	25 (18-31)	B
FA2/FA7/AS	24 (19-31)	B
QTL6/AS	25 (19-27)	B

Appendix A. Complete list of the 666 TYLCV genomes in the with-recombinants data set. Includes genome names, GenBank accession numbers, and geographic region assigned.

Name	Accession	Geographic Region
Australia10-06	KX347102	Oceania
Australia1-03	KX347094	Oceania
Australia11-06	KX347103	Oceania
Australia12-06	KX347104	Oceania
Australia13-06	KX347105	Oceania
Australia14-06	KX347106	Oceania
Australia15-06	KX347107	Oceania
Australia16-06	KX347108	Oceania
Australia17-06	KX347109	Oceania
Australia18-06	KX347110	Oceania
Australia19-06	KX347111	Oceania
Australia20-06	KX347112	Oceania
Australia2-06	GU178819	Oceania
Australia21-06	KX347113	Oceania
Australia22-06	KX347114	Oceania
Australia23-06	KX347115	Oceania
Australia24-06	KX347116	Oceania
Australia25-06	KX347117	Oceania
Australia26-06	KX347118	Oceania
Australia27-06	KX347119	Oceania
Australia28-06	KX347120	Oceania
Australia29-06	KX347121	Oceania
Australia30-06	KX347122	Oceania
Australia3-06	KX347095	Oceania
Australia31-06	KX347123	Oceania
Australia32-06	KX347124	Oceania
Australia33-06	KX347125	Oceania
Australia34-06	KX347126	Oceania
Australia35-06	KX347127	Oceania
Australia36-06	GU178814	Oceania
Australia37-06	GU178813	Oceania
Australia38-06	GU178818	Oceania
Australia39-06	GU178816	Oceania
Australia40-06	GU178815	Oceania
Australia4-06	KX347096	Oceania
Australia41-06	GU178817	Oceania
Australia42-06	GU178820	Oceania
Australia43-07	KX347128	Oceania
Australia44-07	KX347129	Oceania

Australia45-07	KX347130	Oceania
Australia46-09	KX347131	Oceania
Australia47-09	KX347132	Oceania
Australia48-09	KX347133	Oceania
Australia49-09	KX347134	Oceania
Australia50-09	KX347135	Oceania
Australia5-06	KX347097	Oceania
Australia51-09	KX347136	Oceania
Australia52-09	KX347137	Oceania
Australia53-09	KX347138	Oceania
Australia54-10	KX347139	Oceania
Australia55-10	KX347140	Oceania
Australia56-10	KX347141	Oceania
Australia57-10	KX347142	Oceania
Australia58-10	KX347143	Oceania
Australia59-10	KX347144	Oceania
Australia60-10	KX347145	Oceania
Australia6-06	KX347098	Oceania
Australia7-06	KX347099	Oceania
Australia8-06	KX347100	Oceania
Australia9-06	KX347101	Oceania
BurkinaFaso1-09	LM651400	Africa-Europe-Middle East
BurkinaFaso2-09	LM651401	Africa-Europe-Middle East
BurkinaFaso3-09	LM651402	Africa-Europe-Middle East
BurkinaFaso4-09	LM651403	Africa-Europe-Middle East
Cameroon1-07	FM212660	Africa-Europe-Middle East
Cameroon2-07	FM212661	Africa-Europe-Middle East
Cameroon3-07	FM212662	Africa-Europe-Middle East
Cameroon4-07	FM212663	Africa-Europe-Middle East
China100-12	JQ807735	Southeast Asia
China10-08	GU199587	Southeast Asia
China101-12	JX669541	Southeast Asia
China102-12	JX669542	Southeast Asia
China103-12	JX669543	Southeast Asia
China104-12	JX669544	Southeast Asia
China105-12	JX856172	Southeast Asia
China1-06	AM698119	Southeast Asia
China106-12	JX856173	Southeast Asia
China107-12	JX910534	Southeast Asia
China108-12	JX997799	Southeast Asia
China109-12	JX997800	Southeast Asia

China110-12	JX997801	Southeast Asia
China11-08	FJ646611	Southeast Asia
China111-12	JX997802	Southeast Asia
China112-12	KC312655	Southeast Asia
China113-12	KC312656	Southeast Asia
China114-12	KC312657	Southeast Asia
China115-12	KC312658	Southeast Asia
China116-12	KC312659	Southeast Asia
China117-12	KC312660	Southeast Asia
China118-12	KC312661	Southeast Asia
China119-12	KC312662	Southeast Asia
China120-12	KC312663	Southeast Asia
China12-08	FN256257	Southeast Asia
China121-12	KC312664	Southeast Asia
China122-12	KC312665	Southeast Asia
China123-12	KC312666	Southeast Asia
China124-12	KC312667	Southeast Asia
China125-12	KC312668	Southeast Asia
China126-12	KC312669	Southeast Asia
China127-12	KC312670	Southeast Asia
China128-12	KC312671	Southeast Asia
China129-12	KC312672	Southeast Asia
China130-12	KC312673	Southeast Asia
China13-08	GU434142	Southeast Asia
China131-12	KC428753	Southeast Asia
China132-12	KC702798	Southeast Asia
China133-12	KC810892	Southeast Asia
China134-12	KC852147	Southeast Asia
China135-12	KC852149	Southeast Asia
China136-12	KC852150	Southeast Asia
China137-12	KC999844	Southeast Asia
China138-12	KC999845	Southeast Asia
China139-12	KF612971	Southeast Asia
China140-12	KJ125410	Southeast Asia
China14-08	GU434144	Southeast Asia
China141-12	KJ140787	Southeast Asia
China142-12	KJ140788	Southeast Asia
China143-13	KF990604	Southeast Asia
China144-13	KJ879949	Southeast Asia
China145-13	KJ879950	Southeast Asia
China146-13	KM506948	Southeast Asia

China147-13	KM506949	Southeast Asia
China148-13	KM506950	Southeast Asia
China149-13	KM506951	Southeast Asia
China150-13	KM506952	Southeast Asia
China15-09	GU348995	Southeast Asia
China151-13	KM506953	Southeast Asia
China152-13	KM506954	Southeast Asia
China153-13	KM506955	Southeast Asia
China154-13	KM506956	Southeast Asia
China155-13	KM506957	Southeast Asia
China156-13	KM506958	Southeast Asia
China157-13	KM435321	Southeast Asia
China158-13	KM435319	Southeast Asia
China159-13	KM435323	Southeast Asia
China160-13	KM435325	Southeast Asia
China16-09	HM208334	Southeast Asia
China161-13	KM435327	Southeast Asia
China162-13	KM506947	Southeast Asia
China163-13	KF356163	Southeast Asia
China164-13	KF906542	Southeast Asia
China165-13	KF990604	Southeast Asia
China166-13	KJ125411	Southeast Asia
China167-13	KJ546418	Southeast Asia
China168-13	KJ754186	Southeast Asia
China169-13	KJ754187	Southeast Asia
China170-13	KJ754188	Southeast Asia
China17-09	GU563330	Southeast Asia
China171-13	KJ754189	Southeast Asia
China172-13	KJ754190	Southeast Asia
China173-13	KJ754191	Southeast Asia
China174-13	KJ754192	Southeast Asia
China175-13	KJ754193	Southeast Asia
China176-13	KJ754194	Southeast Asia
China177-13	KJ879948	Southeast Asia
China178-13	KU892717	Southeast Asia
China179-14	KX034538	Southeast Asia
China180-14	KX034539	Southeast Asia
China18-09	GU951436	Southeast Asia
China181-14	KX034541	Southeast Asia
China182-14	KX034542	Southeast Asia
China183-14	KX034543	Southeast Asia

China184-14	KX034546	Southeast Asia
China185-14	KX034547	Southeast Asia
China186-14	KX034550	Southeast Asia
China187-14	KX034551	Southeast Asia
China188-14	KP684146	Southeast Asia
China189-14	KP685598	Southeast Asia
China190-14	KM435320	Southeast Asia
China19-09	GU951437	Southeast Asia
China191-14	KM435322	Southeast Asia
China192-14	KM435324	Southeast Asia
China193-14	KM435326	Southeast Asia
China194-14	KM435328	Southeast Asia
China195-14	KJ850344	Southeast Asia
China196-14	KT338293	Southeast Asia
China197-14	KT338294	Southeast Asia
China198-14	KT852577	Southeast Asia
China199-14	KU934104	Southeast Asia
China200-14	KU975396	Southeast Asia
China20-09	GU983859	Southeast Asia
China201-14	KU975397	Southeast Asia
China202-14	KU975398	Southeast Asia
China203-14	KU975399	Southeast Asia
China204-15	KX034540	Southeast Asia
China205-15	KX034544	Southeast Asia
China2-06	AM282874	Southeast Asia
China206-15	KX034545	Southeast Asia
China207-15	KX034548	Southeast Asia
China208-15	KX034549	Southeast Asia
China209-15	KX034553	Southeast Asia
China210-15	KT338295	Southeast Asia
China21-09	HM043732	Southeast Asia
China211-15	KT338296	Southeast Asia
China212-15	KU760888	Southeast Asia
China213-15	KU760889	Southeast Asia
China214-15	KU760890	Southeast Asia
China215-15	KU760891	Southeast Asia
China216-15	KU760892	Southeast Asia
China217	EU031444	Southeast Asia
China218	FN650808	Southeast Asia
China219	GQ352537	Southeast Asia
China220	GQ352538	Southeast Asia

China22-09	HM358879	Southeast Asia
China221	KC999850	Southeast Asia
China222	KX034552	Southeast Asia
China223	HQ702861	Southeast Asia
China224	HQ702862	Southeast Asia
China225	HQ702863	Southeast Asia
China226	JQ004028	Southeast Asia
China227	KC999849	Southeast Asia
China23-09	HM627880	Southeast Asia
China24-09	HM627882	Southeast Asia
China25-09	HM627883	Southeast Asia
China26-10	HM627881	Southeast Asia
China27-10	HM627884	Southeast Asia
China28-10	HM627885	Southeast Asia
China29-10	JF301667	Southeast Asia
China30-10	JF301668	Southeast Asia
China3-06	AM698117	Southeast Asia
China31-10	JF414236	Southeast Asia
China32-10	JF414237	Southeast Asia
China33-10	JF727878	Southeast Asia
China34-10	JF817218	Southeast Asia
China35-10	JF833036	Southeast Asia
China36-11	JQ038233	Southeast Asia
China37-11	JQ038240	Southeast Asia
China38-11	JX070043	Southeast Asia
China39-11	JX456640	Southeast Asia
China40-11	JX456641	Southeast Asia
China4-06	AM698118	Southeast Asia
China41-11	JX456642	Southeast Asia
China42-11	JX456644	Southeast Asia
China43-11	JQ411237	Southeast Asia
China44-11	KM506959	Southeast Asia
China45-11	KM506960	Southeast Asia
China46-11	KC138544	Southeast Asia
China47-11	KC138545	Southeast Asia
China48-11	KC138546	Southeast Asia
China49-11	JX456638	Southeast Asia
China50-11	JX456639	Southeast Asia
China5-07	FN256256	Southeast Asia
China51-11	JX456643	Southeast Asia
China52-11	KC138543	Southeast Asia

China53-11	JX456637	Southeast Asia
China54-11	JF964959	Southeast Asia
China55-11	JN412854	Southeast Asia
China56-11	JN990922	Southeast Asia
China57-11	JN990923	Southeast Asia
China58-11	JN990924	Southeast Asia
China59-11	JN990925	Southeast Asia
China60-11	JN990926	Southeast Asia
China6-07	GU111505	Southeast Asia
China61-11	JN990927	Southeast Asia
China62-11	JN990928	Southeast Asia
China63-11	JQ004045	Southeast Asia
China64-11	JQ004046	Southeast Asia
China65-11	JQ004047	Southeast Asia
China66-11	JQ004048	Southeast Asia
China67-11	JQ004049	Southeast Asia
China68-11	JQ004050	Southeast Asia
China69-11	JQ004051	Southeast Asia
China70-11	JQ004052	Southeast Asia
China7-07	FN252890	Southeast Asia
China71-11	JQ034613	Southeast Asia
China72-11	JQ038232	Southeast Asia
China73-11	JQ038234	Southeast Asia
China74-11	JQ038235	Southeast Asia
China75-11	JQ038236	Southeast Asia
China76-11	JQ038237	Southeast Asia
China77-11	JQ038238	Southeast Asia
China78-11	JQ038239	Southeast Asia
China79-11	JQ326957	Southeast Asia
China80-11	JQ867092	Southeast Asia
China8-07	FN256258	Southeast Asia
China81-11	JX070042	Southeast Asia
China82-11	JX070044	Southeast Asia
China83-11	JX070045	Southeast Asia
China84-11	JX128100	Southeast Asia
China85-11	JX997798	Southeast Asia
China86-11	KC999851	Southeast Asia
China87-12	JX128099	Southeast Asia
China88-12	JX675237	Southeast Asia
China89-12	KC211184	Southeast Asia
China90-12	KC702796	Southeast Asia

China9-08	FN256259	Southeast Asia
China91-12	KC702797	Southeast Asia
China92-12	KC852151	Southeast Asia
China93-12	KC999846	Southeast Asia
China94-12	KC999847	Southeast Asia
China95-12	KC999848	Southeast Asia
China96-12	KM506961	Southeast Asia
China97-12	KJ140788	Southeast Asia
China98-12	KJ140789	Southeast Asia
China99-12	KJ140787	Southeast Asia
CostaRica1-12	KF533857	Americas
CostaRica2-12	KF533856	Americas
CostaRica3-12	KF533855	Americas
Cuba1-11	KM926623	Americas
Cuba2-11	KM926624	Americas
Cuba3-11	KM926625	Americas
Cuba4-11	KM926626	Americas
Cuba5	AJ223505	Americas
DominicanRepublic1-11	KJ913683	Americas
DominicanRepublic2-11	KJ913682	Americas
DominicanRepublic3-94	AF024715	Americas
Egypt1-14	KT921303	Africa-Europe-Middle East
Egypt2	AY594174	Africa-Europe-Middle East
Egypt3	EF107520	Africa-Europe-Middle East
Estonia-08	HF548826	Africa-Europe-Middle East
Ethopia-03	DQ358913	Africa-Europe-Middle East
Ghana-08	EU847740	Africa-Europe-Middle East
Grenada-07	FR851297	Americas
Guatemala-06	GU355941	Americas
Iran10-06	GU076451	Africa-Europe-Middle East
Iran1-06	GU076442	Africa-Europe-Middle East
Iran11-06	GU076440	Africa-Europe-Middle East
Iran12-06	KX347155	Africa-Europe-Middle East
Iran13-06	KX347156	Africa-Europe-Middle East
Iran14-06	KX347158	Africa-Europe-Middle East
Iran15-07	GU076450	Africa-Europe-Middle East
Iran16-07	GU076444	Africa-Europe-Middle East
Iran17-07	GU076445	Africa-Europe-Middle East
Iran18-07	GU076446	Africa-Europe-Middle East
Iran19-07	GU076447	Africa-Europe-Middle East
Iran20-07	KX347157	Africa-Europe-Middle East

Iran2-06	GU076443	Africa-Europe-Middle East
Iran21-08	KX347159	Africa-Europe-Middle East
Iran22-09	JQ928347	Africa-Europe-Middle East
Iran23-09	KX347162	Africa-Europe-Middle East
Iran24-09	KX347163	Africa-Europe-Middle East
Iran25-10	JQ414025	Africa-Europe-Middle East
Iran26-10	JQ928346	Africa-Europe-Middle East
Iran27-10	JQ928348	Africa-Europe-Middle East
Iran28-10	JQ928349	Africa-Europe-Middle East
Iran29-10	KX347160	Africa-Europe-Middle East
Iran30-10	KX347161	Africa-Europe-Middle East
Iran3-06	GU076454	Africa-Europe-Middle East
Iran31-10	JQ231214	Africa-Europe-Middle East
Iran32-11	KC106643	Africa-Europe-Middle East
Iran33-11	KC106636	Africa-Europe-Middle East
Iran34-11	KC106637	Africa-Europe-Middle East
Iran35-11	KC106638	Africa-Europe-Middle East
Iran36-11	KC106640	Africa-Europe-Middle East
Iran37-11	KC106641	Africa-Europe-Middle East
Iran38-11	KC106642	Africa-Europe-Middle East
Iran39-11	KC106644	Africa-Europe-Middle East
Iran40-11	KC106645	Africa-Europe-Middle East
Iran4-06	EU635776	Africa-Europe-Middle East
Iran41-11	KC106646	Africa-Europe-Middle East
Iran42-11	KC106647	Africa-Europe-Middle East
Iran43-11	KC106648	Africa-Europe-Middle East
Iran44-11	KC106649	Africa-Europe-Middle East
Iran45-11	KC106650	Africa-Europe-Middle East
Iran46-11	KC106651	Africa-Europe-Middle East
Iran47-11	KC106652	Africa-Europe-Middle East
Iran48-11	KC106635	Africa-Europe-Middle East
Iran49-12	KX347164	Africa-Europe-Middle East
Iran50-12	KX347165	Africa-Europe-Middle East
Iran5-06	GU076441	Africa-Europe-Middle East
Iran51-12	KX347166	Africa-Europe-Middle East
Iran52-13	KT990213	Africa-Europe-Middle East
Iran53	EU085423	Africa-Europe-Middle East
Iran54	FJ355946	Africa-Europe-Middle East
Iran55	AJ132711	Africa-Europe-Middle East
Iran6-06	GU076448	Africa-Europe-Middle East
Iran7-06	GU076449	Africa-Europe-Middle East

Iran8-06	GU076452	Africa-Europe-Middle East
Iran9-06	GU076453	Africa-Europe-Middle East
Iraq-11	JQ354991	Africa-Europe-Middle East
Israel1-89	X15656	Africa-Europe-Middle East
Israel2	X15656	Africa-Europe-Middle East
Israel3	X76319	Africa-Europe-Middle East
Italy1-04	EU734831	Africa-Europe-Middle East
Italy2-04	EU734832	Africa-Europe-Middle East
Italy3-04	DQ144621	Africa-Europe-Middle East
Japan10-13	AB921568	Southeast Asia
Japan1-05	AB192965	Southeast Asia
Japan11	AB110217	Southeast Asia
Japan12	AB116629	Southeast Asia
Japan13	AB116630	Southeast Asia
Japan14	AB116631	Southeast Asia
Japan15	AB116633	Southeast Asia
Japan16	AB116634	Southeast Asia
Japan17	AB116635	Southeast Asia
Japan18	AB116636	Southeast Asia
Japan19	AB110218	Southeast Asia
Japan20	AB116632	Southeast Asia
Japan2-05	LC099965	Southeast Asia
Japan21	AB014346	Southeast Asia
Japan22	AB014347	Southeast Asia
Japan3-07	AB363566	Southeast Asia
Japan4-07	AB439841	Southeast Asia
Japan5-07	AB439842	Southeast Asia
Japan6-11	KJ585666	Southeast Asia
Japan7-11	KJ466047	Southeast Asia
Japan8-11	KJ466048	Southeast Asia
Japan9-11	KJ585666	Southeast Asia
Jordan10	EF433426	Africa-Europe-Middle East
Jordan1-08	GQ861426	Africa-Europe-Middle East
Jordan2-08	GQ861427	Africa-Europe-Middle East
Jordan3-11	JX444575	Africa-Europe-Middle East
Jordan4-11	JX131286	Africa-Europe-Middle East
Jordan5-13	KM215610	Africa-Europe-Middle East
Jordan6	EF054894	Africa-Europe-Middle East
Jordan7	EF158044	Africa-Europe-Middle East
Jordan8	EU143745	Africa-Europe-Middle East
Jordan9	EF054893	Africa-Europe-Middle East

Kuwait1-08	JF451352	Africa-Europe-Middle East
Kuwait2-10	KJ830841	Africa-Europe-Middle East
Kuwait3-12	KJ830842	Africa-Europe-Middle East
Kuwait4-12	KR108214	Africa-Europe-Middle East
Lebanon1	EF185318	Africa-Europe-Middle East
Lebanon2	EF051116	Africa-Europe-Middle East
Mali	AY502934	Africa-Europe-Middle East
Mauritius1-09	HM448447	Africa-Europe-Middle East
Mauritius2-09	KX347167	Africa-Europe-Middle East
Mauritius3-09	KX347168	Africa-Europe-Middle East
Mauritius4-09	KX347169	Africa-Europe-Middle East
Mauritius5-09	KX347170	Africa-Europe-Middle East
Mauritius6-09	KX347171	Africa-Europe-Middle East
Mauritius7-09	KX347172	Africa-Europe-Middle East
Mexico1-11	JQ303121	Americas
Mexico2-11	JN680353	Americas
MexicoBajaCaliforniaSur-08	HM459851	Americas
MexicoSinaloa1-06	FJ012358	Americas
MexicoSinaloa2-06	DQ631892	Americas
MexicoSinaloa3	EF523478	Americas
MexicoSonora-06	EF210555	Americas
Morocco10-13	LN846614	Africa-Europe-Middle East
Morocco1-02	LN846617	Africa-Europe-Middle East
Morocco11-13	LN846613	Africa-Europe-Middle East
Morocco12-13	LN846600	Africa-Europe-Middle East
Morocco13-14	LN846608	Africa-Europe-Middle East
Morocco14-14	LN846607	Africa-Europe-Middle East
Morocco15-14	LN846610	Africa-Europe-Middle East
Morocco16-14	LN846606	Africa-Europe-Middle East
Morocco17-14	LN846615	Africa-Europe-Middle East
Morocco18-14	LN846605	Africa-Europe-Middle East
Morocco19-14	LN846604	Africa-Europe-Middle East
Morocco20-14	LN846603	Africa-Europe-Middle East
Morocco2-03	LN846616	Africa-Europe-Middle East
Morocco21	EF060196	Africa-Europe-Middle East
Morocco22	LN812978	Africa-Europe-Middle East
Morocco3-10	LN831187	Africa-Europe-Middle East
Morocco4-12	LN846612	Africa-Europe-Middle East
Morocco5-12	LN846611	Africa-Europe-Middle East
Morocco6-12	LN846602	Africa-Europe-Middle East
Morocco7-12	LN846601	Africa-Europe-Middle East

Morocco8-12	LN846599	Africa-Europe-Middle East
Morocco9-13	LN846609	Africa-Europe-Middle East
Netherlands-08	FJ439569	Africa-Europe-Middle East
NewCaledonia1-10	HE603245	Oceania
NewCaledonia2-10	HE603246	Oceania
NewCaledonia3-10	HE603244	Oceania
NewCaledonia4-10	HE603243	Oceania
NewCaledonia5-10	HE603242	Oceania
NewCaledonia6-10	HE603241	Oceania
Oman10-11	JN604485	Africa-Europe-Middle East
Oman1-05	FJ956700	Africa-Europe-Middle East
Oman11-11	JN604488	Africa-Europe-Middle East
Oman12-11	JN604487	Africa-Europe-Middle East
Oman13-11	JN604486	Africa-Europe-Middle East
Oman14-11	JN604485	Africa-Europe-Middle East
Oman15-11	JN604484	Africa-Europe-Middle East
Oman16-11	HE819245	Africa-Europe-Middle East
Oman17-11	HE819243	Africa-Europe-Middle East
Oman18-11	HE819242	Africa-Europe-Middle East
Oman19-11	HE819241	Africa-Europe-Middle East
Oman20-11	HE819240	Africa-Europe-Middle East
Oman2-05	DQ644565	Africa-Europe-Middle East
Oman21-12	KF229726	Africa-Europe-Middle East
Oman22-12	KF229725	Africa-Europe-Middle East
Oman23-12	KF229724	Africa-Europe-Middle East
Oman24-12	KF229723	Africa-Europe-Middle East
Oman25-12	KF229722	Africa-Europe-Middle East
Oman26-12	KF229721	Africa-Europe-Middle East
Oman27-13	HG941641	Africa-Europe-Middle East
Oman28-13	HG969205	Africa-Europe-Middle East
Oman29-13	HG969206	Africa-Europe-Middle East
Oman30-13	HG969207	Africa-Europe-Middle East
Oman3-05	FJ956706	Africa-Europe-Middle East
Oman31-13	HG969208	Africa-Europe-Middle East
Oman32-13	HG969258	Africa-Europe-Middle East
Oman33-13	HG969259	Africa-Europe-Middle East
Oman34-13	HG969260	Africa-Europe-Middle East
Oman35-13	HG969286	Africa-Europe-Middle East
Oman36-13	KF260965	Africa-Europe-Middle East
Oman37-13	KF260966	Africa-Europe-Middle East
Oman38-13	KF260967	Africa-Europe-Middle East

Oman39-13	KF260968	Africa-Europe-Middle East
Oman40-13	KF260969	Africa-Europe-Middle East
Oman4-05	FJ956705	Africa-Europe-Middle East
Oman41-13	HG969254	Africa-Europe-Middle East
Oman42-13	HG969287	Africa-Europe-Middle East
Oman43-13	HG969286	Africa-Europe-Middle East
Oman44-13	HG969285	Africa-Europe-Middle East
Oman45-13	HG969284	Africa-Europe-Middle East
Oman46-13	HG969283	Africa-Europe-Middle East
Oman47-13	HG969282	Africa-Europe-Middle East
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Oman50-13	HG969279	Africa-Europe-Middle East
Oman5-05	FJ956704	Africa-Europe-Middle East
Oman51-13	HG969272	Africa-Europe-Middle East
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Oman61-13	HG941651	Africa-Europe-Middle East
Oman62-13	HG941650	Africa-Europe-Middle East
Oman63-13	HG941649	Africa-Europe-Middle East
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Oman66-13	HG941645	Africa-Europe-Middle East
Oman67-13	HG941642	Africa-Europe-Middle East
Oman68-13	HG941640	Africa-Europe-Middle East
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Oman70-14	LN680631	Africa-Europe-Middle East
Oman7-05	FJ956702	Africa-Europe-Middle East
Oman71-14	LN680630	Africa-Europe-Middle East
Oman72	HG969204	Africa-Europe-Middle East
Oman8-05	FJ956701	Africa-Europe-Middle East
Oman9-11	HE819239	Africa-Europe-Middle East
Portugal1-01	JN859135	Africa-Europe-Middle East

Portugal2-01	JN859137	Africa-Europe-Middle East
Portugal3-02	JN859138	Africa-Europe-Middle East
Portugal4-95	AF105975	Africa-Europe-Middle East
ReunionIsland1-04	AM409201	Africa-Europe-Middle East
ReunionIsland2	AJ865337	Africa-Europe-Middle East
SaudiArabia1-12	KF435136	Africa-Europe-Middle East
SaudiArabia2-13	KF435137	Africa-Europe-Middle East
SaudiArabia3-14	KT033709	Africa-Europe-Middle East
SaudiArabia4-14	KT033713	Africa-Europe-Middle East
SaudiArabia5-14	KT033715	Africa-Europe-Middle East
SaudiArabia6-14	KT355023	Africa-Europe-Middle East
SaudiArabia7-14	KU248482	Africa-Europe-Middle East
SouthKorea10-09	GU126513	Southeast Asia
SouthKorea1-08	HM130912	Southeast Asia
SouthKorea11-09	JQ013090	Southeast Asia
SouthKorea12-09	JQ013091	Southeast Asia
SouthKorea13-09	JQ013089	Southeast Asia
SouthKorea14-10	AB613208	Southeast Asia
SouthKorea15-10	AB613209	Southeast Asia
SouthKorea16-11	AB636411	Southeast Asia
SouthKorea17-11	AB636264	Southeast Asia
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SouthKorea19-11	AB636412	Southeast Asia
SouthKorea20-11	AB669434	Southeast Asia
SouthKorea2-08	HM130913	Southeast Asia
SouthKorea21-11	AB636409	Southeast Asia
SouthKorea22-12	JX961665	Southeast Asia
SouthKorea23-12	JX961666	Southeast Asia
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SouthKorea25-12	KF225312	Southeast Asia
SouthKorea26-12	JX961668	Southeast Asia
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SouthKorea28-16	KY111368	Southeast Asia
SouthKorea29	HM856909	Southeast Asia
SouthKorea30	HM856911	Southeast Asia
SouthKorea3-08	HM130914	Southeast Asia
SouthKorea31	HM856912	Southeast Asia
SouthKorea32	JN183873	Southeast Asia
SouthKorea33	HM856913	Southeast Asia
SouthKorea34	HM856914	Southeast Asia
SouthKorea35	HM856915	Southeast Asia

SouthKorea36	HM856917	Southeast Asia
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SouthKorea38	JN183872	Southeast Asia
SouthKorea39	JN183874	Southeast Asia
SouthKorea40	JN183875	Southeast Asia
SouthKorea4-08	HM856873	Southeast Asia
SouthKorea41	JN183876	Southeast Asia
SouthKorea42	JN183879	Southeast Asia
SouthKorea43	HM856910	Southeast Asia
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SouthKorea6-09	GQ141873	Southeast Asia
SouthKorea7-09	GU325634	Southeast Asia
SouthKorea8-09	GU325632	Southeast Asia
SouthKorea9-09	GU325633	Southeast Asia
Spain1-00	AJ489258	Africa-Europe-Middle East
Spain2-03	KC953602	Africa-Europe-Middle East
Spain3-11	KT099158	Africa-Europe-Middle East
Spain4-11	KT099157	Africa-Europe-Middle East
Spain5-97	AF071228	Africa-Europe-Middle East
Spain6-99	AF271234	Africa-Europe-Middle East
Spain7-99	AJ519441	Africa-Europe-Middle East
Sudan1-07	GU180085	Africa-Europe-Middle East
Sudan2-11	JX483704	Africa-Europe-Middle East
Sudan3-11	JX483705	Africa-Europe-Middle East
Sudan4-11	JX483707	Africa-Europe-Middle East
Sudan5-11	JX483708	Africa-Europe-Middle East
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Sudan7-96	AY044138	Africa-Europe-Middle East
Sudan8-96	AY044139	Africa-Europe-Middle East
Sweden-09	HF548825	Africa-Europe-Middle East
Tunisia-05	EF101929	Africa-Europe-Middle East
Turkey-04	AJ812277	Africa-Europe-Middle East
USAArizona-06	EF210554	Americas
USACalifornia-07	EF539831	Americas
USAFlorida10-15	KY971333	Americas
USAFlorida11-15	KY971332	Americas
USAFlorida1-15	KY971326	Americas

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USAGeorgia7-15	KY971354	Americas
USAGeorgia8-15	KY971362	Americas
USAGeorgia9-15	KY971363	Americas
USAHawaii1-09	GU322424	Oceania
USAHawaii2-09	GU322423	Oceania
USAHawaii3-10	HM988987	Oceania
USAPuertoRico-01	AY134494	Americas
USATexas-06	EF110890	Americas
Venezuela-09	KF477277	Americas

Appendix B. List of the recombinants detected by RDP4.

Name	Geographic Region
BurkinaFaso1-09	Africa-Europe-Middle East
BurkinaFaso2-09	Africa-Europe-Middle East
BurkinaFaso3-09	Africa-Europe-Middle East
BurkinaFaso4-09	Africa-Europe-Middle East
Cameroon1-07	Africa-Europe-Middle East
Cameroon2-07	Africa-Europe-Middle East
Cameroon3-07	Africa-Europe-Middle East
Cameroon4-07	Africa-Europe-Middle East
Ethopia-03	Africa-Europe-Middle East
Ghana-08	Africa-Europe-Middle East
Iran10-06	Africa-Europe-Middle East
Iran1-06	Africa-Europe-Middle East
Iran11-06	Africa-Europe-Middle East
Iran15-07	Africa-Europe-Middle East
Iran17-07	Africa-Europe-Middle East
Iran18-07	Africa-Europe-Middle East
Iran19-07	Africa-Europe-Middle East
Iran2-06	Africa-Europe-Middle East
Iran22-09	Africa-Europe-Middle East
Iran25-10	Africa-Europe-Middle East
Iran27-10	Africa-Europe-Middle East
Iran28-10	Africa-Europe-Middle East
Iran3-06	Africa-Europe-Middle East
Iran31-10	Africa-Europe-Middle East
Iran32-11	Africa-Europe-Middle East

Iran33-11	Africa-Europe-Middle East
Iran34-11	Africa-Europe-Middle East
Iran35-11	Africa-Europe-Middle East
Iran36-11	Africa-Europe-Middle East
Iran37-11	Africa-Europe-Middle East
Iran38-11	Africa-Europe-Middle East
Iran39-11	Africa-Europe-Middle East
Iran40-11	Africa-Europe-Middle East
Iran4-06	Africa-Europe-Middle East
Iran41-11	Africa-Europe-Middle East
Iran42-11	Africa-Europe-Middle East
Iran43-11	Africa-Europe-Middle East
Iran44-11	Africa-Europe-Middle East
Iran45-11	Africa-Europe-Middle East
Iran46-11	Africa-Europe-Middle East
Iran47-11	Africa-Europe-Middle East
Iran48-11	Africa-Europe-Middle East
Iran52-13	Africa-Europe-Middle East
Iran53	Africa-Europe-Middle East
Iran54	Africa-Europe-Middle East
Iran55	Africa-Europe-Middle East
Iran6-06	Africa-Europe-Middle East
Iran7-06	Africa-Europe-Middle East
Israel3	Africa-Europe-Middle East
Italy1-04	Africa-Europe-Middle East
Italy2-04	Africa-Europe-Middle East
Jordan2-08	Africa-Europe-Middle East
Jordan5-13	Africa-Europe-Middle East
Jordan6	Africa-Europe-Middle East
Jordan7	Africa-Europe-Middle East
Jordan8	Africa-Europe-Middle East
Kuwait3-12	Africa-Europe-Middle East
Kuwait4-12	Africa-Europe-Middle East
Lebanon1	Africa-Europe-Middle East
Mali	Africa-Europe-Middle East
Morocco12-13	Africa-Europe-Middle East
Morocco13-14	Africa-Europe-Middle East
Morocco14-14	Africa-Europe-Middle East
Morocco15-14	Africa-Europe-Middle East
Morocco16-14	Africa-Europe-Middle East
Morocco18-14	Africa-Europe-Middle East

Morocco19-14	Africa-Europe-Middle East
Morocco20-14	Africa-Europe-Middle East
Morocco22	Africa-Europe-Middle East
Morocco3-10	Africa-Europe-Middle East
Morocco4-12	Africa-Europe-Middle East
Morocco5-12	Africa-Europe-Middle East
Morocco6-12	Africa-Europe-Middle East
Morocco7-12	Africa-Europe-Middle East
Morocco8-12	Africa-Europe-Middle East
Morocco9-13	Africa-Europe-Middle East
Oman10-11	Africa-Europe-Middle East
Oman11-11	Africa-Europe-Middle East
Oman12-11	Africa-Europe-Middle East
Oman13-11	Africa-Europe-Middle East
Oman14-11	Africa-Europe-Middle East
Oman15-11	Africa-Europe-Middle East
Oman16-11	Africa-Europe-Middle East
Oman17-11	Africa-Europe-Middle East
Oman18-11	Africa-Europe-Middle East
Oman2-05	Africa-Europe-Middle East
Oman25-12	Africa-Europe-Middle East
Oman26-12	Africa-Europe-Middle East
Oman28-13	Africa-Europe-Middle East
Oman29-13	Africa-Europe-Middle East
Oman30-13	Africa-Europe-Middle East
Oman3-05	Africa-Europe-Middle East
Oman31-13	Africa-Europe-Middle East
Oman34-13	Africa-Europe-Middle East
Oman35-13	Africa-Europe-Middle East
Oman36-13	Africa-Europe-Middle East
Oman37-13	Africa-Europe-Middle East
Oman38-13	Africa-Europe-Middle East
Oman39-13	Africa-Europe-Middle East
Oman4-05	Africa-Europe-Middle East
Oman41-13	Africa-Europe-Middle East
Oman42-13	Africa-Europe-Middle East
Oman43-13	Africa-Europe-Middle East
Oman44-13	Africa-Europe-Middle East
Oman45-13	Africa-Europe-Middle East
Oman46-13	Africa-Europe-Middle East
Oman47-13	Africa-Europe-Middle East

Oman48-13	Africa-Europe-Middle East
Oman49-13	Africa-Europe-Middle East
Oman50-13	Africa-Europe-Middle East
Oman5-05	Africa-Europe-Middle East
Oman51-13	Africa-Europe-Middle East
Oman52-13	Africa-Europe-Middle East
Oman53-13	Africa-Europe-Middle East
Oman54-13	Africa-Europe-Middle East
Oman55-13	Africa-Europe-Middle East
Oman56-13	Africa-Europe-Middle East
Oman57-13	Africa-Europe-Middle East
Oman58-13	Africa-Europe-Middle East
Oman60-13	Africa-Europe-Middle East
Oman6-05	Africa-Europe-Middle East
Oman61-13	Africa-Europe-Middle East
Oman62-13	Africa-Europe-Middle East
Oman63-13	Africa-Europe-Middle East
Oman64-13	Africa-Europe-Middle East
Oman65-13	Africa-Europe-Middle East
Oman66-13	Africa-Europe-Middle East
Oman68-13	Africa-Europe-Middle East
Oman69-14	Africa-Europe-Middle East
Oman7-05	Africa-Europe-Middle East
Oman71-14	Africa-Europe-Middle East
Oman72	Africa-Europe-Middle East
Oman8-05	Africa-Europe-Middle East
Oman9-11	Africa-Europe-Middle East
Portugal1-01	Africa-Europe-Middle East
Portugal2-01	Africa-Europe-Middle East
Portugal4-95	Africa-Europe-Middle East
ReunionIsland2	Africa-Europe-Middle East
SaudiArabia1-12	Africa-Europe-Middle East
SaudiArabia2-13	Africa-Europe-Middle East
SaudiArabia3-14	Africa-Europe-Middle East
SaudiArabia4-14	Africa-Europe-Middle East
SaudiArabia5-14	Africa-Europe-Middle East
SaudiArabia6-14	Africa-Europe-Middle East
SaudiArabia7-14	Africa-Europe-Middle East
Spain5-97	Africa-Europe-Middle East
Spain6-99	Africa-Europe-Middle East
Spain7-99	Africa-Europe-Middle East

Sudan1-07	Africa-Europe-Middle East
Sudan2-11	Africa-Europe-Middle East
Sudan3-11	Africa-Europe-Middle East
Sudan4-11	Africa-Europe-Middle East
Sudan5-11	Africa-Europe-Middle East
Sudan6-94	Africa-Europe-Middle East
Sudan7-96	Africa-Europe-Middle East
Sudan8-96	Africa-Europe-Middle East
Sweden-09	Africa-Europe-Middle East
Cuba1-11	Americas
Cuba2-11	Americas
DominicanRepublic1-11	Americas
DominicanRepublic2-11	Americas
Venezuela-09	Americas
Japan10-13	SE Asia
Japan15	SE Asia
Japan16	SE Asia
Japan17	SE Asia
Japan18	SE Asia
Japan19	SE Asia
Japan20	SE Asia
Japan21	SE Asia
Japan22	SE Asia
Japan5-07	SE Asia
NewCaledonia1-10	Oceania

Appendix C. List of genomes in the without-recombinants data set.

Name	Accession
Australia10-06	KX347102
Australia1-03	KX347094
Australia11-06	KX347103
Australia12-06	KX347104
Australia13-06	KX347105
Australia14-06	KX347106
Australia15-06	KX347107
Australia16-06	KX347108
Australia17-06	KX347109
Australia18-06	KX347110
Australia19-06	KX347111
Australia20-06	KX347112

Australia2-06	GU178819
Australia21-06	KX347113
Australia22-06	KX347114
Australia23-06	KX347115
Australia24-06	KX347116
Australia25-06	KX347117
Australia26-06	KX347118
Australia27-06	KX347119
Australia28-06	KX347120
Australia29-06	KX347121
Australia30-06	KX347122
Australia3-06	KX347095
Australia31-06	KX347123
Australia32-06	KX347124
Australia33-06	KX347125
Australia34-06	KX347126
Australia35-06	KX347127
Australia36-06	GU178814
Australia37-06	GU178813
Australia38-06	GU178818
Australia39-06	GU178816
Australia40-06	GU178815
Australia4-06	KX347096
Australia41-06	GU178817
Australia42-06	GU178820
Australia43-07	KX347128
Australia44-07	KX347129
Australia45-07	KX347130
Australia46-09	KX347131
Australia47-09	KX347132
Australia48-09	KX347133
Australia49-09	KX347134
Australia50-09	KX347135
Australia5-06	KX347097
Australia51-09	KX347136
Australia52-09	KX347137
Australia53-09	KX347138
Australia54-10	KX347139
Australia55-10	KX347140
Australia56-10	KX347141
Australia57-10	KX347142

Australia58-10	KX347143
Australia59-10	KX347144
Australia60-10	KX347145
Australia6-06	KX347098
Australia7-06	KX347099
Australia8-06	KX347100
Australia9-06	KX347101
China100-12	JQ807735
China10-08	GU199587
China101-12	JX669541
China102-12	JX669542
China103-12	JX669543
China104-12	JX669544
China105-12	JX856172
China1-06	AM698119
China106-12	JX856173
China107-12	JX910534
China108-12	JX997799
China109-12	JX997800
China110-12	JX997801
China11-08	FJ646611
China111-12	JX997802
China112-12	KC312655
China113-12	KC312656
China114-12	KC312657
China115-12	KC312658
China116-12	KC312659
China117-12	KC312660
China118-12	KC312661
China119-12	KC312662
China120-12	KC312663
China12-08	FN256257
China121-12	KC312664
China122-12	KC312665
China123-12	KC312666
China124-12	KC312667
China125-12	KC312668
China126-12	KC312669
China127-12	KC312670
China128-12	KC312671
China129-12	KC312672

China130-12	KC312673
China13-08	GU434142
China131-12	KC428753
China132-12	KC702798
China133-12	KC810892
China134-12	KC852147
China135-12	KC852149
China136-12	KC852150
China137-12	KC999844
China138-12	KC999845
China139-12	KF612971
China140-12	KJ125410
China14-08	GU434144
China141-12	KJ140787
China142-12	KJ140788
China143-13	KF990604
China144-13	KJ879949
China145-13	KJ879950
China146-13	KM506948
China147-13	KM506949
China148-13	KM506950
China149-13	KM506951
China150-13	KM506952
China15-09	GU348995
China151-13	KM506953
China152-13	KM506954
China153-13	KM506955
China154-13	KM506956
China155-13	KM506957
China156-13	KM506958
China157-13	KM435321
China158-13	KM435319
China159-13	KM435323
China160-13	KM435325
China16-09	HM208334
China161-13	KM435327
China162-13	KM506947
China163-13	KF356163
China164-13	KF906542
China165-13	KF990604
China166-13	KJ125411

China167-13	KJ546418
China168-13	KJ754186
China169-13	KJ754187
China170-13	KJ754188
China17-09	GU563330
China171-13	KJ754189
China172-13	KJ754190
China173-13	KJ754191
China174-13	KJ754192
China175-13	KJ754193
China176-13	KJ754194
China177-13	KJ879948
China178-13	KU892717
China179-14	KX034538
China180-14	KX034539
China18-09	GU951436
China181-14	KX034541
China182-14	KX034542
China183-14	KX034543
China184-14	KX034546
China185-14	KX034547
China186-14	KX034550
China187-14	KX034551
China188-14	KP684146
China189-14	KP685598
China190-14	KM435320
China19-09	GU951437
China191-14	KM435322
China192-14	KM435324
China193-14	KM435326
China194-14	KM435328
China195-14	KJ850344
China196-14	KT338293
China197-14	KT338294
China198-14	KT852577
China199-14	KU934104
China200-14	KU975396
China20-09	GU983859
China201-14	KU975397
China202-14	KU975398
China203-14	KU975399

China204-15	KX034540
China205-15	KX034544
China2-06	AM282874
China206-15	KX034545
China207-15	KX034548
China208-15	KX034549
China209-15	KX034553
China210-15	KT338295
China21-09	HM043732
China211-15	KT338296
China212-15	KU760888
China213-15	KU760889
China214-15	KU760890
China215-15	KU760891
China216-15	KU760892
China217	EU031444
China218	FN650808
China219	GQ352537
China220	GQ352538
China22-09	HM358879
China221	KC999850
China222	KX034552
China223	HQ702861
China224	HQ702862
China225	HQ702863
China226	JQ004028
China227	KC999849
China23-09	HM627880
China24-09	HM627882
China25-09	HM627883
China26-10	HM627881
China27-10	HM627884
China28-10	HM627885
China29-10	JF301667
China30-10	JF301668
China3-06	AM698117
China31-10	JF414236
China32-10	JF414237
China33-10	JF727878
China34-10	JF817218
China35-10	JF833036

China36-11	JQ038233
China37-11	JQ038240
China38-11	JX070043
China39-11	JX456640
China40-11	JX456641
China4-06	AM698118
China41-11	JX456642
China42-11	JX456644
China43-11	JQ411237
China44-11	KM506959
China45-11	KM506960
China46-11	KC138544
China47-11	KC138545
China48-11	KC138546
China49-11	JX456638
China50-11	JX456639
China5-07	FN256256
China51-11	JX456643
China52-11	KC138543
China53-11	JX456637
China54-11	JF964959
China55-11	JN412854
China56-11	JN990922
China57-11	JN990923
China58-11	JN990924
China59-11	JN990925
China60-11	JN990926
China6-07	GU111505
China61-11	JN990927
China62-11	JN990928
China63-11	JQ004045
China64-11	JQ004046
China65-11	JQ004047
China66-11	JQ004048
China67-11	JQ004049
China68-11	JQ004050
China69-11	JQ004051
China70-11	JQ004052
China7-07	FN252890
China71-11	JQ034613
China72-11	JQ038232

China73-11	JQ038234
China74-11	JQ038235
China75-11	JQ038236
China76-11	JQ038237
China77-11	JQ038238
China78-11	JQ038239
China79-11	JQ326957
China80-11	JQ867092
China8-07	FN256258
China81-11	JX070042
China82-11	JX070044
China83-11	JX070045
China84-11	JX128100
China85-11	JX997798
China86-11	KC999851
China87-12	JX128099
China88-12	JX675237
China89-12	KC211184
China90-12	KC702796
China9-08	FN256259
China91-12	KC702797
China92-12	KC852151
China93-12	KC999846
China94-12	KC999847
China95-12	KC999848
China96-12	KM506961
China97-12	KJ140788
China98-12	KJ140789
China99-12	KJ140787
CostaRica1-12	KF533857
CostaRica2-12	KF533856
CostaRica3-12	KF533855
Cuba3-11	KM926625
Cuba4-11	KM926626
Cuba5	AJ223505
DominicanRepublic3-94	AF024715
Egypt1-14	KT921303
Egypt2	AY594174
Egypt3	EF107520
Estonia-08	HF548826
Grenada-07	FR851297

Guatemala-06	GU355941
Iran12-06	KX347155
Iran13-06	KX347156
Iran14-06	KX347158
Iran16-07	GU076444
Iran20-07	KX347157
Iran21-08	KX347159
Iran23-09	KX347162
Iran24-09	KX347163
Iran26-10	JQ928346
Iran29-10	KX347160
Iran30-10	KX347161
Iran49-12	KX347164
Iran50-12	KX347165
Iran5-06	GU076441
Iran51-12	KX347166
Iran8-06	GU076452
Iran9-06	GU076453
Iraq-11	JQ354991
Israel1-89	X15656
Israel2	X15656
Italy3-04	DQ144621
Japan1-05	AB192965
Japan11	AB110217
Japan12	AB116629
Japan13	AB116630
Japan14	AB116631
Japan2-05	LC099965
Japan3-07	AB363566
Japan4-07	AB439841
Japan6-11	KJ585666
Japan7-11	KJ466047
Japan8-11	KJ466048
Japan9-11	KJ585666
Jordan10	EF433426
Jordan1-08	GQ861426
Jordan3-11	JX444575
Jordan4-11	JX131286
Jordan9	EF054893
Kuwait1-08	JF451352
Kuwait2-10	KJ830841

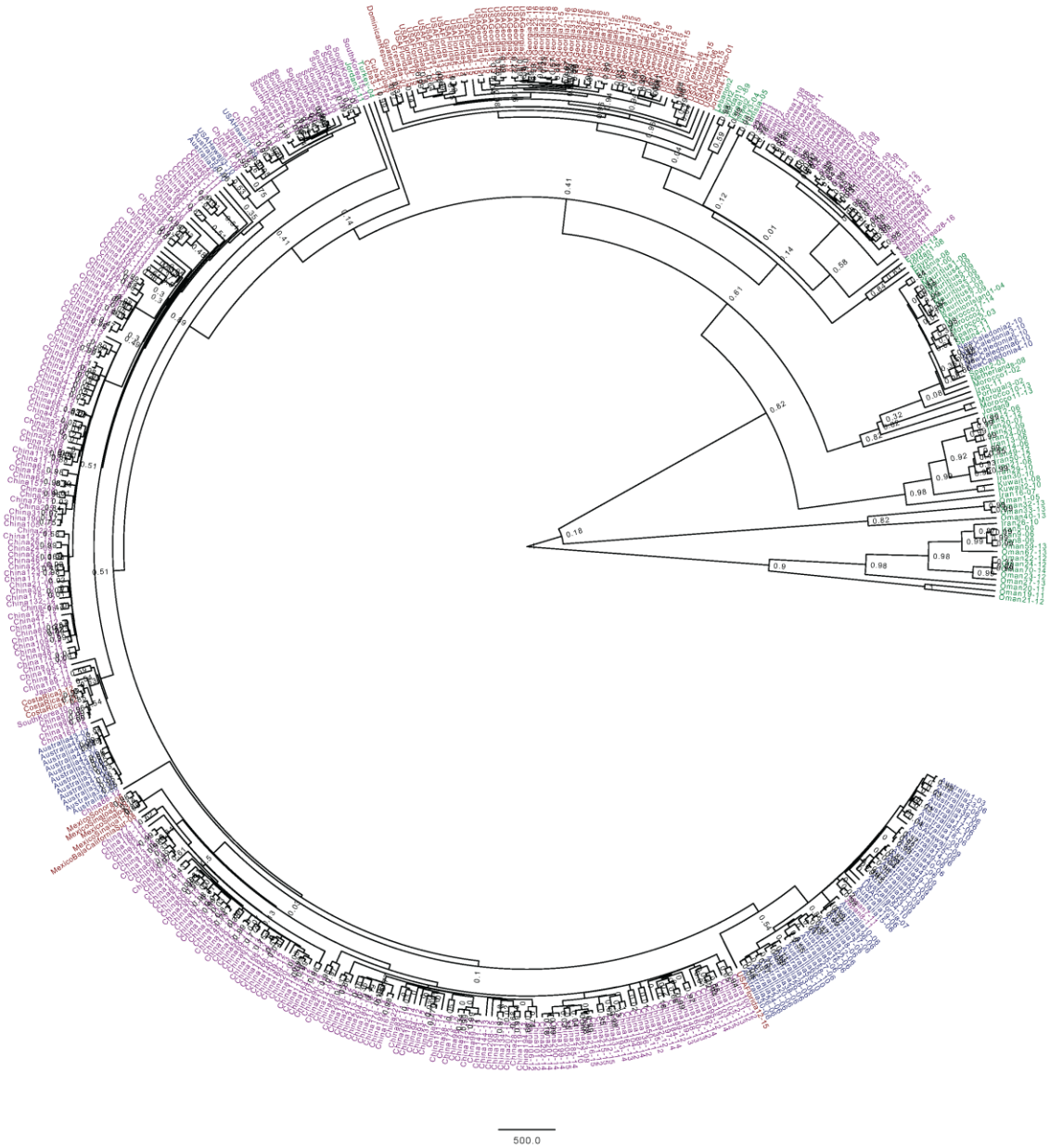
Lebanon2	EF051116
Mauritius1-09	HM448447
Mauritius2-09	KX347167
Mauritius3-09	KX347168
Mauritius4-09	KX347169
Mauritius5-09	KX347170
Mauritius6-09	KX347171
Mauritius7-09	KX347172
Mexico1-11	JQ303121
Mexico2-11	JN680353
MexicoBajaCaliforniaSur-08	HM459851
MexicoSinaloa1-06	FJ012358
MexicoSinaloa2-06	DQ631892
MexicoSinaloa3	EF523478
MexicoSonora-06	EF210555
Morocco10-13	LN846614
Morocco1-02	LN846617
Morocco11-13	LN846613
Morocco17-14	LN846615
Morocco2-03	LN846616
Morocco21	EF060196
Netherlands-08	FJ439569
NewCaledonia2-10	HE603246
NewCaledonia3-10	HE603244
NewCaledonia4-10	HE603243
NewCaledonia5-10	HE603242
NewCaledonia6-10	HE603241
Oman1-05	FJ956700
Oman19-11	HE819241
Oman20-11	HE819240
Oman21-12	KF229726
Oman22-12	KF229725
Oman23-12	KF229724
Oman24-12	KF229723
Oman27-13	HG941641
Oman32-13	HG969258
Oman33-13	HG969259
Oman40-13	KF260969
Oman59-13	HG969256
Oman67-13	HG941642
Oman70-14	LN680631

Portugal3-02	JN859138
ReunionIsland1-04	AM409201
SouthKorea10-09	GU126513
SouthKorea1-08	HM130912
SouthKorea11-09	JQ013090
SouthKorea12-09	JQ013091
SouthKorea13-09	JQ013089
SouthKorea14-10	AB613208
SouthKorea15-10	AB613209
SouthKorea16-11	AB636411
SouthKorea17-11	AB636264
SouthKorea18-11	AB636410
SouthKorea19-11	AB636412
SouthKorea20-11	AB669434
SouthKorea2-08	HM130913
SouthKorea21-11	AB636409
SouthKorea22-12	JX961665
SouthKorea23-12	JX961666
SouthKorea24-12	JX961667
SouthKorea25-12	KF225312
SouthKorea26-12	JX961668
SouthKorea27-12	JX961669
SouthKorea28-16	KY111368
SouthKorea29	HM856909
SouthKorea30	HM856911
SouthKorea3-08	HM130914
SouthKorea31	HM856912
SouthKorea32	JN183873
SouthKorea33	HM856913
SouthKorea34	HM856914
SouthKorea35	HM856915
SouthKorea36	HM856917
SouthKorea37	HM856919
SouthKorea38	JN183872
SouthKorea39	JN183874
SouthKorea40	JN183875
SouthKorea4-08	HM856873
SouthKorea41	JN183876
SouthKorea42	JN183879
SouthKorea43	HM856910
SouthKorea44	HM856916

SouthKorea45	HM856918
SouthKorea46	HQ260984
SouthKorea47	JN183878
SouthKorea5-08	JN680149
SouthKorea6-09	GQ141873
SouthKorea7-09	GU325634
SouthKorea8-09	GU325632
SouthKorea9-09	GU325633
Spain1-00	AJ489258
Spain2-03	KC953602
Spain3-11	KT099158
Spain4-11	KT099157
Tunisia-05	EF101929
Turkey-04	AJ812277
USAArizona-06	EF210554
USACalifornia-07	EF539831
USAFlorida10-15	KY971333
USAFlorida11-15	KY971332
USAFlorida1-15	KY971326
USAFlorida12-15	KY971328
USAFlorida13-15	KY971327
USAFlorida14-15	KY971323
USAFlorida15-15	KY971322
USAFlorida2-15	KY971325
USAFlorida3-15	KY971320
USAFlorida4-15	KY971324
USAFlorida5-15	KY971321
USAFlorida6-15	KY971337
USAFlorida7-15	KY971336
USAFlorida8-15	KY971335
USAFlorida9-15	KY971334
USAGeorgia10-15	KY971361
USAGeorgia11-15	KY971360
USAGeorgia1-15	KY971368
USAGeorgia12-15	KY971359
USAGeorgia13-15	KY971355
USAGeorgia14-15	KY971365
USAGeorgia15-15	KY971369
USAGeorgia16-15	KY971356
USAGeorgia17-15	KY971371
USAGeorgia18-15	KY971367

USAGeorgia19-15	KY971353
USAGeorgia20-16	KY971352
USAGeorgia21-16	KY971351
USAGeorgia2-15	KY971366
USAGeorgia22-16	KY971350
USAGeorgia23-16	KY971349
USAGeorgia24-16	KY971348
USAGeorgia25-16	KY971347
USAGeorgia26-16	KY971346
USAGeorgia27-16	KY971343
USAGeorgia28-16	KY971342
USAGeorgia29-16	KY971344
USAGeorgia30-16	KY971372
USAGeorgia31-16	KY971345
USAGeorgia3-15	KY971365
USAGeorgia32-16	KY971338
USAGeorgia33-16	KY971341
USAGeorgia34-16	KY971340
USAGeorgia35-16	KY971339
USAGeorgia4-15	KY971364
USAGeorgia5-15	KY971358
USAGeorgia6-15	KY971357
USAGeorgia7-15	KY971354
USAGeorgia8-15	KY971362
USAGeorgia9-15	KY971363
USAHawaii1-09	GU322424
USAHawaii2-09	GU322423
USAHawaii3-10	HM988987
USAPuertoRico-01	AY134494
USATexas-06	EF110890

Appendix D. Bayesian tree constructed with the without-recombinants data set – circular tree. Genomes are color-coded by region: green is Africa-Europe-Middle East, blue is Oceania, pink is Southeast Asia, and red is Americas.



Appendix E. Bayesian tree constructed with the with-recombinants data set – circilar view. Genomes are color-coded by region: green is Africa-Europe-Middle East, blue is Oceania, pink is Southeast Asia, and red is Americas.