Genetska raznolikost genotipova trsova ušenca (Daktulosphaira vitifoliae Fitch) u Hrvatskoj

Čajić, Marko

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UNIVERSITY OF ZAGREB

FACULTY OF AGRICULTURE

MARKO ČAJIĆ

Genetic Diversity of Grape Phylloxera (*Daktulosphaira vitifoliae* Fitch) Genotypes in Croatia

DIPLOMA THESIS

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Menthor: prof. dr. sc. Ivan Pejić

Zagreb, 2016

This Diploma thesis is evaluated and defended on _____

and graded with _____ in front of Committee composed of:

1. Prof. dr. sc. Ivan Pejić _____

2. Prof. dr. sc. Božena Barić_____

3.Prof.dr.sc.Astrid Forneck(BOKU,Austria)_____

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Genetic Diversity of Grape Phylloxera (*Daktulosphaira vitifoliae* Fitch) Genotypes in Croatia

Summary

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) is known to be a major pest for European and worldwide viticulture (Powel et al., 2013). Introduction of the pest from America to Europe in the second half of the 19th century led to the economic losses due to the destruction of vineyards. At the beginning of the 20th century American vines and their hybrids were used as rootstocks for European grapevine varieties to achieve a partial phylloxera tolerance. As a result, the hybrids showed less or no damage from phylloxera, they were initially thought to be resistant. With the increasing damage in the last few decades, it was realized that rootstocks are rather phylloxera tolerant, than completely resistant. The cases of phylloxera overcoming plant resistance leading to economic loss on the rootstock AxR1 in California are well known.

With regard to feeding and development, pest occurs in two forms - phylloxera, which feeds and lives on the root (root-feeding phylloxera) and phylloxera which feeds and lives on the feeding (leaf-feeding phylloxera). Although damage by leaf-feeding phylloxera is often neglected, it does lead to reduction of photosynthesis and an additional sink tissue for the plants, it also affects the size of the population forms that inhabits and is doing the damage to the roots.

The aim of this thesis was to determine the genetic diversity of grape phylloxera leaf feeding populations in Croatia and compare it with the similar data representing phylloxera genotypes from Burgenland, Austria, expecting a lot of variation within Croatian genotypes due to the diversity of the Croatian climate. The field sampling of grape phylloxera indiviudals has been conducted on the leaf infested plants, in Croatia on fall 2015. The samples were collected in Dubrovačko–Neretvanska, Splitsko–Dalmatinska, Šibensko-Kninska, Primorsko-Goranska, Virovitičko-Podravska and Zagrebačka county. The isolation of the DNA from L5 stage of pest and the genotyping of individuals with standardized set of 7 SSR markers, and the analysis of PCR products on capillary sequencer ABI 3130 xl were conducted at the University of BOKU (Austria).

In total, 120 individuals of phylloxera were genotyped and analyzed. A high genetic variability of Croatian populations was assessed, especially among individuals of the coastal area. The results confirmed a high proportion of individuals resulting from asexual reproduction (1/3), but among them the dominant genotype (clone) was not observed.

Key words: grape phylloxera, genotype, genetic diversity, SSR

Genetska raznolikost genotipova trsova ušenca (*Daktulosphaira vitifoliae* Fitch) u Hrvatskoj

Sažetak

Trsov ušenac (*Daktulosphaira vitifoliae* Fitch) je poznat štetnik vinogradarstva u Europi i diljem svijeta. Introdukcija štetnika iz Amerike u Europu u drugoj polovici 19. stoljeća dovela je do propadanja vinograda i velikih ekonomskih gubitaka. Početkom dvadesetog stoljeća američke sorte i njihovi hibridi uvode se kao podloge za europske sorte vinove loze za postizanje otpornosti na trsova ušenca, obzirom da ekonomska šteta proizlazi iz uništavanja korijena plemenite loze. Podloge su pokazivale malu ili nikakvu štetu, te se prvotno mislilo da su rezistentne. Porastom šteta u posljednjih par desetljeća, došlo se do zaključka da su podloge vjerojatnije tolerantne, nego u potpunosti rezistentne na trsova ušenca. U praksi je dobro poznat slučaj u kojemu je trsov ušenac prevladao rezistentnost biljke te doveo do ekonomskih gubitaka na podlogama AxR1 u Kaliforniji.

Obzirom na ishranu i razvoj, štetnik se pojavljuje u dvije forme,trsov ušenackoji se hrani i obitava na korijenu (*root-feeding phylloxera*) iforma koja se hrani i obitava na listu (*leaf-feeding phylloxera*). Iako se štete izazvane ishranom štetnika na listu obično smatraju zanemarivima, one ipak dovodi do smanjene fotosinteze i dodatnih venuća tkiva na biljci, te utječu i na veličinu populacije forme koja obitava i pravi štetu na korijenu.

Cilj ovoga rada je utvrditi genetski diverzitet populacija trsovog ušenca koje se hrane listom sa područja Hrvatske, te ga usporediti sa diverzitetom analognih populacija iz Gradišća (Burgenland) u Austriji. Uzimanje uzoraka jedinki trsova ušenca obavljeno je na listu zaraženih biljaka u jesen 2015. Uzorci su sakupljeni na dvadesetjednoj lokaciji u Dubrovačko-Neretvanskoj, Splitsko-Dalmatinskoj, Šibensko–Kninskoj, Primorsko–Goranskoj, Virovitičko-Podravskoj i Zagrebačkoj županiji. Izolacija DNA iz L5 stadija štetnika i genotipizacija jedinki standardiziranim setom od 7 SSR markera, te analiza PCR produkata na kapilarnom sekvenceru ABI 3130 xl provedena je na Sveučilištu BOKU (Austrija).

Ukupno je genotipizirano i analizirano 120 jedinki trsova ušenca. Utvrđena je visoka genetska varijabilnost populacija iz Hrvatske, a posebno među jedinkama iz priobalnog područja. Rezultati su potvrdili visok udio jedinki proisteklih iz aseksualnog razmnožavnja (1/3), ali među njima nije uočen dominantni genotip (klon).

Ključne riječi: trsov ušenac, genotip, genetski diverzitet, SSR

Contents

1. Introduction 1
2. Bibliography review 2
2.1. Grape phylloxera history 3
2.2. Grape phylloxera history in Croatia 5
2.3. Life cycle of grape phylloxera 6
2.4. Phylloxera biology 8
2.5. Presence of grape phylloxera 9
2.6. Genetic variation of grape phylloxera 11
2.7. Grafting with resistant rootstock 12
2.8. The choice of rootstock 14
3. Research aim 16
4. Materials and methods 17
4.1. Materials 17
4.2. Methods 20
4.2.1.Tissue collection 20
4.2.2. DNA extraction 21
4.2.3. PCR –Reaction mixture 22
4.2.4. PCR 23
4.2.5. PCR products' check 24
4.2.6. Fragment evaluation by ABI 3130 xl 25
4.2.7. Data scoring and analysis of diversity 28
5. Results and discussion 29
5.1. Genotypic data of 7 SSR loci for all analyzed phylloxera individuals 29
5.2. Allelic polymorphism 32
5.3. Allele frequencies 35
5.4. Analysis of genetic similarity among phylloxera individuals of different geographic origin 37
6. Conclusions 40
7. List of references 41

1. Introduction

About 150 years ago from America to Europe entered a pest named grape phylloxera (*Daktulosphaira vitifoliae* Fitch), that has caused drying and decay of vineyards in Europe and around the world. Phylloxera is one of the most important pests that on the turn of the 19th and 20th century entered to Europe from the New World, causing significant economic losses and deterioration of the former economy. Consequently, this also caused great demographic changes that lead to significant emigration from Europe to New World countries. While America's genotypes of*Vitis spp*. had developed tolerance to this aphid due to long evolutionary coexistence, European „noble grape“ (*Vitis vinifera* L.) is completely susceptible to this pest. The main damage is done by feeding on the roots of *Vitis vinifera* L., resulting in formation of tuberosities and nodosities on plant roots. The first attempts to overcome the problems were attempts to create interspecies hybrids but, they have not been proven acceptable.The most practical way to prevent damage of this pest was found in grafting European grapevines on American rootstocks.However, cases of phylloxera overcoming plant resistance leading to economic loss e.g. on the rootstock Ax#R1 in California are well known (Granett, 1985). This drew the attention of scientists to start research on the life cycle biotypes of phylloxera and studies on the genetic structure of the pest (Powel et al., 2013) of the pest.

However, the genetic structure of phylloxera of Croatia have never been analyzed. The adaptation and reproduction of every living organism, as well as phylloxera, largely depend on adapting to the conditions of the new habitat. As environmental conditions of viticulture in Croatia are very different between continental and coastal areas, it would be important to study populations originating from different agro-ecological conditions to analyse their mode of reproduction and to genereally get information of their genetic structure.

Apart from researching the biological and morphological characteristics of populations of phylloxera,today the analysis of genetic diversity using molecular markers is possible as well.In numerous studies on different plant and animal species a relationship (correlation) between the biological and morphological diversity was detected with the one revealed by molecular markers.

Therefore, the aim of this thesis was to analyze the genetic diversity of the Croatian populations of phylloxera by molecular markers.

2. Bibliography review

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) is known to be a major pest for the European and worldwide viticulture (Powel et al., 2013). The Phylloxeridae are a small family, containing 75 species. Grape phylloxera is the most economically destructive and geographically widespread pest species of commercial grapevines, on which it is an obligate biotroph of *Vitis* species, occurring in almost all viticultural regions around the world (Powell, 2012). Root feeding phylloxera is different genetically from leaf feeding phylloxera (Bao et al., 2014). The parasite infests the root system and lives off *Vitis ssp.* and is gall-forming. The root-feeding stages cause the highest economic damage, leading to tuberosity and nodosity formation. Grape phylloxera feeds monophagously on *Vitis* species. Root-galling grape phylloxera disrupts water and nutrient uptake, loss of leaf surface area, yield reduction, and depending on other biotic and abitoc factors grapevine death within 4-7 years of infestation, as a result of nodosities on nonlignified roots and tuberosites on older lignified roots (Powel et al., 2013). Currently there is no cure for root-feeding phylloxera and unlike other grape diseases such as powdery or downy mildew, there is no chemical control or response. The only successful mean of controlling phylloxera is grafting more susceptible European *vinifera* vines on phylloxera resistant American rootstock (usually hybrid varieties created from the *Vitis berlandieri*, *Vitis riparia* and *Vitis rupestris* species) (Robinson, 2011).



Figure1. Grape phylloxera

(http://ncsmallfruitsipm.blogspot.hr/2010/07/grape-phylloxera-update.html - 2016)

2.1. Grape phylloxera history

In the late 19th century the phylloxera epidemic destroyed most of the vineyards for wine grapes in Europe, most notably in France. Phylloxera was introduced to Europe when avid botanists in Victorian England collected specimens of American grapevines in the 1850s. Because phylloxera is native to North America, the native grape species are at least partially resistant. By contrast, the European wine grape *Vitis vinifera* is very susceptible to the insect. The epidemic devastated vineyards in Britain and then moved to the European mainland, destroying most of the European grape growing industry. In 1863, the first vines began to deteriorate inexplicably in the southern Rhône region of France. The problem spread rapidly across the continent. In France alone, total wine production fell from 84.5 million hectolitres in 1875 to only 23.4 million hectolitres in 1889. Some estimates hold that between two-thirds and nine-tenths of all European vineyards were destroyed (http://www.winepros.com.au, 2016). Introduction of the pest from America to Europe led to economic losses due to the destruction of vineyards. Many vineyards were completely collapsed, so in those years there were recorded thousands of suicides among French winegrowers (Maceljski, 2002). In France, one of the desperate measures of grape growers was to bury a live toad under each vine to draw out the "poison". Areas with soils composed principally of sand or schist were spared, and the spread was slowed down in dry climates, but gradually the aphid spread across the continent. A significant amount of research was devoted to finding a solution to the phylloxera problem, and two major solutions gradually emerged: grafting cuttings onto resistant rootstocks and hybridization (http://www.winepros.com.au, 2016).

With this ended the golden age of European viticulture,and vineyards across the Old Continent began to deteriorate massively. In 1863, the cuttings of some American species of the genus Vitis infected with phylloxera were brought from America to the Botanical Garden in England. The pest originated from the valley of the Mississippi river, which at that time was not known outside of North America (Maletić et al., 2008).

These almost microscopic, pale yellow insects, related to aphids, feed on the roots and leaves of grapevines. On *Vitis vinifera L.*, resulting in deformations on roots ("nodosities" and "tuberosities") and secondary fungal infections can girdle roots, gradually cutting off the flow of nutrients and water to the vine (Robinson, 2011).In the late 19th and early 20th century phylloxera destroyed all the vineyards in Europe.This has prompted many countries to cooperate and in 1878 the Berlin Conference on suppression phylloxera is formed, the world's

first phytosanitary document (Maceljski et al.,2006).This Convention is considered as the beginning of organized international cooperation in the implementation of plant quarantine and the forerunner of today's Plant Protection Convention, which was signed by Croatia as well. And in our area at the time, a number of regulations were adopted and mass actions were undertaken to reduce the consequences of the appearance of phylloxera. At the beginning of the twentieth century, American grapevines and their hybrids were used as rootstocks for European grapevine varieties to achieve a partial phylloxera resistance. As a result, hybrids showed less or no damage from phylloxera, they were initially thought to be resistant. With the increasing damage in the last few decades, it has been realized that the rootstocks are phylloxera tolerant, rather than completely resistant. However, cases of phylloxera biotzpes overcoming plant resistance leading to economic loss e.g. on the rootstock Ax#R1 in California are well known (Granett, 1985, Forneck et al. 2016).



Figure 2. Brown leaves and sparse foliage in Cabernet Sauvignona vineyard demaged by grape phylloxera, feeding on roots. Photo by Jack Kelly Clark. University of California (http://westernfarmpress.com/monitor-vineyards-problems-phylloxera- 2016)

Root-galling grape phylloxera disrupts water and nutrient uptake, loss of leaf surface area, yield reduction, and depending on other biotic and abitoc factors vine death within 4-7 years of infestation, as a result of nodosities on nonlignified roots and tuberosites on older lignified roots (Powel et al., 2013).

Although the damage by leaf-feeding phylloxera is often neglected, it does lead to reduction of photosynthesis and an additional sink tissue for the plants it also affects the size of the population forms that inhabits and is doing the damage to the roots (Nabity et al., 2013).

By the end of the 19th century, hybridization became a popular avenue of research for stopping the phylloxera louse. Hybridization is the breeding of *Vitis vinifera* with resistant species. Most native American grapes are naturally phylloxera resistant (*Vitis aestivalis, rupestris,* and *riparia* are particularly so, while *Vitis labrusca* has somewhat weak resistance to it) but have aromas that are off-putting to palates accustomed to European grapes. The intent of the cross was to generate a hybrid vine that was resistant to phylloxera but produced wine that did not taste like the American grape. Ironically, the hybrids tend not to be especially resistant to phylloxera, although they are much more hardy with respect to climate and other vine diseases. The new hybrid varieties have never gained the popularity of the traditional ones. In the EU they are generally banned or at least strongly discouraged from use in quality wine, although they are still in widespread use in much of North America, such as Missouri, Ontario, and upstate New York (https://en.wikipedia.org/wiki/Phylloxera, 2016).

On grafted vine suppression is not needed. But between 1960 and 1965 in some countries of Central Europe, including Croatia, some vineyard experts have developed a theory about the absence of phylloxera, and if it appeared, it could be suppressed by chemical and mechanical means. Therefore, it was proposed to raise some vineyards without grafting European vines. Entomological experts disagreed with that theory, and were warning that phylloxera was found on non-grafted individual vines, and mainly on inadmissibilityof introducing widespread use of chemical agents for the treatment of soil (Maceljski, 2002).

2.2. Grape phylloxera history in Croatia

In Croatia, the phylloxera was firstly established in Brdovec near Zagreb in 1880, and soon in other areas (Maceljski et al.,2006). In Dalmatia this pest occurs later than in continental Croatia and Western Europe. For that reason, there was an increased demand for Dalmatian vines. Exports were increased, mainly in France, prices of grapes and wine were growing, which lead to a burgeoning viticulture and economy. Overthe next twenty years, during this period, there was a large increase in the area and production of wine.Official statistics record that in the late 19th century, there were more than 90 000 ha under the vines only in Dalmatia, and throughout Croatia 170 000 ha. It should be noted the fact that in 1908 almost the entire surface of the the Island of Susak (96%) was covered with vines (Maletić et al., 2008). Between people, the term 'phylloxera' is used to refer to an extremely high harmfulness ofother pests as well, similarlyas the term of plague is used for various other diseases (Maceljski,2002).Introduction

of pests from America to Europe has led to a deterioration of the vineyards and economic losses. After this there were some great economic and demographic changes, thanks to the areas where viticulture has been the dominant branch, which lead to a mass exodus of the population (Maletić et al., 2008). At the beginning of the twentieth century the American varieties and their hybrids were used as the basis for the European grapevine varieties for partial resistance to phylloxera. Hybrids showed little or no damage from phylloxera, and originally it was thought they were resistant. With increasing damage in the last few decades, it was concluded that the rootstocks are likely tolerant, but not completely resistant to phylloxera.Although the damage caused by pests feeding on the leaf usually considered negligible, they nevertheless lead to reduced photosynthesis and additional wilting on the plant tissue (Nabity et al., 2013).Phylloxera that feeds on the roots is genetically different from the one that feeds on the leaf (Bao et al., 2014).



Figure 3. Mass exsodus from the island of Korčula to South America and Australia because of the phylloxera threat. (In one day, from the port of Prigradica, 1200 people moved out and it was recorded as the saddest day in the history of the island.) (http://www.korcula-barilo.com/blato - 2016)

2.3. Life cycle of grape phylloxera

Grape phylloxera has a complex life cycle. The classic life cycle is holocyclic, which means that it includes sexual and asexual reproduction (Powell et al., 2013). Its cycle is termed anholocyclic when only asexual morphs are found on either roots or leaves (Forneck et al., 2001). During spring and summer, phylloxera reproduces parthenogenetically on roots and leaves. When the growing season ends, winged phylloxera capable of sexual reproduction (sexuparae) occur. Sexuparae can asexually produce offspring, more specifically, males and

females, which in turn mate. Then, females (oviparae) lay single eggs that overwinter (Forneck&Huber, 2009). When hatching out, these eggs give rise to fundatrices, which resume asexual reproduction on roots or leaves (Powell et al., 2013).

Nymphs form protective galls on the undersides of grapevine leaves of some *Vitis spp.* and overwinter under the bark or on the vine roots; these leaf galls are typically only found on the leaves of American grapevines. American grapevine species (such as *Vitis labrusca*) have evolved to have several natural defenses against phylloxera. The roots of the American grapevines exude a sticky sap that repels the nymph form when it tries to feed from the vine by clogging its mouth. If the nymph is successful in creating a feeding wound on the root, American grapevines respond by forming a protective layer of tissue to cover the wound and protect it from secondary bacterial or fungal infections (Robinson, 2011).

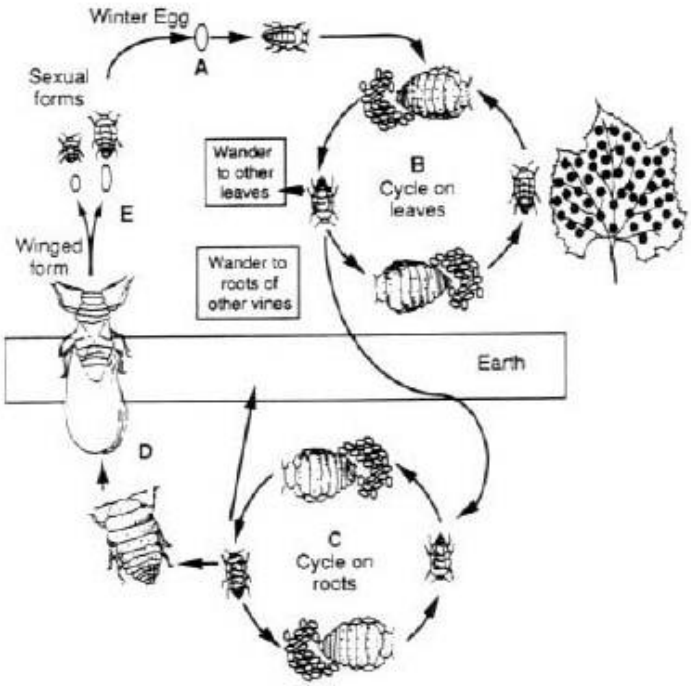


Figure 4. Life cycle of grape phylloxera, *Daktulosphaira vitifoliae* (Fitch), (after Williams, 1938). (A) Winter egg, (B) foliar form (Gallicola), (C) root form (radicola), (D) winged adult, (E) sexual stage.

(https://archive.fo/20120716060151/http://ohioline.osu.edu/hyg-fact/2000/2600.html - 2016)

2.4. Phylloxera biology

Phylloxera is a small aphid, 0.7-1.4 mm long, sometimes up to 2 mm. Yellow-brown in color, the stiletto passes through the proboscis, which pierces the plant tissue, from which they suck the juices. There are several strains that differ morphologically and in way of living. It feeds with the juices by sucking them from the leaves of American grapevines and directly reproducing hybrids. Therefore, it is found in the stock nursuries and the "directors" (Isabela, etc.). In the stock nurseries it can reduce growth and slow the woodening of the sprout, which is becoming more sensitive to the frost. It cannot damage the root of the majority of species of American grapevines, which are therefore used as the rootstock to European lineage. Yet there is a theoretical possibility that the phylloxera can adjust to the roots of the American grapevines as well because it was noted that it very slowly adapts to the European grapevine leaves (with natural selection). In Croatia it is constantly maintained and present on the American grapevine and hybrids. It would therefore be wrong to plant the vines in Europe (on its own root, without grafting) (Maceljski et al., 2006).



Figure 5. Grape phylloxera (http://bugguide.net/node/view/794982- 2016)

Nowadays, the European grapes can only be grown on some sandy terrains along the Danube and on some of our islands without grafting on the American grapevines, because in these fields phylloxera has no possibilities for development. In France, it is considered that phylloxera has no conditions for the development on the sandy soil that contains less than 5% loam particles or during the winter if it is under water for more than 40-60 days. In Croatia, in all other cultivated vineyards grafted vine is grown (Maceljski, 2002).

2.5. Presence of grape phylloxera

The presence on the leaves is seen by swellings on the underside of the leaves, where aphids live. On the upper side of the leaves there is a small hole. The swellings of grape phylloxera are easily differed from the swellings of the grape bud mite, which are located on the face of the leaf, and on the underside are sunken, that depression is often covered with woolly coating (Maceljski et al., 2006).



Figure6. Grape phylloxera on leaf (http://entoweb.okstate.edu/ddd/insects/grapephylloxera.htm-2016)

On the European grapevine, it sucks on the root, making a swelling of different types of nodosities and tuberosities (Maceljski et al., 2006). These bumps hinder the circulation of the juices, and the vine dries. However, it should be mentioned that in the mid-eighties in Italy some infected leaves with phylloxera were found on European grapevines. Soon after, the phenomenon was observed in Istria on some cultivars of grapevines. Although for now, this

phenomenon is not harmful, it indicates that the phylloxera can adapt to different conditions, so if it could adapt to European grapevine leaves, there is the possibility of adapting to the root of the American rootstocks. In that case, the problem of a phylloxera would again appear for viticulture, but we hope that this will not occur in the foreseeable future (Maceljski, 2002).

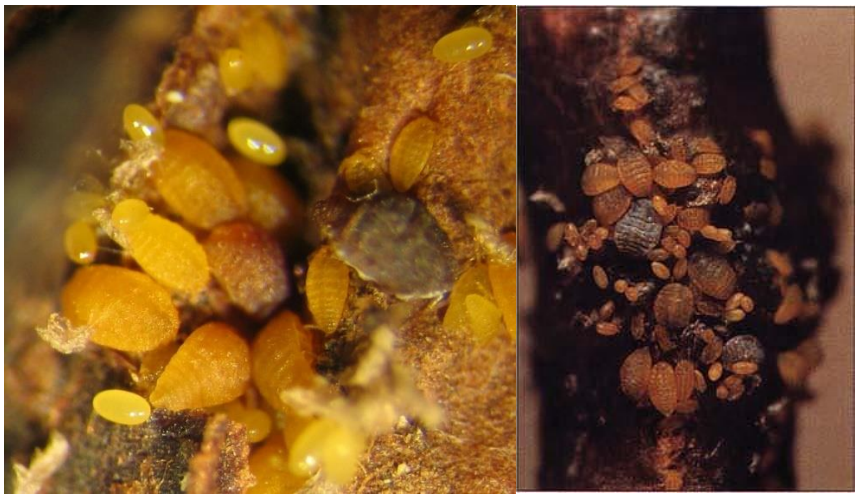


Figure 7 and 8. Grape phylloxera on root
(http://apelasyon.com/Yazi/425-bagcilikta-neden-anac-kullaniyoruz- 2016)
(http://calag.ucanr.edu/Archive/?article=ca.v050n04p9– 2016)

The development of phylloxera is noted on the American and European grapevines. The winter egg overwinters on the American lineage on the aerial parts, from which the founders of colony come out, forming themselves on the underside of leaves. Several generations of these aphids, called aphids gall wasp, develop on the leaves. Part of the gall wasp then migrate to the root. In the fall the sexual forms occur, and the females deposit winter eggs. On the European grapevine, all the generations of aphids develop on the roots, and are parthenogenetic. They overwinter without sexual generation and larvae. The phylloxera in Croatia has 4-9 generations per year (Maceljski, 2002).

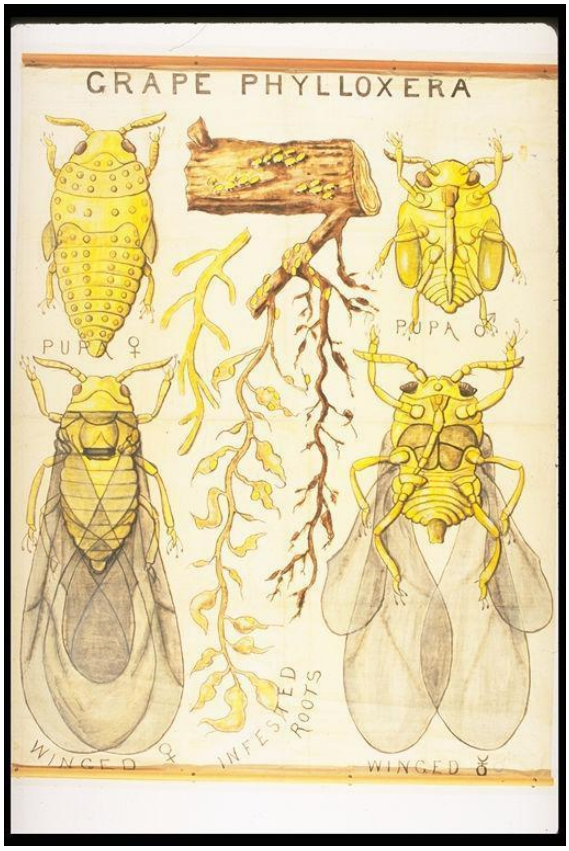


Figure 9. Grape phylloxera winged male and female, pupal forms and infested roots (http://entomology.k-state.edu/images/cv-riley-collection/grape-phyloxera.-winged-male-and-female.-pupal-forms-and-infested-roots.-6-forms.jpg - 2016)

The root of the most types of the American grapevines (*Vitis riparia*, *V. berlandieri*, *V. rupestris*, etc.) and the root of many hybrids are tolerant to phylloxera's attack so it does not cause any damage to the roots. But there are some rootstocks and some hybrids, whose root is sensitive or even vulnerable to the attack of phylloxera (Maceljski, 2002). Although in practice, there is a well-known case in which the phylloxera overcame the resistance of the plants and led to the economic losses on the rootstock AxR1 in California (Granett, 1985).

2.6. Genetic variation of grape phylloxera

The abandoned vineyards or hedges with the vegetative shoots of the American rootstock cultivars host high phylloxera population densities on the leaves. They can be potential selection pools and sources of more aggressive biotypes emerging by the sexual recombination and parthenogenetic reproduction. The biotypes of phylloxera are defined by their ability to infest *Vitis ssp.* and may contain various genotypes. Understanding the biology

of an insect is the essential key for pest management. It is important for the prediction of emergence, the establishment of populations, and risks of phylloxera overcoming resistance and their spread. *D. vitifoliae* reproduce by cyclical parthenogenesis with numerous generations through summer, followed by a sexual cycle, though in moderate climates an anholocyclic reproduction seems to be predominant (Forneck & Huber, 2009). Genetic variation and the mode of reproduction can be investigated using the population's genetic statistics if the genotypes are known. Various studies of phylloxera genotypes on different *Vitis ssp*. have been performed worldwide (Corrie & Hoffmann, 2004; Vorwerk & Forneck, 2006).

2.7. Grafting with resistant rootstock

The use of a resistant, or tolerant, rootstock, developed by Charles Valentine Riley in collaboration with J. E. Planchon and promoted by T. V. Munson, involved grafting of a *Vitis vinifera* scion onto the roots of a resistant *Vitis aestivalis* or another American native species (https://en.wikipedia.org/wiki/Phylloxera). This is the preferred method of phylloxera control today, because the rootstock does not interfere with the development of the wine grapes (technically, the genes responsible for the grapes are not in the rootstock but in the scion), and it furthermore allows the customization of the rootstock to soil and weather conditions, as well as the desired vigor. Not all rootstocks are equally resistant. Between the 1960s and the 1980s in California, many growers used a rootstock called AxR1. Even though it had already failed in many parts of the world by the early twentieth century, it was thought to be resistant by the growers in California. Although phylloxera initially did not feed heavily on AxR1 roots, within twenty years, mutation and selective pressures within the phylloxera population began to overcome this rootstock, resulting in the eventual failure of most vineyards planted on AxR1. The replanting of the afflicted vineyards continues today. Many have suggested that this failure was predictable, as one parent of AxR1 is in fact a susceptible *V. vinifera* cultivar. But the transmission of phylloxera tolerance is more complex, as is demonstrated by the continued success of 41B, an F1 hybrid of *Vitis berlandieri* and *Vitis vinifera*. The full story of the planting of AxR1 in California, its recommendation, the warnings, financial consequences, and subsequent recriminations remain to be told. Modern phylloxera infestation also occurs when the wineries are in need of fruit immediately, and choose to plant ungrafted vines rather than wait for the grafted vines to be available. The use of resistant American rootstock to guard against phylloxera also brought about a debate that remains unsettled to this day: whether self-

rooted vines produce better wine than those that are grafted. Of course, the argument is essentially irrelevant wherever phylloxera exists. Had American rootstock not been available and used, there would be no *V. vinifera* wine industry in Europe or in most places other than Chile, Washington State, and most of Australia. Cyprus was spared by the phylloxera plague, and thus its wine stock has not been grafted for phylloxera resistant purposes. With the arrival of phylloxera to the European vineyards in the sixties of the 19th century, a new chapter of modern viticulture begins. The solution to combat phylloxera was found in grafting noble grapevine to the rootstock of American grapevine species and hybrids. From many species of the genus *Vitis* from American groups, as rootstock are pointed out, there are 3 types and their hybrids that today are classified into four groups:

I. American species of the genus *Vitis* and selections

For the first renewal of vineyards the species from North American groups subgenus *Euvitis* were used (number of chromosomes is identical for all types, 2n = 38). Out of all the species of this group of *Vitis,* three types were distinguished: *V. riparia*, *V. rupestris* and *V. berlandieri* and their selections (Mirošević, 2007)

II. American-american hybrids

The original American species as a rootstock had a number of problems: poor adaptability to most wine-growing soil, different ability to rooting, different affinity to the noble grape varieties, and large differences in resistance to drought and lime. All of this resulted in the next step, related to the mutual intersections of American species. This group is divided into 3 sub-groups:

- *V.riparia x V. rupestris*
- *V. berlandieri x V. riparia*
- *V. berlandieri x V. rupestris*

III. European-American hybrids

With these crossings the rootstocks that are resistant to phylloxera were wanted, but with the retained quality of the European vines. The rootstocks of this group submitted the highest levels of lime tolerance, but they are not so well resistant to phylloxera. From these selections some directly generic hybrids were made.

IV. The complex hybrids

Seeking the best possible rootstock at the end of the 19th Century, it began with the intersections among already created hybrids, some of them are in present time used as the rootstock (Fercal, 1616 Couderc) (Mirošević& Karoglan Kontić, 2008).

2.8. The choice of rootstock

The choice of rootstock is essential in the establishing vineyards. The rootstock with its characteristics affects the scion in a way that it regulates its vigor and ripening. Also, the selection of the rootstock depends on the soil type of the future vineyard. Various soil types demand different rootstocks. There is no ideal rootstock.

Resistance of rootstock to phylloxera

Viala and Ravaz (1892) have created a scale of resistance to phylloxera. According to the table below, zero (0) in the scale means maximum sensitivity, while twenty (20) indicates absolute resistance (immunity) (Mirošević & Karoglan Kontić, 2008).

Table 1. Resistance of species and hybrids to phylloxera (source: Mirošević & Karoglan Kontić, 2008)

Stage of resistance	Species and hybrids	Stage of resistance	Species and hybrids
20	-	14	*V. solonis* i dr.
19	*V. rotundifolia*	13	Taylor« i dr.
18	*V. riparia, V. rupestris, V. riparia x V. rupestris, i*	12	»Jacquez«, »Herbemont« i dr.
17	*dr.*	11	
	V. berlandieri	< 10	–
	V. berlandieri x V. riparia, V. berlandieri		*V. labrusca* »Izabela«, »Concord«, »Otelo« i dr.
16	*x V. rupestris, i dr.*	–	*V. vinifera* x *V. rupestris*
15	*Rupestris du Lot, V. rupestris metalica i dr V. cinerea, V. aestivolis, V. candicans*	0	(»Aramon«) i dr. *V. vinifera*

Tolerance to lime in the soil

One of the important features of the rootstock is the tolerance to lime because the noble grapes were traditionally grown on karst terrains, which are characterized by a high content of lime. The hybrids of the American species (generally tolerant to phylloxera) with the European (vinifera) vines, as well as some related complex crossings have resulted in rootstocks with increased tolerance to lime (Table 2).

Table 2. Tolerance of some rootstocks to the lime in the soil (source: Mirošević & Karoglan Kontić, 2008)

Rootstock	Total lime (%)	Rootstock	Total lime (%)
Riparia	< 15	*Riparia*	6
3309	25	3306, 3309	11
R. du Lot	30	R. du Lot	15
420 A, 5BB, 110 R, SO_4	35	99 R, 110 R, SO_4	17
140 Rg	46	5BB, 420 A, 1103 P	20
41 B	55	161-49,1447	25
333 EM	60	333EM, 140 R	30
		41 B,	40
		FERCAL	60

Besides resistance/tolerance to phylloxera, a good rootstock needs to have a flexibility (adaptability) to the environmental conditions (climate and soil), a good grafting affinity to *V. vinifera* varieties, as well as a good capability of rooting. Also, essential features are the resistance to nematodes (vectors of viruses), magnesium resistance and drought tolerance.

3. Research aim

The aim of this thesis is to assess grape phylloxera leaf feeding populations form different regions of Croatia using standardized set of SSR microsatellite markers and compare them among themselves, as well as with the comparable data representing phylloxera genotypes from Burgenland, Austria. Expecting a lot of variation among Croatian genotypes due to the diverse climate in Croatia, the populations sampled around Zagreb will be compared to those originating from the coastal region in order to test the hypothesis that the genotypes from Zagreb area are more similar to Burgenland than to the coastal area.

4. Materials and methods

4.1. Materials

The field sampling of grape phylloxera individuals has been conducted on some leaf infested plants, in Croatia on fall 2015 from October 21 till November 11. The leaf samples were collected (Figure 10) in Dubrovačko–Neretvanska (brown), Splitsko–Dalmatinska, Šibensko-Kninska (dark red), Primorsko-Goranska (green), Istarska (purple), Virovitičko-Podravska (blue) and Zagrebačka (yellow) County. Each sampling location was represented by 2 – 4 infested vines. The leaf samples were collected and stored in some Falcon tubes containing 70 % Ethanolat 4 ° C. Each Falcon tube contained 3 – 5 leaves from the single vine. Later, in the lab from these leaves (representing single vine), 5 phylloxera individual DNA samples were collected (approximately 15 per location). In total, there were more than 400 DNA samples, but for further analysis we used 107 samples. Additional 13 samples representing Burgenland, Austria have been joined for the genetic analysis, from which the DNA has already been extracted (Table 3).



Figure 10. Locations of field sampling in Croatia

Table 3: The origin, codes and number of collected samples.

Location of the sample	**County**	**Code of sample**	**Dendrogram code**	**Number of samples**
Jazbina	Zagrebačka	J_01_1_1, J_01_1_2, J_01_2_1, J_01_2_3, J_01_2_4, J_01_2_5, J_01_3_2, J_01_3_3, J_01_3_5, J_02_2_1, J_02_2_3, J_02_2_5, J_02_3_2	A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13	13
Sedlarica	Virovitičko - Podravska	Se_09_1_3, Se_09_1_4, Se_09_1_5, Se_09_2_1 Se_09_2_2	A47, A48, A49, A50, A51	5
Aršanj		A_10	-	-
Otrovanjski breg		O_11	-	-
Klas		Kl_12	-	-
Beter	Zagrebačka	B_03_1_1, B_03_1_3, B_03_1_4, B_03_2_3, B_03_2_5, B_03_3_4, B_03_3_5	A14, A15, A16, A17, A18, A19, A20	7
Novo selo okičko		NO_04_2_4, NO_04_2_5, NO_04_3_4	A21, A22, A23	3
Stankovo		S_05_1_1, S_05_1_2, S_05_1_3, S_05_1_4, S_05_2_1, S_05_2_2, S_05_2_3, S_05_2_4, S_05_3_1, S_05_3_2 S_05_3_3, S_05_3_4 S_05_3_5	A24, A25, A26, A27, A28, A29, A30, A31, A32, A33, A34, A35, A36	13
Repišće		R_06_1_1, R_06_1_2 R_06_1_3, R_06_1_4, R_06_2_4, R_06_2_5, R_06_3_4, R_06_3_5	A37, A38, A39, A40, A41, A42, A43, A44	8
Poljanica okička		PO_07_3_1, PO_07_3_4	A44, A45	2
Klake		K_08	-	-

Poreč	Istarska	P_13_1_3	C1	1
Kadumi		Ka_14_3_3, Ka_14_3_5	C2, C3	2
Istra – cemetery		U_15_1_1, U_15_1_2, U_15_1_5, U_15_1_6, U_15_1_7, U_15_2_1, U_15_2_2, U_15_2_3, U_15_2_6, U_15_2_8, U_15_3_1, U_15_3_3 U_15_3_4, U_15_3_5, U_15_3_6	C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15, C16, C17, C18	15
Vrbnik – Island of Krk	Primorsko - Goranska	V_17_1_6, V_17_1_8, V_17_1_9, V_17_2_0, V_17_2_1, V_17_3_1, V_17_3_4	C20, C21, C22, C23, C24, C25, C26	7
Island of Krk		Kk_16_1_1	C19	1
Čibača	Dubrovačko - Neretvanska	Ci_20	-	-
Vid		Vd_21_26, Vd_21_4_6	C45, C46	2
Krvavac		Kv_22_1_1, Kv_22_1_2, Kv_22_1_3, Kv_22_1_6, Kv_22_1_7, Kv_22_1_8, Kv_22_2_2	C47, C48, C49, C50, C51, C52, C53	7
Opuzen		Us_23_1_3, Us_23_2_6, Us_23_2_7	C54, C55, C56	3
Primošten	Šibensko - Kninska	Pr_18_1_6, Pr_18_1_7, Pr_18_2_2, Pr_18_2_6, Pr_18_2_7, Pr_18_3_2, Pr_18_3_3, Pr_18_3_6, Pr_18_4_1, Pr_18_4_2, Pr_18_4_3	C27, C28, C29, C30, C31, C32, C33, C34, C35, C36, C37	11
Mujići		Mu_19_1_1, Mu_19_2_4, Mu_19_2_7, Mu_19_3_1, Mu_19_3_7, Mu_19_4_1, Mu_19_4_3	C38, C39, C40, C41, C42, C43, C44	7

4.2. Methods

In the lab, the phylloxera was excised from the leaf galls and collected into 96-well platesfor DNA extraction. This is followed by the polymerase chain reaction (PCR). The PCR products were then loaded onto the ABI (Applied Biosystem) 3130 xl for electrophoresis. Finally, the data were analyzed and evaluated by an appropriate software.

4.2.1.Tissue collection

The L5 stadium of grape phylloxera was used for DNA extraction according to Lin and Walker (1996). The first step was to cut out the galls and to collect L5 stadium phylloxera. The excised insects (Fig. 11) were placed into 96 well plates for the subsequent extraction of DNA.
The alcohol and the eggs during the isolation gradually change the color from originally yellow to brown and black (Figure 11).

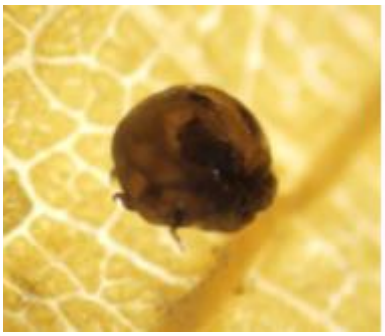


Figure 11: L5 stadium of the phylloxera turning color from yellow to brown in 70% alcohol (photo by Dockner, 2016)

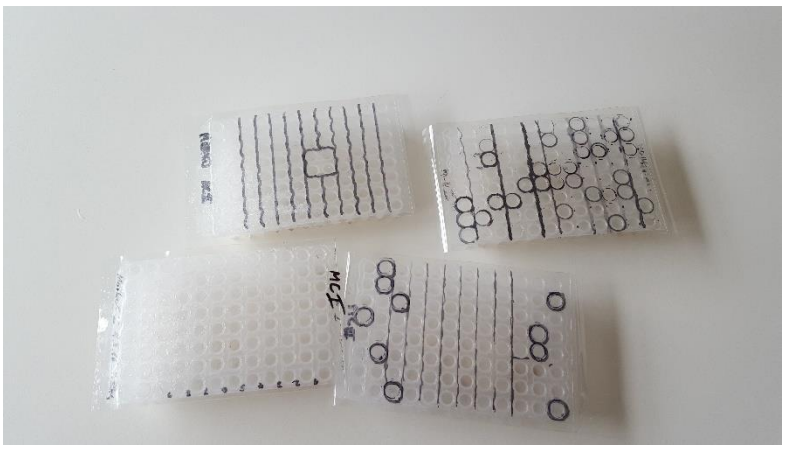


Figure 12. Collected samples in plates (photo by M. Čajić)

4.2.2. DNA extraction

The original DNA extraction protocol of Lin and Walker (1996) was slightly modified by Vorwerk et al. (2007) and optimized according to Dockner (2016) and Riaz pers.comm. 2014. This DNA extraction protocol is applied to single adults, and/or eggs in plates, and consists of the following steps:

1. First, prepare a 5% Chelex buffer in ddH_2O (0,05g Chelex Raison 100 in 1ml); the stock may last 1 week.
2. Then turn on the heating block to 90°C to preheat
3. Then grind samples with a cold pistil (cooled in liquid nitrogen) before adding 200µl of 5% CHELEX in each well, then grind again, and close the plate tight with adhesive film and vortex carefully!
4. After incubation for 20 min turn on heating block to 90°C.
5. Then centrifuge the plate at maximum speed (14 000rpm) 10min.
6. Take out the supernatant carefully (without chelex in it). The Solution is ready to use.
7. Measure the DNA content with a nanodrop and a preparation of dilutions.
8. The last step is to freeze and accurately label samples.



Figure 13. Example of the extracted DNA in plates (photo by M. Čajić)

4.2.3. PCR –Reaction mixture

The PCR reaction is performed for all the samples with the components listed in Table 4 according to Forneck et al. 2016 (accepted). For genotyping 107 phylloxera genotypes 7 SSR fluorescently labeled primers have been used (Table 5).

First, the fresh master mix was prepared for each particular SSR primer pair based on the volumes listed in Table 2, except for the DNA which was applied to each single well of the 96-plate individually with a 8-chanel micro pipette. After pipetting was completed and the plate was sealed, the plate was vortexed and then put in the Thermal cycler to execute the PCR cycling program.

Table 4. Reaction mixture 10 µl for PRC

Substance	Working concentration	Volume
DNA	10 ng/ µl	1 µl
Buffer Taq	5 x	2 µl
dNTP's	2 mM	1µl
Primer F	10 pmol	0,5µl
Primer R	10 pmol	0,5µl
Taq	5 Units/ µl	0,05µl
ddH_2O		4,95µl
Total		10 µl

Table 5: Primer Sequences. The table shows the primer sequence, its repetition sequence, and the primer development reference.

Primer	Primer sequence	T_{A*} (°C)	Repeat sequence	Reference
Dvit 6 F	TGGACGATGGTTTTCATAGC	56	(AAT)9	VORWERK and FORNECK, 2006
Dvit 6 R	TTGATTGTCATTGGTTTTGC			
DV 4 F	TCATATAACCGTTCCCCCTG	58	(GTT)9	DOCKNER, 2016.
DV 4 R	AATTACCGTGTTTTCACCGC			
DV 8 F	TAGACTAGCGCAACGATGG	58	(TG)8	DOCKNER, 2016.
DV 8 R	CCAGCACGTTGAAATCTGTA			
PhyIII_36 F	CGTCCTTCTTGCGTGATATTTT	58	(TAA)11	RIAZ et al., 2014
PhyIII_36 R	GGCGGAATAAATGAGAAAAGTG			
PhyIII_55 F	CGTATGATCGTCACAGAGGAAA	60	(ATT)11	RIAZ et al., 2014
PhyIII_55 R	CGATTCCGCTTTAAACAATACC			
DVSSR8 F	GGTCGTCCCAGTAAACGTAATC	58	(GCA)6	LIN et al., 2006
DVSSR8 R	TGTTTGATAACGGTGATGGTGG			

* Annealing temperature

4.2.4. PCR

The PCR reaction begins with the denaturation of the DNA at 95 ° C for 5 minutes, then it is followed by 35cycles with the following temperature profile:

- 92 ° C for 45 seconds,
- 56 – 60 ° C for 45 seconds (see Table 5)
- 72 ° C for 60 seconds

The final step of PCR (elongation) was at 72 ° C for 10 minutes, and in the end it is stored at temperature 4 ° C or 8 ° C.

After PCR was completed, plates with PCR products have been sealed and stored for subsequent capillary electrophoresis (Figure 14).



Figure 14. PCR plates with samples (photo by M. Čajić)

4.2.5. PCR products' check

Products of PCR amplification were tested on 1,5 % agarose gel electrophoresis before loading them on Applied Biosystems 3130 and 3130 xl. The agarose gel was prepared by following steps:

1. use 250 ml 1xTBE + 3,75 g NEEO Agarose
2. then cook in microwave until the agarose is completely melted
3. then cool it a bit under running water
4. add 12,5 µl Peqgreen (4-5µl/100ml)
5. pour into the the biggest tray (used with 2 x 50 slots comb and 1 x 36 slots-comb)
6. wait until it is solid
7. carefully take out the combs and pipette PCR product mixed with 5 x loading dye
8. use 3µl PCR + 0,6µl 5x loading dye and a ladder for control
9. run at 150 V for 40 min

Preparation of 10 x TBE by 108 g Tris base, 55g of boric acid, 7,44g EDTA (disodium salt) or 40 ml 0,5 M EDTA Ph 8,0. After that add 800 ml dH^2O and stir plate until it is dissolved. Then add dH^2O to 1 L.

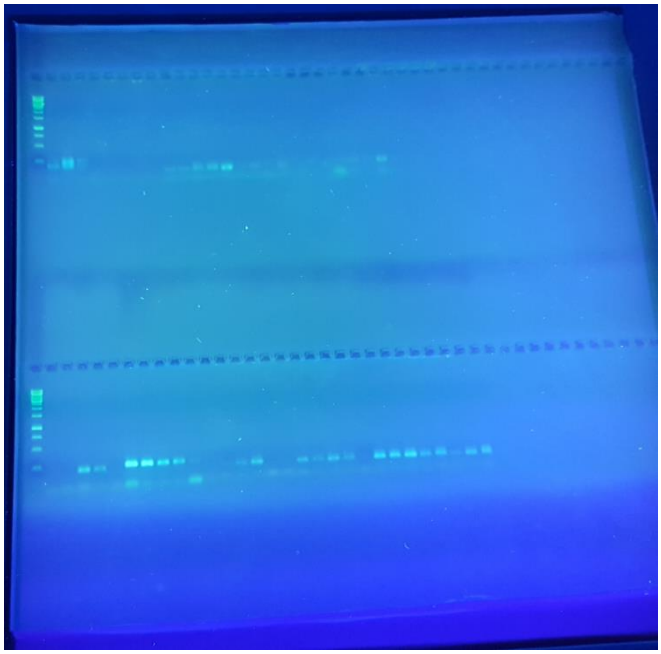


Figure 15. An example of the results, the agarose gel (photo by M. Čajić)

4.2.6. Fragment evaluation by ABI 3130 xl

The PCR samples are assessed on Applied Biosystems 3130xl. The samples are prepared for sequencing in 96- or 384-well plates. The fluoresced labeled DNA is loaded into the capillary for a short period of time and electrophoresis is performed by electro kinetic injection. A laser beam activates the fluorescence, which is spectrally separated by a reflective spectrograph. The light columns are recorded and broken down by the software (Dockner, 2016).

Depending on the device type, the machine has a capillary of up to 96 capillaries. ABI is fully automated. The ABI is a sequencing device and has an even better resolution with an accuracy of one base pair. Furthermore, the ABI is multi-colored, so primers with the same allele size can be analyzed simultaneously (Dockner, 2016.)

Primer combinations for loading the ABI

III: PhyIII30(Hex) + PhyIII55(Fam) + PhyIII36(Hex)

IV: Dvit6 (Fam) + DV4 (Hex) + DVSSR4 (Fam) + DV8 (Fam)



Figure 16. 96 well plates with PCR before loading on ABI (Applied Byosistems 3130 and 3130 xl)(photo by M. Čajić)

Preparations for running ABI

Dilutions of each primer/ PCR with ddH^2O: depending on gel pictures of PCR (Table 6):

Table 6. The dilution of each primer

Primer	Dilution
PhyIII30	2µl PCR + 48µl ddH^2O
PhyIII36	2µl PCR + 48µl ddH^2O
PhyIII55	2µl PCR + 48µl ddH^2O
Dvit6	2µl PCR + 7µl ddH^2O
DV4	2µl PCR + 28µl ddH^2O
DV8	2µl PCR + 38µl ddH^2O
DVSSR4	2µl PCR + 28µl ddH^2O



Figure 17. Samples ready for loading on ABI (photo by M. Čajić)

Steps under the hood (fume cupboard) on ice:

Mix: HiDi and ROX- Size standard (in 1,5ml Tube):

1. HiDi 10µl/well + ROX: 0,35µl/well
2. Mix in Sequencer Platte vorlegen: 10µl/well
3. Add 2µl diluted PCR-product to 10µl Mix (of HiDI and Rox) pipette according to the primer combination!
4. Centrifuge at 2000 rpm 4°C
5. Then incubate for 2 minutes at 95°C
6. Centrifuge again at 4°C with 2000 rpm
7. Load the ABI, with injection time of 10 seconds!

4.2.7. Data scoring and analysis of diversity

The all data from ABI was exported to Notepad, then transferred to Excel and the results were evaluated by the following methods. The row data are used to perform following statistical analyses:

Allele polymorphism:

The number of alleles per locus (Na), observed heterozygosity (Ho), expected heterozygosity (He), polymorphism information content (PIC) and allele frequencies for all loci were calculated using The Excel Microsatellite Toolkit 3.1.1. (Park 2001). The calculations were made for each population separately and then for all the populations combined. Expected heterozygosity was calculated using an unbiased formula from allele frequencies assuming Hardy-Weinberg equilibrium (equation 8.4, Nei 1987). Polymorphic information content (PIC) is a measure of informativeness related to expected heterozygosity and likewise it is calculated from the allele frequencies (Botstein *et al.* 1980).

Analysis of genetic similarity:

Allele values were translated to binary data based on which a similarity matrix was calculated using the dissimilarity index – in proportion to the shared alleles (Bowcock et al. 1994). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The dendrogram was drawn to the scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree and analyses were conducted in MEGA6 (Tamura et al., 2013).

In order to visualize genetic relationships among presumed geographic groups of phylloxera genotypes in a better manner, the principal coordinate analysis (PCoA) was performed using GenAlEx 6.5 (Peakall and Smouse, 2012).

Analysis of molecular variance (AMOVA):

The AMOVA has been performed on genotypic data for two Croatian subpopulations (A and C) to test how they differentiate and if these groups are significantly different. AMOVA has been performed by the software GenAlEx 6.5 (Peakall and Smouse, 2012).

5. Results and discussion

5.1. Genotypic data of 7 SSR loci for all analyzed phylloxera individuals

The genotyping data for 120 individuals at 7 SSR loci are presented in the Table 7. Apart from total data, for further data analysis all samples were divided into three different groups (group A: Croatia – continental area; group B: Austria – Burgenland and group C: Croatia – coastal area).

It can be noted that many samples share the same genotype at 7 SSR loci. In total there were 40 individuals (33.3 %) clustered in 17 groups of individuals with identical multilocus genotype (IMG). In most IMG groups (11 out of 17) the individuals with the identical genotype were sampled at the same grapevine plant. However, in several cases (6) individuals had identical genotype when collected from different plants but the same location, even in two cases from two different geographic locations (C03/C44 and A24-36/A44). The individuals with identical SSR genotypes have been observed in all sampling areas (groups A, B and C) and nearly equally represented in both, continental and coastal area.

Out of total 120 analyzed individuals, there were 80 (66,6%) unique genotypes which make a non-redundant set of genotypes. These results are also visible from the dendrogram as clusters with genetic similarity value 1 (Fig. 18).

Apearance of repeated multilocus genotypes has been reported also in the studies of Vorverk and Forneck (2006), Riaz et al. (2014) and Forneck et al. (2015) what confirms phylloxera's reproduction diversity, i.e. sexual and asexually produced genotypes.

The size of IMG groups ranged from 2 – 11 individuals and the largest one consisted of 11 individuals collected from 4 different vines from two neighbouring sampling locations about 30 km south of Zagreb (Fig. 18). However, results from this study do not support the existance of any particular genotype ("superclone" of leaf-feeding phylloxera) that would be spread across many different locations.

Table 7. Genotypic data of 7 SSR loci for 120 analyzed phylloxera individuals.

Sample code	Dendrogram code	PhyIII30		PhyIII55		PhyII36		DV8		Dvit6		DV4		DVSSR4	
J_01_1_1	A1	132	132	127	130	195	207	143	147	202	208	210	222	251	251
J_01_1_2	A2	129	132	124	130	195	195	145	145	208	208	216	216	251	253
J_01_2_1	A3	129	135	127	130	195	204	143	143	202	202	216	219	251	251
J_01_2_3	A4	129	132	124	130	195	195	145	145	208	208	216	228	251	253
J_01_2_4	A5	132	132	124	127	195	204	145	145	202	202	222	222	251	251
J_01_2_5	A6	132	132	127	130	195	204	145	145	202	205	216	219	251	251
J_01_3_2	A7	132	135	124	130	195	195	143	143	208	208	216	222	241	253
J_01_3_3	A8	132	135	124	130	195	195	143	147	208	208	210	222	245	251
J_01_3_5	A9	132	135	124	130	195	195	143	143	208	208	216	222	241	253
J_02_2_1	A10	129	129	121	130	195	195	143	143	202	202	219	222	241	251
J_02_2_3	A11	129	129	115	127	195	204	143	143	208	208	219	222	245	251
J_02_2_5	A12	132	132	115	127	195	195	145	145	205	208	216	216	251	253
J_02_3_2	A13	132	135	130	130	195	204	143	145	202	202	222	222	245	251
B_03_1_1	A14	132	135	130	130	195	204	143	143	202	202	222	222	245	245
B_03_1_3	A15	132	135	130	130	195	204	143	145	202	202	222	222	245	251
B_03_1_4	A16	132	135	130	130	195	204	143	145	202	202	222	222	245	251
B_03_2_3	A17	132	132	124	127	195	204	145	145	202	208	210	222	245	249
B_03_2_5	A18	132	132	124	127	195	204	145	145	202	208	210	222	245	249
B_03_3_4	A19	132	132	115	130	195	195	143	145	202	202	216	216	251	251
B_03_3_5	A20	132	132	115	130	195	195	143	145	202	202	216	216	251	251
NO_04_2_4	A21	132	132	115	130	195	195	143	145	202	202	216	216	251	251
NO_04_2_5	A22	132	132	115	130	195	195	143	145	202	202	216	216	251	251
NO_04_3_4	A23	129	132	130	130	195	195	143	145	202	205	222	228	245	255
S_05_1_1	A24	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_1_2	A25	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_1_3	A26	132	132	124	127	195	204	145	147	202	208	219	219	251	253
S_05_1_4	A27	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_2_1	A28	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_2_2	A29	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_2_3	A30	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_2_4	A31	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_3_1	A32	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_3_2	A33	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_3_3	A34	132	132	124	127	195	207	145	147	202	208	219	222	251	253
S_05_3_4	A35	132	132	124	130	195	204	145	145	202	202	222	222	251	251
S_05_3_5	A36	132	132	124	130	195	204	145	145	202	202	222	222	251	251
R_06_1_1	A37	129	132	115	124	195	204	143	143	202	202	222	222	251	255
R_06_1_2	A38	129	132	115	124	195	204	143	143	202	202	222	222	251	255
R_06_1_3	A39	129	132	115	124	195	204	143	143	202	202	222	222	251	255
R_06_1_4	A40	129	132	115	124	195	204	143	143	202	202	222	222	251	255
R_06_2_4	A41	132	135	124	127	204	204	145	147	208	208	222	222	251	251
R_06_2_5	A42	132	135	124	127	204	204	145	147	208	208	222	222	251	251
R_06_3_4	A43	129	132	115	127	195	204	145	147	202	208	222	222	251	251
R_06_3_5	A44	132	132	124	130	195	204	145	145	202	202	222	222	251	251
PO_07_3_1	A45	132	135	124	127	204	204	145	147	208	208	222	222	255	255
PO_07_3_4	A46	132	135	124	127	204	204	145	147	208	208	222	222	245	251
Se_09_1_3	A47	132	132	127	130	195	195	145	145	205	205	222	222	251	253
Se_09_1_4	A48	132	132	127	130	195	195	145	145	205	205	222	222	251	253
Se_09_1_5	A49	132	132	127	130	195	195	145	145	205	205	222	222	251	253
Se_09_2_1	A50	132	132	127	130	195	195	145	145	205	205	222	222	251	253
Se_09_2_2	A51	132	132	127	130	195	195	145	145	205	205	222	222	251	251
MB17	B1	129	132	124	130	195	195	145	145	202	202	222	228	251	251
MB27	B2	132	135	124	130	198	198	143	143	208	208	219	219	251	251
MB19	B3	132	132	124	130	198	198	143	143	208	208	219	219	251	253
MB20	B4	132	135	124	130	198	198	143	143	208	208	219	219	251	253
MB21	B5	132	135	124	130	198	198	143	143	208	208	219	219	251	253
MB22	B6	132	135	124	130	198	198	143	143	208	208	219	219	251	253
MB23	B7	132	135	124	130	195	198	143	143	208	208	219	219	251	253
MB28	B8	132	135	124	127	195	195	143	143	202	205	210	219	251	251

MB29	B9	129	132	130	133	195	195	143	145	202	205	219	222	251	253
MB24	B10	129	129	124	130	195	195	145	145	205	208	219	219	251	251
MB25	B11	129	129	124	124	195	195	143	145	205	205	219	222	251	251
MB26	B12	132	135	124	130	198	198	143	143	208	208	219	219	251	253
MB30	B13	129	135	124	127	195	195	143	145	202	208	219	219	251	251
P_13_1_3	C1	132	141	124	124	204	204	147	147	196	196	222	222	243	243
Ka_14_3_3	C2	132	132	121	127	195	207	143	143	205	208	216	222	241	251
Ka_14_3_5	C3	129	132	127	130	195	195	143	145	202	208	216	219	245	251
U_15_1_1	C4	129	132	130	130	195	204	143	143	202	208	216	216	249	249
U_15_1_2	C5	129	132	130	130	195	204	143	143	202	208	216	216	249	249
U_15_1_5	C6	129	132	130	130	195	204	143	143	202	208	216	216	249	249
U_15_1_6	C7	129	132	130	133	195	204	143	143	202	208	219	219	251	251
U_15_1_7	C8	129	132	130	133	195	204	143	143	202	208	219	219	251	251
U_15_2_1	C9	129	132	130	130	195	204	143	143	202	208	216	216	249	249
U_15_2_2	C10	132	132	127	130	195	195	145	147	202	208	213	213	251	251
U_15_2_3	C11	132	132	124	127	195	198	145	145	196	205	216	219	249	249
U_15_2_6	C12	132	132	121	127	198	204	143	145	205	205	219	219	251	253
U_15_2_8	C13	132	132	121	124	195	195	143	145	202	202	219	219	241	251
U_15_3_1	C14	132	132	124	127	195	195	145	145	205	205	222	222	249	249
U_15_3_3	C15	132	132	130	130	195	204	145	145	205	208	219	219	251	251
U_15_3_4	C16	132	132	130	130	195	204	145	145	205	208	210	219	241	249
U_15_3_5	C17	132	132	130	130	195	195	143	145	205	205	210	210	249	251
U_15_3_6	C18	132	132	121	127	198	204	143	145	205	205	222	222	251	251
Kk_16_1_1	C19	129	132	130	130	195	204	145	145	205	208	213	213	239	251
V_17_1_6	C20	132	132	127	127	195	204	143	145	202	208	219	219	241	251
V_17_1_8	C21	132	132	127	127	195	204	143	145	202	208	219	219	241	251
V_17_1_9	C22	132	132	127	127	195	204	143	145	202	208	219	219	241	251
V_17_2_0	C23	132	132	127	127	195	204	143	145	202	208	219	219	251	251
V_17_2_1	C24	132	132	127	127	195	204	145	145	202	208	210	210	251	251
V_17_3_1	C25	132	132	127	127	195	204	143	145	202	208	216	216	239	249
V_17_3_4	C26	132	132	127	127	195	204	143	145	202	202	216	216	239	249
Pr_18_1_6	C27	129	132	124	124	195	195	143	145	202	202	216	225	251	253
Pr_18_1_7	C28	132	132	124	127	195	195	143	145	202	202	216	225	253	253
Pr_18_2_2	C29	129	135	124	124	195	195	143	145	202	202	216	222	251	251
Pr_18_2_6	C30	129	132	124	124	195	195	143	143	202	202	216	225	253	253
Pr_18_2_7	C31	123	129	124	127	195	195	143	145	202	202	216	225	253	253
Pr_18_3_2	C32	123	129	124	127	195	195	143	143	202	208	216	216	253	253
Pr_18_3_3	C33	123	129	124	127	195	195	143	143	202	208	216	216	253	253
Pr_18_3_6	C34	132	132	124	130	195	195	143	143	202	202	216	225	253	253
Pr_18_4_1	C35	123	129	124	127	195	195	143	145	202	208	216	216	253	253
Pr_18_4_2	C36	123	129	124	127	195	195	143	145	202	208	216	216	253	253
Pr_18_4_3	C37	123	129	130	130	195	195	143	143	202	208	216	216	253	253
Mu_19_1_1	C38	123	129	124	130	195	195	143	143	202	208	216	216	253	253
Mu_19_2_4	C39	129	132	121	130	195	195	145	147	204	207	222	222	241	251
Mu_19_2_7	C40	129	129	124	127	195	198	143	143	202	208	219	219	251	251
Mu_19_3_1	C41	132	132	121	130	195	207	143	143	204	207	216	222	241	251
Mu_19_3_7	C42	129	132	130	130	195	195	143	145	202	202	216	225	253	253
Mu_19_4_1	C43	129	129	130	130	195	195	145	147	204	207	222	222	241	251
Mu_19_4_3	C44	129	132	127	130	195	195	143	145	202	208	216	219	245	251
Vd_21_2_6	C45	129	129	124	127	195	198	143	143	202	208	210	219	251	251
Vd_21_4_6	C46	129	135	127	127	192	195	143	143	205	208	210	210	251	251
Kv_22_1_1	C47	129	129	124	124	195	195	143	145	205	208	219	225	251	251
Kv_22_1_2	C48	129	129	124	124	195	195	143	145	205	208	219	225	251	251
Kv_22_1_3	C49	129	129	124	124	195	195	143	145	205	208	219	225	251	251
Kv_22_1_6	C50	129	129	127	127	195	195	143	145	205	208	219	219	251	251
Kv_22_1_7	C51	129	129	127	127	195	195	143	145	205	208	219	219	251	251
Kv_22_1_8	C52	129	129	127	127	195	195	143	143	202	208	219	219	251	251
Kv_22_2_2	C53	123	123	124	124	195	195	143	145	205	208	219	225	251	251
Us_23_1_3	C54	129	129	124	139	195	195	143	145	202	208	222	222	251	251
Us_23_2_6	C55	129	135	127	133	195	198	145	145	202	208	219	222	251	253
Us_23_2_7	C56	129	135	127	133	195	195	143	145	202	208	216	222	251	251

5.2. Allelic polymorphism

Number of alleles per locus across the total set of samples (N=120) ranged from 3 to 8. Minimum polymorphism was observed at locus DV8, and maximum at locus DVSSR4 with the average vaule of 5.9 allels per locus through all 7 loci (Table 8). In a similar study (Forneck et al., 2015) on a sample of 226 unique phylloxera genotypes originating from 10 different geographic populations from Austria analyzed by 6 SSRs, the number of allels per locus varied between 2 and 3. Riaz et al. (2014) in their study with 28 SSRs and 10 very different phylloxera strains observed 2 – 6 allels per locus. Even though applied SSR markers in the studies above mentioned were not identical to those from our study, the obtained results suggest that the diversity of Croatian phylloxera populations is substantial.

The observed allele polymorphism among different geographic areas was rather different. In particular, the individuals originating from the Croatian coastal area (N=56) showed the highest polymorphism having on average 5.4 allels per locus and the most polymorphic locus was DVSSR4 with 7 different allels. This was rather higher compared to the average of 4 allels per locus in almost the same sample size of the continental area of Croatia (N=51) and in comparison with 13 samples from Burgenland.

The highest polymorphism in the coastal area might be the consequence of the longest history of this pest in Croatia because its spread to other parts of the country started from this area.

Table 8. The number of alleles, expected and observed heterozygosity and polymorphism information content (PIC) calculated for total of 120 individuals (Total) and three geographically distinct groups (A) Croatia - continental area, (B) Burgenland – Austria and (C) Croatia - coastal area.

	Locus	Number of alleles	Fragment range (bp)	Ho	He	PIC
Total (N =120)	**PhyIII30**	5	123/141	0,46	0,57	0,51
	PhyIII55	7	115/139	0,70	0,73	0,67
	PhyIII36	5	192/207	0,47	0,50	0,44
	DV8	3	143/147	0,42	0,56	0,45
	Dvit6	6	196/208	0,46	0,65	0,58
	DV4	7	210/228	0,31	0,73	0,68
	DVSSR4	8	239/255	0,42	0,61	0,57
	Mean	**5,9**		**0,46**	**0,62**	**0,56**

A– CRO continental area (N =51)	**Locus**	**Number of alleles**	**Spread (bp)**	**Ho**	**He**	**PIC**
	PhyIII30	3	129-135	0,39	0,40	0,37
	PhyIII55	5	115-130	0,90	0,72	0,66
	PhyIII36	3	195-207	0,59	0,49	0,38
	DV8	3	143-147	0,33	0,53	0,45
	Dvit6	3	202-208	0,18	0,55	0,48
	DV4	5	210-228	0,25	0,47	0,43
	DVSSR4	6	241-255	0,49	0,53	0,50
	Mean	**4**		**0,45**	**0,53**	**0,47**

B - AUT - Burgenland (N = 13)	**Locus**	**Number of alleles**	**Spread (bp)**	**Ho**	**He**	**PIC**
	PhyIII30	3	129-135	0,77	0,68	0,58
	PhyIII55	4	124-133	0,92	0,62	0,51
	PhyIII36	2	195-198	0,08	0,52	0,38
	DV8	2	143-145	0,23	0,41	0,32
	Dvit6	3	202-208	0,31	0,57	0,49
	DV4	4	210-228	0,31	0,34	0,31
	DVSSR4	2	251-253	0,54	0,41	0,32
	Mean	**2,9**		**0,45**	**0,51**	**0,41**

C - CRO - coastal area (N = 56)	**Locus**	**Number of alleles**	**Spread (bp)**	**Ho**	**He**	**PIC**
	PhyIII30	5	123-141	0,45	0,61	0,53
	PhyIII55	6	121-139	0,46	0,73	0,67
	PhyIII36	5	192-207	0,45	0,42	0,38
	DV8	3	143-147	0,54	0,53	0,43
	Dvit6	6	196-208	0,75	0,70	0,63
	DV4	6	210-225	0,36	0,76	0,71
	DVSSR4	7	239-253	0,32	0,69	0,64
	Mean	**5,4**		**0,47**	**0,63**	**0,57**

Expected (H_E) and observed heterozygosity (H_O)

Generally, the observed heterozigosity over 7 SSR loci in this study (H_O=0.46) was lower than expected one (H_E=0.62) (Table 8). Forneck et al. (2015) in a similar study conducted in Austria have found the opposite (H_O> H_E). This could be due to a smaller sample size and the samples originating from more diverse locations, which did not enable the natural gene flow. However, the absolute values were similar.

Both expected and observed heterozigosity of group C (Croatia - coastal area) were slightly higher than corresponding values of group A (Croatia - continetal area) and B (Austria - Burgenland) that were rather similar.

In case of locus **Dvit6** and group A, the observed heterozigosity was rather low (H_O=0.18) compared to the expected one (H_E=0.55), and in case of locus **PhyIII36** and group B, the observed heterozigosity was very low (H_O=0.08) compared to the expected one (H_E=0.52). This might be caused by existance of null allels, however, this was not tested. Considering overal large number of detected allels it can be assumed existance of null allels as well.

Polymorphism information of applied SSR markers

Based on the number of different allels and calculated PIC values that ranged between 0.44 to 0.68, it can be stressed that all applied SSR markers were very informative (Table 8). Based on the overall data (n=120), the most informative was locus DV4 with an value of 0.68 while the least informative was locus PhyIII36 with 0.44. The average PIC for all 7 loci was 0,56. These values are in line with values observed in the study of Riaz et al., 2014.

As in the case of previously described parameters, highest PIC values have been recorded for genotypes from group C (Croatia – coastal area). Considering also their properties in PCR and electrophoresis, all applied SSR markers can be recommended for similar future studies.

5.3. Allele frequencies

The following Table 9 shows the observed allele frequencies over the total sample size for respective geographic regions. This data point to the different genetic structure of the analyzed phylloxera populations. It is of interest to analyze the presence/absence of the unique allels for a particular region, as well as the frequency of a particular allele across different regions.

It can be noted that some allels were not found in all sampling regions (yellow highlight). Group C representing the phylloxera individuals from Croatia – coastal area had 10 unique allels that were not present neither in Croatia – continental area nor Burgenland. In the same time, Croatia – continental area used to have two unique allels across 7 SSR loci, while 13 samples from Burgenland (group B) did not posses any allel which was not already present in the Croatian samples.

It is interesting that in Table 9 just 2 allels (195, 198) are detected at the locus PhyIII36 and both were equally frequent (50 %). Alelle 204 at the same locus had freqency of 35.29 % in Croatia – continental area and was not present in Austria – Burgenland. In case of loci DV4 allele 222 was dominant in the population with occurance of 70,59 % in Croatia – continental area, while only with 11,54 % in Burgenland – Austria and 15,18 % in Croatia – coastal area. Some allels of DV4 were found in Croatia but not in Austria. Also, for locus DV8 one allele was present with high percentage (61,76 %) in Croatia – continental area while in Burgenland it was only in 26,92 %. Generally, more different alleles were present in individuals originating from Croatia, but this has be to taken with caution because the size of the compared samples was not equal.

Table 9. The frequencies of the detected alleles at applied 7 SSR markers, computed for total of 120 individuals (Total) and three geographically distinct groups (A) Croatia - continental area, (B) Burgenland – Austria and (C) Croatia - coastal area.

Locus	Groups	Allele							
		123	**129**	**132**	**135**	**141**			
PhyIII30	**Croatia - continental area**	0,00	12,75	75,49	11,76	0,00			
	Austria - Burgenland	0,00	26,92	42,31	30,77	0,00			
	Croatia - coastal area	8,04	39,29	48,21	3,57	0,89			
	Total	3,75	26,67	59,17	10,00	0,42			
		115	**121**	**124**	**127**	**130**	**133**	**139**	
PhyIII55	**Croatia - continental area**	10,78	0,98	29,41	19,61	39,22	0,00	0,00	
	Austria - Burgenland	0,00	0,00	50,00	7,69	38,46	3,85	0,00	
	Croatia - coastal area	0,00	5,36	26,79	35,71	27,68	3,57	0,89	
	Total	4,58	2,92	30,42	25,83	33,75	2,08	0,42	
		192	**195**	**198**	**204**	**207**			
PhyIII36	**Croatia - continental area**	0,00	62,75	0,00	35,29	1,96			
	Austria - Burgenland	0,00	50,00	50,00	0,00	0,00			
	Croatia - coastal area	0,89	74,11	5,36	17,86	1,79			
	Total	0,42	66,67	7,92	23,33	1,67			
		143	**145**	**147**					
DV8	**Croatia - continental area**	29,41	61,76	8,82					
	Austria - Burgenland	73,08	26,92	0,00					
	Croatia - coastal area	56,25	39,29	4,46					
	Total	46,67	47,50	5,83					
		196	**202**	**204**	**205**	**207**	**208**		
Dvit6	**Croatia - continental area**	0,00	60,78	0,00	12,75	0,00	26,47		
	Austria - Burgenland	0,00	19,23	0,00	19,23	0,00	61,54		
	Croatia - coastal area	2,68	40,18	2,68	17,86	2,68	33,93		
	Total	1,25	46,67	1,25	15,83	1,25	33,75		
		210	**213**	**216**	**219**	**222**	**225**	**228**	
DV4	**Croatia - continental area**	3,92	0,00	16,67	6,86	70,59	0,00	1,96	
	Austria - Burgenland	3,85	0,00	0,00	80,77	11,54	0,00	3,85	
	Croatia - coastal area	7,14	3,57	33,04	32,14	15,18	8,93	0,00	
	Total	5,42	1,67	22,50	26,67	38,33	4,17	1,25	
		239	**241**	**243**	**245**	**249**	**251**	**253**	**255**
DVSSR4	**Croatia - continental area**	0,00	2,94	0,00	10,78	1,96	66,67	10,78	6,86
	Austria - Burgenland	0,00	0,00	0,00	0,00	0,00	73,08	26,92	0,00
	Croatia - coastal area	2,68	8,04	1,79	1,79	14,29	49,11	22,32	0,00
	Total	1,25	5,00	0,83	5,42	7,50	59,17	17,92	2,92

5.4. Analysis of genetic similarity among phylloxera individuals of different geographic origin

Considering the similarity of the climate and soil types, as well as the cultivars in viticulture between Croatia – continental part and Austria – Burgenland, it was hypothesized that the individuals from these two regions might be more genetically similar to each other than to the individuals of the Adriatic coast origin.

In order to test this, for all possible pairs of 120 genotyped individuals, the coefficient of genetic similarity (GS) has been calculated. These values have been used as input data for cluster analysis (Neighbor-Joining algorhitm) and its result is shown in the Figure 18 in the form of a dendrogram.

It can be noted that there is no clear differentiation of genotypes according to geographic origin. Also, the existance of 17 clusters with GS value of 1 is visible, which means that all the genotypes consistuting these clusters have identical genotype across all 7 SSR loci. This was already explained earlier in Chapter 5.1.

Considering the level of genetic similarity (GS values close to 1 are more genetic similar), it is obvious that most of the individual genotypes from the same geographic origin (A, B or C) were clustered together. Clustering based on a relatively small number of SSR loci (such as in this study) and branching of clusters with axis values bellow 0.8 is not very reliable. Thus, the interpretation of grouping particular genotypes from different geographic regions when GS value is low shoud be taken with reserve. Anyway, it can be noted that majority (12/13) of genotypes from Burgenland (marked with a red asterisk) was joined within a single cluster with 17 genotypes from Croatia – coastal area and 7 genotypes from Croatia – continental area. It is very interesting that Croatian genotypes constituting this cluster are sampled at very divergent locations, 16 out of them being from the very south of Croatia (Dubrovnik area).

Dispersion of all 120 analyzed genotypes by the principal coordinate analysis (PCoA) placed the majority of genotypes from Burgenland in one quadrant of the coordinate system along with the Croatian genotypes predominantly from the coastal area of Croatia (Fig. 19). This was in contrast with the expectation mentioned above.

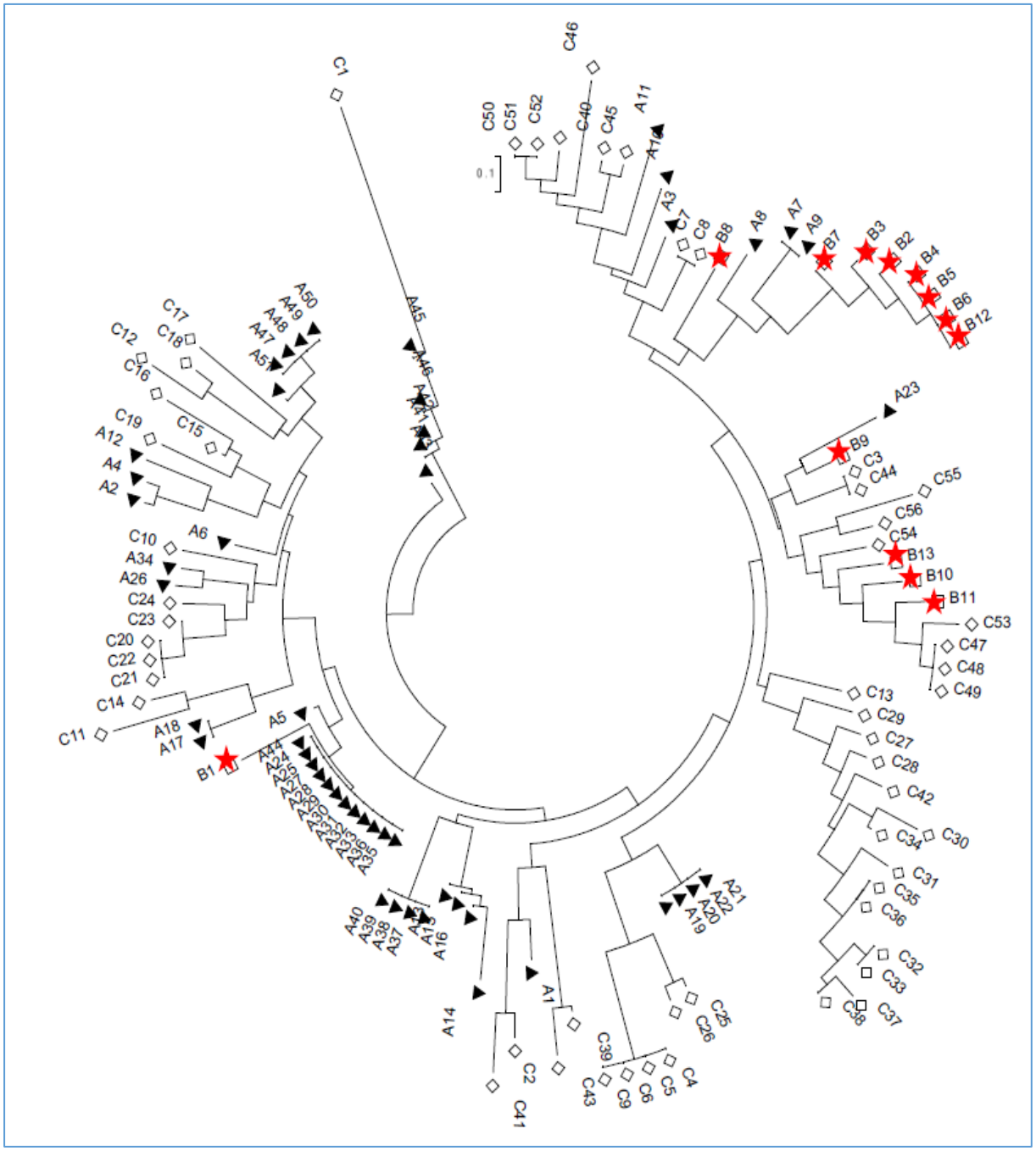


Figure18. Dendrogram based on genetic similarity indices computed out of SSR data for 120 phylloxera individuals originating from Croatian continental area (A1 – A51), Austria – Burgenland (B1 – B13★) and Croatian coastal area (C1 – C56).

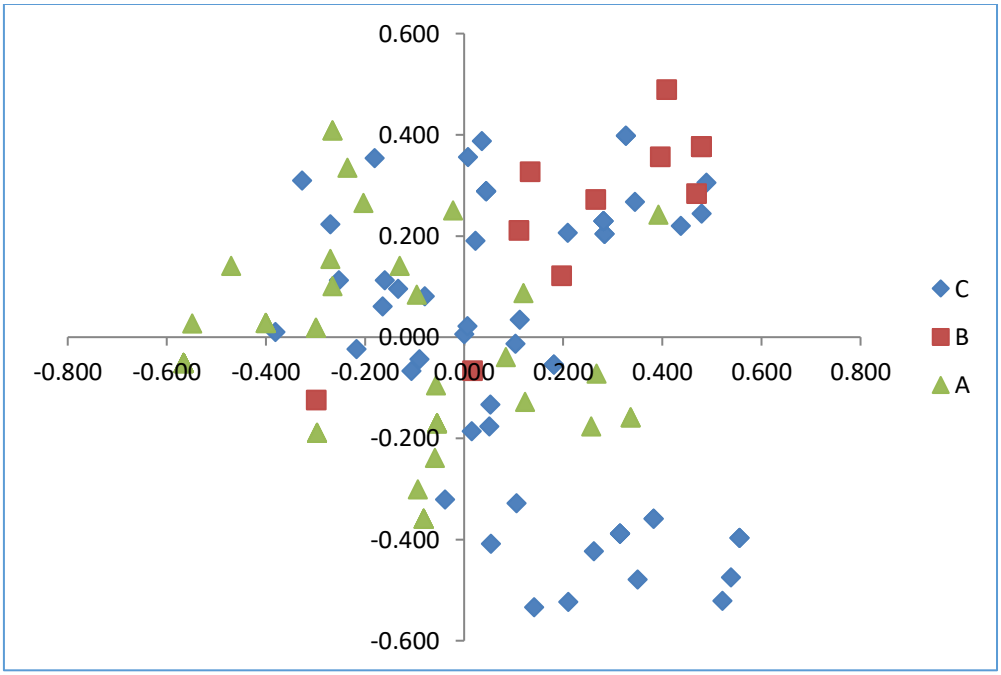


Figure 19. Diagram of dispersion as outcome of the principal coordinate analysis (PCoA) based on genetic similarity indices computed out of SSR data for 120 phylloxera individuals originating from the Croatian continental area (A - green), Austria – Burgenland (B - red) and the Croatian coastal area (C - blue).

Analysis of molecular variance (AMOVA)

The data from the table 10 proves genetic differentiation among two different genotypic data sets. Higher observed genetic diversity of coastal populations and their particular genetic structure might be result of more favorable (mild) climate conditions that enable easier both sexual and asexual reproduction.

Table 10. Summary AMOVA table shows significant differentiation of phylloxera populations originating from Croatian continental (A, n=51) and coastal (C, n=56).

Source	df	SS	MS	Est. Var.	%
Among Pops	**1**	**24.573**	**24.573**	**0.207**	**9%**
Among Indiv	105	259.063	2.467	0.425	19%
Within Indiv	107	173.000	1.617	1.617	72%
Total	213	456.636		2.249	100%

F-Statistics	Value	P(rand >= data)
Fst	0.092	0.001
Fis	0.208	0.001
Fit	0.281	0.001

Probability, P(rand >= data), for Fst, Fis and Fit is based on standard permutation across the full data set.

6. Conclusions

1. The recorded average number of allels, allel frequencies and computed gene diversity parameters in this study proves high level of diversity of Croatian population of leaf-feeding phylloxera when compared with similar studies. Among the analyzed sub-groups, the highest diversity had genotypes originating from the coastal area. In addition, analysis of molecular variance showed that genetic structure of Croatian subpopulations (continental and coastal ones) were significantly different.

2. All 7 applied SSR loci had high polymorphism information content and proved to be suitable for the analysis of genetic structure of leaf-feeding phylloxera populations.

3. A large proportion (1/3) of the total 120 genotyped individuals had identical multilocus genotype, which proved that a significant ratio of individuals came out of asexual propagation. However, there was no prevalence of any particular clone across the sampled locations.

4. In spite of the similarity of climate, the soil types and the cultivars in viticulture between Croatia – continental part and Austria – Burgenland, the expected genetic similarity of phylloxera genotypes from these regions was not confirmed. On the contrary, it seems that the genotypes from Burgenland have more genetic similarity with the genotypes from the Croatian coast.

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