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CENTRO DE CIÊNCIAS AGRÁRIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM
PRODUÇÃO VEGETAL E BIOPROCESSOS ASSOCIADOS

DISTRIBUTION AND GENETIC DIVERSITY OF *Xylella fastidiosa* ASSOCIATED WITH OLIVE QUICK DECLINE IN SOUTHEASTERN BRAZIL

NÁGELA GOMES SAFADY

Araras
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SUMÁRIO

LIST OF TABLES

Page

Chapter 1

Chapter 2

Chapter 3

Appendix

LIST OF FIGURES

Chapter 3

Final considerations

DISTRIBUTION AND GENETIC DIVERS ITY OF *Xylella fastidiosa* ASSOCIATED WITH OLIVE QUICK DECLINE IN SOUTHEASTERN BRAZIL

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ABSTRACT

Xylella fastidiosa (XF) is a bacterium capable of colonizing a large number of plants, but is mainly known to cause disease in economically important crops such as citrus, grapevines, coffee and more recently olive trees. XF colonizes the xylem vessels, forming biofilm and thus blocking the passage of sap and water from roots to leaves. General symptoms related to diseases caused by these bacteria are leaves wilt, burning and subsequent leaf necrosis, leaves chlorosis and in same pathosystem plant death. The bacterium, which until recently was restricted to the American continent, in 2013 was reported in southeast Italy causing severe symptoms in olive trees leaving to dead of over one milion plants and infected eleven million. This desease was call as Olive Quick Decline Syndrome (OQDS). Since then XF has also been reported in other European countries, such as France, Spain and Portugal, infecting sweet cherry, almond, lavender and ornamental plants. Lately, in mid-2016, similar symptoms as observed in disease olive plants in Italy were also found in olive plants in the state of Minas Gerais in Brazil. The identification of XF in symptomatic olive trees in Brazil leave us to some questions about the; geographic distribution of infected plants, genetic diversity of pathogen, and natural infectivity of possible vectors involved on natural transmission. Therefore, the objetives of this work were; 1. to analyze the distribution of XF in olive orchards in southeastern Brazil; 2. to determined the genetic diversity of strains isolated from symptomatic trees; 3. to verify the natural infectivity of possible vectors. Targeting the objectives 1 and 2 aproximatley 500 samples with OQDS-like symptoms were analized for the presence of XF by using specific primers and bacterial isolation in agar medium growth following the genetic diversity studies. Those samples were sampled from twenty-five orchards located in 15 cities of states of São Paulo and Minas Gerais plus one from Espirito Santo. XF was identified in 21 of the 26 orchards analyzed. A total of 204 isolates were cultured from the symptomatic samples. Twenty-one representative strains of whole population were typed by both Multilocus Sequence type (MLST) and Simple Sequence Reapts (SSR) and the total population by the last methodology. Sixty-five percent of strains were identified as ST16 (previsouly reported in coffee plants) but others already described such as ST70 (from in hibiscus) and ST13 (from citrus) were found causing OQDS in olive plants. Beside these, new STs like the 84, 85 and 86, all belonging to subsp. *pauca* of *X. fastidiosa* were also identified in disease olive plants. Population genetic analysis using twelve SSR markers clustered the total population (204 strains) from 16 different orchards representing 12 different municipalities in only three subpopulations. Except the strains from SPA1, MG18, MGA7, MG10 and MGA4 all the remaining strains were placed in a specific subpopulation. In the SPA1 (S22°42'10.71"/W45°41'32.91") was

found the largest number of private alleles and heterozygosis and also two different STs (84 and 86). The unbiased heterozygosity (H_{Nei}), ranged from 0 to 0.53 (Avg 0.20). Analyzes of possible migration routes have shown the orchards MG-A1 and MG-A2 as the largest migrant donors. *X. fastidiosa* was detected in at least 37% of potential vectors in commercial orchard SP-A1.

Key words: vector-borne disease, bacteria, perennial crop, OQDS, *Olea europaea*

DISTRIBUIÇÃO E DIVERSIDADE GENÉTICA DE *Xylella fastidiosa* ASSOCIADA A SÍNDROME DO DECLÍNIO RÁPIDO DA OLIVEIRA NO SUDESTE BRASILEIRO

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RESUMO

A *Xylella fastidiosa* (XF) é uma bactéria capaz de colonizar um grande número de plantas, mas é mais conhecida por causar doenças em culturas economicamente importantes, como frutas cítricas, videiras, café e, mais recentemente, oliveiras. O XF coloniza os vasos do xilema, formando biofilme e, assim, bloqueando a passagem de seiva e água das raízes para as folhas. Os sintomas gerais relacionados com doenças causadas por estas bactérias são as folhas murchas, queimadas e subsequentemente a necrose das folhas, a clorose das folhas e, no mesmo patossistema, a morte das plantas. A bactéria, que até recentemente era restrita ao continente americano, em 2013 foi relatada no sul da Itália, causando sintomas severos em oliveiras deixando mortos de mais de um milhão de plantas e infectados onze milhões. Esta doença foi chamada de Síndrome de Declínio Rápido de Oliva (OQDS). Desde então, XF também tem sido relatado em outros países europeus, como França, Espanha e Portugal, infectando cerejeira, amendoeira, lavanda e plantas ornamentais. Ultimamente, em meados de 2016, sintomas semelhantes aos observados em plantas olivícolas na Itália também foram encontrados em olivais no estado de Minas Gerais no Brasil. Portanto, os objetivos deste trabalho foram; 1. analisar a distribuição de XF em pomares de oliva no sudeste do Brasil; 2. Determinar a diversidade genética de cepas isoladas de plantas sintomáticas; 3. verificar a infecciosidade natural de possíveis vetores. Tendo como alvo os objetivos 1 e 2, aproximadamente 500 amostras com sintomas do tipo OQDS foram analisadas quanto à presença de XF utilizando iniciadores específicos e isolamento bacteriano em meio de crescimento em ágar seguindo os estudos de diversidade genética. Essas amostras foram coletadas de 25 pomares localizados em 15 municípios dos estados de São Paulo e Minas Gerais e um do Espirito Santo. XF foi identificada em 21 dos 26 pomares analisados. Um total de 204 isolados foram cultivados a partir das amostras sintomáticas. 21 cepas representativas da população total foram diagnosticadas tanto por marcadores Multilocus Sequence Type (MLST) quanto por Simple Sequence Reapts (SSR) e a população total pela última metodologia. Sessenta e cinco por cento das cepas foram identificadas como ST16 (anteriormente relatadas em cafeeiros), mas outras já descritas, como ST70 (em hibisco) e ST13 (citros), foram encontradas causando OQDS em plantas de oliva. Além destes, novos STs como os 84, 85 e 86, todos pertencentes à subespécie. *pauca* de *X. fastidiosa* também foram identificados em plantas olivícolas. A análise genética populacional utilizando doze marcadores SSR agrupou a população total (204 cepas) de 16 pomares diferentes representando 12 municípios diferentes em apenas três subpopulações. Com excessão das amostras coletadas das localidades SPA1, MGA4, MGA10, MG18 e MGA7, todas as cepas restantes foram colocadas em apenas uma subpopulação específica. Em SP-A1 (S22

° 42'10.71 "/ W45°41'32.91") foi encontrado o maior número de alelos privados e heterozigose e também dois diferentes STs (84 e 86). A heterozigose não-viesada de Nei (HNei) variou de 0 a 0,53 (média de 0,20). Análises de possíveis rotas migratórias mostraram os pomares MG-A1 e MG-A2 como os maiores doadores migrantes. Insetos vetores potentiais transmissores de *X. fastidiosa* na área SP-A1 apresentaram taxa infectividade ao redor de 37%.

Palavras-chave: insetos vetores, bactérias, culturas perenes, OQDS, *Olea europaea*

INTRODUCTION

Olive tree (*Olea europaea* L.), a fruit tree that belongs to the Oleaceae family, has originated from the shores of the Mediterranean Sea and is now grown in several other locations where the climate is favorable, such as Italy, Portugal, South Africa, China, Australia, Mexico, United States, Peru, Chile and Brazil (IOC, 2007). The Mediterranean climate is the most appropriate for growing olive trees, with annual average temperature varying from 15 to 25^{o}C. For fruiting the temperature should vary between 25 and 35^{o}C, also requiring a period of drought during flowering whereas the average of temperature should be around 15^{o}C (VIEIRA NETO et al., 2008; ALBA et al.2013).

The introduction of olive trees in Brazil occurred in the 1800s, but difficulties such as the lack of knowledge of management, non-adaptation of the cultivar to the climatic conditions and cultivation of only one genetic variety contributed to the insussesfull on consolidation of this crop. However, in the last decades, advances in the olive tree studies in Brazil have allowed the implantation of this crop in regions with favorable microclimate, such as montain chains in southeastern and plateaus at soulth region of southeastern Brazil (ALBA et al., 2013). Nowadays, Rio Grande do Sul in south Brazil is the main state producer, followed by the states of Minas Gerais and São Paulo in the southeastern. The olive tree cultivated area of Rio Grande do Sul is approximately 3.464 ha (IBRAOLIVA, 2019) while in São Paulo and Minas Gerais, the commercial production of olive trees, which is mostly limited to the high-altitude regions, covers about 70 municipalities, totaling 400,000 plants in 1000 ha (BERTONCINI; TERAMOTO; PRELA-PANTANO, 2010). This regions know as “Serra da Mantiqueira” is about 500 km long, with altitudes varying from 1000 to 2,798 meters and microclimate that provides sufficient hours of cold needed for olive trees flowering (ALBÉRICO ALVARENGA et al., 2017).

In Minas Gerais, the first olive trees were introduced in the city of Maria da Fé in 1930 by Portuguese immigrants (BERTONCINI; TERAMOTO; PRELA-PANTANO, 2010). Although initially olive cultivation has not been expanded due to lack of knowledge and inadequate implantation, currently the olive cultivation covers approximately 50 municipalities in Minas Gerais, with 180 olive growers (SMA, 2017). This spread is due to the initiative of the Minas Gerais Research and Agricultural Company (EPAMIG), a national reference in studies with olive trees in the country, which has an extensive active olive tree germplasm bank with 68 cultivars (VIEIRA NETO et al., 2008). In the State of São Paulo, olive trees were introduced by the São Paulo State Department of Agriculture and Food Supply (SAA) in the city of São Bento do Sapucaí (BERTONCINI, 2012). The cultivated area currently encompasses municipalities such as Campos do Jordão and neigbours county, Águas da Prata, Santo Antônio do Pinhal, Monte Verde, Silveiras, and Cunha (BERTONCINI; TERAMOTO; PRELA-PANTANO, 2010).

The olive fruit is used for consumption *in natura* or oil extraction. Olive oil is called Extra Virgin Olive Oil (EVOO) when produced entirely by mechanical means, under low temperature avoiding nutrient degradation and without adding any solvents. Many benefits of EVOO consumption for human health are known, for example incidence reduction of diabetes type II (SALAS-SALVADÓ et al., 2011), induction of cancer cell death (ESCRICH; SOLANAS; MORAL, 2014; LEGENDRE; BRESLIN; FOSTER, 2015) and prevention of stroke in people 65 and older (SAMIERI et al., 2011). The consumption of olive oil has been increasing over the years due to awareness campaigns of its health benefits and the contribution of different aromas and flavors in the gastronomy (WEST, M., 2019). Spain, Italy, and the United States are the largest olive oil consumers and Spain, Italy, Greece and Portugal, the main producer, responsible for two-thirds of world production. In terms of imports, the United States, Brazil, and Japan are the three largest in the world, having imported in 2018 310,000, 78,000, and 55,000 tons, respectively (IOOC, 2018). In 2018 the total world olive oil consumption and production was approximately 3,000,000 tons (IOOC, 2018). Although per capita consumption of olive oil in Brazil is still low in relation to other countries such as Portugal, the consumption of olive oil in Brazil increased by 50% in the last ten years. Brazil is the second largest importer of olive oil and table olive in the world. Exampling, in 2018 110 thousand tons of table olive and 78 thousand tons of olive oil were imported, almost 30 thousand tons more compared to the year 2015

(IOOC, 2018). As an expanding market and with high commercial value this product has become an important alternative for small farmers in Brazil. According to the Association of Olives Growers of Mantiqueira (Assoolive) and the Brazilian Institute of Olive Growing (Ibraoliva), in the 2017 Brazil produced 105 thousand liters of olive oil and in 2019 the total production was around 230 thousand liters (IBRAOLIVA, 2019). The quality of olive oil produce in Brazil has increasing also. Recently, May 2019, in the New York World Olive Oil Competition, the world's largest and most prestigious olive oil contest with 26 countries participating, Brazilian olive oils were awarded with eight gold medals, two silver medals, 11 bronze medals and, a Best in Class award (BEST OLIVE OILS, 2019). As a whole, olive culture is a promising business opportunity for the small farmers established in highland regions, such as the mountainous areas at the Serra da Mantiqueira.

The expansion of olive oil industry in Brazil found problems than can be associated with non-adaptated varieties to tropical clime (warm temperature and humidity) resulting in irregular flowering and frutification as well as the occurrence of phytopathogenic diseases, mainly caused by fungi, among them, cercospora leaf spot (*Pseudocercospora cladosporioides*), Anthracnose (*Colletrotrichum* sp.); and, Verticillium (*Verticillium dahliae*). However, recently, the bacteria *Xylella fastidiosa* was diagnosticated in some olive plants with leaves dissecation in the Serra da Mantiqueira (COLETTA-FILHO et al. 2016) whose symptoms were similar to described to a disease known as Olive Quick Decline Sindrome (OQDS). First reported in southeastern Italy and associated to presence of *X. fastidiosa* in symptomatic plants (SAPONARI et al., 2013), this disease spread quickly around that region. By 2015 it had already affected approximately one million olive trees causing major economic damage, infecting mainly centennial olive trees from the region of Apulia (SAPONARI et al., 2013). Lately, Saponari et al. (2017) confirmed through extensive experimentation the association of OQDS symptoms with infection of the bacteria *Xylella fastidiosa*, as did that the plant-to-plant transmission occur mainly through leafhopper insects (*Philaenus spumarius*), which feed on the olive xylem (CARIDDI et al., 2014).

However, information about *Xylella fastidiosa* and olive tree patossystem in Brazil was still completely unknown. Therefore, the spatial distribution of bacteria in producing regions in southeastern Brazil, prevalence of strains and associated symptoms, as well as possible sources of origin were the object of study of this research.

OBJECTIVES

General objectives:

I. Evaluate the spatial distribution of *Xylella fastidiosa* in olive plants in the different producing regions of the states of São Paulo and Minas Gerais as also the natural infectivity of a vector population;

II. Verify the occurrence of genetic variability of the bacterium *Xylella fastidiosa*, present in different varieties of olive trees with symptoms of OQDS in municipalities of the state of São Paulo and Minas Gerais;

III. Define possible migratory routes between the different producing regions, inferring about possible sources of origin of the primary infection.

Hypotheses:

(i) *X. fastidiosa* is narrowly spread in a few olive orchards in southeastern region of Brazil

(ii) *X. fastidiosa* subsp. *pauca* is the unique subsp. infecting olive trees in Brazil

(iii) A clonal population of the bacteria is infecting the olive orchards

(iv) Natural transmission by insect vectors is occuring in the olive orchards

LITERATURE REVIEW

1. Xylella fastidiosa: new disease of old pathogen

Xylella fastidiosa is a Gram-negative bacterium with fastidious growth habits specifically in the xylem vessels of host plants and digestive tracts of insect vectors (JANSE; OBRADOVIC, 2010). *X. fastidiosa* is known for its ability to infect and colonize many cultivated, ornamental and wild plants, which includes more than 350 plant species, but most of the host plants are asymptomatic (JANSE; OBRADOVIC, 2010; EFSA, 2016). Bacterial population growth and the formation of polysaccharide matrix in the xylem vessels associated with dissemination through the vessels membranes causes blockage and reduces sap flow in the xylem (ALMEIDA et al., 2005; HILL, 1995). This blockade leads to the appearance of symptoms related to hydric stress such as leaf wilting with progressive scorching in the majority of successful colonization and plant stunting .

The main vector of this bacteria under natural conditions are insects popularly known as sharpshooter leafhoppers (Cicadellidae subfamily Cicadellinae) and spittlebugs (Cercopoidea, families Aphrophoridae, Cercopidae, and Clastopteridae), that, when fed on the xylem of *X. fastidiosa* infected plant, acquires the bacterium and transmits to other plants later on (ALMEIDA et al., 2005; JANSE; OBRADOVIC, 2010). Transmission is possible because the bacterium can adhere, multiply and form a persistent biofilm in the interior of the foregut of vectors, more precisely to the cuticular lining of the precibarium and cibarium. Although *X. fastidiosa* forms a biofilm on the vector's foregut, there is no latent period needed for transmission and the bacterium can be transmitted by the vector immediately after acquisition (PURCELL; FINLAY, 1979). Strong evidence indicates that environmental conditions may influence the establishment of the infection or even to act on the remission of symptoms (HENNEBERGER et al., 2004). An example of this environmental effect on the *X.*

fastidiosa - host patosystem, although not reproduced under controlled conditions, is the minor latency period, high severity and broad distribution of CVC in the North / Northwest region of São Paulo compared to the southeastern region (COLETTA-FILHO et al., 2013). The long latency period of *X. fastidiosa* in most of susceptible host could be contributed for the transmission of this pathogen for long distance by infected vegetative material undoubtedly. The natural transmission by the cosmopolitan vector associate to recombinant feature of *X. fastidiosa* shows an opportunity for this bacterium to colonize new environment and hosts (COLETTA-FILHO; BITTLESTON; ALMEIDA, 2011).

The first report of *X. fastidiosa* causing problems in crops of economic interest occurred in vineyards in California in 1887, causing the disease known as Pierce's disease (PD) (HEWITT, 1958; DAVIS; PURCELL; THOMSON, 1978). Nowadays is know that PD is caused by *X. fastidiosa* subsp. *fastidiosa*. Diseases caused by *X. fastidiosa* used to occur strictly in the Americas (HOPKINS; PURCELL, 2002; REDAK et al., 2004). However, recently diseases caused by this bacterium has been reported in other continents such as the Asiatic by SU et al., (2012) in Taiwan and by Amanifar et al., (2014) in Iran, and in Europa by Saponari et al., (2013). *X. fastidiosa* was first reported in Europe, specifically in southeast Italy where the bacterium is responsible by the Olive Quick Decline Syndrome (OQDS) in olive tress (SAPONARI et al., 2013). Following, *X. fastidiosa* was reported in Spain, Portugal, and France, mostly in ornamental and wild plants (EPPO, 2019). Population analysis showed that a single genotype of the *X. fastidiosa* subsp. *pauca* (ST53) was related to disease in olive trees in Italy and its introduction was associated with the entrance of imported infected plant material from Central America, specifically ornamental coffee trees (GIAMPETRUZZI et al., 2017; LEGENDRE et al., 2015; MARCELLETTI; SCORTICHINI, 2016). On the other hand, in France and Spain distinct *X. fastidiosa* genotypes were detected (*X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *sandy*) suggesting that multiples introductions may have occurred (DENANCÉ et al., 2017; LANDA; MARCO-NOALES; LÓPEZ, 2017).

In Brazil, the first report of *X. fastidiosa* causing disease in crop was done by FRENCH and KITAJIMA (1978) in prunus (*Prunus domestica*) whereas the subsp. *multiplex* is the main agent. Lately, the *X. fastidiosa* subsp. *pauca* was described in sweet orange trees (*Citrus sinensis*) causing the Citrus Variegated Chlorosis (CVC), disease the caused one of the greatest economic damage to the Brazilian citrus

industry. Typical symptoms of CVC consist of irregular chlorotic spots along the leaf that subsequently evolve to necrotic lesions. At an advanced stage of the disease the fruits present with small size, hardened and early maturation (BOVÉ, AYRES, 2007). Beyond that, *X. fastidiosa* subsp. *pauca* was reported in coffee tress causing symptoms like shortening of the internodes, leaf abscission with retention offew narrow-shaped leaves at the most distal ending and also the reduction in fruit size (PARADA-FILHO et al., 1995; LIMA et al., 1998). More recently *X. fastidiosa* subsp. *pauca* was also identified in olive trees in the Serra da Mantiqueira region (in the states of São Paulo and Minas Gerais) (COLETTA-FILHO et al., 2016) whose symptoms are similar to observed in olive trees in southeast Italy, that are drying of branches with wilted, yellowed and dried leaves; Progressive leaf desiccation (burn) beginning at the apex towards the base of the leaves (Figure 1) Results of studies aiming to understand this pathosystem will be shown in the Chapters 1, 2 and 3 bellow.

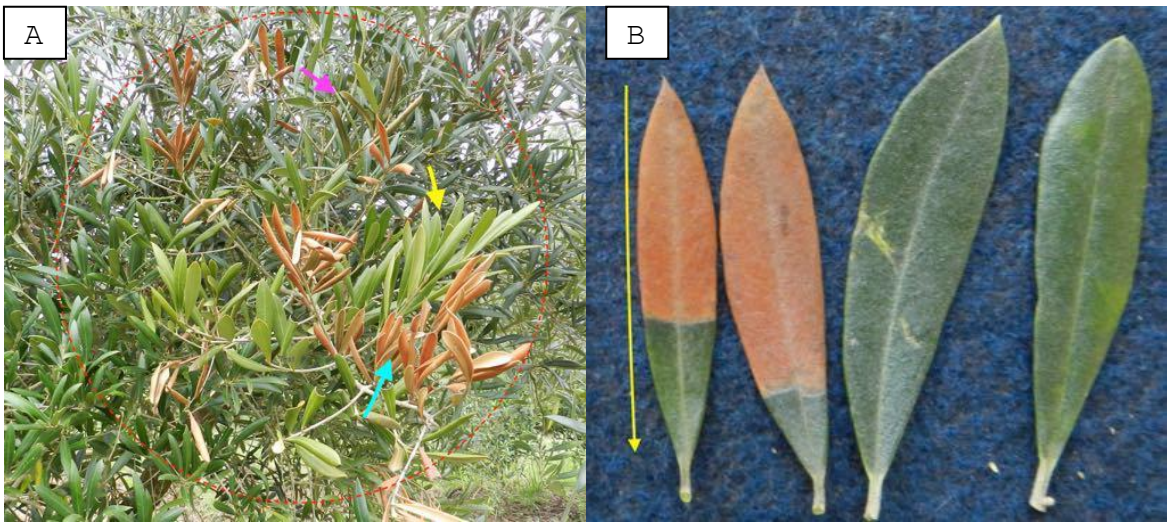

Figure 1: OQDS symptoms observed in olive plants in Serra da Mantiqueira, southeastern Brazil. **A**. dryness of branches with yellowish leaves (yellow pointer), withered (purple pointer) and dried (blue pointer). **B.** Progressive leaf desiccation beginning at the apex towards the base of the leaves.

2. Xylella fastidiosa: genetic markers in inter and intra subspecies

Xylella fastidiosa is currently classified into five different subspecies with biological and genetic differences, usually associated with the specific hosts. They are: subspecies i. *fastidiosa*, initially found in grapevines, but also present in *Prunus dulcis*,

Medicago sativa, among others; ii. *sandyi*, associated with *Nerium oleander*; iii. multiplex, associated with *Quercus* spp., *Ulmus americana, Platanus occidentalis*, *Liquidambar styraciflua, Carya illinoinensis, Prunus* sp.; iv. *pauca*, associated with *Citrus sinensis, Coffea arabica*, and *Olea europaea*; and *v. morus* associated with *Morus rubra* (ALMEIDA; NUNNEY, 2015). The genetic classification of *X. fastidiosa* in subspecies was initially proposed through 16S rDNA gene sequences and DNA-DNA hybridization (SCHAAD et al., 2004). However, new data were incorporated into the classification using multigenic sequences known as Multi Locus Sequence Types (MLST) (SCHUENZEL et al., 2005; YUAN et al., 2010a). MLST is a technique that monitors variations in internal fragments of genes of basic functions and constant expression level (housekeeping), thus conserved inside the species (ENRIGHT; SPRATT, 2009). Any mutation that occurs in these sequences (substitution, addition, or deletion of at least one nucleotide) is considered as a different allele. The allelic profile of a strain is defined by the analysis of all loci and each allelic profile defines a Sequence Type – ST (FEIL et al., 2004). In *X. fastidiosa* seven housekeeping genes are used to determine the allelic profile: *leuA* responsible for amino acid biosynthesis, *petC* associated with electron transport, *mal*F responsible for the transport of carbohydrates, *cysG* gene responsible for biosynthesis of heme and porphyrin, *hol*C associated with replication, *nuo*L associated with aerobic respiration and *gltT* associated with the transport of amino acids (SCALLY et al., 2005). All information regarding MLST in *X. fastidiosa* is available in the database "*Xylella fastidiosa* MLST website" (https://pubmlst.org/xfastidiosa/), a platform developed by Keith Jolley and located at the University of Oxford, UK (JOLLEY; CHAN; MAIDEN, 2004). In this platform, it is possible to obtain all the alleles of the seven housekeeping genes in FASTA format, as well as the ST of each allelic profile. New alleles and ST of *X. fastidiosa* identified in population studies in different parts of the world are constantly added to this platform, and it is assigned a sequential number to the later added to the MLST database. For each new allele and ST is assigned a sequential number to the number to the later added.

Although the MLST technique is very useful for subspecies analysis, it provides little information at the species or subspecies level (ALMEIDA et al., 2008). For studies at the level of strains (intra-subspecies) the markers based on Short Sequence Repeats (SSR) have a higher resolution and have been used successfully in studies of populations of *X. fastidiosa* (ALMEIDA et al., 2008; COLETTA-FILHO;

BITTLESTON; ALMEIDA, 2011; COLETTA-FILHO; FRANCISCO; ALMEIDA, 2014; FRANCISCO et al., 2017; LI et al., 2004). These markers are characterized by short nucleotide sequences (1 to 15 base pairs), repeated in tandem and are frequently found in eukaryotic and prokaryotic genomes. SSR are highly polymorphic markers and is characterized by the presence of multiple alleles (ELLEGREN, 2004). SSR is assumed to be neutral molecular marker, therefore, free of selection effect when located in non-coding regions of the genome. Their frequency and distribution reflect the mutational processes that occur in the population (ELLEGREN, 2004). The occurrence of variability may occur by slipped-strand mispairing in combination with inadequate DNA mismatch repair pathways (MRAZEK; GUO; SHAH, 2007). The evolution of the microsatellites happens through Stepwise Mutation Model- SMM, in which mutations in this region involve the deletion or insertion of a repeat unit (motif) (KIMURA; OTA, 1975). Difference in length of SSR can be used to determine the relatedness of individuals. More genetically related individuals would present smaller differences in SSR fragment length than less related individuals (LI et al., 2004; VAN BELKUM et al., 1998). SMM is thought to be an important source of bacterial variability and adaptation (review in VAN BELKUM et al., 1998). The identification of differences in fragments length is possible because the DNA sequences bordering the repeats are generally conserved, which can be explained by an entirely gene duplication. (VAN BELKUM et al., 1997). Examples of the use of SSR markers in genetic studies of populations of *X. fastidiosa* can be found for subsp. *fastidiosa* infecting vines in the Napa Valley-California region where 83 different genotypes were found in 93 isolates, showing a high degree of polymorphism (COLETTA-FILHO; BITTLESTON; ALMEIDA, 2011). In the spatiotemporal analysis of the population of *X. fastidiosa* in sweet orange with CVC in Brazil, it was shown that the bacterial population is structured in multiple scales (micro and macro). Of the isolates analyzed, 45% presented different genotypes (COLETTA-FILHO; FRANCISCO; ALMEIDA 2014). The association of neutral and housekeeping molecular markers such as VNTR and MLST have been widely applied in studies aiming to understand the molecular ecology and evolution of *Xylella fastidiosa* in different environments. Studying genetics of plant pathogen populations is important because it offers the opportunity to understand evolutionary processes involved in agroecological pathosystems and outline possible pathogen control strategies.

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CHAPTER 1. Distribution and genetic diversity of *Xylella fastidiosa* based on housekeeping genetic markers

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1. Abstract

In Brazil, the host expansion of *Xylella fastidiosa* subsp. *pauca* was recently demonstrated with the report of diseased olive trees (O*lea europaea*), whose symptoms were associated with olive quick decline syndrome (OQDS) previously described in Southeast Italy. We employed both PCR-based techniques and culture medium isolation to investigate the geographic distribution of *X. fastidiosa* as well as the genetic signatures of 21 strains isolated from 11 olive orchards in both São Paulo and Minas Gerais states in Brazil. *X. fastidiosa* subsp. *pauca* was detected in 83% of the orchards examined in the region and was positively diagnosed in 43.7% of all sampled plants with typical scorching symptoms. Of the 21 strains characterized with fast-evolving microsatellite (SSR) markers, 20 different multilocus microsatellite genotypes (MLMGs) were observed with the overall allelic diversity of H_{Nei} = 0.38. Principal component analysis (PCoA) using the SSR markers clustered all strains, except for three, in one cluster evidencing limited range genetic diversity. Multilocus sequence typing (MLST) analysis showed the prevalence of a sequence type (ST16) in 75% of the samples. Three novel STs (84, 85, and 86) belonging to *X. fastidiosa* subsp. *pauca* cluster were detected. These results denote that genetically diverse strains of *X. fastidiosa* subsp. *pauca* are widely present in olive orchards in Southeastern Brazil, which is consistent with the long history of this bacterium in that region.

2. Introduction

The xylem-inhabiting bacterium *Xylella fastidiosa* is the etiological agent of some of the most important plant diseases that have recently emerged worldwide (ALMEIDA; NUNNEY 2015). This pathogen can infect over 350 plant taxa, but not always causing disease symptoms (EFSA, 2016). Furthermore, the bacterium plant-to-plant dispersion occurs naturally by hundreds of species polyphagous xylem-sap feeding insects (SICARD et al. 2018). Also, environmental conditions (PURCELL, 2013) and agricultural practices (SICARD et al. 2018) could affect the severity of the diseases caused by *X. fastidiosa*. The species *X. fastidiosa* is divided in five clades (subspecies) based on DNA-DNA hybridization, ITS sequences, and more recently based on multilocus sequence typing (MLST) using the partial sequences of seven housekeeping genes (SCHAAD et al. 2004; YUAN et al. 2010; https://pubmlst.org/xfastidiosa/). The *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *sandyi* and X. *fastidiosa* subsp. *morus* were first found in North America (SCALLY et al. 2005; NUNNEY et al. 2013), albeit subsp. *multiplex* is also present in South America and subsp. *fastidiosa* is hypothesized to have originated from Central America (COLETTA-FILHO et al., 2017; NUNNEY et al. 2010). On the other hand, *X. fastidiosa* subsp. *pauca* was identified as being restricted to South America, infecting citrus and coffee plants (SCALLY et al. 2005). However, recent studies demonstrated that subsp. *pauca* has expanded to different regions and hosts in Europe and Central America (NUNNEY et al. 2014b; SAPONARI et al. 2017). The risks of introducing new *X. fastidiosa* strains in areas already experiencing epidemics of this pathogen favors an increase in genetic diversity, the emergence of new diseases and host range expansion (SICARD et al., 2018). Moreover, human-altered natural habitats could induce evolutionary changes in pathogens that may also drive the invasion of plant pests to novel hosts (HUFBAUER et al. 2012). The unintentional introduction of *X. fastidiosa* subsp. *fastidiosa* by anthropogenic activities

by moving exotic plant species to a new geographic area in combination with favorable environmental conditions resulted in the Pierce's disease of grapevine outbreak in Southeast California, as discussed by Nunney et al. (NUNNEY et al. 2010). Recently a new outbreak caused by the *X. fastidiosa* subsp. *pauca* has affected olive trees in Southeast Italy. Evidence indicates that the introduction of infected plant material carrying a single *X. fastidiosa* genotype and favorable environmental conditions were sufficient for the emergence of the olive quick decline syndrome (OQDS) disease (SICARD et al. 2018). The young olive oil industry in southeastern Brazil occupies areas located at high altitudes, such as the mountain chain known as "Serra da Mantiqueira", with small orchards surrounded by native vegetation. This scenery could favor the migration of *X. fastidiosa* from native vegetation to crops (PURCELL; SAUNDERS, 1999). Albeit olive crops are a recent agricultural activity, *X. fastidiosa* subsp. *pauca* has already been associated with olive plants sampled in Brazil (COLETTA-FILHO et al., 2016). Due to the Brazilian populations of *X. fastidiosa* subsp. *pauca* are genetically diverse (COLETTA-FILHO et al. 2017), and the favorable environment conditions in olive orchards for pathogen reproduction and spreading, it is necessary to investigate the spatial distribution of these bacteria through in the olive production areas in southeastern Brazil and the genetic diversity of the strains present in plants with OQDS-like symptoms. Here we present data on the genetic diversity and dispersion of *X. fastidiosa* in olive orchards in Southeastern Brazil.

3. Material and Methods

3.1 Sampling

A total of 395 plants from olive orchards located in the southeastern region of Brazil, São Paulo and Minas Gerais states, were sampled at different altitudes and variable landscape vegetation for diagnosis of *X. fastidiosa* (Table 1). Only plants with suspicious symptoms of OQDS like described by Saponari et al. (2017) were selected. Branches with three to five millimeters in diameter containing suspicious symptomatic leaves, i.e., scorching symptoms, were transferred to the laboratory for diagnosis, bacterial isolation, and molecular typing of *X. fastidiosa*

3.2 *Xylella fastidiosa* diagnoses and isolation

The presence of *X. fastidiosa* in the samples was determined by PCR using the RST 31/33 *X. fastidiosa*-specific primer set (MINSAVAGE, et al. 1994) with amplification conditions specified by the authors; approximately 100 ng of total DNA was used in a final volume of 20 µL per reaction. For DNA extraction we used petiole and midribs of green leaves sampled from asymptomatic portions of symptomatic branches, using a CTBA-based protocol adapted from Murray and Thompson (1985). *X. fastidiosa* was tentatively isolated using the same portion of olive tissue used for the PCR-based diagnosis. First, the branches were superficially disinfested with 70% ethanol for 2 min, immersed in a 2% bleach solution for 2 minutes, and washed 3 times in sterilized water. After dried on a sterile bench in a laminar flow hood, the branches were cut in the middle, the internal cut ends were squeezed and the sap was blotted onto BCYE agar plates (WELLS et al. 1981), followed by incubation at 28°C for 15 to 30 days. The identification as *X. fastidiosa* was carried out based on *in vitro* fastidious growth, convex format and whiteness of colonies, which were streaked it onto PD3 agar plates and incubated at 28°C for 7 days, following PCR assays using the RST 31/33 primers for confirmation the authenticity of bacterium.

3.3 *Xylella fastidiosa* typing

The single sequence repeats (SSR) and multilocus sequence typing (MLST) methodologies were used to type 21 strains of *X. fastidiosa* isolated from olive trees from 11 different orchards at nine geographic regions. The number of strains used for typing was defined based on proportion of successful isolation from each geographic area, where each isolate represents one infected olive plant. The bacteria were triple passaged on axenic PD3 medium, and total DNA was extracted using the Wizard DNA extraction kit (Promega Corporation, Madison, WI). Twelve previously described SSR loci (LIN et al. 2005; FRANCISCO et al. 2017) were grouped in four sets, and the amplifications were optimized to amplify *X. fastidiosa* DNA using multiplexed primers (Table 2- Chapter 2). The primer-set were selected based on subspecies-specificity of SSR in *X. fastidiosa* (COLETTA-FILHO et al., 2014), as well as, the presence of multiple peaks based on previous tests. Before fragment analysis, the reactions were diluted 20x in sterile milli-Q water, and 1 µL was added into 10 µL of Hi-Di formamide and 0.1 µL of the GeneScan™ 500-LIZ Size Standard (both from Life Technologies, Foster City, CA), and the mixture loaded onto the gel. Capillary electrophoresis was run on an ABI 3730XL DNA sequencer (Life Technologies), and the peak size in base

pairs was estimated by Peak Scanner version 1.0 software (Life Technologies). For the MLST methodology, seven loci (*leuA, petC, malF, cysG, holC, nuoL,* and *gltT*) were amplified from triple passage *X. fastidiosa* cells in axenic medium using the same methodology reported by Yuan et al. (YUAN et al. 2010). An aliquot of the reaction was used to confirm of the amplicon size in agarose gels (1%), and the remaining (approximately 20 µL) sample was used for PCR cleanup using the ExoSAP- IT kit (Thermo Fisher Scientific, Whalthan, MA). The amplicon was directly sequenced on both strands (ABI 3130 sequencer, Life Technologies). The forward and reverse sequences of each isolate were assembled by the CLC Genomics Workbench platform (QIAGEN). SNPs were manually checked on the electropherograms.

3.4 Genetic analysis.

The allele size for each SSR locus was used to define the haplotypes within the set of *X. fastidiosa* isolates. The same multilocus microsatellite genotypes (MLMGs) were considered as a clone or haplotype as determined by GENODIVE version 2.0b23 (MEIRMANS; VAN TIENDEREN 2004). Nei's measure of genetic diversity corrected for the population size was calculated as $H = [(1 - \sum pi_2) \times (n/n-1)]$, where p_i was the frequency of allele *i* at the locus and n was the number of individuals (NEI, 1978) determined using the SSR locus results. The principal coordinate analysis (PCoA) was used to plot patterns within a multivariate SSR dataset (e.g., multiple loci and multiple samples) by using GeneAlex version 6.5 (PEAKALL; SMOUSE, 2012). Reference sequences of each MLST locus was downloaded from the *X. fastidiosa* MLST database (<http://pubmlst.org/xfastidiosa/>) and aligned with the sequences obtained i this work using Mega v.6.06 software (TAMURA et al. 2013). The MLST profile of each isolate was defined using the information described in the *X. fastidiosa* MLST databases (http://pubmlst.org/xfastidiosa/). The identification of a new allele for each MLST locus, as well as the construction of a new allelic profile, which was assigned a sequence type (ST) number, were performed according to the instructions found at http://pubmlst.org/xfastidiosa and generously provided by the curator of MLST database, Dr. Lenard Nunney (UC Riverside). Concatenated sequences of the seven MLST loci were used to determine the genetic relationships among all known *X. fastidiosa* strains and novel STs obtained in this study. The phylogenetic reconstructions were based on Bayesian interferences using the Markov chain Monte

Carlo method with 100,000 interactions and 1,000 bootstraps using MrBayes v.3.2 (HUELSENBECK ; RONQUIST, 2001). Hasegawa-Kishino-Yano with an invariable site and a gamma-distributed rate (HKY+I+G) was defined as the best-fit model of DNA evolution by PAUP v.4 (SWOFFORD, 2002) and used in this analysis. To represent the genetic relationship among *X. fastidiosa* STs, we built a NeighborNet phylogenetic network using SplitTree v.4 (HUSON; Bryant 2006). The genetic distances among *X. fastidiosa* STs were inferred using the neighbor-joining method implemented in Mega v.6.06 software (TAMURA et al. 2013). The evolutionary distances were computed using HKY+I+G and 1,000 bootstraps. The genetic relationship of the new alleles found for the genes *cysG* and *nuoL* were also reconstructed using the neighbor-joining method with 1,000 bootstraps implemented in Mega v.6.06 software.

3.5 Nucleotide sequence accession numbers

All MLST data are available at the *Xylella fastidiosa* MLST database. The gene sequences for the novel MLST alleles obtained in the present study are available at GenBank under the following accession numbers: *cys*G allele 34 = MH706701 and *nuo*L allele 20 =MH706702.

4. Results

4.1 Distribution of *X. fastidiosa* and symptoms.

A total of 24 different olive orchards located in geographic regions with altitudes ranging from 800 to 1780 m were evaluated for the presence of disease symptoms similar to those described for OQDS. Samples (n = 395) were collected from diseased branches and the asymptomatic portions were used for diagnosis. *X. fastidiosa* detected in 20 out of 24 sampled orchards (83%) against a successful isolation of 55.1% (13 off 24 orchards) (Table 1, Figure 1). Although the olive trees sampled from Minas Gerais areas A6, A7, A8, A14, A15, and A17 were PCR-positive for *X. fastidiosa*, the bacterial isolation in artificial culture medium was unsuccessful. On the other hand, PCR-negative diagnosis was obtained for some samples from Minas Gerais areas A10, A13, and A18 but the isolation of *X. fastidiosa* was very successful (Table 1). Taking into account, the overall *X. fastidiosa* incidence was 43.7%, considering both PCR and axenic isolation methods, which means that the regular leaf scorch is no

specific OQSD symptoms in Brazil. Moreover, 82% of the samples with positive diagnosis for *X. fastidiosa* showed OQDS foliar symptoms identical to those previously described in Southeast Italy (SAPONARI et al. 2017), such as leaves wilting followed by scorching from the apex to the base and later, a general leaf scorching, always restrict to few branches on canopy at the beginning of disease. Another 18% of the *X. fastidiosa*-positive samples showed wilted and/or yellowish coloring leaves but no desiccated, which could denote the initial stages of infection (Figure 2). Samples from Minas Gerais areas A1 to A4 correspond with 34% of total analyzed as consequence of higher number of OQDS-like symptomatic plants resulting in infection rate ranging from 2.7 to 84.2% (Table 1). Although 100% of *X. fastidiosa* infection were obtained for samples collected in Minas Gerais areas A8, A15, A17 and São Paulo area A3, the limited number of sampled plants in these areas may have introduced bias into the diagnosis (Table 1). At the same trend, the few samples taken from Minas Gerais A11 and A16 areas and from São Paulo-A5 could have introduced bias on negative results for presence of *X. fastidiosa* in these areas.

Table 1. General information about *Xylella fastidiosa* (XF) subsp. *pauca* diagnosis and surveyed olive orchards in southeastern Brazil.

Origin (State/area])[a]	**Latitude / Longitude**	**Altitude (m)**	**Number of plants[b]**	**Xf positive plants[c]**	**Plants with Xf isolation[d]**	**Landscape Vegetation**
MG- A1	22º18'56.55''/ 45º22'30.49''	1300	57	48 (84.2)	16	Araucaria forest
MG- A2	22º18'51.67''/ 45º22'40.82''	1310	22	16 (72.7)	4	Araucaria forest and stone fruits
MG- A3	22º18'45.01''/ 45º22'23.40''	1329	23	5 (21.7)	1	Araucaria forest
MG- A4	22º18'45.01''/ 45º22'23.40''	1318	33	19 (57.5)	4	Araucaria forest
MG- A5	22°35'46.45''/ 45°55'25.88''	1235	39	6 (15.3)	2	Araucaria and ombrophilous forests
MG- A6	22°10' 47.57''/ 44°36'17.71''	1200	14	7 (50.0)	0	Araucaria and ombrophilous forests
MG- A7	22°59'52.60''/ 44°40'27.31''	1400	4	0 (0.0)	0	Araucaria and ombrophilous forests
MG- A8	22°01'5.55''/44°39'32.90''	1500	4	4 (100)	0	Araucaria and ombrophilous forests
MG- A9	22°01'8.58''/44°37'23.17''	1300	9	6 (66.6)	0	Araucaria and ombrophilous forests
MG- A10	22°36'53.30''/ 45° 24'32.87''	1780	10	0 (0.0)	**1**	Araucaria and ombrophilous forests

MG- A11	22°30'29.87"/ 44°18'7.09"	1250	1	0 (0.0)	0	Ombrophilous forests
MG- A12	21°57'31.16"/ 46° 21'19.96"	1380	12	7 (58.3)	0	Ombrophilous forests, grass
MG- A13	21°1'54.44"/4 6°35'3.77"	1050	12	1 (8.3)	**3**	Araucaria and ombrophilous forests
MG- A14	22°18'16.85"/ 44°58' 34.03"	1065	3	1 (33.3)	0	Small area of ombrophilous forests, grasses
MG- A15	20°41'7.35"/4 3°28' 9.34"	854	1	1 (100)	0	Ombrophilous forests
MG- A16	19°40'20.63"/ 43°35'11.67"	987	1	0 (0.0)	0	Ombrophilous forests
MG- A17	20°17'10.78"/ 43°48'37.02"	883	1	1 (100)	1	Ombrophilous forests, grasses
MG- A18	22°35'8.84"/4 5°50'33.50"	1329	6	0 (0.0)	**3**	Araucaria and ombrophilous forests
SP- A1	22°42'10.71"/ 45°41'32.91"	1602	40	16 (40.0)	4	Araucaria and ombrophilous forests
SP- A2	23°45'37.30"/ 47°44'45.47"	690	60	18 (30.0)	3	Small area of tropical forests
SP- A3	22°37'41.72"/ 44°53'55.70"	615	5	5 (100.0)	NR	Ombrophilous forests, grasses
SP- A4	22°30'13.54"/ 46°36'45.39"	1054	2	0 (0.0)	NR	Araucaria forest
SP- A5	23°31'13.64"/ 46°56'11.21"	755	20	3 (15.0)	1	No vegetation, grasses
SP- A6	23°16'22.23"/ 47°9'9.42"	883	16	3 (18.7)	2	Pine forest, eucalyptus, grasses
Overall			395	167 (43.7)	45	

[a] Areas (orchards) in Minas Gerais and São Paulo states of Brazil used for sampling.
[b] Total number of tested plants showing olive quick decline syndrome (OQDS) like-symptoms.
[c] Number of *Xylella fastidiosa* PCR-positive plants using the RST31/RST33 primers. The percentage is shown in brackets.
[d] Number of plants with successful *X. fastidiosa* isolation in BCYE medium. *X. fastidiosa* with a positive isolation but
negative PCR amplification are shown in bold.
[e] NR - no tentative isolation

[f] Considering both positive PCR amplification and successful isolation of *X. fastidiosa*

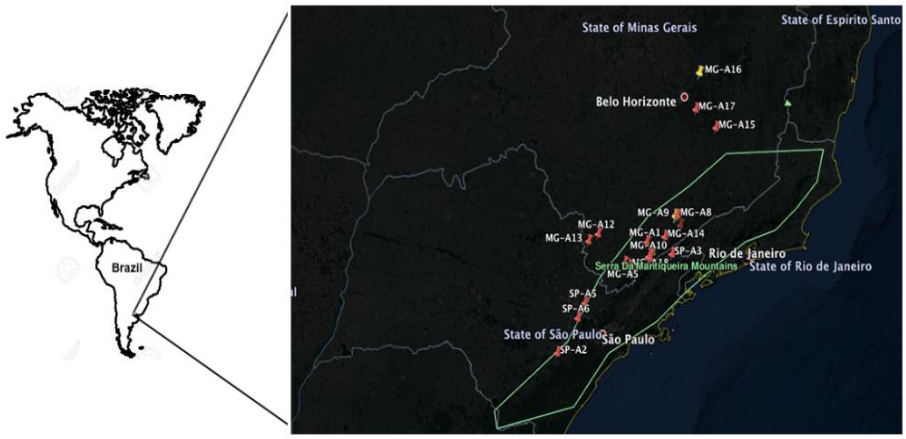

Figure 1. Sampling site locations in the São Paulo and Minas Gerais States of Brazil. The red and yellow pins indicate *X. fastidiosa*-positive and -negative olive orchards, respectively.

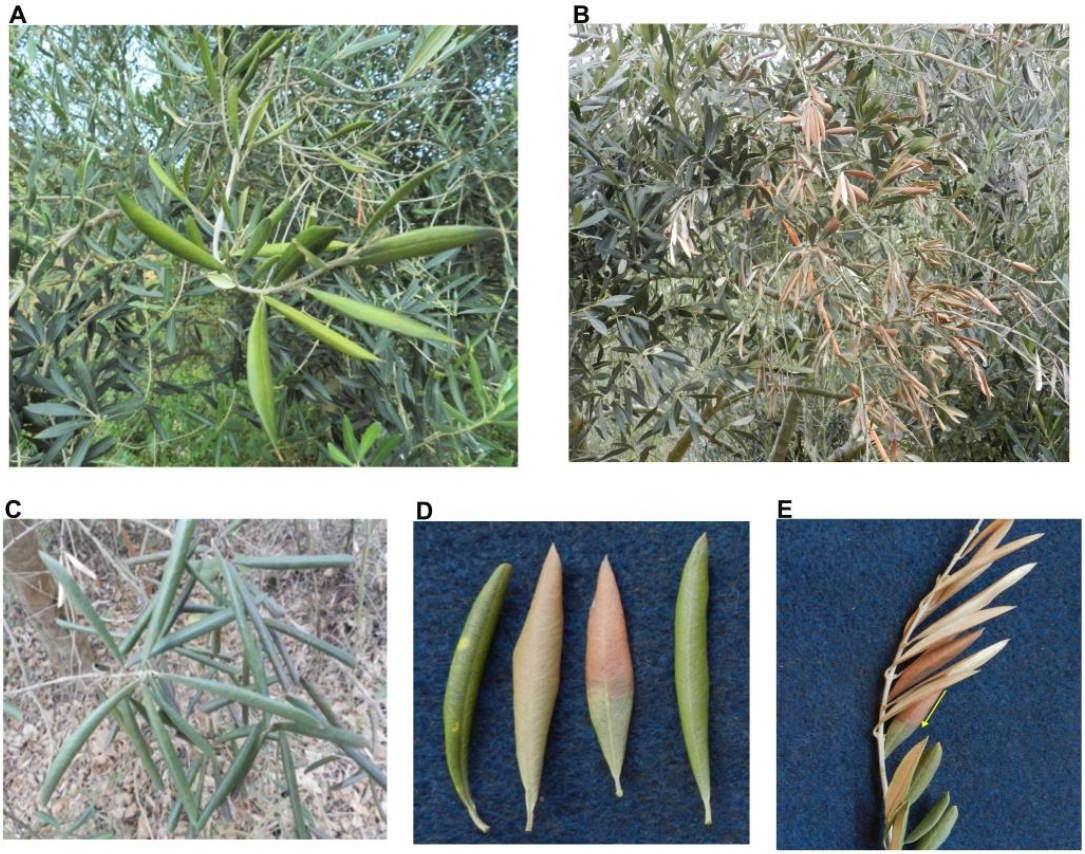

Figure 2. **Symptoms of olive quick decline syndrome (OQDS) observed in olive trees infected with *Xylella fastidiosa* subsp. *pauca* in** southeastern

Brazil. First symptoms started with leaves chlorosis (A) and / or leaves wilting (C) both restrict to one or few branches following by leaves desiccation restrict to the branch (B) that start from the top to the base (D). On leaves the progressive desiccation also started from apex to the base (D). The desiccated leaves remain attached to the branch.

***4.2 X. fastidiosa* diversity and phylogenetic analysis.** Of the 12 SSR loci used, only two (CSSR42 and GSSR12) were monomorphic. On the other hand, the amplification of loci COSSR4, OSS17 and COSSR5 resulted in highly polymorphic alleles for all *X. fastidiosa* strains independently of the geographic origin of the pathogen, totalizing 21, 20 and 18 different alleles, respectively (Table 2). Overall, the genetic diversity for this region was HNei = 0.39 and the total number of alleles was n=56. Strains isolated from São Paulo area A1 showed the highest values for genetic diversity (HNei = 0.38) and number of different alleles (n = 32), even compared with distinct geographic regions analyzed together, such as Minas Gerais areas A5, A17, A13, A18 and São Paulo areas A5 and A16, which exhibited an HNei = 0.18. Similar value obtained with strains from Minas Gerais areas A1 to A4 (HNei = 0.20) and the lowest diversity index (HNei = 0.07) was found for strains isolated from São Paulo area A2 (Table 2).

Table 2. Genetic diversity of *X. fastidiosa* strains isolated from olives trees in southeastern Brazil.

Locus	**Genetic diversity of *Xylella fastidiosa* strains according to geographic origin[a]**				
	All regions[b]	**Maria da Fé**	**S.B. Sapucai**	**Pilar do Sul**	**Other[c]**
COSSR4	0.83 (9)[d]	0.60 (5)	0.17 (2)	0.17 (2)	0.50 (3)
CSSR42	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)
OSSR19	0.38 (6)	0.00 (1)	0.67 (4)	0.00 (1)	0.17 (2)
OSSR14	0.29 (3)	0.00 (1)	0.17 (2)	0.00 (1)	0.17 (2)
OSSR17	0.77 (8)	0.68 (5)	0.50 (3)	0.17 (2)	0.17 (2)
GSSR4	0.22 (4)	0.00 (1)	0.67 (4)	0.00 (1)	0.00 (1)
COSSR6	0.55 (5)	0.46 (4)	0.50 (3)	0.00 (1)	0.17 (2)
GSSR12	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)
COSSR1	0.37 (5)	0.00 (1)	0.58 (3)	0.00 (1)	0.50 (3)
COSSR3	0.30 (4)	0.00 (1)	0.50 (3)	0.00 (1)	0.17 (2)
CSSR18	0.22 (4)	0.00 (1)	0.67 (4)	0.00 (1)	0.00 (1)
COSSR5	0.78 (6)	0.68 (4)	0.16 (2)	0.50 (3)	0.50 (3)
AVG	**0.39**	**0.20**	**0.38**	**0.07**	**0.19**

[a] Nei's genetic diversity.
[b] All *X. fastidiosa* strains listed in Table 2.

[c]From other regions (Consolação, Itabirito, Andradas, Gonçalves, Cabreuva, Itatiba – strain per each region)
[d]In brackets are the number of alleles

PCoA analysis segregated the strains in only one cluster composed by 85.7% of strains, except the XF156.1, XF367.1, and XF369.2, from São Paulo A1, which were placed outlying (Figure 3). The MLST analysis showed that strains characterized as ST16 was frequently found in the majority of the areas analyzed charactering a clonal dispersion of the pathogen, except for the areas A1 and A5 in São Paulo state whereas three novel STs (84 and 86 – A1 and ST85 - A5) were obtained (Table 3).

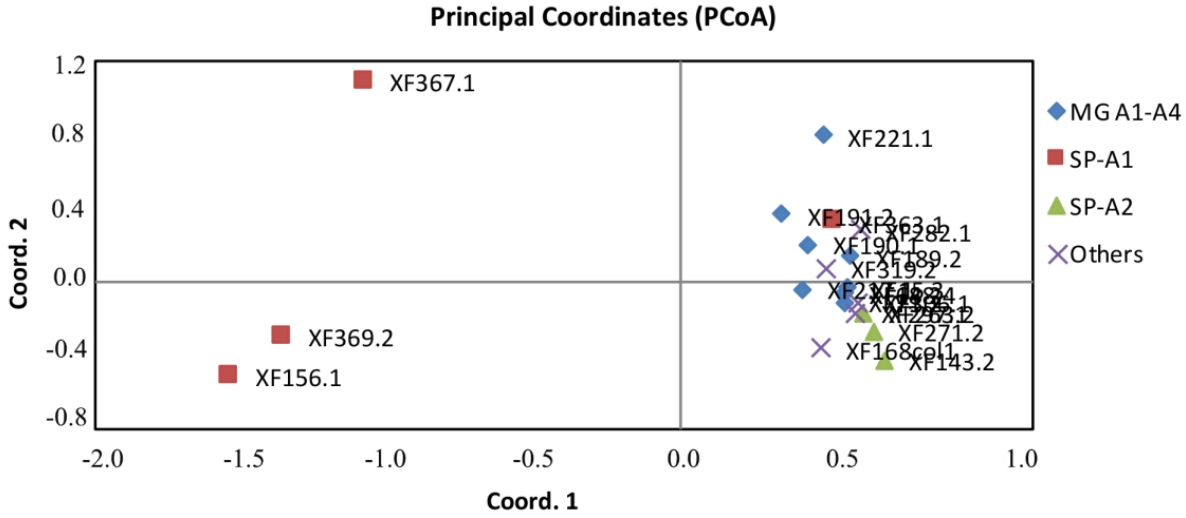

Figure 3. **PCoA analysis of SSR markers obtained for *Xylella fastidiosa* strains isolated from olive trees in southeastern Brazil.** Red square represents strains from São Paulo A1, green triangle from São Paulo A2, blue diamond from Minas Gerais A1 to A4, and purple axis from another regions.

Table 3. Genetic typing by MLST and SSR of *Xylella fastidiosa* (XF) subsp. *pauca* isolates used in this study

Isolate	Origin[b]	Variety	MLST profile[c] / Sequence type
XF15.3	Minas Gerais- A1	Negroa	7, 6, 8, 10, 11, 8, 8 / ST16
XF64.2	Minas Gerais- A1	Coratina	7, 6, 8, 10, 11, 8, 8 / ST16
XF71.2	Minas Gerais- A1	Rodapés	7, 6, 8, 10, 11, 8, 8 / ST16
XF188.4	Minas Gerais- A2	Grappolo	7, 6, 8, 10, 11, 8, 8 / ST16
XF189.2	Minas Gerais- A2	Grappolo	7, 6, 8, 10, 11, 8, 8 / ST16
XF190.1	Minas Gerais- A2	Grappolo	7, 6, 8, 10, 11, 8, 8 / ST16
XF191.2[a]	Minas Gerais- A2	Grappolo	7, 6, 8, 10, 11, 8, 8 / ST16
XF217.1	Minas Gerais- A4	Arbequina	7, 6, 8, 10, 11, 8, 8 / ST16
XF282.1[a]	Minas Geras- A5	Grappolo	7, 6, 8, 10, 11, 8, 8 / ST16

XF297.1[a]	Minas Gerais- A13	Grappolo	7, 6, 8, 10, 11, 8, 8 / ST16
XF308.1	Minas Gerais- A18	Grappolo	7, 6, 8, 10, 11, 8, 8 / ST16
XF319.2	Minas Gerais- A17	unknow	7, 6, 8, 10, 11, 8, 8 / ST16
XF156.1[a]	São Paulo- A1	Grappolo	7, 6, 7, ***34***, 10, ***20***, 8 / **ST84**
XF363.1	São Paulo- A1	Grappolo	7, 6, 7, ***34***, 10, ***20***, 8 / **ST84**
XF369.2	São Paulo- A1	Grappolo	7, 6, 7, ***34***, 10, ***20***, 8 / **ST84**
XF367-1[a]	São Paulo- A1	Grappolo	7, 6, 8, **10**, 11, ***20***, 8 / **ST86**
XF143.2	São Paulo- A2	Arbosana	7, 6, 8, 10, 11, 8, 8 / ST16
XF263.2	São Paulo- A2	Grappolo	7, 6, 8, 10, 11, 8, 8 / ST16
XF271.2 [a]	São Paulo- A6	Ascolana	7, 6, 8, 10, 11, 8, 8 / ST16
XF168.1[a]	São Paulo- A5	Koroneiki	7, 6, 8, 10, **10**, 8, 8 / **ST85**

[a]MLST loci amplified from total DNA of plants
[b]MG - Minas Gerais State; SP - São Paulo State
[c]Numbers identify loci leu*A,* pet*C,* mal*F,* cys*G,* hol*C,* nuo*L,* and glt*T* in the sequence. **In bold are novel alleles (20 and 34) and sequence types (ST 84 , 85, and 86)**

For ST84 and ST86, a new allele was identified for the locus nuo*L* (allele #20) based on one SNP, which resulted in the substitution of C by a T at position 113 (Table 4). The new allele (#34) of locus cys*G* was characterized by three SNPs observed at positions 266 (substitution of C by a T), 522 (G by A) and 587 (a replacing of C or G by A) (Table 4). An evidence for recombination event was observed for this locus at position 389 as confirmed by both DNAsp and GARD software. No new allele was observed for the ST85. Phylogenetic analysis clustered both novel alleles for *cysG* (#34) and *nuoL* (#20) loci together with the alleles from *X. fastidiosa* subsp. *pauca* from citrus and coffee plants (Figure 4. 1A and B).

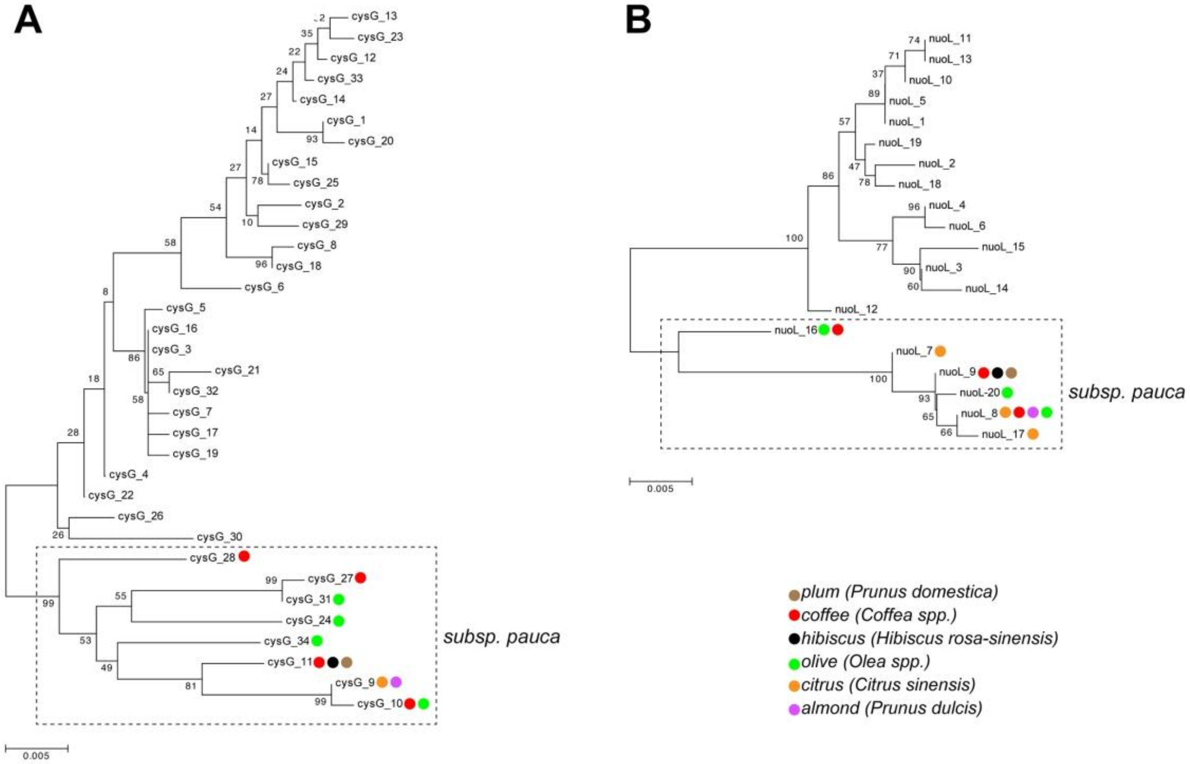

Figure 4. **Single-locus phylogenetic trees placing the new alleles *cys*G (A) and *nuo*L(B) found in *Xylella fastidiosa* subsp. *pauca* strains infecting olive trees in Brazil**. The colored circles show the different plants species where the *X. fastidiosa* strains with *cys*G and *nuo*L alleles were isolated. The numbers indicated the bootstrap support

Table 4. SNPs and potential recombination sites among the *X. fastidiosa* subsp. *pauca* strains for the *cysG* locus

		Position in *cysG*- total of 606 bp																																				
Sequence Type (ST)	Allele	23	28	30	68	71	83	135	159	186	206	209	216	236	237	238	266	276	281	282	284	303	323	335	348	349	371	380	389	476	503	510	514	521	522	548	554	587	599
11, 12, 13, 64, 65, 69, 78	9	C	G	T	C	C	C	T	G	G	A	C	G	C	A	G	C	G	G	C	G	G	A	T	A	A	C	T	G	G	A	G	G	G	C	G	C	C	G
16, 66, 85*, 86*	10	A	G	T	C	C	C	T	G	G	A	C	G	C	A	G	C	G	G	C	G	G	A	T	A	A	C	T	G	G	A	G	G	G	C	G	C	C	G
14, 68, 70, 71	11	C	A	T	C	C	A	C	A	G	A	C	G	C	A	G	C	G	G	C	G	G	A	T	A	A	C	T	G	C	G	G	G	G	T	G	T	G	G
53	24	C	A	T	C	C	A	C	A	A	G	T	C	C	G	C	C	G	G	T	G	A	G	C	A	G	C	C	G	C	G	G	G	A	T	G	C	G	A
73	27	C	A	C	C	C	A	C	A	G	G	T	G	T	G	C	C	A	G	C	A	A	G	C	A	G	A	C	T	G	A	G	G	G	C	G	C	C	G
74	28	C	A	T	T	T	C	C	A	G	G	T	G	C	G	C	C	G	A	C	G	A	G	C	G	G	C	C	T	C	G	A	A	G	C	G	C	G	G
80	31	C	A	T	C	C	A	C	A	G	G	T	G	T	G	C	C	A	G	C	A	A	G	C	A	G	A	C	T	G	A	G	G	G	C	G	C	C	G
84*	**34**	C	A	T	C	C	A	T	G	G	A	C	G	T	G	C	**T**	G	A	C	G	A	G	C	A	A	C	C	G	C	G	G	G	G	C	**A**	C	**A**	G

Concatenated sequences of the seven housekeeping genes totalized 4161 nucleotides placed the novel STs from Brazilian's olives trees into the *X. fastidiosa* subsp. *pauca* clades. Surprisingly, the STs 85 and 86 grouped with ST16 (isolate from coffee and olive trees) and ST66 (isolate from coffee trees). In contrast, the ST84 was closely related to STs from *X. fastidiosa* causing citrus disease in Brazil and Argentina and also almond disease in Argentina (ST86). A clear split can be observed between the

STs originated from South and Central Americas clade formed by the *X. fastidiosa* subsp. *pauca* strains (dashed circles), even when common host, such as coffee, was shared between both continents (Figure 5).

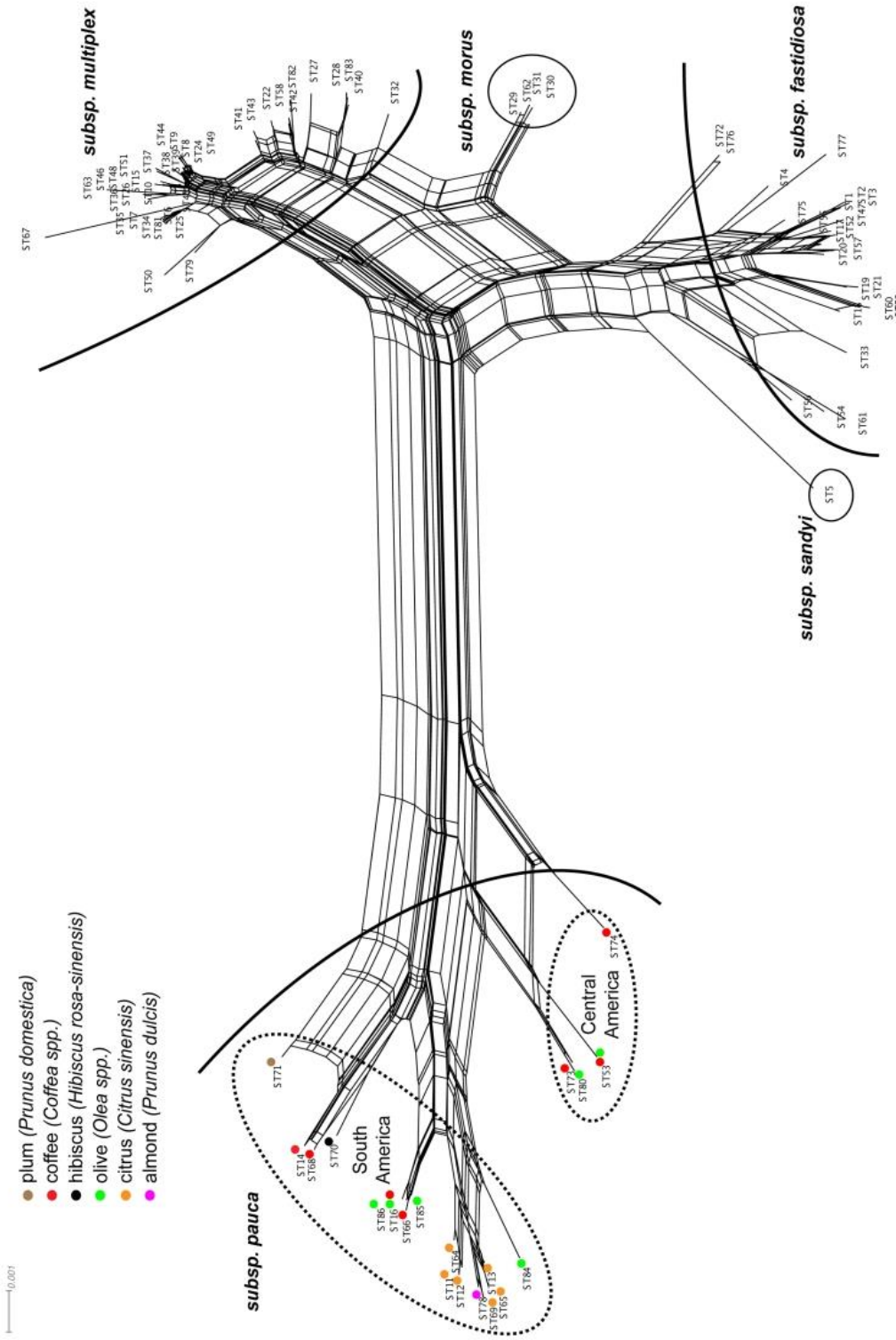

Figure 5. Phylogeny neighbor-net with all the ST from the MLST database, including the novel STs 84, 85 and 86

5. Discussion

The outbreaks in olive trees infection by *X. fastidiosa* both in Southeast Italy in 2013 and in southeastern Brazil in 2016 (São Paulo and Minas Gerais states) were caused by different strains of *X. fastidiosa* subsp. *pauca* classified as ST53 (ELBEAINO et al., 2014), and ST16 (COLETTA-FILHO et al., 2016), respectively, according with MLST methodology. Both STs had coffee plants as a common host, but ST53 was also found to infect oleander plants (*Olea oleaster*) in Central America (NUNNEY et al., 2014) and also in ornamental plant species in Southeast Italy beyond olive trees (LOCONSOLE et al. 2016). Recently, novel STs belonging to subsp. *pauca* were intercepted in Europe (ST73 and ST74) in coffee plants, most likely originated from Central America (LOCONSOLE et al. 2016; JACQUES et al. 2016). Another subsp. *pauca* ST was found in oleander plants (*Nerium oleander*) in the Balearic Islands (ST80) (EFSA Journal, 2018). Together, these results definitively change the phylogeography and host restrictions of *X. fastidiosa* subsp. *pauca* to citrus and coffee plants in South America and highlight the expansion of this subspecies to new hosts and environments. These novel hosts also give rise to a discussion regarding the *pauca* nomenclature proposed by Schaad et al. (2004) based on the narrow host range of this bacterium (pau' ca L. fem. adj. *pauca* few). The movement of asymptomatic, but infected plant material, from areas wherein the pathogen occurs to environments that are ecologically prone to harboring and spreading the bacteria to potential hosts was the main factor underlying the movement of *X. fastidiosa* subsp. *pauca* to a new geographic scenario, i.e. Europe (ALMEIDA; NUNNEY 2015). Furthermore, the introduction of an exotic vector-borne phytopathogen may also favor, for the first years, a clonal wave-spreading dispersion of the pathogen, as observed for the outbreak of *X. fastidiosa* (ST53) in Southeast Italy (ELBEAINO et al., 2014; BLEVE et al, 2016; MANG et al. 2016) or *Candidatus* Liberibacter asiaticus psyllid vectored bacteria in Brazil (COLETTA-FILHO, *personal communication*). However, as *X. fastidiosa* is naturally competent (KUNG; ALMEIDA, 2014), the presence of a local pathogen

reservoir plays an important role for intra- or intersubspecific homologous recombination (NUNNEY et al. 2012; BARBOSA et al. 2015), which may contributes to genome evolution and adaptation to new hosts (NUNNEY et al. 2014b; COLETTA-FILHO et al. 2017). Commercial olive farming in southeastern Brazil began around the 2000's in areas with high altitudes (800 to 1900 meters height) and mild temperatures, favoring flowering and fructification. This recent agricultural activity is spread over the 'Serra da Mantigueira' and neighboring areas for approximately 2000 ha. Most of these areas are surrounded by native vegetation (Table 1), with minimal spray of insecticides for pests management, scenarios that may potentialize the vector's diversity and population size (FROZA, 2017). Despite a recent report of *X. fastidiosa* in olive plants in southeastern Brazil, 83% of the sampled orchards were infested by the bacteria as observed by PCR and/or isolation, although the frequency of positive samples was variable among individual orchards (Table 1). An explanation for the long-distance dispersion of *X. fastidiosa* could be through anthropogenic action using infected vegetative material. Supporting this first statement, we determined that the vertical transmission of *X. fastidiosa* by rooting asymptomatic branches sampled from symptomatic olive plant was around 13% (data not shown). Additionally, a unique ST (ST16) was assessed in 15 out of 20 typed strains and it was present in 7 out of 9 geographic regions (Table 3). Albeit, a natural reservoir for the ST16 of *X. fastidiosa* located nearby the sampled orchards cannot be discarded and need to be explored in future works. Beyond the ST16, we found three new STs related to *X. fastidiosa* subsp. *pauca* colonizing OQDS symptomatic olive trees in two areas of São Paulo state (A1 and A5). Interestingly, the area A1 hosted two different STs (ST84 and ST86) identified in the four *X. fastidiosa* strains analyzed. ST84 represents a novel sequence type with two novel alleles for the MLST loci *cys*#34 and *nuoL*#20, which have not been described yet. However, two other alleles in the ST84 (*malF*#7 and *holC*#10) are exclusive from *X. fastidiosa* subsp. p*auca* associated with CVC in Brazil and the three others alleles from coffee-associated *X. fastidiosa* (http://pubmlst.org/xfastidiosa/; COLETTA-FILHO et al. 2017). The ST86 profile comprises all MSLT loci from ST16 except for the novel allele *nuoL*#20, while ST85 differs from ST16 only by the presence of the allele *holC*#10 (Table 3). The allele combinations observed for the ST84 (Table 4) support the intraspecies recombination hypothesis initially proposed by Almeida et al. (ALMEIDA et al. 2008) which was later confirmed (NUNNEY et al. 2012; COLETTA-FILHO, et al. 2017) where the ST (strains) from coffee plants are the potential donor

subspecies. The host jump of *X. fastidiosa* from coffee to olive plants probably carried by xylem-sap-feeder sharpshooters following by anthropogenic activity, which may have spread unwittingly the bacteria through contaminated vegetative material, is a plausible hypothesis to explain the prevalence of ST16 in the symptomatic olive trees. Supporting this hypothesis PCoA analysis using the SSR loci placed the majority *X. fastidiosa* strains into a unique cluster regardless of geographic origin, except the strains characterized as ST84 from São Paulo A1 (Figure 3). A natural reservoir located nearby the olive orchards could also contribute to genotypic diversification on specific area such as found in São Paulo areas A1 and A5. None *X. fastidiosa* subsp. *pauca* reservoir, such as coffee- and citrus orange-infected plants, were found close to olive orchards in A1 and A5 areas. Several species of native plants are described to host *X. fastidiosa* but with miscellaneous or no visible symptoms (PURCELL; SAUNDERS 1999), which difficult the identification in the field. On the other hand, asymptomatic plants are more attractive for the vectors and provide an effective inoculum sources for *X. fastidiosa* acquisition and spread (MARUCCI et al. 2005; DAUGHERTY et al., 2017). In addition, the variability of vector populations must be considerate also a potential driver for the genetic variability. Coincidently, the Shannon index for genetic diversity of leafhoppers species collected by yellow trap was significative higher for São Paulo area A1 compared to other areas included in this paper (FROZA, 2017). The geographic origin of *X. fastidiosa* subsp. *pauca* is still uncertain, but South America is suggested as a possible center of origin that was later introduction into Central America (NUNNEY et al. 2014). This hypothesis is supported by the split of *X. fastidiosa* strains into two major clades (Figure 5), consistent with previously results obtained by Giampetruzzi et al. (2017). Recently, the number of known STs related to *X. fastidiosa* subsp. *pauca* in South America strongly increased from 5 (STs 11, 12, 13, 14 and 16 – (NUNNEY et al. 2012) to 9 (STs 64, 65, 66, 68, 69, 70, and 71- COLETTA-FILHO et al. 2017), and now, 3 new STs (ST 84, 85 and 86), which reinforce this hypothesis. Analysis of the dataset from more variable genomic regions (SSRs) indicate 20 different multilocus microsatellite genotypes (MLMGs) of 21 analyzed strains (Table 3), while only 4 STs were observed. Such differences were also observed by Almeida et al. (ALMEIDA et al. 2008) and Yuan et al. (YUAN et al. 2010). Overall, the genetic diversity index observed for *X. fastidiosa* subsp. *pauca* from olives (HNei = 0.39) was lower than those of *subsp. pauca* strains from citrus (HNei = 0.46) (COLETTA-FILHO et al., 2014) or coffee (HNei = 0.60)

(FRANCISCO et al. 2017). This result can be interpreted as the consequence of a recent outbreak of OQDS (Table 2). The higher diversity index obtained for the strains from São Paulo A1 (HNei = 0.38) agreed with richness of the MLST alleles resulting in two novel STs (84 and 86) for samples from this region. In the past five years, the phylogeographic map and host range of *X. fastidiosa* subsp. *pauca* has changed considerably. In this paper, we confirmed that only *X. fastidiosa* subsp. *pauca* was found in olive plants with OQDS symptoms in Southeastern Brazil, so far. Despite the recent report *X. fastidiosa* subsp. *pauca*-infected plants were widespread over the sampled geographic regions. In addition, strains with clonal MLST profile (ST16) and similar SSR pattern were widely distributed through the olive orchards, with few exceptions (Table 3, Figure 3). These findings highlight the importance of exploring the population diversity in *X. fastidiosa* subsp. *pauca* to better understand the spatial distribution in olive orchards and also the probable mode of dispersion. Because of the high incidence and widespread distribution of *X. fastidiosa* subsp. *pauca* in olives in Southeastern Brazil it is necessary the implementation of a strategy of management of OQDS similar to implemented for CVC in Brazil (ALMEIDA, 2013).

6. Conclusion

- *Xylella fastidiosa* is widely distributed in the olive groves of the Serra da Mantiqueira (about 80% of the olive orchards analyzed in the states of Minas Gerais and São Paulo)
- The largest observation of infected plants occurred in the city of MG-A1 and SP-A1
- Only strains of *X. fastidiosa* subsp. *pauca* were identified associated with olive trees with symptoms of OQDS in southeastern Brazil
- The highest genetic diversity was observed in SP-A1 and MG-A18

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CHAPTER 2. Population structure of *Xylella fastidiosa* in olive trees in southeastern Brazil

1. Abstract

The bacteria *Xylella fastidiosa* is considerate as a non-specificity plant pathogen infecting a hundred plant species and causing disease in dozens of them. In South America, Brazil, *X. fastidiosa* subsp. *pauca* is known mainly to cause the Citrus Variegated Chlorosis (CVC) in sweet orange, but also was described causing the olive quick decline syndrome (OQDS) disease, specifically in the southeastern region, so far. In this chapter, the population structure of 177 *X. fastidiosa* isolates obtained from olive plants located at 16 different geographic regions was analyzed by 12 Single Sequence Repeats (SSR) loci multiplexed in 4 sets for the PCR and electrophoresis. The forward primers were labeled with different dyes and the amplicons run by capillarity electrophoresis. Off a total of 158 isolates, 64 different multilocus microsatellite genotypes (MLMGs) were observed, i.e., 40%. Unbiased Nei's genetic diversity corrected by the population size (HNei) index ranged from 0.00 to 0.55 (AVG of 0,24 for all populations), lower than obtained by subsp*. pauca* populations from citrus and coffee (Francisco et al. 2017). FST index (Wright's fixation index) for all pairwise comparison of populations ranged from 0.075 ($P>0.01$) to 0.968 ($P<0.001$), meaning a strong subdivision among some populations, even with a recent outbreak. Discriminant Analysis of Principal Components (DAPC) and PCoA via Nei Unbiased Genetic Distance clustered the samples into three main genetic groups, while model-based Bayesian clustered into two. Two orchards geographically closed were identified as the core contributors of migrants to the other populations, even the most distant ones, suggesting an anthropogenic activity in the dissemination of bacteria throughout southeastern Brazil.

2. Introduction

The introduction of olive trees (*Olea europaea*) in Brazil occurred more than 200 years ago, but difficulties such as lack of knowledge on management, non-adaptation of the introduced genotype to climatic conditions, the use of few genetic varieties, contributed for the non-establishment of the commercial production in the country. However, in the last two decades, advances in the knowledge about the olive plant physiology and the use of different varieties allowed the implantation of this crop in Brazil but in restrict regions with favorable microclimate. High altitude areas, such as mountain chains in “Serra da Mantiqueira” in the southeastern Brazil located in the states of Minas Gerais and the southeast State (Rio Grande do Sul), are the main areas for olive production. In Southeastern Brazil the olive production, characterized by small areas, is spread through approximately 70 municipalities, 60% of which are in Minas Gerais and 30% located in São Paulo (BERTONCINI; TERAMOTO; PRELA-PANTANO, 2010). Olive crop production in southeastern Brazil has been affected by phytosanitary problems such as the disease known as the Olive Quick Decline Syndrome (OQDS) caused by *Xylella fastidiosa* subsp. *pauca* (COLETTA-FILHO; FRANCISCO; ALMEIDA, 2014), similarly to early described in Apulia region, southeastern Italy (SAPONARI et al., 2013). But, differently from Italy, the extension of the economic damage of OQDS in olive crops in southeastern Brazil is unknown. On the other hand, olive plants with presence of *X. fastidiosa* subsp*. pauca* were found widely spread in orchards in Southeastern Brazil (SAFADY et al., 2019). Genetic characterization of 21 strains by Simple Sequence Repeats (SSR) and analysis by Principal Coordinate - PCoA showed the presence of three genetic clusters, and by Multi Locus Sequence Types (MLST) of four Sequence Types (ST) evidencing limited range of genetic diversity of these bacteria through the sampled area (SAFADY et al., 2019). However, questions concerning the population structure as determined by the geographic origin of bacteria and migration route remain open. In the present study, we analyzed the genetic diversity of *X. fastidiosa* subsp. *pauca* isolated from olives

plants with OQDS at the population level by using the fast evolving SSR markers to test the hypothesis of genetic structuration of this pathogen by spatial-scale taking advantage of wide sampling strategy.

3. Material and methods

Bacteria isolation and origin

X. fastidiosa was isolated of olive trees containing OQDS symptomatic leaves collected from 13 areas in Minas Gerais State and four in Sao Paulo, totalizing 204 isolates (Table 1). Branches with three to five millimeters in diameter were used for isolation by using the same procedure adopted by Safady et al, (2019). Branches were superficially disinfested with 70% ethanol followed by immersion in 2% bleach solution (2 minutes each), and at final washed in sterilized water. After dried, the branches were cut in the middle, squeezed and the sap was blotted onto BCYE agar plates (WELLS et al., 1981), followed by incubation at 28oC for 15 or more. The identification as *X. fastidiosa* was carried out based on in vitro fastidious growth, convex format and whiteness of colonies, which were streaked it onto PD3 agar plates and incubated at 28oC for 7 days, following PCR assays using the RST 31/33 primers (MINSAVAGE et al., 1994) for confirming the authenticity of the bacterium.

Table 1: Origin of *Xylella fastidiosa* subsp. *pauca* isolates used in this study.

Origin (State/area)	No.of plants	No. of XF isolates	Latitude S / Longitude W[b]	Altitude (m)
MINAS GERAIS				
MGA1[a]	18	36	22º18'56.55"/ 45º22'30.49"	1300
MGA2	5	17	22º18'51.67"/ 45º22'40.82"	1310
MGA3	4	8	22º18'53.01"/ 45º22'23.40"	1318
MGA4	2	7	22º18'45.01"/ 45º22'23.40"	1329
MGA5	3	13	22°35'46.45"/ 45°55'25.88"	1235
MGA7	2	5	22°59'52.60"/44°40'27.31"	1400
MGA10	2	7	22°36'53.30"/45° 24'32.87"	1780
MG13	5	19	21°1'54.44"/46°35'3.77"	1050
MGA14	2	5	22°18'16.85"/44°58' 34.03"	1065
MGA17	2	5	20°17'10.78"/43°48'37.02"	883
MGA18	3	9	22°35'8.84"/45°50'33.50"	1329
MGA19	2	5	21º 50' 10"/45º 24' 02"	928
SÃO PAULO				

SPA1[a]	4	14	22°42'10.71"/45°41'32.91"	1602
SPA2	3	13	23°45'37.30"/47°44'45.47"	690
SPA5	1	5	23°31'13.64"/46°56'11.21"	755
SPA6	2	9	23°16'22.23"/47°9'9.42"	883
TOTAL:	60	177		

[a]MG - Minas Gerais State; SP - São Paulo State / [b]Latitude -South/ Longitude- West

DNA extraction

DNA extraction of *X. fastidiosa* grown at culture medium was performed by using the Wizard DNA extraction kit (Promega), with some modifications. Bacteria from the third passage were scraped and added in a 1.5ml tube with 500 uL of 0.1% TE buffer (1M Tris HCl pH 7.5, 0.5M EDTA, pH 8) and mixed by repeated pipetting. Six microliters of proteinase K (20mg / mL) was added, mixed, and incubated for 1 hour at 37 °C. Then, 600 uL of Nuclei Lysis Solution was added and maintained at 80°C for 5 min, then cooled to room temperature. Next, 200 uL of Protein Precipitation Solution was added, vortexed and incubated on ice for 5 minutes and centrifuged at 13000 rpm for 3 minutes at 4°C. The upper phase of solution was transferred to a 1.5ml tube and 600ul of isopropanol was added and gently mixed. The tubes were kept at minimal 12 hours at -20°C and then centrifuged at 13000rpm for 10 minutes. The supernatant was discarded by inversion of the tube which was placed at 50°C for 20 minutes to dry. Then 50ul of 1/10 TE + RNAse was added for resuspension of DNA

Amplification of SSR and genotyping

Twelve microsatellite loci (SSR) mapped in the *X. fastidiosa* genomes were used (Table 2). These loci were selected based on the results obtained in previously published papers such as the generation of only one amplicon, discrimination power, and preferentially with origin in the genome of *X.f. subsp. pauca* (FRANCISCO et al., 2017; LIN et al., 2005). The forward primers were labeled with different dyes FAM (blue), PET (red), VIC (green), NED (yellow) which possibility run PCR in sets of multiplexed three primers (Table 2). The thermocycler was programmed following the steps of initial denaturation at 95ºC for 5 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 58ºC for 30 seconds and extension at 72ºC for 45 seconds, following the last extension step at 72ºC for 10 minutes. Before fragment analysis, the reactions were diluted 20x in sterile milli-Q water, and 1 µL was added into 10 µL of Hi-Di formamide and 0.1 µL of the

GeneScan™ 500-LIZ Size Standard (both from Life Technologies, Foster City, CA), and the mixture loaded onto the gel. The capillary electrophoresis was performed an ABI 3730XL DNA Analyzer (Applied Biosystems, USA) sequencer in the LacTAD facility (www.lactad.unicamp.br). The peak size (in base-pair) of the fragments were analyzed using the software PeakScanner version 1.0 software (Life Technology). Final data table of amplified fragments used in the analysis can be found in Table 1 of the Appendix.

Table 2. General information on the primers of the microsatellite-SSR locus used to analyze the genetic diversity of the population of *Xylella fastidiosa*

Multiplex[1]	Primer[2]	Dye[3]	Forward sequence(5'-3') /Reverse sequence(5' - 3')	Motif[4]	concentration (µM)[5]
	COSSR4	**FAM**	CAAGGTGACCGCTAGCCTAT /GCTGTCATTGGGTGATGC	(CAATACAC)	0.1
	CSSR42	**PET**	ATTACGCTGATTGGCTGCAT/ GTTTCATTACGCGGAACAC	(TGTTATC)	0.2
	OSSR19	**NED**	GCTGTGAACTTCCATCAATC C/GCAAGTAGGGGTAAATGTGAC	(CAGGATCA)	0.2
	OSSR14	**VIC**	GGCGTAACGGAGGAAACG/A TGAACACCCGTACCTGG	(TGATCCATCCCTGTG)	0.27
	OSSR17	**FAM**	AGTACAGCGAACAGGCATTG /AGCAACCAGGACGGGAAC	(TGCCTG)	0.27
	GSSR4	**PET**	GCGTTACTGGCGACAAAC/G CTCGTTCCTGACCTGTG	(ATCC)	0.27
	COSSR6	**PET**	TGCTGCGCGATAACCAAGT/ GCATCCAATCAGCCCTAACCT	(GTGATGCG)	0.27
	GSSR12	**VIC**	TTACGCTGATTGGCTGCATT G/GTCAAACACTGCCTATAGAGCG	(TATCTGT)	0.2
	COSSR1	**FAM**	GAAACAAGATGGCGGTTGC/ CATTTAAACGGGCGGCATA	(ATTGCTG)	0.27
	COSSR3	**FAM**	AAGTATTCGCTACGCTACGC/ GTGTGTTATGTGTGCCATTCGT	(CTGATGTG)	0.38
	CSSR18	**NED**	GTGCTTCCAGAAGTTGTG/G ACTGTTCTCTTCGTTCAG	(GCCAA)	0.38
	COSSR5	**VIC**	ACACTGACACAACAGCCACC A/AATGGTGGGTGTGATGGTTTC	(CATACAGA)	0.27

[1] Set of primers used in multiplex PCR.

[2] COSSR, CSSR, OSSR and GSSR: microsatellite markers based on strains of coffee (CO), sweet orange (C), oleander (O) and grapevine (G).

[3] Markers with fluorophores: FAM (blue), NED (red with black vision), PET (red), VIC (green).

[4] Motif: Nucleotide sequence that repeats in tandem in a microsatellite region

[5] Final Primer Concentration in the PCR Reaction

Overall statistic

As previously shown by Coletta-Filho et al., (2014) and by Francisco et al., (2017) for *X. fastidiosa* subsp. *pauca* from citrus and coffee, the changes in the length of the SSR fragments observed in the present work it was also due to an insertion or deletion

of a motif following the Stepwise Mutation Model - SMM, that was adopted in the statistical analyses conducted here. This model also assumes that alleles that differ from a smaller number of motifs are more related and have a more recent common ancestor than those which have a higher number of motif units (POKHRIYAL et al., 2012). Indexes for general genetic information were obtained such as; (i) number of alleles per locus, (ii) allelic patterns, (iii) private allele by population, and (iv) number of genotypes by population, by using the software GenAlex (PEAKALL; SMOUSE, 2012).

Population genetic analysis

For the determination of the number of genetically homogeneous subpopulations (K) both multivariate and model-based Bayesian clustering approaches were used. The model-based Bayesian method was implemented in STRUCTURE 2.2. This software makes use of randomizations based on Monte Carlo simulations via Markov Chain Monte Carlo (MCMC) using the Gibbs algorithm. The "admixture" model was chosen with K value varying from 1 to 16. In the runs, a 100000 burn-in period was adopted, followed by 200,000 replicates of MCMC (Monte Carlo Markov Chain), alpha (initial value = 1.0, max = 10.0, DP = 0.025) and allele frequency correlated between as subpopulations (previous mean = 1.0, previous SD = 0.05, $\lambda = 1.0$). The input data was all the fragment lengths of the 177 DNA samples without informing the geographical origin (Table 1- Appendix). The output data from STRUCTURE was analyzed by the web-based front-end Structure Harvester (EARL; VONHOLDT, 2012) and Clumpak - Cluster Markov Packager Across K, (KOPELMAN et al., 2016). Both web-based softwares summarize the output data from Structure and present the best K. Principal Coordinates Analysis (PCoA) was constructed using GenAlex software, taking as input a matrix giving the genetic distance among all individuals and Nei's unbiased genetic distance among all populations (NEI, 1978).

Migration rate and population size

The effective population size (θ), which for haploid is $2N_e\mu$, being Ne = effective population size and μ = mutation rate for each loci, and migration rates among populations ($M = 2N_em$) of *X. fastidiosa* isolated from olive trees were inferred through the software MIGRATE version 3.7.2 (BEERLI, P., ; FELSENSTEIN, J., 2001). The Bayesian estimate performed was based on Monte Carlo simulations via Markov

Chains (MCMC), programmed for microsatellite data (Brownian motion) assuming the stepwise mutation model (SMM). 5 runs of 5000 samples were performed, and consisted of a long markov chain and static heating regime with four temperatures (1.0, 1.3, 2.6 and 3.9). Prior distribution for theta parameter and 2Neμ was uniform with a minimum = 0.0, mean =10.0 and the maximum = 20.0. Theta and M parameters were calculated using the percentile approach for the approximate confidence interval.

4. Results

Genetic and genotypic diversity

The number of alleles per locus ranging from two to 12, totaling 91 alleles. The OSSR17 locus was the most polymorphic among all and CSSR42 being the least polymorphic (data not shown). The number of private alleles by population (total n = 43) was higher in SPA1 (n = 12) and in MGA18 (n = 10). Even considering MGA1, MGA2, MGA3 and MGA4 as the same population, due to the geographical proximity, the number of private alleles observed (n = 13) was similar to obtained for SPA1 population. The highest total number of alleles per population was observed in MGA1 (n = 45), followed by SPA1 (n = 35) and MGA18 (n = 29). Observed heterozygous levels were highest in SPA1 (Hnei = 0.5), followed by MGA18 (Hnei = 0.47) and MGA7 (Hnei = 0.41) (Table 3 and Figure 1). The overall genetic diversity Hnei was 0.19.

Table 3. Genotypic, allelic and genetic diversity of *Xylella fastidiosa* populations isolated from olive trees in different geographic areas of the state of São Paulo and Minas Gerais (Brazil).

POP	MG A1	MG A2	MG A3	MG A4	MG A5	MG A7	MG A10	MG A13	MG A14	MG A17	MG A18	MG A19	SP A1	SP A2	SP A5	SPA 6
N	36	17	8	7	13	5	7	19	5	5	9	5	14	13	5	9
NaT	45	22	21	13	21	23	18	21	12	13	29	12	35	18	12	15
No.Pa	5	2	2	4	1	4	11	1	2	0	9	1	11	0	1	0
H	0,33	0,20	0,21	0,03	0,23	0,41	0,20	0,21	0,16	0,03	0,47	0	0,5	0,14	0	0,09
uH	0,34	0,21	0,24	0,04	0,25	0,52	0,23	0,22	0,68	0,03	0,53	0	0,54	0,15	0	0,06
GeT	22	5	4	2	6	3	2	6	1	2	5	1	7	4	1	3

Pop: Population name
N: number of individuals
NaT: No. of Different Alleles
No.Pa: No. of private alleles

H: heterozygosity (Hnei)
uH: unbiased heterozygosity- corrected by the number of individuals
Get: No. of different genotypes

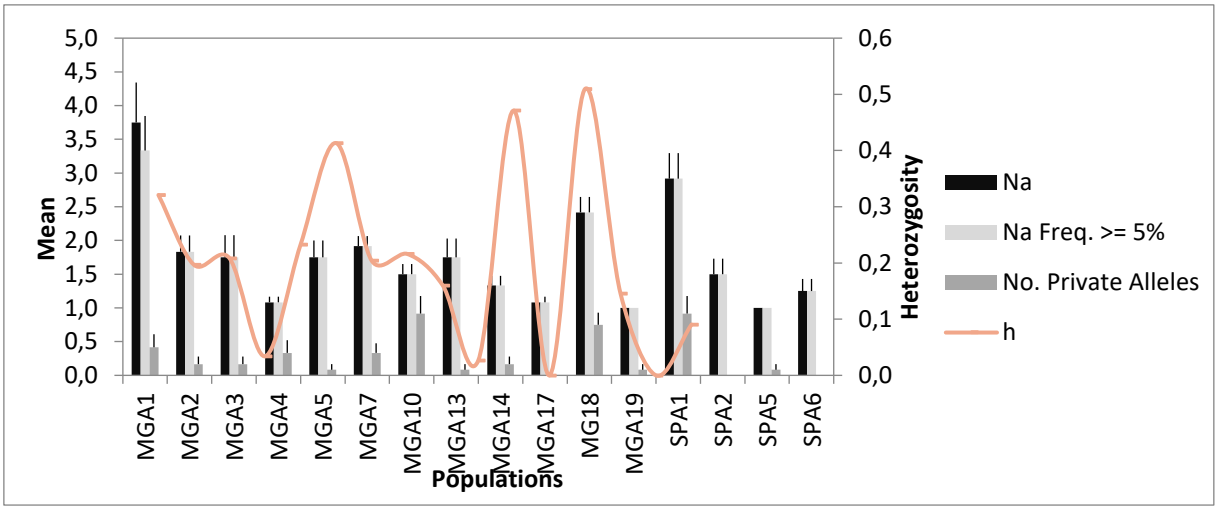
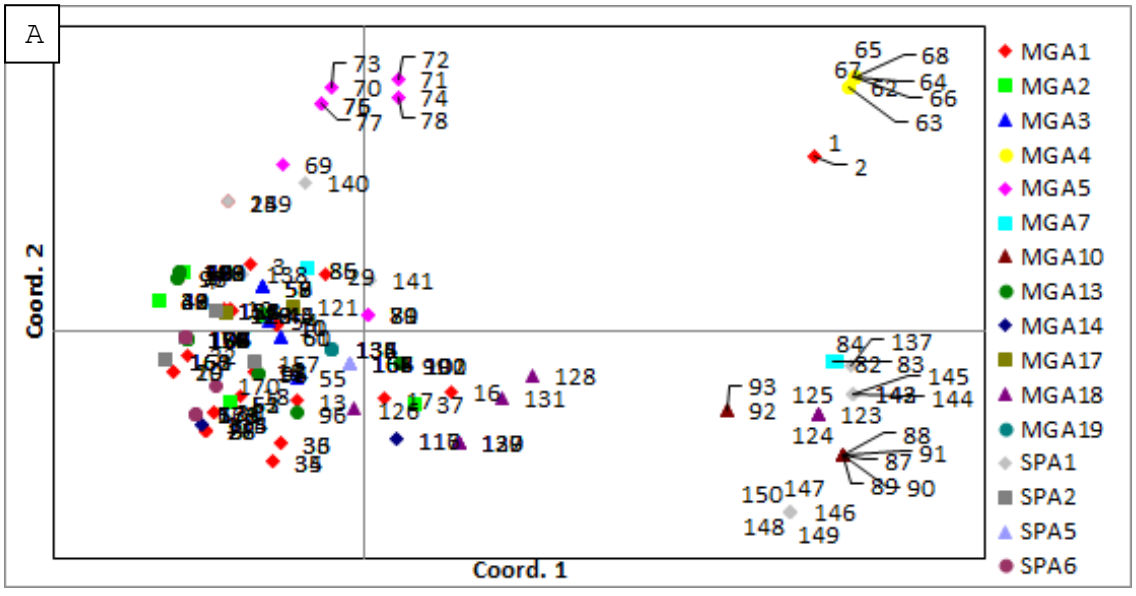


Figure 1: Mean allelic patterns of *Xyllela fastidiosa* isolated from olive trees. Na is the number of alleles, Na Freq. >= 5% is the number of alleles in a frequency >=5%, h is heterozygosity.

Genetic differentiation among populations

Three clusters (n = 3) were obtained by Principal Coordinate Analysis (PCoA) analysis constructed with a pairwise population matrix by using both genotypic genetic distance (Figure 2A) and Nei Unbiased Genetic Distance (Figure 2B) among all *X. fastidiosa* strains considering their geographic origins. Also, single component clusters were observed, but with the majority of populations presented in only one cluster (Figure 2).



B

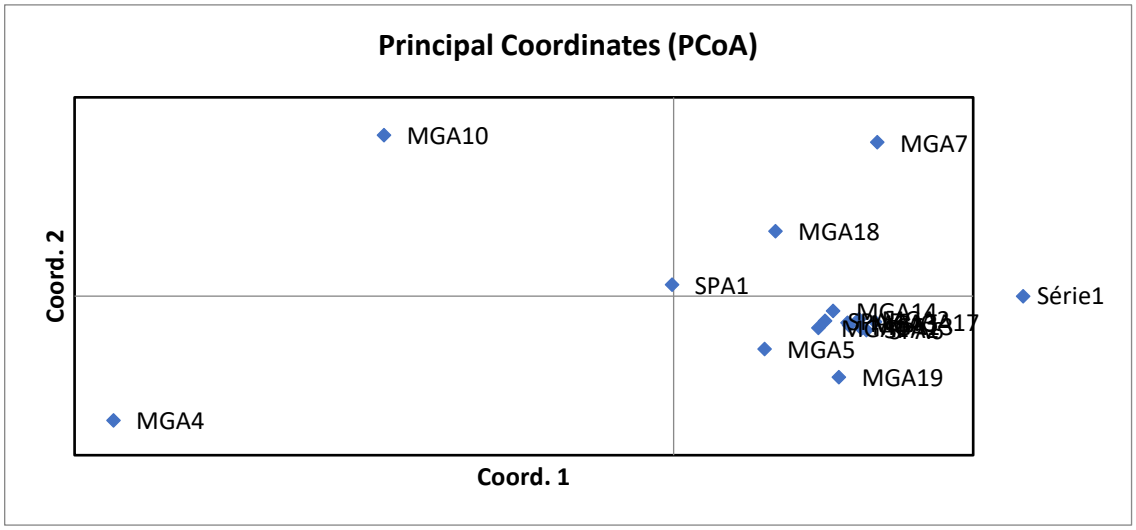


Figure 2: Principal Coordinate (PCoA) constructed with a pairwise genotypic genetic distance (A) and Unbiased Nei Genetic Distance (B) among all *X. fastidiosa* isolates from olive plants.

Differently from the Principal Coordinates analysis, the number of clusters obtained with the genetic clusters analysis by the Bayesian method to infer the number of genetically homogeneous groups or subpopulations (K) among the sampled isolates using the STRUCTURE software (HUBISZ et al., 2009), identified two clusters (K=2) based on the delta values by Evanno et al. (2015) (Figure 3). One of the clusters (K-1) represents 81% of the total (Figure 4). Analysis considering K=3 were also run based on the PCOA results.

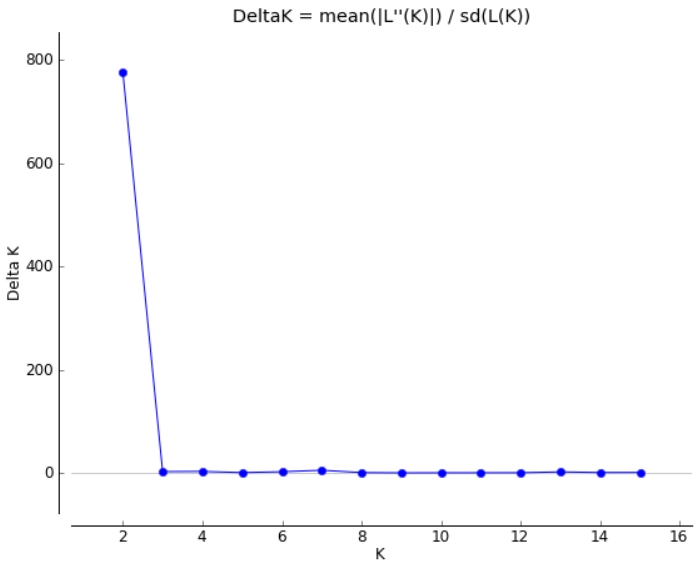


Figure 3. STRUCTURE HARVESTER graphic output of Delta K and Mean L(K);

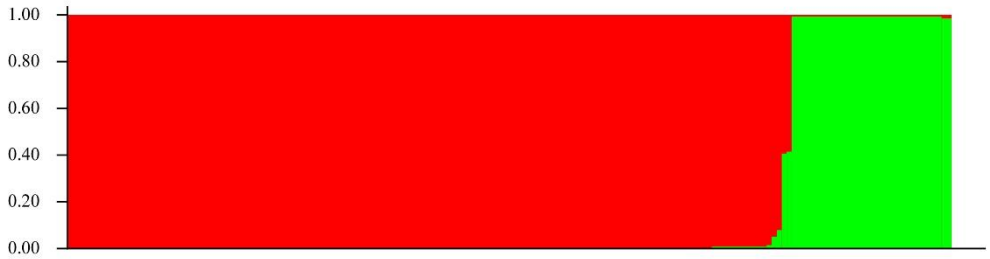


Figure 4: STRUCTURE bar plot of Bayesian assignment of individuals to two clusters (K=2).

Figure 5: Clustering of *Xylella fastidiosa* isolates sampled from olive plants in southwest Brazil by using Bayesian method inferred by Structure and assignment of individual (strains) to the *K* genetic groups. In *x* axis are the geographic origin of strains (see Table 1, please). The length of each colored bar (*y* axix) indicates the estimated probability of assignment to each of the defined K genetic groups. A. K =2 optimal number of clusters. B. K = 3 optimal K value for a number of clusters. Same color means genetically similar groups of genotypes.

Through AMOVA analysis with 9999 permutations, of the total genetic diversity, 37% of the observed variations occurred within individuos and 63% among geographic areas (ΦSTAT = 0.631, $P \leq 0.001$).

Migration rate and population size.

Estimates of population size of *X. fastidiosa* infecting olive trees in 16 different regions (Θ = 0.09 -) were not significantly different. Migration rates between geographic populations were symmetrical for MGA1 e MGA5, SPA2, SPA5, SPA6, in which the numbers of migrants donated and received were similar. In relation to SPA1, MGA1 contributed more than twice as many migrants than it received ($2Nem_{MGA1 \rightarrow SPA1}$ = 13,0 migrates / generation; $2Nem_{SPA1 \rightarrow MGA1}$ = 5 migrates / generation). In general, MGA1 and MGA2 contributed the largest number of migrants to populations SPA1, SPA5, SPA6, MGA5 and MGA19 and with an important contribution to MGA3 (Figure 5). The smallest number of migrants were received by MGA4 from SPA1 ($2Nem_{SPA1 \rightarrow MGA4}$ = 1.5), while SPA2 received the largest number of migrants mainly from MGA1($2Nem_{MGA1 \rightarrow SPA2}$ = 14.5). On the other hand, the population that contributed with less migrants to the other populations was MGA19 ($2Nem_{MGA19 \rightarrow MGA7}$ = 2.8), while the largest contributors were MGA1 ($2Nem_{MGA1 \rightarrow MGA4}$ = 5.6; $2Nem_{MGA1 \rightarrow MGA5}$ = 15.5) and MGA2 ($2Nem_{MGA2 \rightarrow MGA4}$ = 2.8 - $2Nem_{MGA2 \rightarrow MGA6}$ = 16). When considering MGA1 and MGA2 as the same geographical population duo to their proximity, the number of migrants contribution to other geographic populations is even larger, for example: $2Nem_{MGA1+MGA2 \rightarrow SPA2}$ = 35.1.

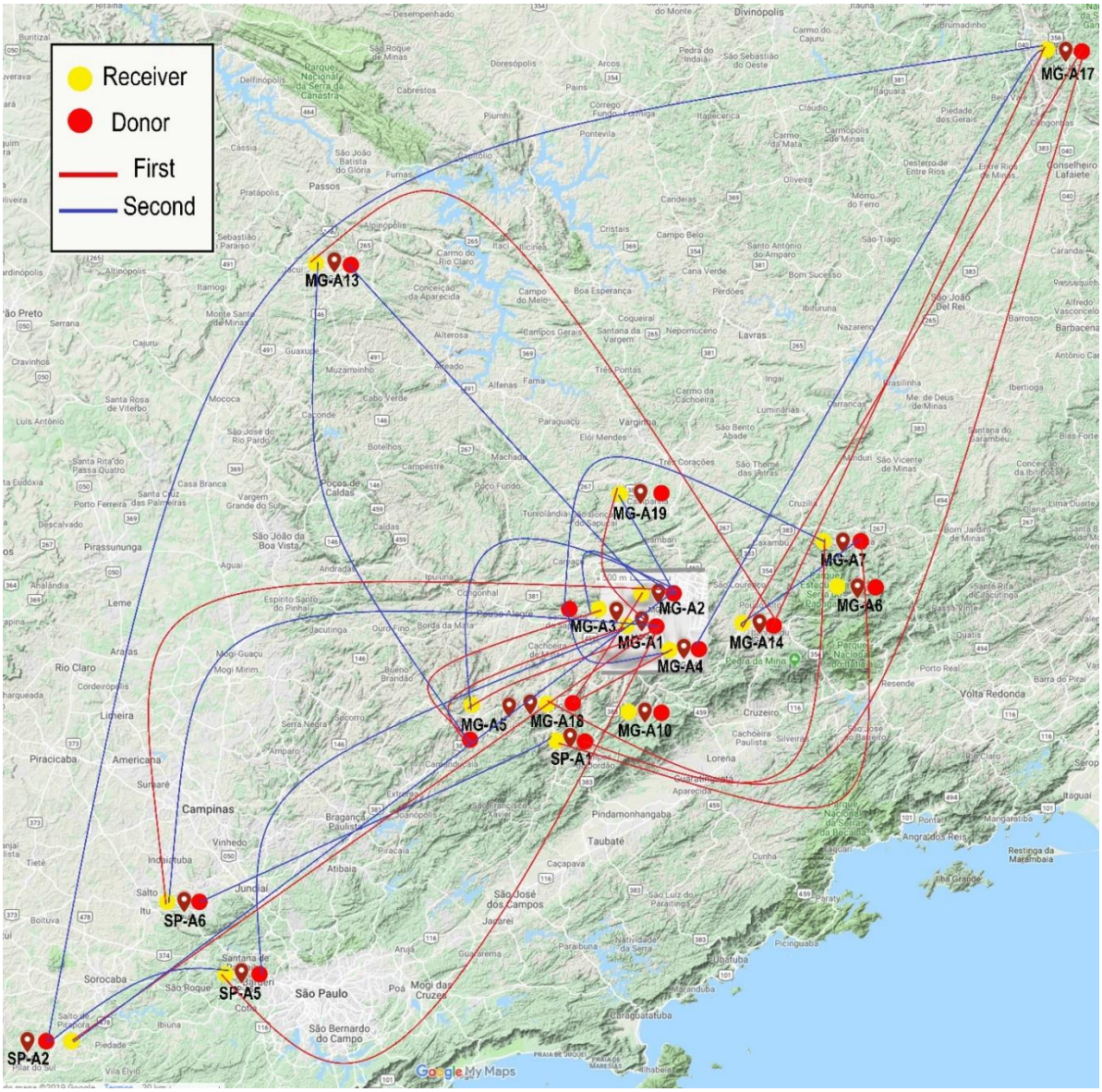


Figure 6. Map of *Xylella fastidiosa* migration route in olive orchads in southeastern Brazil. Red lines are the first major migrante contributor and the blue lines are the second major contributor. Yellow circles are migrante receiver and the red circles are the contributors.

5. Discussion

The overall unbiased genetic diversity index of Nei (heterozygosity index) Hnei = 0.19, was smaller to the obtained for the population of *X. fastidiosa* subsp. *pauca* isolates from citrus (Hnei = 0.46) (COLETTA-FILHO; FRANCISCO; ALMEIDA, 2014) and from coffee (Hnei =0.60) (FRANCISCO et al., 2017) which can be explained by the recent outbreak of OQDS. Also, the heterozygosity observed in the *X. fastidosa* population studied in this work was slightly inferior to yearly observed (Hnei = 0.22) by using a smaller number of isolates (SAFADY et al., 2019). The broader sampling

strategy did in the present work allowed to explore *X. fastidiosa* strains in geographic population with clonal foundation as the populations SPA5, MGA14 and MGA19 which contributed to drop the heterozygosity of whole population. However, *X. fastidiosa* populations from MGA7, MGA18 and SPA1 shown heterozygosity index similar to populations with ancient outbreaks as *X. fastidiosa* causing Citrus Variegated Chlorosis and Coffee leaf scorch. Based on capacity of recombination of these bacteria (POTNIS et al., 2019; VANHOVE et al., 2019) is hope that in future works the heterozygosity index to *X. fastidiosa* from olives will be similar to obtained for populations obtained from citrus and coffee hosts (COLETTA-FILHO; FRANCISCO; ALMEIDA, 2014; FRANCISCO et al., 2017).

Although the sampling were conducted in 16 different orchards geographically distant (except MGA1, MGA2, MGA3, and MGA4), the statistical analysis of genetic dataset aiming population structure allowed clustered all individuals into three genetic clusters, but with the majority into only one cluster. Some population (MGA17, MGA18 and SP1) were individually segregate meaning genetically different founder population. In view of the short time of the epidemic we hypothesized that the different inoculum sources could be most reasonable explanation for these ‘isolated’ population apart from the main cluster. However, recombination and specific environment conditions shaping those populations could not be discarded (HENNEBERGER et al., 2004; NUNNEY et al., 2012; SCALLY et al., 2005). On the other hand, the main cluster formed by 14 populations suggests a clonal wage of dissemination of the pathogen through the regions (orchards) as already discussed previously (SAFADY et al., 2019). However, in the present work, even using faster evolved SSR markers as the MLST previously used, a clear pattern of clonality among all populations of *X. fastidiosa* infecting olive trees in southeastern Brazil was observed. Despite genetically different strains were observed with each population, which could result in the genetic structuration of population in the future, as observed for citrus and coffee plants.

The populations of MGA1, MGA2, MGA3 and MGA4 are geographically close and, historically, this region is where the first olive trees were established in Southeastern Brazil. Due to the geographical proximity between the orchards and since olive trees are cultivated in the open, migration between these four populations may be occurring through insect vectors. Although MGA1 heterozygosis is lower than in SPA1, MG18 and MGA7, this population presented the highest number of alleles and, besides that, MGA1 (Hnei = 0.33) and MGA2(Hnei =22) contributed a larger

number of migrants to the other populations and was the region where the largest number of *X. fastidiosa* -positive plants was found (SAFADY et al. 2019). The low contribution of migrants from MGA1 to MGA4 can be explained by the isolation of this region from other orchards since MGA4 is located in a heterogeneous environment with native plants that may be contributing as an inoculum reservoir for the bacteria. The largest contributor of migrants to SPA1 was MGA7, located more than 200km apart. In addition, MGA1 and MGA2 also contributed to this region with similar numbers of migrants and are located 90km away from SPA1. This exchange of migrants between distant regions can be explained by the movement of contaminated propagating material.

The results observed in this chapter agree with the previous chapter in which there was a higher percentage of *X. fastidiosa* ST16 isolated from olive trees in southeastern Brazil, which was first reported in MGA1. (COLETTA-FILHO; FRANCISCO; ALMEIDA, 2014; SAFADY et al., 2019).

6. Conclusion

- The populations of *Xylella fastidiosa* in southeastern Brazil were grouped into two groups.
- 14 geographical populations were clustered together, suggesting a clonal spread of the bacteria in the orchards.

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CHAPTER 3: Diagnosis of *Xylella fastidiosa* in sharpshooters captured in olive SP-A1 orchard.

1. Abstract

Xylella fastidiosa (XF) bacteria have been identified in olive orchards in southeastern Brazil with symptoms similar to those seen in an epidemic known as the Olive Quick Decline Syndrome (OQDS) that led to the decline of thousands of olive trees in Italy. XF is mainly transmitted by vector insects that feed on the sap of plant xylem where the bacteria is found. By feeding on plants colonized by XF, insects begin to transmit it to other plants, since XF bacteria can colonize the digestive tract of insects. In Italy, the insect vector species identified as responsible for transmitting the OQDS was Philaenus spumarius. In the orchards of Brazil, nineteen species of possible vectors insects were found, with predominance of the species *Clastoptera* sp. 1, *Macugonalia cavifrons* and *Scopogonalia* sp. 1. Studies have shown that the greatest diversity of insects was found in SP-A1 (S22 ° 42'10.71 "W45 ° 41'32.91"), where is also the highest diversity of XF isolates. For this reason, the objective of this study was to diagnose different species of possible vector insects in the region of SP-A1 for the presence of bacteria. Total DNA extraction from leafhoppers and real-time polymerase chain reaction (PCR) were performed with XF bacteria-specific primers and PCR with housekeeping petC and nuoL gene primers as a suggestion for diagnosing XF in leafhoppers. The results showed that the highest number of XF positive insects were *Scopogonalia paula* and *Subrasaca bimaculata* species and that the use of petC primer is efficient for XF diagnosis in total insect DNA.

2. Introduction

The natural transmission of bacteria *Xylella fastidiosa* occurs mainly by insect vectors belonging to two groups: sharpshooter leafhoppers (Cicadellidae, Cicadellinae) and spittlebugs (superfamily Cercopoidea) (REDAK, et al. 2004, ALMEIDA, et al. 2005a,). When feed in xylem vessels of *X. fastidiosa*-infected plants, these insects are able to acquire the bacteria and latter transmit to other plants (HOPKINS; PURCELL, 2002). Although the transmission efficiency increase proportional to feeding time, a latency period between acquisition and transmission is not required (PURCELL; FINLAY, 1979). Once acquired, *X. fastidiosa* is persistently retained in the digestive tract (stomodeus) of the adult phase of these vectors, specifically in the region of the cibarium and pre-cibarium, where it has the capacity to multiply and colonize. The persistence of the bacteria in the digestive tract of nymphs is lost in each change of instar, once the cuticular lining of the stomodeum, where the bacterium lodges, is changed in ecdysis (HILL, 1995). Forty-eight species of insect vectors are currently known as responsible for the transmission of *X. fastidiosa* in major crops of economic importance. In Brazil, studies on the diversity of insect vectors carried out in citrus and coffee orchards, two crops known to be affected by this pathogen. Insect species frequently found in these orchards are *Acrogonia citrina, Dilobopterus costalimai, Oncometopia facialis and Bucephalogonia xanthophis* in citros (KRUGNER, et al. 2000; LOPES; KRUGNER, 2013) and *Bucephalogonia xanthophis, Oncometopia facialis, Dilobopterus costalimai* and *Homalodisca ignorata* in coffee crops (MARUCCI, et al. 2008). In olive crops, the species *Philaenus spumarius* was identified as the main vector responsible for the *X. fastidiosa* outbreak in Italy (SAPONARI, et al. 2014; CORNARA, et al. 2017). In a recent survey of insect diversity in olive orchards in the Serra da Mantiqueira, where olive trees with symptoms similar to Olive Quick Decline Syndrome (OQDS) with the presence of *X. fastidiosa* were identified, Froza (2017) identified 44 species of insects predominant in the orchards, 19 belonging to the group of possible vectors of the pathogen (Cicadellinae, Cercopidae and Clastopteridae). In addition, the species *Clastoptera* sp. 1 *Macugonalia cavifrons* and *Scopogonalia* sp. 1, were predominant in all observed orchards (FROZA, 2017), but no information about natural infectivity of these insects is known. The objective of this wok is to determine the natural infection by *X. fastidiosa*

of these individuals sampled in a orchard with high vector richeness as determine by (FROZA, 2017).

3. Material and methods

3.1 Insects sampling

Adesive yellow traps - 30 x 10 cm (ISCA ®, hot melt) were used to capiture insect species in SP- A1 orchard located at S22°42'10.71" and W45°41'32.91". This specific area was previously characterized as high vector richeness area as determined by (FROZA, 2017)..

Nine yellow-traps were installed in the orchard at the periphery of olive plant canopy. The insects identified in the traps as potential vector were individually placed in plastic microtubes containing absolute alcohol in order to conserve them. In total, 158 insect samples were analysed, including the species *Clastoptera* sp. 1, *Macugonalia cavifrons, Subrasaca bimaculata, Paratubana luteomaculata* and *Scopogonalia paula*. Sharpshooters raised in laboratory were used as negative control.

3.2 DNA extraction from insects

DNA extraction from the insects was done following the Promega kit of extraction and purification Wizard® Genomic DNA Purification kit protocol with some modifications. In order to test the extraction protocol, experiments were carried out using one to five specimes. Based on fact that *X. fastidiosa* colonize only in the cibarium and pre-cibarium the insect's head was disconnected from the body by a blade and used for DNA extraction. After placed in microtubes with two stainless 3 mm steel beads, 100 ul of STE buffer (10mM TRIS-HCl, 1mM EDTA, 25mM NaCl) was added and then macerated with TissueLyser® for 2min at 30 Hertz. After macerated, 15µl de proteinase K (200µg/ml) and 2µl de SDS 20% was added to the microtube and maintained for 30 minutes at 37°C. The microtube was spinned and 100 µl of the solution was transferred to another microtube. Then, 150µl of Nuclei Lysis Solution (Wizard®) was added and the tube maintained for five minutes at 80°C. After, the tube was cooled at room temperature and 60 µl of Protein Precipitation Solution (Wizard®), was added, vortexed and incubated in ice for 5 minutes. The microtube was centrifuged at 13,000 rpm for 3 minutes at 4°C. The upper phase was transferred to a new tube following by addition of 200 µl of isopropanol. After mixing the tube was maintened by

at 4°C for 10 hours (overnight). The tube was centrifuged at 13000 rpm for 5 minutes, discarded the supernatant, added 150 µl of alcohol 70. After rapidly vortexed, the tube was centrifuged as did early, the upper phase discarded and the tube left dry up side down on absorbent paper. Finally, 50 µl of 1/10 TE + RNAse were added to resuspend the DNA. The samples were quantified using NanoDrop ™ 2000 / 2000c Spectrophotometers (ThermoFisher Scientific).

3.3 *Xylella fastidiosa* diagnosis

DNA samples were first diluted at concentration of 50 – 100 ng/µl, aiming to reduce possible polymerase chain reaction (PCR) inhibitors. Three different PCR-based stratagies were tested for diagnosis of *X. fastidiosa* in the insects; by using the *X. fastidiosa* universal primers RST31 / 33 (MINSAVAGE, et al., 1993) following the same cycling describe by the authors and a total volume of 13µl containing 100ng of DNA, 1x FIREPol 5X Master Mix (Solis BioDyne) and 1mM of each forward and reserve primers. The same material was also tested using the MLST primers (Yuan et al., 2010) amplied by both Platinum TAQ DNA polymerase (ThermoFisher Scientific) and FIREPol 5X Master Mix. Details about the reactions volume as components please see chapter 1. Results of all tested reaction were observed on 1% agarose gel electrophoresis. Finally, the real-time Quantitative PCR (qPCR) was also performed using the primers CVC1 / CCSM1 as described by OLIVEIRA, et al. (2002). qPCR results are shown in Ct values, which represents the number of cycles required to capture the fluorescent probe signal by the thermal cycler. Ct value is inversely proportional to the number of fragments available for amplification. Samples with Ct values bellow 36 are considerated as positive for the presence of *X. fastidosa* (FADEL et al., 2014).

4. Results

Protocol for diagnosis of *X. fastidiosa* in sharpshooter's head.

PCR experiments aiming diagnosis of *X. fastidosa* using the RST31/33 primers was unsusccefull with one amplification in all 30 tested samples (data no shown). Although several set primers were published aiming *X. fastidiosa* diagnosis (POOLER et al., 1997; FRANCIS et al., 2006; BULLETIN EOPP, 2016) we decide to teste the seven MLST primers (YUAN et al., 2010) as a diagnostic tool for the presence of

Xylella fastidiosa in the vector. Out of seven primers, only three (cysG, petC and nuoL) showed positive results for the presence of the bacteria (Figure 1). In a subsequent experiment, other DNA samples from insects collected in the field were submitted to PCR using these three selected set-primers (Figure 2). Amplification of *X. fastidiosa* The cysG primer was less sensitive compared to petC and nuoL sets (Figures 2A, B), while the petC set was more stable for diagnosis of *X. fastidiosa* from total DNA (Fig. 2C). In some cases, the total DNA of samples amplified with nuoL showed nonspecific fragments (Figure 3). Based on this information we selected the petC set for diagnosis of *X. fastidiosa* in insects. Amplifications using both high-fidelity TAQ polymerase enzyme or recombinant TAQ polymerase present at master mix kit (FIREPol 5x Master Mix - Solis BioDyne) were similar (Figure 4).

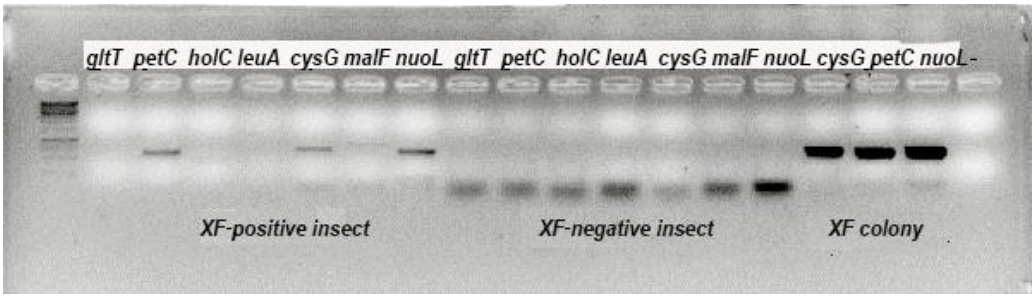

Figure 1: Agorose gel electrophoresis of DNA fragment amplified by seven housekeeping genes. Total DNA of *X. fastidiosa*-infected sharpshooter' head (*X. fastidiosa*-positive; Sample 31) and from health insect (*X. fastidiosa*-negative) was amplified by the seven MLST primers set (*gltT, petC, holC, leuA, cysG, malF, nuoL*).

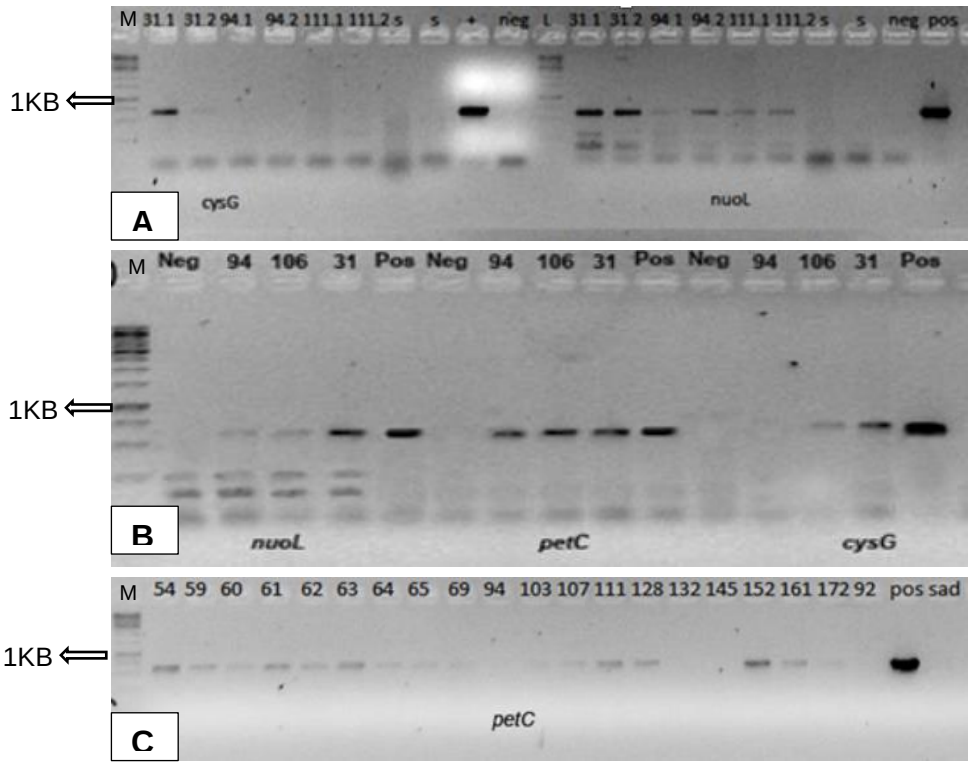

Figure 2: Agorose gel electrophoresis of DNA fragment amplified by three housekeeping genes. **A.** Amplification by the primers *cysG* and *nuoL.* The extension .1 means concentration at 50 ng/ul and .2 (100 ng/ul). **B.** Amplification of total insect DNA using the primers *nuoL*, *petC* and *cysG.* **C.** Amplification of total DNA from twenty samples using the housekeeping gene primer *petC.* M- Ladder 1Kb Genruller.

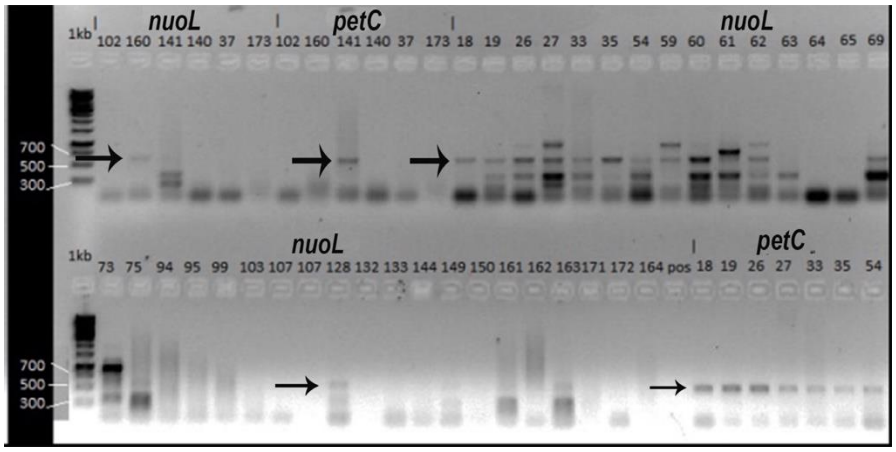

Figure 3: Agorose gel electrophoresis of DNA fragment amplified by the primers petC and nuoL. Arrows shown the expected amplicon sizes of 533bp (petC) and 557 (nuoL), respectivelly. Non-specific fragments are present for nuoL amplification of samples 19, 26, 27, 33, 35, 54, 59, 60, 61, 62, and 69.

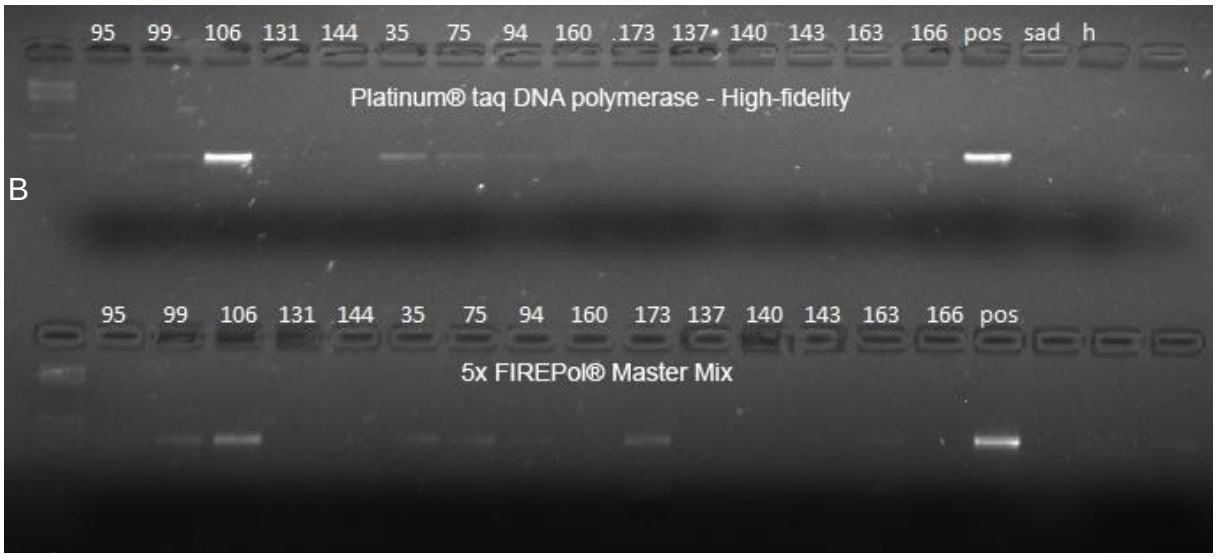

Figure 4. Agorose gel electrophoresis of DNA fragment amplified by the primers petC. A. using a high-fidelity (Platinum™ Taq DNA Polymerase- Invitrogen). B. Regular TAQ polymerase (FIREPol 5x Master Mix- Solis BioDyne).

Detection of Xylella fastidiosa in insect vectors

Based on stablished protocol the FIREPol 5x Master Mix was used to amplify a fragment of *pet*C gene in *X. fastidiosa* using total DNA obtained from 72 specimes of

sharpshooters collect in SP-A1 orchard. At comparation the real-time quantitative PCR using the protocol described by Oliveira et al., (2002) was run using the same samples (Table 1). Standard PCR with petC primer showed a rate of 37% of positive insects inwhile 67% of the analyzed samples by qPCR were positive. In general both tolls share the same positive results in 44% os samples, but samples with Ct values bellow to 29 share amplification of *X. fastidiosa* in 100% with tradicional PCR. Interesting insects of the species *Subrasaca bimaculata* and *Scopogonalia paula* presented the highest percentage of *X. fastidiosa* -positive samples (100% and 72%, respectively).

Table 1: Detection of *Xylella fastidiosa* in insect vectors using Real-time qPCR with and Standard PCR with *nuoL* and *petC* primers.

Sample name	Species	qPCR (Ct)	*petC*
18	*Scopogonalia paula*	Nr[2]	Pos
19	*Scopogonalia paula*	Pos (29.28)	Pos
22	*Scopogonalia paula*	Nr	Pos
26	*Scopogonalia paula*	Pos (25.66)	Pos
27	*Scopogonalia paula*	Pos (29.17)	Pos
128	*Scopogonalia paula*	Nr	Pos
131	*Scopogonalia paula*	Pos (29.92)	Pos
132	*Scopogonalia paula*	Neg (Und)	Neg
133	*Scopogonalia paula*	Pos (30,38)	Pos
134	*Scopogonalia paula*	Neg (38,35)	Neg
135	*Scopogonalia paula*	Neg (38,56)	Neg
48	*Subrasaca bimaculata*	Pos (26,84)	Pos
50	*Subrasaca bimaculata*	Pos (24,13)	Pos
54	*Subrasaca bimaculata*	Pos (30,62)	Pos
59	*Subrasaca bimaculata*	Pos (29,31)	Pos
60	*Subrasaca bimaculata*	Pos (29,04)	Pos
61	*Clastoptera* sp. *1*	Pos (28,34)	Pos
62	*Clastoptera* sp. *1*	Pos (28,37)	Pos
63	*Clastoptera* sp. *1*	Pos (28,57)	Pos
64	*Clastoptera* sp. *1*	Pos (27,99)	Pos
65	*Clastoptera* sp. *1*	Nr	Pos
69	*Clastoptera* sp. *1*	Nr	Pos
75	*Clastoptera* sp. *1*	Pos (34,07)	Neg
94	*Clastoptera* sp. *1*	Pos (34,14)	Neg
97	*Clastoptera* sp. *1*	Pos (32,57)	Neg
98	*Clastoptera* sp. *1*	Pos (31,59)	Neg
99	*Clastoptera* sp. *1*	Pos (29,41)	Pos
100	*Clastoptera* sp. *1*	Pos (32,32)	Neg
101	*Clastoptera* sp. *1*	Pos (31,34)	Neg

102	*Clastoptera* sp*. 1*	Neg (Und)	Neg
103	*Clastoptera* sp*. 1*	Pos (31,00)	Neg
105	*Clastoptera* sp*. 1*	Neg (37,59)	Neg
106	*Paratubana luteomaculata*	Pos (29,64)	Pos
107	*Paratubana luteomaculata*	Neg (Und)	Neg
166	*Macugonalia cavifrons*	Neg (Und)	Neg
167	*Macugonalia cavifrons*	Pos (30,37)	Neg
168	*Macugonalia cavifrons*	Pos (29,23)	Nr
169	*Macugonalia cavifrons*	Pos (31,37)	Neg
170	*Macugonalia cavifrons*	Pos (29,59)	Neg
171	*Macugonalia cavifrons*	Neg (37,74)	Neg
172	*Macugonalia cavifrons*	Pos (30,72)	Pos
173	*Macugonalia cavifrons*	Pos (33,91)	Neg
174	*Macugonalia cavifrons*	Neg (36,61)	Neg
175	*Macugonalia cavifrons*	Neg (38,13)	Neg
176	*Macugonalia cavifrons*	Pos (31,25)	Neg
177	*Macugonalia cavifrons*	Pos (31,73)	Neg
179	*Macugonalia cavifrons*	Pos (31,13)	Neg
180	*Macugonalia cavifrons*	Neg (36,78)	Neg
31	*Macugonalia cavifrons*	Nr	Pos
32	*Macugonalia cavifrons*	Pos (33,56)	Neg
33	*Macugonalia cavifrons*	Nr	Pos
34	*Macugonalia cavifrons*	Nr	Neg
35	*Macugonalia cavifrons*	Pos (33,11)	Pos
76	*Macugonalia cavifrons*	Neg (Und)	Neg
77	*Macugonalia cavifrons*	Pos (32,60)	Neg
78	*Macugonalia cavifrons*	Pos (30,69)	Neg
80	*Macugonalia cavifrons*	Pos (31,29)	Neg
81	*Macugonalia cavifrons*	Pos (29,83)	Neg
82	*Macugonalia cavifrons*	Neg (38,17)	Neg
136	*Macugonalia cavifrons*	Neg (38,03)	Neg
137	*Macugonalia cavifrons*	Neg (Und)	Neg
138	*Macugonalia cavifrons*	Pos (30,89)	Neg
139	*Macugonalia cavifrons*	Neg (36,59)	Neg
140	*Macugonalia cavifrons*	Neg (Und)	Neg
141	*Macugonalia cavifrons*	Neg (Und)	Neg
142	*Macugonalia cavifrons*	Pos (20,64)	Pos
143	*Macugonalia cavifrons*	Neg (38,47)	Neg
144	*Macugonalia cavifrons*	Pos (29,25)	Pos
149	*Macugonalia cavifrons*	Pos (30,29)	Neg
150	*Macugonalia cavifrons*	Pos (29,49)	Neg
Neg	*Macugonalia cavifrons*	Neg (Und)	Neg
Neg	*Macugonalia cavifrons*	Neg (Und)	Neg

Nr – not performed; Und – Undeterminated (Not detected by qPCR)

5. Discussion

In this chapter, we analyze potential *X. fastidiosa*-vectors collected in the orchard located in SP-A1. *X. fastidiosa* MLST housekeeping gene primers were used to identify the presence of bacteria in these insects. In order to validate the results of selected MLST petC primer, the real-time PCR with CVC-1/CCSM-1 (OLIVEIRA et al., 2002) was also performed. Samples with CT values below 29 were also positive by standard PCR with 100% of agreement. When CT results were greater than 31, the result with common PCR was negative. All results of undeterminated CT by qPCR were negative with the use of both primers, as well as negative control insect samples. There is no information about natural infectivity of sharpshooter leafhoppers or spittlebugs by *X. fastidiosa* in Brazil. Natural infectivity of competent vectors *Phyllenius spumaris* by *X. fastidiosa* ranged from 26 to 71% in southeastern Italy (CORNARA et al., 2016). The presence of *X. fastidiosa* bacteria was identified in at least one sample of in all five insect species collected in this experiment, confirming the ability of these sap-feeding insects to be a vector of the bacteria. The highest percentage of insects diagnosed as *X. fastidiosa*-positive belongs to species *Scopogonalia paula, Subrasaca bimaculata e Clastoptera sp. 1*. The high level of infective vectors (37% by PCR and 67% by qPCR) could explain the spread of *X. fastidosa* throught the olive orchards.

6. Conclusion

- *Xylella fastidiosa* is being acquired by vector insects in an OQDS olive tree orchard in São Paulo state, with high infectivity (67% by qPCR).
- Real time PCR using CVC1 / CCSM1 primers is more effective for diagnosing *X. fastidiosa* in vector insects when compared to conventional PCR using RST31 / 33 primers and housekeeping gene primers

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FINAL CONSIDERATIONS

Olive trees with symptoms similar to those seen in southeastern Italy were also identified in southeastern Brazil (Figure 2- chapter 1) which are consequence of infection by *X. fastidiosa* subsp. *pauca* (SAPONARI et al., 2013; COLETTA-FILHO et al., 2016a). Olive production in southeastern Brazil is restricted to small properties spread by the montain chain “Serra da Matiqueira”. Although the olive oil industry has no impact on Brazilian economy, this economic activity has been expanding for the past 10 years, as well as the consumption of olive oil, representing an interesting source of incomes for small farmers (IOOC, 2018). After the first identification of *X. fastidiosa* in few orchards located at Serra da Mantiqueira, questions such as the distribution of bacteria throught orchards of southeastern Brazil and the genetic diversity of bacteria were raised, which were the main objectives of this work.

X. fastidiosa was found in 84% of analyzed orchards but in approximately 45% of suspicious analysed samples. The main symptom related to *X. fastidiosa* infection in olive oil trees is the foliar dissecation that is easly confounded with symptoms related to fungy infections like *Alternaria alternata* and *Verticiliun dahlia* (LOPES-ESCIDERO ;BLANCO, M., 2010; ALAN; MUNIS, 2019). Both symptoms could initially confounded the sampling. Hovewer, dissecated leaves in olive trees infected by *X. fastiodiosa* no drop easly as did those related to fungy infection. Also, in *X. fastidiosa* infected shoots the leaves show wilting and a progressive dessication from top to the base over the time (Figure 1 – chapter one)

In general, the great majority of typed strains by MLST were placed in a main cluster (Table 3, Fig 5 – chapter 1). Fifteen off 20 typed strains shown allelic combination related to ST16 that was early reported in coffee infected plants (YUAN et al., 2010) and recently in olive plants (COLETTA-FILHO et al., 2016b). Few strains shown allelic combinations resulting in novel ST as the ST84 (n=3) and ST86 (n=1)

both from orchards in Sao Paulo State (SP) area A1 and ST85 (n=1) from SPA5. ST85 and 86 were closely retated to ST16 and 66, previously isolated from coffee and the ST84 was closely related to strains from citrus (Fig. 5. chapter 1). Interestingly, in recent survey after the publication of data from chapter 1, we also found ST84 infecting olive trees located at MG-A10, approximately 70km away from SP-A1 (Chapter 1). Besids that, in olive orchards in the areas SP-A1, MG-A7 e MG-A18 we identify the ST-70 of *X. fastidiosa.* This ST was originally reported in hibiscus (*Hibiscus rosa-sinensis*) in the midwest region of Brazil (COLETTA-FILHO et al., 2016b). Finally, in olive leaves with OQDS like-symptoms sampled in Espírito Santo State (ES-A1: 19° 56' 46" S / 40° 46' 18" W) the *X. fastidiosa* isolate from symptomatic plant was characterized as ST13. This sequence type was previously identified in citrus causing CVC (COLETTA-FILHO et al., 2017). This was the unique case of infection in olive plants by *X. fastidiosa* from citrus off all analized samples here. However, in Argentine TOLOCKA et al., 2017 identified the *X. fastidiosa* ST69 in olive trees with OQDS symptoms. The ST69 was previously identified in citrus plants in that country and is closely related to ST13 (COLETTA-FILHO et al., 2017). Tanking together all these information we can affirm that differently of host specificity observed for *X. fastidiosa* subsp. *pauca* from citrus and coffee with no cross inoculation between the respective hosts (FRANCISCO et al., 2016), the olive plants are potentially infected by all strains (STs) of *X. fastidiosa* subsp. *pauca* know so far. Summarizing, in Brazil by STs 13, 16, 70, 84, 85, 86 (COLETTA-FILHO et al., 2016; SAFADY et al 2019), in Argentine the ST69 (TOLOCKA et al., 2017) and in Italy the ST53 (SAPONARI et al 2017). Beyond the subsp. *pauca* the subsp. *multiplex* was reported in California (USA) infecting olive plants also but with less severity symptoms, no systemic infection and apparently with no economic impact (KRUGNER et al., 2014). Hovewer, these information have direct impact on management of sharpshooters in olive orchards closely the coffee or sweet orange plantations once all these three plant species are susceptible to *X. fastidiosa* subsp. *pauca* and share potential vectors (MARUCCI et al. 2008; FROZA 2017).

Simillar to MLST results, analyses of the population structure using all the 204 *X. fastidiosa* isolates typed by 12 SSR loci placed the great majority of strains in a unique cluster plus three other clusters formed by few isolates (Figs. 3 and 4 – chapter 2; Fig.1 this chapter). Still in agreement with MLST results, the greatest heterozygosis calculated from SSR loci were observed in SP-A1 (Hnei=0.5), MG-A8 (Hnei=0.47) and MG-A7 (Hnei = 0.41), the same orchards where different Sequence Types were

identified (MG-A7 and MG-A18 = ST70 and 16; SP-A1 = ST 16, 70, 84 e 86) (Table 1 – chapter 2). But, the general hetegozity index obtained for populations of *X. fastidiosa* from olive (Hnei = 0,19) was smaller than obtained for population from citrus (Hnei = 0.46) (COLETTA-FILHO; FRANCISCO; ALMEIDA, 2014) and from coffee (Hnei =0.60) (FRANCISCO et al., 2017) which can be explained by the recent outbreak of OQDS.

The high number of strains from different orchards in a unique cluster, if by MLST or SSR markers, suggested a clonal spread of bacteria that could be occurred with untentional transite of asymptomatic vegetative material colonized by *X. fastidiosa*. The possible migratory routes shown that most migrants originate from populations located at MG-A1 and MG-A2 areas (Fig. 5, chapter 2). However, reservoir of *X. fastidiosa* in native plants (HILL; PURCELL, 1995) or in planted plants like sweet orange and coffee (TOLOCKA et al., 2017; COLETTA-FILHO et al., 2016[a]) which are visited by potential vectors plus the occurrence of natural recombination (VANHOVE et al., 2019) can explain the occurrence of novel alleles and consequently new sequence types.

Finally, the survey of *X. fastidiosa* in potential vectors collected in the orchard SP-A1 resulted in a natural rate of infectivity of 37.5% by PCR and 67% by qPCR with 44% of agreement for both methodologies (Table 1- chapter 3). The species of vectors related to transmission of *X. fastidiosa* plant-to-plant and their competence has been studied in Brazilian's olive orchards.

However, based on results obtained in this work raises the importance of propagative material on the transmission of *X. fastidiosa* as weel as the management of vectors aiming to reduz the capacity of individuals on transmission of bacteria plant-to-plant.

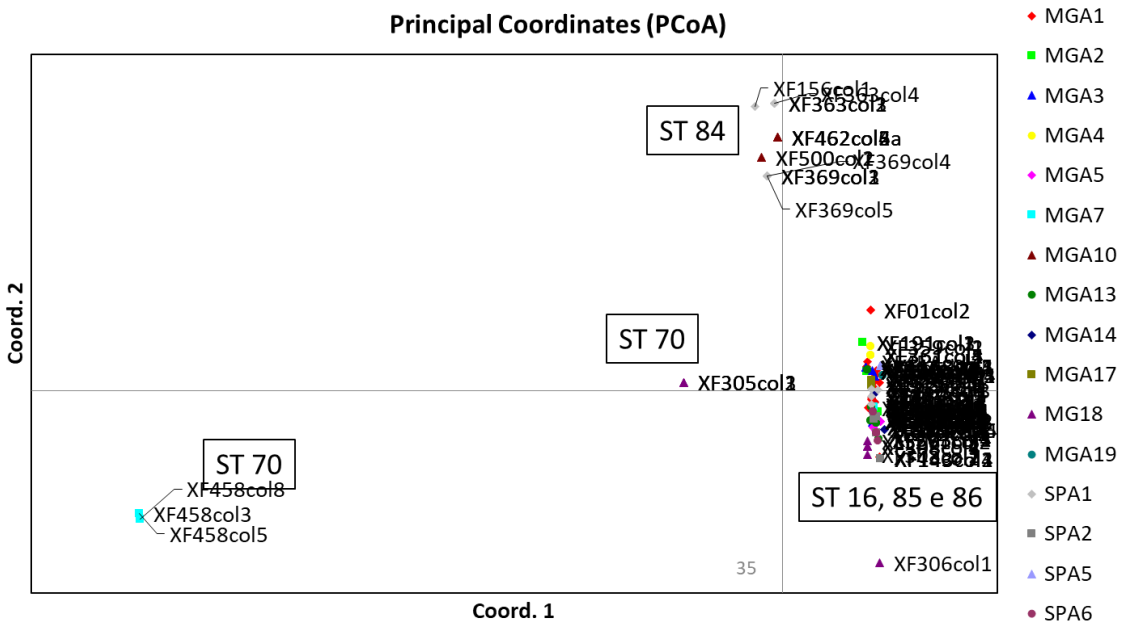

Figure 1. Association of results obtained by 12 polymorphic loci (SSR) and seven MLST housekeeping genes used for typing *Xylella fastidiosa* isolate from sixteen olive oil orchards in Southeastern Brazil.

CONCLUSION

Based on results obtained we can concluded that;

(v) *Xylella fastidiosa* is endemically spread into olive orchards in southeastern region of Brazil.

(vi) *Xylella fastidiosa* subsp. *pauca* was the unique subsp. found in olive plants with OQDS, so far.

(vii) Olive plants were susceptible to natural infection by different sequence type (ST) of *Xylella fastidiosa* subsp. *pauca* from different host plant such as coffee, citrus or hibiscus.

(viii) A clonal population of *Xylella fastidiosa* subsp. *pauca* was found in orchards geographic distante.

(ix) *Xylella fastidiosa*-positive competent vectors were found in a commercial olive orchard.

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Distribution and genetic diversity of xylella fastidiosa associated with olive quick decline in southeastern brazil

APPENDIX

Table 1: Microsatellite data of *Xylella fastidiosa* isolated from olive trees that were used in population genetics analysis.

			LOCUS[4]											
NUMBER[1]	NAME[2]	ORIGIN[3]	COSSR4	CSSR42	OSSR19	OSSR14	OSSR17	GSSR4	COSSR6	GSSR12	COSSR1	COSSR3	CSSR18	COSSR5
			Fragment size											
1	XF01col2	MGA1	307	287	255	372	247	455	312	336	297	294	316	202
2	XF01col2	MGA1	307	287	255	372	247	455	312	336	297	294	316	202
3	XF04col1	MGA1	299	287	239	357	289	439	361	336	283	342	291	218
4	XF07col1	MGA1	323	287	239	357	289	439	361	336	290	342	291	218
5	XF15col1	MGA1	331	287	239	357	295	439	361	336	290	342	291	210
6	XF15col2	MGA1	331	287	239	357	295	439	361	336	290	342	291	210
7	XF15col3	MGA1	331	287	239	357	295	439	361	336	290	342	291	210
8	XF39col1	MGA1	331	287	239	357	283	439	361	336	290	342	291	226
9	XF39col2	MGA1	331	287	239	357	283	439	361	336	290	342	291	226
10	XF43col1	MGA1	347	287	239	357	289	439	361	336	290	342	296	234
11	XF43col2	MGA1	347	287	239	357	289	439	361	336	290	342	296	234
12	XF45Acol1	MGA1	331	287	239	357	277	439	354	336	290	342	291	218
13	XF45Acol3	MGA1	299	287	239	357	283	439	361	336	276	342	291	226
14	XF48Ecol1	MGA1	331	287	239	357	301	439	361	336	290	342	296	218
15	XF48Ecol2	MGA1	331	287	239	357	301	439	361	336	290	342	296	218
16	XF49col1	MGA1	323	287	239	357	283	435	361	336	283	350	286	218
17	XF49col2	MGA1	323	287	239	357	283	435	361	336	290	350	286	218
18	XF50col1	MGA1	331	287	247	357	283	439	361	336	290	342	291	218
19	XF56col1	MGA1	331	287	239	357	283	439	361	336	290	342	291	218
20	XF56col2	MGA1	331	287	239	357	283	439	361	336	290	342	291	218
21	XF64col1	MGA1	331	287	239	357	265	439	354	336	290	342	291	218
22	XF64col2	MGA1	331	287	239	357	289	439	361	336	290	342	291	218
23	XF64col4	MGA1	331	287	239	357	265	439	347	336	290	342	291	218
24	XF69col1	MGA1	323	287	239	357	289	439	368	336	290	342	291	218
25	XF69col2	MGA1	323	287	239	357	289	439	368	336	290	342	291	218
26	XF71col1	MGA1	331	287	239	357	283	439	361	336	290	342	291	226
27	XF71col2	MGA1	331	287	239	357	283	439	361	336	290	342	291	226
28	XF71col3	MGA1	331	287	239	357	283	439	361	336	290	342	291	226
29	XF73col1	MGA1	339	287	239	357	253	439	354	336	297	342	291	218
30	XF327col1	MGA1	331	287	239	357	277	439	361	336	290	342	291	226
31	XF327col2	MGA1	331	287	239	357	277	439	361	336	290	342	291	226
32	XF330col1	MGA1	331	287	239	357	265	439	361	336	290	342	291	218
33	XF331col1	MGA1	331	287	239	357	277	439	361	336	290	350	291	226
34	XF331col2	MGA1	331	287	239	357	283	439	361	336	290	350	291	226
35	XF331col3	MGA1	331	287	239	357	283	439	361	336	290	350	291	226
36	XF331col4	MGA1	331	287	239	357	277	439	361	336	290	350	291	226
37	XF187col1	MGA2	331	287	239	357	277	443	361	336	297	342	296	234
38	XF188col2	MGA2	331	287	239	357	289	439	361	336	290	342	291	218
39	XF188col3	MGA2	331	287	239	357	289	439	361	336	290	342	291	218
40	XF188col4	MGA2	331	287	239	357	289	439	361	336	290	342	291	218
41	XF188col5	MGA2	331	287	239	357	289	439	361	336	290	342	291	218

42	XF188col6	MGA2	331	287	239	357	289	439	361	336	290	342	291	218
43	XF189col1	MGA2	331	287	239	357	277	439	368	336	290	342	291	234
44	XF189col2	MGA2	331	287	239	357	277	439	368	336	290	342	291	234
45	XF189col3	MGA2	331	287	239	357	277	439	368	336	290	342	291	234
46	XF190col1	MGA2	299	287	239	357	289	439	361	336	290	342	291	218
47	XF190col2	MGA2	299	287	239	357	289	439	361	336	290	342	291	218
48	XF190col3	MGA2	299	287	239	357	289	439	361	336	290	342	291	218
49	XF190col4	MGA2	299	287	239	357	289	439	361	336	290	342	291	218
50	XF190col5	MGA2	299	287	239	357	289	439	361	336	290	342	291	218
51	XF191col1	MGA2	275	287	239	357	283	439	361	336	290	342	291	210
52	XF191col2	MGA2	275	287	239	357	283	439	361	336	290	342	291	210
53	XF191col3	MGA2	275	287	239	357	283	439	361	336	290	342	291	210
54	XF98col1	MGA3	323	287	239	357	295	439	368	336	290	342	291	226
55	XF99col1	MGA3	323	287	239	357	277	439	361	336	283	342	291	210
56	XF217col1	MGA3	291	287	239	357	289	439	354	336	290	342	291	226
57	XF217col2	MGA3	291	287	239	357	289	439	354	336	290	342	291	226
58	XF217col3	MGA3	291	287	239	357	289	439	354	336	290	342	291	226
59	XF217col4	MGA3	291	287	239	357	289	439	354	336	290	342	291	226
60	XF221col1	MGA3	323	287	239	357	253	439	385	336	290	342	291	210
61	XF221col2	MGA3	323	287	239	357	253	439	385	336	290	342	291	210
62	XF359col1	MGA4	315	287	255	372	247	451	312	336	325	294	311	194
63	XF359col2	MGA4	315	287	255	372	247	451	312	336	325	294	311	194
64	XF361col1	MGA4	315	287	255	372	247	451	312	336	332	294	311	194
65	XF361col2	MGA4	315	287	255	372	247	451	312	336	332	294	311	194
66	XF361col3	MGA4	315	287	255	372	247	451	312	336	332	294	311	194
67	XF361col4	MGA4	315	287	255	372	247	451	312	336	332	294	311	194
68	XF361col5	MGA4	315	287	255	372	247	451	312	336	332	294	311	194
69	XF280col1	MGA5	315	287	239	372	289	439	361	336	290	342	291	218
70	XF280col2	MGA5	315	287	239	372	289	439	368	336	290	342	291	218
71	XF280col3	MGA5	315	287	239	372	289	439	368	336	283	342	291	218
72	XF280col4	MGA5	315	287	239	372	289	439	368	336	283	342	291	218
73	XF280col5	MGA5	315	287	239	372	289	439	368	336	290	342	291	218
74	XF282col1	MGA5	307	287	239	372	289	439	368	336	297	342	291	218
75	XF282col2	MGA5	307	287	239	372	289	439	368	336	290	342	291	218
76	XF282col3	MGA5	307	287	239	372	289	439	368	336	290	342	291	218
77	XF282col4	MGA5	307	287	239	372	289	439	368	336	290	342	291	218
78	XF282col5	MGA5	307	287	239	372	289	439	368	336	297	342	291	218
79	XF424col2	MGA5	339	287	239	357	283	439	354	336	283	358	291	218
80	XF424col3	MGA5	339	287	239	357	283	439	354	336	283	358	291	218
81	XF424col1	MGA5	339	287	239	357	283	439	354	336	283	358	291	218
82	XF458col3	MGA7	243	294	497	248	301	267	368	343	297	286	306	202
83	XF458col5	MGA7	243	294	497	248	301	267	368	343	297	286	306	202
84	XF458col8	MGA7	243	294	497	248	301	267	368	343	297	286	301	202
85	XF481col2	MGA7	331	287	239	357	277	439	368	336	297	342	291	218
86	XF481col1	MGA7	331	287	239	357	277	439	368	336	297	342	291	218
87	XF462col2a	MGA10	243	287	278	234	235	450	327	336	275	284	326	170
88	XF462col3	MGA10	243	287	278	234	235	450	327	336	275	284	326	170
89	XF462col4	MGA10	243	287	278	234	235	450	327	336	275	284	326	170
90	XF462col6	MGA10	243	287	278	234	235	450	327	336	275	284	326	170
91	XF462col5a	MGA10	243	287	278	234	235	450	327	336	275	284	326	170

92	XF500col1	MGA10	243	287	286	234	265	439	337	336	262	284	360	170
93	XF500col2	MGA10	243	287	286	234	265	439	337	336	262	284	360	170
94	XF296col1	MGA13	339	287	247	357	283	439	361	336	290	342	291	218
95	XF296col3	MGA13	339	287	247	357	283	439	361	336	290	342	291	218
96	XF296col5	MGA13	339	287	247	357	283	439	361	336	290	342	291	219
97	XF297col1	MGA13	339	287	239	357	289	439	361	336	290	342	291	218
98	XF297col2	MGA13	339	287	239	357	289	439	361	336	290	342	291	218
99	XF300col1	MGA13	323	287	247	357	265	439	361	336	283	342	296	218
100	XF300col2	MGA13	323	287	247	357	265	439	361	336	283	342	296	218
101	XF300col3	MGA13	323	287	247	357	265	439	361	336	283	342	296	218
102	XF300col4	MGA13	323	287	247	357	265	439	361	336	283	342	296	218
103	XF302col1	MGA13	323	287	239	357	295	439	361	336	290	342	291	218
104	XF302col2	MGA13	323	287	239	357	295	439	361	336	290	342	291	218
105	XF302col3	MGA13	323	287	239	357	295	439	361	336	290	342	291	218
106	XF302col4	MGA13	323	287	239	357	295	439	361	336	290	342	291	218
107	XF302col5	MGA13	323	287	239	357	295	439	361	336	290	342	291	218
108	XF303col1	MGA13	347	287	239	357	289	439	361	336	290	342	291	218
109	XF303col2	MGA13	347	287	239	357	289	439	361	336	290	342	291	218
110	XF303col3	MGA13	347	287	239	357	289	439	361	336	290	342	291	218
111	XF303col4	MGA13	347	287	239	357	289	439	361	336	290	342	291	218
112	XF303col5	MGA13	347	287	239	357	289	439	361	336	290	342	291	218
113	XF489col1	MGA14	331	287	239	357	283	439	361	336	290	342	291	210
114	XF489col2	MGA14	331	287	239	357	283	439	361	336	290	342	291	210
115	XF489col3	MGA14	331	287	239	357	283	439	361	336	290	342	291	210
116	XF489col4	MGA14	362	287	239	357	283	439	361	336	290	343	306	170
117	XF489col5	MGA14	362	287	239	357	283	439	361	336	290	343	306	170
118	XF319col1	MGA17	323	287	239	357	289	439	361	336	290	342	291	202
119	XF319col2	MGA17	323	287	239	357	289	439	361	336	290	342	291	202
120	XF319col3	MGA17	323	287	239	357	289	439	361	336	290	342	291	202
121	XF319col4	MGA17	323	287	239	357	289	439	361	336	283	342	291	202
122	XF319col5	MGA17	323	287	239	357	289	439	361	336	290	342	291	202
123	XF305col1	MG18	243	287	295	267	271	279	340	343	318	334	331	170
124	XF305col2	MG18	243	287	295	267	271	279	340	343	318	334	331	170
125	XF305col3	MG18	243	287	295	267	271	279	340	343	318	334	331	170
126	XF306col1	MG18	323	287	239	357	295	439	361	336	360	350	291	234
127	XF308col1	MG18	323	287	247	357	301	439	361	336	297	350	291	226
128	XF308col2	MG18	323	287	247	357	301	439	368	343	297	350	291	226
129	XF308col3	MG18	323	287	247	357	301	439	361	336	297	350	291	226
130	XF308col4	MG18	323	287	247	357	301	439	361	336	297	350	291	226
131	XF308col5	MG18	323	287	247	357	301	439	354	336	297	350	291	226
132	XF483col1	MGA19	339	287	239	357	259	447	361	336	290	342	296	218
133	XF483col2	MGA19	339	287	239	357	259	447	361	336	290	342	296	218
134	XF483col3	MGA19	339	287	239	357	259	447	361	336	290	342	296	218
135	XF483col4	MGA19	339	287	239	357	259	447	361	336	290	342	296	218
136	XF483col5	MGA19	339	287	239	357	259	447	361	336	290	342	296	218
137	XF156col1	SPA1	243	287	303	237	223	451	305	336	240	294	306	178
138	XF367col1	SPA1	323	287	239	357	283	439	368	336	290	342	291	218
139	XF367col2	SPA1	323	287	239	357	289	439	368	336	290	342	291	218
140	XF367col3	SPA1	323	287	239	357	289	451	368	336	290	342	291	218
141	XF367col5	SPA1	323	287	239	357	283	439	368	336	283	342	296	218

142	XF363col1	SPA1	243	287	279	237	223	447	305	336	240	294	301	178
143	XF363col2	SPA1	243	287	279	237	223	447	305	336	240	294	301	178
144	XF363col3	SPA1	243	287	279	237	223	447	305	336	240	294	301	178
145	XF363col4	SPA1	243	287	279	237	223	447	305	336	240	294	301	178
146	XF369col1	SPA1	243	287	287	237	235	431	361	336	247	286	281	178
147	XF369col2	SPA1	243	287	287	237	235	431	361	336	247	286	281	178
148	XF369col3	SPA1	243	287	287	237	235	431	361	336	247	286	281	178
149	XF369col4	SPA1	243	287	287	237	235	431	361	336	247	286	281	178
150	XF369col5	SPA1	243	287	287	237	235	431	361	336	247	286	281	178
151	XF143col1	SPA2	347	287	239	357	289	439	361	336	290	342	291	234
152	XF143col2	SPA2	347	287	239	357	289	439	361	336	290	342	291	234
153	XF143col3	SPA2	347	287	239	357	289	439	361	336	290	342	291	234
154	XF143col4	SPA2	347	287	239	357	289	439	361	336	290	342	291	234
155	XF263col1	SPA2	339	287	239	357	295	439	361	336	290	342	291	218
156	XF263col2	SPA2	339	287	239	357	295	439	361	336	290	342	291	218
157	XF263col3	SPA2	339	287	239	357	283	439	361	336	290	342	286	218
158	XF263col4	SPA2	339	287	239	357	295	439	361	336	290	342	291	218
159	XF263col5	SPA2	339	287	239	357	295	439	361	336	290	342	291	218
160	XF265col1	SPA2	331	287	239	357	295	439	361	336	290	342	291	218
161	XF265col2	SPA2	331	287	239	357	295	439	361	336	290	342	291	218
162	XF265col3	SPA2	331	287	239	357	295	439	361	336	290	342	291	218
163	XF265col4	SPA2	331	287	239	357	295	439	361	336	290	342	291	218
164	XF168col1	SPA5	323	287	231	357	265	439	354	336	290	342	291	226
165	XF168col2	SPA5	323	287	231	357	265	439	354	336	290	342	291	226
166	XF168col4	SPA5	323	287	231	357	265	439	354	336	290	342	291	226
167	XF168col5	SPA5	323	287	231	357	265	439	354	336	290	342	291	226
168	XF168col3	SPA5	323	287	231	357	265	439	354	336	290	342	291	226
169	XF271col1	SPA6	331	287	239	357	295	439	361	336	290	342	291	210
170	XF271col2	SPA6	347	287	239	357	295	439	361	336	290	342	291	210
171	XF271col3	SPA6	331	287	239	357	295	439	361	336	290	342	291	210
172	XF271col4	SPA6	331	287	239	357	295	439	361	336	290	342	291	210
173	XF271col5	SPA6	331	287	239	357	295	439	361	336	290	342	291	210
174	XF272col1	SPA6	339	287	239	357	295	439	361	336	290	342	291	218
175	XF272col2	SPA6	339	287	239	357	295	439	361	336	290	342	291	218
176	XF272col3	SPA6	339	287	239	357	295	439	361	336	290	342	291	218
177	XF272col4	SPA6	339	287	239	357	295	439	361	336	290	342	291	218

1: Representative number

2: Isolated name. XF: Xylella fastidiosa; col: Colonie

3: Region where the bacteria was isolated

4: SSR locus