Corso di dottorato di ricerca in:

"Scienze e biotecnologie agrarie"

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# *"Breeding for multiple disease resistances in table grape"*

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# Index

# Abstract

Nowadays, the value of the table grape production is increasing and Italy is one of the main producers in Europe and in the world. Despite this, there are not many high quality cultivars with resistance to powdery and / or downy mildews. 'Lasta' is a table grape cultivar characterized by a medium dense bunch and oval green yellow berries. It was obtained in Serbia by crossing 'Muscat de St. Vallier' and 'Lyana', which are both table grapes, and it seems not susceptible to *Plasmopora viticola*. The source of this resistance is still unclear: the pedigree of 'Lasta' shows that it could derive from *Vitis rupestris* and 'Seibel 4614'; the screening with the SSRs markers usually used for the detection of the known resistance genes did not give the expected allelic profile. For this reason, other markers, available for the locus Rpv3, were used to analyse more strictly the region of interest and verify the presence of a different haplotype of the gene. Furthermore, the progeny of the controlled cross 'Picolit x Lasta' was obtained to generate a map using different types of markers, such as microsatellites, already known to map the 19 linkage groups of grape, and SNPs, detected by ddRADseq technique. The progeny was also tested in a greenhouse experiment for resistance / susceptibility using leaf discs sprayed with a suspension of the pathogen. These phenotypic data were analysed through MapQTL 5.0 (Van Ooijen, 2004) to detect possible QTL associated to the resistance trait. The analyses showed that QTL could be localized on the lower arm of linkage group 18.

Another important aspect of table grape breeding is the pomological evaluation of populations, obtained from different crosses with resistant parents ('20/3' and 'Lasta'), that have been previously screened for the presence of resistance loci. Cluster and berries weight, soluble solid content (SSC), firmness and berries dimensions and shape were analysed for two consecutive years in order to understand the influence of the different parents on the pomological traits of the progenies. Finally, some individuals of these populations were selected on the basis of both genotypic and phenotypic profile because they could have many chances to succeed in table grape market.

# Other PhD activities

- Phenotypic and genotypic evaluation of apple progenies resistant to scab;
- Genotyping of grape progenies obtained from crosses made in 2014, 2015 and 2016.

## Poster presentations:

- Monte C., Brisotto S., Testolin R., Cipriani G., "Agronomic evaluation of apple progenies resistant to scab (*Venturia inaequalis*)", XII Giornate Scientifiche SOI 2018;
- Monte C., Foria S., Testolin R., Cipriani G., "Molecular characterization of the downy mildew resistance in the table grape Lasta", XII ICGBG 2018.

## Winter / summer schools:

- Winter school "Plant biotechnology and environmental sustainability" Canazei (TN), January 2017;
- Summer school "Analisi e gestione dei dati in biologia applicata" (organizzata dal collegio di dottorato UNIUD), Paluzza (UD), September 2017;
- Summer school "System biology" (organizzata dal collegio di dottorato UNIUD), Udine, September 2018.

# 1. Introduction

## 1.1 Main diseases affecting *Vitis vinifera*

Most of the high-quality varieties cultivated all around the world mainly belong to *Vitis vinifera* species. They are produced through vegetative propagation and so they are subject to several diseases, because they can not coevolve rapidly with the pathogen. In particular, two of the most economically relevant diseases of grapevine cultivation are powdery and downy mildew.

### 1.1.1 Powdery mildew

Powdery mildew is caused by *Erysiphe necator* (also called *Uncinula necator*), an obligate biotrophic ascomycete that originated in Eastern and Central United States and seems to have been introduced into Europe in the 1850s together with some American wild *Vitis* species (Riaz et al., 2011; Blanc et al., 2012). The spread across the Eurasian continent and the Mediterranean basin was very rapid, and nowadays *E. necator* can be found in all temperate grape–growing regions.

The pathogen can infect and reproduce itself only in photosyntethic active tissues, such as leaves, inflorescences, young clusters, stems, shoots, and appears as a whitish mycelia on their surface. Severe infections could have some dramatic effects on the production: yield reductions or losses, compromised fruit and wine quality, delay of maturation, berry cracking, cluster rot. Moreover, these bad conditions of the green tissues can facilitate the attack of other pathogens or make the plant more sensitive to environmental stresses.

**Figure 1.** Symptoms of powdery mildew on leaves (left; centre) and bunches (right) (from: Angeli D., Pertot I., 2017. L'oidio della vite. Istituto Agrario di San Michele all'Adige. Safe Crop Centre).

*E. necator* can germinate and develop in the host plant through appressoria, that permit to force penetration of the cuticle of green tissues by a penetration peg and form haustoria into the epidermal cells. These intracellular structures absorb nutrients from host cells and at the same time secrete proteins for suppressing host defences (Qiu et al., 2015). After 5–25 days the ascomycete produces conidiophores on plant surface that release conidia as spores which are spread by the wind to start a new cycle of secondary infection. *E. necator* usually shifts to sexual reproduction at the end of the epidemic season, after many cycles of asexual reproduction (Zendler et al., 2017). From late summer or fall, on the basis of the climate conditions, the pathogen can overwinter in the bark of the plants through ascospores that are enclosed in cleistothecia (chasmothecia) and will be released in spring for the primary infection of the next year. The vegetative mycelium may also colonize dormant buds to overwinter.

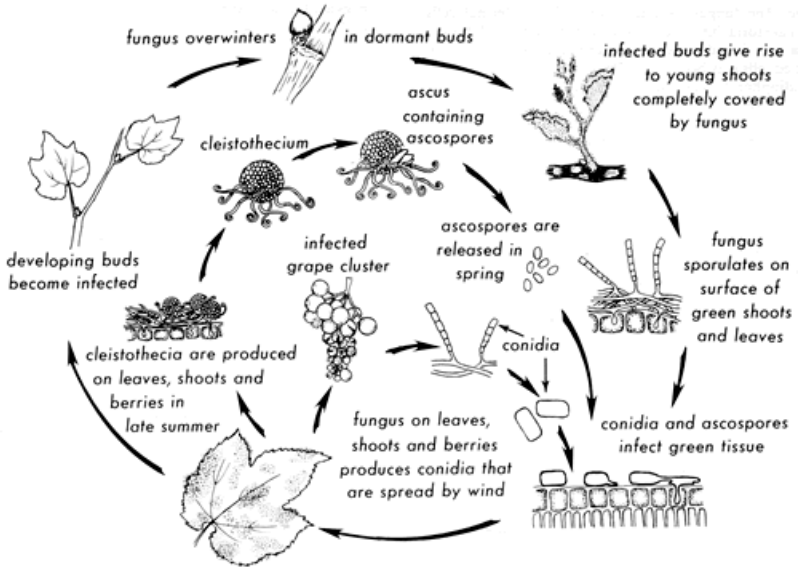


**Figure 2.** Life cycle of *Erysiphe necator* (from: https://evineyardapp.com/blog/2015/08/31/powdery_mildew/).

### 1.1.2 Downy mildew

Downy mildew is also one of the major threats that affect grapevine and it is caused by the oomycete *Plasmopora viticola*. The pathogen is native to the Southeastern United States and was first reported in Europe (France) in the second half of the nineteenth century, when there was an intensive importation of cuttings of American wild grapes used for introducing *Phylloxera* resistance in the susceptible European vines. Since then, it has been rapidly propagated across Europe, becoming now a major problem in all temperate or warm regions (Galet, 1977; Gessler et al., 2011).

All the green parts of the host plants may be infected by *P. viticola*, causing quantitative and qualitative crop losses. On the leaves, symptoms of primary infections are typically yellowish and

translucent oily spots on the upper surface and massive whitish sporulations on the lower side. If the infection occurs on inflorescences and young bunches, they will be covered by the whitish mycelia, especially with high humidity rate conditions, leading to the most serious damages for the production. Shoots are less receptive to the attacks of the pathogen compared to the other green tissues also because of their lignification process that is in progress.

**Figure 3.** Symptoms of downy mildew on upper (left) and lower (center) surface of leaves, and on bunches (right) (from: https://www.apsnet.org/edcenter/intropp/lessons/fungi/Oomycetes/Pages/DownyMildewGrape.aspx)

During spring, in warm and humid weather, the asexual sporangia of *P. viticola*, germinated from oospores, start an infection by releasing zoospores that are splashed by rain into the canopy of the host plant, where they swim to reach stomata. Here the zoospores attack and form germ tubes that penetrate the substomatal cavity. Subsequently, these germ tubes swell into infection vesicles (Kiefer et al., 2002). During the colonization of the intercellular space of the mesophill, primary hyphae appear from infection vesicles and quickly develop branches and haustoria (Yu et al., 2012), by which they can absorb nutrients from the host cells and also exchange signals involved in the establishment of compatibility (Greenville-Brigg and van West, 2005). In this stage hyphae are able to grow without causing visible signs of the disease (Bellin et al., 2009). After an incubation period of several days, sporangiophores emerge through the stomata and form sporangia (Gindro et al., 2003) that can start a new secondary infection on other host plants. At the end of autumn, the formation of numerous oospores within fallen leaves and berries allow *P. viticola* to overwinter (Burruano, 2000), leading to a primary infection in the following epidemic season.

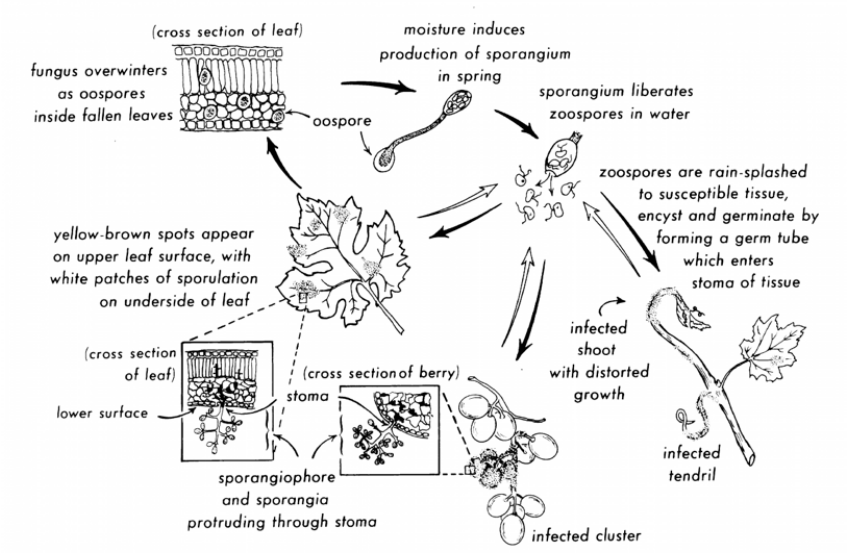


**Figure 4.** Life cycle of Plasmopora viticola (from: https://ohioline.osu.edu/factsheet/plpath-fru-33)

## 1.2 Use of agrochemicals

Although only 3.3 % of the total crop production area in Europe is taken up by viticulture, more than 65 % of fungicides used in agriculture is utilized for grapevine cultivation to avoid attaks of the most common and devastating pathogens. These intensive applications have a concomitant impact on the environment and human health: chemicals contained in pesticides and fungicides could be dangerous for soil, water, humans, livestock and wildlife (Skevas et al., 2012).

The European policy provided specific regulations trying to balance the need of reducing agrochemicals utilization and protection, yield and quality of the crop. Regulations 1107/2009/CE, 1185/2009/CE and directives 2009/127/CE and 2009/128/CE are the basis of the European regulatory framework. In particular, directive 2009/128/CE was issued to achieve a sustainable use of pesticides in all EU member States; its main objectives are:

- Decrease of the risks and impacts of agrochemicals products on human health and environment;
- Promotion of the application of Integrated Pest Management (IPM) and of alternative non-chemical approaches or techniques;
- Protection of the users of these substances, and also of the consumers;
- Defense of the aquatic environment and potable water;
- Preservation of the biodiversity and protection of the ecosystems.

The directive identify the main actions that member States have to follow and carry out through the following directions: provide adequate training to users and dealers; inspect pesticide

application equipment; prohibit aerial spraying; limit pesticide use in sensitive areas; give informations and raise awareness about pesticide risks. Each member State has a specific national plan for putting into practice all determined objectives and actions; in Italy it was defined through Ministerial Decree 22/1/14 and published on 12/2/14, when the plan became law.

These strict regulations on the application of pesticides make plant breeding for disease resistance the most attractive way to control the spread of plant, and in particular grapevine, pathogens without affecting environment and human health (Blasi et al., 2011).

## 1.3 Plant immunity and resistance genes

Physical and chemical barriers of plants are not always sufficient to counteract pathogen attacks, so other two mechanisms are involved in this process. The first line of defense is the PAMP–triggered immunity (PTI), which consists in the recognition of pathogen–associated molecular patterns (PAMPs) associated with specific responses to avoid pathogen growth. When plant pattern recognition receptors (PRRs) recognize PAMPs, there would be the induction of the production of reactive oxygen species (ROS) in a oxidative burst (Zhang et al., 2007) and of salicilic acid, jasmonic acid and ethylene defense hormones (Jones and Dange, 2006). Also the increase of ion fluxes across the cellular membrane and the accumulation of callose between the cell wall and the plasma membrane are classical steps of PTI.

Many pathogens can overcome this resistance mechanisms, but the plant has a second tier of defense called effector–triggered immunity (ETI) that recognizes specific effectors produced by the pathogen (avirulence, Avr proteins) through resistance (R) genes triggering to a signal transduction that activates a strong resistance response. ETI usually leads to hypersensitive response (HR), in which infected and also a few surrounding cells undergo to rapid cell death. It also activates salicilic acid–dependent signaling, tissue reinforcement and antibiotic production at the site of infection. The activation of salicilic acid signaling leads to a long lasting systemic response known as systemic acquired resistance (SAR) that makes the plant more responsive to future pathogen infections. Most of the signaling responses induced by PTI and ETI are the same but PTI is temporally slower and of lower amplitude whereas ETI is more specific and intensive.

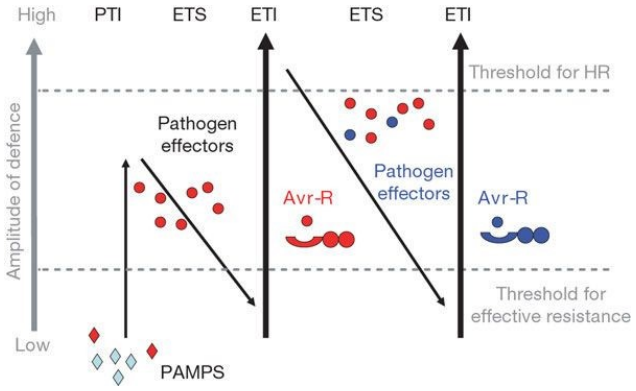


**Figure 5.** A zig zag model for the plant immune system (from: https://www.nature.com/articles/nature05286/figures/1)

The recognition between R gene products and Avr proteins that induces R gene–mediated resistance may occur: directly ("gene–for–gene model") or indirectly ("guard hypothesis"). "Gene–for–gene model" was proposed by Harold Flor in the late 1940s, and affirmed that resistance and avirulence are both characters determined by complementary pairs of dominant genes, one in the host and the other in the pathogen (Hammond–Kosack and Jones, 1997). By contrast, "guard hypothesis" predicts that the initiation of resistance might not necessarily involve the direct interaction between R and Avr proteins: a plant protein (guardee) interacts with a R protein and may also be targeted and modified by a pathogen effector; only when the R protein detects an attack, a modification of its guardee, or recognizes the product of the pathogen attack, the resistance is activated (McDowell and Woffenden, 2003).

Basically, R genes may be divided into eight major classes, depending on their amino acid motif organization and on their membrane spanning domains (Gururani et al., 2012). The classes are listed below:

- NBS–LRR–TIR: it is a cytoplasmic protein consisting of a nucleotide–binding site (NBS), a C–terminal leucine rich repeat (LRR) and a N–terminal domain (TIR) that has homology to the intracellular domain of the *Drosophila* Toll and mammalian interleukin–1–receptors;
- NBS–LRR–CC: it is similar to the previous one, but has a coiled coil (CC) domain at the amino terminus of the protein;
- LRR–TrD: it consists of a transmembrane domain (TrD) attached to extracellular leucine rich repeats (eLRR);
- LRR–TrD–Kinase: it has an intracellular serine–threonine kinase domain linked to the transmembrane domain and the extra cytoplasmic LRR;
- TrD–CC: it is composed by a transmembrane domain and a putative coiled coil domain;

- TIR–NBS–LRR–NLS–WRKY: it has the same structure of the genes of the first class but is extended with a putative nuclear localization signal (NLS) and a WRKY domain at the C–terminus;
- LRR–TrD–PEST–ECS: the extracellular LRR and the transmembrane domain are fused to a PEST (Pro-Glu-Ser-Thr) domain for protein degradation and the proteins motifs ECS to target the protein for receptor mediated endocytos;
- Enzymatic R–genes: they can encode for kinases, reductases or also for different proteins that in some cases have very different functions.

Among these groups, most of the R genes that have been studied belongs to the NBS–LRR classes. LRR domain that is present in the majority of the R proteins structure, is involved in the recognition specificity for pathogen effectors, or may also facilitate the interaction of R gene products with other proteins participating in signal transduction (Dixon et al., 1996). NBS domain is usually found in numerous ATP and GDP binding proteins, suggesting that R proteins require nucleotide triphosphate binding to function (Bent, 1996).

### 1.3.1 Sources of esistance to powdery and downy mildew

Sources of disease resistance are usually found where pathogens and host–plants can coevolve (Riaz et al., 2011). Thus, many North American *Vitis* species like *V. riparia*, *V. rupestris*, *V. cinerea*, *V. lincecumii*, *V. labrusca*, *V. aestivalis*, *V. berlandieri*, and *Muscadinia rotundifolia* have high levels of resistance to both powdery and downy mildews, and had been used to introgressed these resistances in European *Vitis vinifera* cultivars for long time.

Although Chinese *Vitis* species and the two pathogens *E. necator* and *P. viticola* did not coevolve, also several species from China and Central Asia show resistance to both mildews. Maybe, pathogens isolates have been in existance in these regions for a much longer period and wild grape species could have evolved these R genes (Riaz et al., 2013). Another possible explanation is that *Vitis* species have acquired a broad resistance to mildews by facing down other fungal diseases.

Up until now, many resistance loci have been evaluated and mapped, and most of them belong to NBS–LRR category. For example, for powdery mildew resistance, Ren1 is the first locus that was identified in two accessions of *V. vinifera* from Central Asia, ‘Kishmish vatkana’ and ‘Dzhandzhal kara’. Its mediated resistance allows the formation of a germ tube and the establishment of an appressorium, but suppressed hyphal development and conidiophore production (Hoffman et al., 2008; Dry et al., 2009).

Ren4 locus is located on chromosome 18 of the Chinese species *V. romanetii* and its mediated resistance involves rapid PCD of penetrated cells and callose encasement of haustorium that blocks

nutrient uptake. Other two loci were found in the Chinese species *V. piasezkii*: Ren6 locus on chromosome 9 provides total immunity by giving rise to rapid PCD at the point of *E. necator* penetration; Ren7 resides on chromosome 19 and its action is less effective because it limits hyphal development and conidiation after the establishment of the pathogen.

Mapped on the upper side of chromosome 14 of *M. rotundifolia*, Ren5 acts by delaying and then stopping mycelium growth after the formation of the first appressorium (Blanc et al., 2012). Ren3 and Ren9 are two distinct loci that resides on the first half of chromosome 15 of *Muscadinia*.

Run1 in located on chromosome 12 of *M. rotundifolia* and rapidly induces PCD in penetrated epidermal cells. Some evidences demonstrate that it cosegregates with Rpv1, a locus involved in resistance to *P. viticola*.

Another source of downy mildew resistance is Rpv3 locus from *V. rupestris* that is located on the lower arm of chromosome 18 and acts by mounting a localized hypersensitive response.

From *V. amurensis* three loci have been mapped and introgressed in European cultivated vines: Rpv8, on the upper side of LG 14; Rpv10, on chromosome 9; and Rpv12, on chromosome 14, that acts similarly to Rpv3 but it limits more significantly the pathogen sporulation.

| R–locus | Chromosome | R–gene type | References |
|---|---|---|---|
| Rpv1 / Run1 | 12 | TIR–NBS–LRR | Feechan et al., 2013 |
| Rpv2 | 18 | NBS–LRR | Wiedermann – Merdinoglu et al., 2006 |
| Rpv3 | 18 | TIR–NBS–LRR | Bellin et al., 2009; Casagrande et al., 2011 |
| Rpv8 | 14 | NBS–LRR | Blasi et al., 2011 |
| Rpv10 | 18 | NBS–LRR | Schwander et al., 2012 |
| Rpv12 | 14 | CC–NBS–LRR | Venuti et al., 2013 |
| Ren1 | 13 | CC–NBS–LRR | Hoffmann et al., 2008 |
| Ren3 | 15 | Unknown | Van Heerden et al., 2014 |
| Ren4 | 18 | NBS–LRR | Ramming et al., 2010 |
| Ren6 | 9 | Unknown | Pap et al., 2016 |
| Ren7 | 19 | Unknown | Pap et al., 2016 |
| Ren9 | 15 | Unknown | Zendler et al., 2017 |

**Table 1.** Summary of known R–loci for powdery and downy mildew, with informations about chromosome localization and R–gene structure.

## 1.4 ddRADseq technique

Several methods have emerged for developing new molecular markers, leading to the construction of more saturated linkage maps. Most of them have been used even more frequently

because they can increase the number of samples analyzed and maintan the same costs of a normal sequencing approach by reducing the fraction of sequenced genome. One of this methods, restriction – site associated DNA sequencing (RADseq), allows the identification of polymorphic variants neighboring particular restriction enzyme recognition sites (Baird et al., 2008), and has been utilized for SNPs detection in many plant species, also with no prior knowledge of a reference genome. A variation on this sequencing protocol was established by Peterson et al. (2012) and was called double digest restriction–site associated DNA (ddRADseq or ddRAD); it can be considered one of the genotyping by sequencing (GBS) techniques that are used for new markers discovery and genotyping studies. This technique utilizes two restriction nucleases to digest the target genome and then inspects the resulting fragments for SNP variants using next generation sequencing (NGS) technologies.

Thus, ddRADseq is suitable for high–throughput applications, rapid, inexpensive and requires little starting material (Peterson et al., 2012).

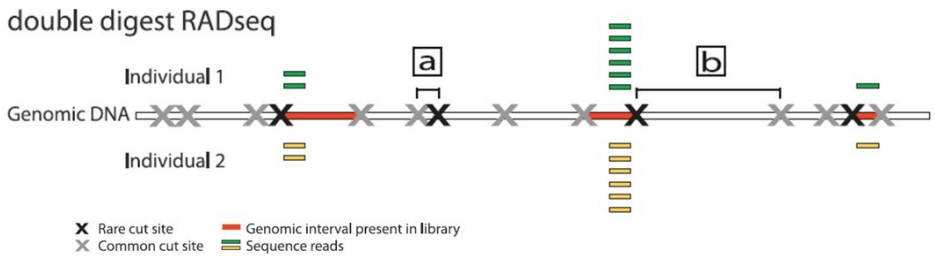


**Figure 6.** Use of a two enzyme double digest followed by precise size selection that excludes regions flanked by either very close (a) and very distant (b) restriction enzymes recognition sites, recovering a library consisting of only segments close to the target site (red segments) (from: Peterson et al., 2012).

## 1.5 Mapping

Linkage maps are a particular class of genomic maps that show position and relative distance between molecular markers or genes on the basis of their genetic association (linkage). Crossing–over events can explain this association because they occur rarely when genes or markers are very close along the chromosome, and during alleles segregation particular combinations would likely be maintained in the progeny. In fact, Collard et al. (2005) affirm that genes or molecular markers that are stricktly linked in the parents would be inherited together in the offspring more frequently than ones that are more far away. Frequency of allelic combinations gives information about the relative position of different genetic elements and can be utilized to map them on the chromosomes.

In linkage maps the distance between various loci is expressed in centiMorgans (cM), that represent the recombination rate between them (Kumar et al., 1999); this genetic distance does not

correspond to the physical one but varies along the chromosome (Kunzel et al., 2000; Tanksley et al., 1992; Young, 1994).

The first important step for the construction of a linkage map is obtaining a mapping population, generally from two parents that should genetically differ for one or more traits of interest. This choise is necessary for following the inheritance of the trait in the progeny. In grapevine, as in other fruit crops or pluriennal species in general, mapping populations usually consist of full–sib families ($F_1$) derived from a cross between two highly heterozygous parents, also to minimize the occurrence of common alleles.

The size of the population is another relevant aspect to consider; for preliminary studies of genetic mapping it generally varies from 50 to 250 individuals (Mohan et al., 1997). In grape, the number of individuals has ranged from 46 to 181, with most maps based on about 100 because is a good compromise between costs and estimation of the correct markers order and distances.

A second step in map production is the identification of the best molecular markers to use in order to easily characterize the polymorphisms. The use of different types of markers permits the construction of very dense genetic maps covering also the whole genome; in grapevine, SSRs and SNPs are commonly utilized. Then, molecular markers allow to screen the parents and the entire population; resulting genotyping data would be statistically analized and utilized for mapping. LOD value (or LOD score) is the parameter usually needed to calculate the association between the different markers. It is defined as the logarithm of the 'odd ratio', that is the ratio between linkage and no linkage. A LOD score of 3 or more is generally utilized for map production because it means that the probability to have association is very high.

Finally, the markers that result associated are grouped in so called 'linkage groups', which represent parts or whole chromosomes (Collard et al., 2005).

## 1.6 'Lasta'

'Lasta' is a table grape cultivar descendent of 'Villard blanc' characterized by large, oval, firm and green–yellow berries and medium–sized clusters. It has a medium vigor, hermafroditic flowers and usually ripens in late August. This interspecific cross was recently obtained in Serbia from 'Muscat de St. Vallier' and 'Lyana', which are both white table grapes resistant to *P. viticola*: 'Muscat de St. Vallier' is a french bred resulted from 'Seyve Villard 12-129' x 'Muscat Hamburg', whereas 'Lyana' was obtained in Moldova by crossing 'Chaush belyi' and 'Pierrelle'.

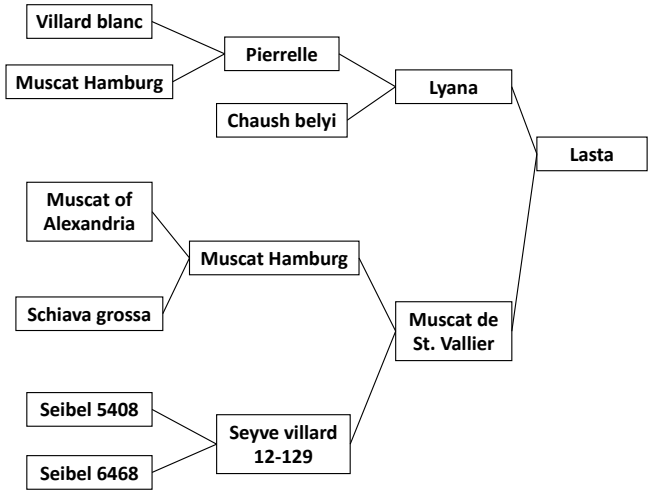


**Figure 7.** Pedigree of 'Lasta', according to the "Vitis International Variety Catalogue" (http://www.vivc.de)

'Lasta' seems to be not susceptible to *Plasmopora viticola*, as it can be seen in the field, but there are no clear evidences of the source of this resistance. In fact, its pedigree shows that it could derive from *Vitis rupestris* and 'Seibel 4614' but the screening with the SSRs markers usually used for the detection of the known resistance genes did not give the expected allelic profile.

## 1.7 Breeding in table grape

Nowadays, the value of table grape production has increased because of increased consumption due also to the important nutritional qualities of the fruits, like high vitamin levels, that are very sought–after from the consumers. Italy is one of the main producers of table grape in Europe and also in the world; other countries involved in this worldwide market are for example China, Turkey, United States and Chile.

Grape varieties are highly susceptible to powdery and downy mildews and the damages affecting bunches and berries lead to qualitative and economical losses. Currently, an intensive use of fungicides from bloom to veraison is still necessary for vineyard protection but it has negative effects on environment and human health. An efficient way to reduce chemical inputs would be the integration of genetic resistance into grapevine cultivars through pyramiding, that is the sequencial accumulation of genes into a single line or cultivar. Thus, one purpouse of table grape breeding is the development of new cultivars with various combinations of resistance loci in order to gain durable resistance and prohibit the breakdown of it by the pathogen. The resistance sources can be optimally combined only if inheritance, genetic control and mechanisms of resistance to these mildews have been completely understood.

In table grape breeding it is also important to combine these resistances with agronomical characteristics that influence consumer acceptability such as seedlessness, berry weight, maturity level, soluble solids concentration (SSC), berry firmness. Seedlessness, one of the primary targets of breeding, and berry weight are controlled by a major QTL, co–localized with SdI (seed development inhibitor) locus (Cabezas et al., 2006; Mejía et al., 2007; Costantini et al., 2008), and other smaller QTLs that are specific for each trait. This relation could not be so favorable in breeding programs because seedless berries are smaller than seeded ones and this character is not always compatible with commercial requirements.

Berry texture is another fundamental property that determines eating quality of table grapes (Sato et al., 1997) because freshness can be easily tested through berry firmness. In particular, crispness is an excellent texture for table use and cultivars with this characteristic are important genetic material for grape breeding (Rolle et al., 2011).

So, marker assisted selection (MAS) helps breeders to early identify the individuals that carry the allele combination needed (Karaaagac et al., 2012) and leads to select new cultivars with all the desirable traits.

# 2. Aim of the thesis

The objectives of my PhD thesis were:

- To study the resistance to downy mildew in table grape cultivar ‘Lasta’. Because none of the known genes seems to be involved, on the basis of the molecular markers available, we would like to map the resistance to understand if it is due to a new gene or to a new allelic form of the genes that are already described;
- To analyze the phenotypic, agronomic characteristics of some table grape offspring obtained by crossing two resistant cultivars or a commercial variety with a resistant one. The study would investigate the influence of the parents on the agronomic value of the progenies. This will lead to the final goal of finding new good commercial varieties carrying resistance genes, in order to limit the use of pesticides in the near future.

# 3. Materials and methods

## 3.1 Plant material

As shown in Table 2, different crosses were made with 'Lasta' and some sensitive cultivars to obtain the mapping population that is needed: 'Chardonnay', 'Cabernet sauvignon' and 'Picolit' are three wine grapes that are useful to evaluate the inheritance of the resistance, as 'Italia', which permits also to observe how the phenotypic characteristics of table grapes would be transmitted.

| Code | Female parent | Male parent |
|---|---|---|
| **Vc546** | Italia | Lasta |
| **Vc548** | Lasta | Lasta |
| **Vc549** | Lasta | Chardonnay |
| **Vc550** | Cabernet sauvignon | Lasta |
| **Vc554** | Picolit | Lasta |
| **Vc555** | Lasta | Italia |

**Table 2.** Crosses that were made to obtain the mapping population. Female and male parents were indicated for each cross.

First of all, plants that were used as female parents had to be emasculated: in this phase it is very important to avoid any injury of the stigma and pistil in order to maximize the success of the pollination. Then, the inflorescences had to be protected against random pollination by wrapping them with paper bags. After collecting the pollen from male parent, we started the pollination. The best moment to do it is when the stigmatic fluid appears on the stigma and so it can support the adhesion of the pollen grains and form the pollen tube. The seeds were harvested when the physiological ripening of berries was reached and then, they were extracted from the berries. Several treatments were carried out to break dormancy. First of all, the seeds were washed in running water to remove all residual sugars and mucilage; once dried, they were put at 4 °C for few weeks. At around mid–December, they were rehydrated by soaking them in water for 24 hours; then, they were soaked in a solution of hydrogen peroxide for other 24 hours for cleaning and sterilizing: this step permits also to facilitate their uniform germination. Afterwards, the seeds were accurately washed to remove residual hydrogen peroxide, dried on blotting paper and planted. Maintained at 4 °C for one month, until mid–January, they were moved into a barn to start vernalization process. Finally, in March the seeds were put in a glasshouse at 18-20°C for germination and the plants were grown and maintained at the Experiment Farm "A. Servadei" owned by the University of Udine.

**Figure 8.** Pictures related to the different phases of the crosses: inflorescences (left) that were protected with paper bags (middle), and young plantlets (right) in the greenhouse (pictures taken at Experiment Farm "A. Servadei", Udine).

From each plantlet we collected a sample of a young leaf that was lyophilized, grinded through the TissueLyser II (Qiagen), and maintained at 4 °C.

Among the crosses made in 2016, the one between 'Picolit' and 'Lasta' (Vc554) was selected as mapping population to study the resistance in 'Lasta' because it is the one from which the highest number of plantlets were obtained. This was possible because 'Picolit' has only female flowers and do not need to be emasculated before the pollination, and this might have led to higher success rate in pollination.

For the agronomical evaluation of new table grape varieties, three different progenies were observed:

- Vc113 ('Italia' x '20/3');
- Vc114 ('Matilde' x '20/3');
- Vc315 ('Lasta' x 'Kishmish vatkana').

Lasta', '20/3' and 'Kishmish vatkana' cultivars can be very useful in breeding programs because they show resistance to powdery and / or downy mildew. 'Italia' and 'Matilde' are two commercial varieties. 'Italia' was obtained at the beginning of nineteenth century and is one of the most productive and most consumed table grapes in our country. It is characterized by late ripening, around the half or end of September. Its huge bunches have conic-pyramidal shape carrying big berries with thick green-yellowish skin; the crunchy and juicy flesh tastes of the sweet aroma of Muscat. 'Matilde' is a vigorous and productive variety that ripens early, usually around the second or third decade of August; it was recently selected by crossing 'Italia' and 'Cardinal'. Bunches are big and compact, with huge yellow berries that have a compact crunchy flesh and an aromatic and sweet taste.

## 3.2 DNA Extraction and quantification

DNA extraction of grinded leaf tissue was done using CTAB protocol adapted to grapevine (Steenkamp et al., 1994; Lodhi et al., 1994; Di Gaspero and Cipriani, 2002). For the samples of the mapping population, the extraction was performed in eppendorf tubes, while for the samples of the second part in 96 - well plates.

The protocol was characterized by the following steps:

- Warm up a suitable amount of 2.5 % CTAB buffer (1M Tris–HCl) supplemented with 2 % v/v of β-mercaptoethanol in a warm bath at 65 °C under a fume hood; add 1–2 % polyvynilpolypyrrolidone powder to the grinded tissue;
- Add 810 µl of chloroform : isoamyl alcohol (24 : 1 v/v), vortex and keep mixing for 3 minutes;
- Centrifuge at 13000 rpm for 15 minutes;
- Under the fume hood, collect the aqueous phase into a 1.5–ml centrifuge tube (or a 96–well plate);
- Add 50 µl of 3M Na Acetate, 150 µl of 4M NaCl, 540 µl of cold isopropanol. Swirl gently and put at -20 °C for at least 30 minutes;
- Centrifuge at 13000 rpm for 10 minutes (3200 rpm for 15 minutes, for plates) at 4 °C and discard the supernatant;
- Wash twice with 500 µl of 70 % EtOH; centrifuge each time for 5 minutes at 13000 rpm (for 15 minutes at 3200 rpm) and discard the liquid. Let the EtOH to evaporate in the shaking oven at 37 °C;
- Resuspend the pellet with 300 µl of TE supplemented with 1 µl of RNAse A (10 mg / ml) and leave at 37 °C overnight (or one hour);
- Add 100 µl of 10 M $NH_4$ Acetate and 1000 µl of 100 % ethanol, and invert the tubes (or the plates) several times;
- Put at -80 °C for 10 minutes;
- Centrifuge at 13000 rpm for 15 minutes (3200 rpm for 20 minutes) and discard the supernatant;
- Wash with 500 µl of 70 % EtOH; centrifuge for 5 minutes at 13000 rpm (for 15 minutes at 3200 rpm) and discard the liquid. Let the EtOH to evaporate at 37 °C;
- Resuspend the pellet in 100 µl of water.

Once extraction was completed, DNA quantification was carried out through a particular spectrophotometer (NanoDrop–Thermo Scientific) that gives us the concentration of the sample and

an estimation of its purity through absorbance measures at 260, 280 and 230 nm and their ratios (260 / 280 and 260 / 230).

Samples of Vc554 population were tested also for DNA integrity with electrophoresis on 1 % agarose gels. This step is crucial because purity and integrity are important features needed for next analysis.

## 3.3 Phenotyping

Phenotyping was executed on the individuals of the progeny of 'Picolit' x 'Lasta' and on the parents themselves in 2017 and 2018. In the first year the evaluation was made through leaf discs bioassay, whereas in the second year also with *in vitro* inoculation of whole leaves. The protocols are basically the same for both analysis. Leaves from sensitive plants that show disease were collected and the spores of the pathogen that resides on them were propagated, collected and used to obtain a suspension concentrated at $10^5$ sporangia / ml. The third, fourth and fifth leaf beneath the apex were detached from each plantlet grown in the greenhouse and rinsed with sterile water; leaf discs of 1 cm diameter were excised with a cork borer. Eight leaf discs, or the whole leaf, of each individual were plated onto wet filter paper in Petri dishes with the abaxial surface up. Then, they were sprayed with the *P. viticola* suspension through a nebuliser (Peressotti et al., 2010; Casagrande et al., 2011). Petri dishes were incubated at 20–22 °C and the disease progress was revealed from 1 to 7 days after infection.

Leaves and leaf discs were scored at 7 dpi for the resistance level to downy mildew using OIV452 descriptors (OIV 2009), visual indexes that assign values from 1 (very susceptible) to 9 (totally resistant), as shown in the table below.

| Scoring | Resistance level |
|---|---|
| **1** | Abundant sporulation densely covering the whole disc area, absence of plant necrosis |
| **3** | Abundant sporulation present in large patches, absence of plant necrosis |
| **5** | Limited sporulation present in intercostal patches, plant necrotic flecks or speckles |
| **7** | Sparse sporulation, necrotic spots |
| **9** | No sporulation, absence of necrosisor necrotic points |

**Table 3.** Scoring of downy mildew resistance level adapted from the criteria of the Office International de la Vigne et du Vin (OIV; Anonymous 2009).

## 3.4 Evaluation of agronomical traits

Agronomical traits were scored during harvesting season in 2017 and 2018. Samples were collected during one day at the end of August, when ripening was completed for most of the individuals. Three bunches were harvested for each plant.

Each bunch was weighted to evaluate the production. Then, two samples of ten fruits randomly chosen from the three clusters were also weighted. One set of fruits was measured with a caliper on its three dimensions to determine the size and the shape of the grapes. Then, a penetrometer with a needle of 2 mm diameter was used to evaluate the firmness of the ten fruits by measuring the force needed to break the skin. Finally, solid sugar content (SSC) was determined through a digital refractometer in Brix degrees. Sensory evaluation was carried out only on samples that seems to have good characteristics, and all individuals were evaluated on the presence of seeds.

Statistical analysis were carried out on collected data using Microsoft Office Excel software. Inside each family all the traits were averaged and ordered to create bar graphs. The same procedure was used to examine the parental lines. For grapes shape the parameter utilized is the ratio between the length and the mean width. Mean values of each trait of Vc113 and Vc114 were compared in order to roughly understand if the different male parent used in the two populations can influence the yield production.

## 3.5 SNPs detection

The individuals selected for DNA integrity and purity (260 / 280 higher than 1.8; 260 /230 comprised between 1.8 and 2.0) were analyzed using ddRAD (double digest Restriction Associated DNA) technique by IGA Technology Services (Udine) to detect SNPs. This approach permits the reduction of the complexity of the genome by using a double restriction enzime digest instead of working with the whole sequenced genome. These SNPs were used to analyze the parents and the progenies.

## 3.6 PCR (Polimerase chain reaction) and fragment analysis

To genetically characterize the samples through microsatellite markers, a touch down PCR followed by a fragment analysis were performed. Several PCR protocols were used on the basis of the different primers, which were all 5'–labeled with 6–FAM or HEX fluorescent dye.

The composition of the 10 µl reaction mix was always the same:

- 1 µl of 10X $Mg^{2+}$ buffer (5 PRIME–10X HotMaster Taq Buffer);
- 0.8 µl of dNTPs (2.5 mM) (Sigma; Deoxinucleotide Set, 100 mM);

- 2 µl of solution of forward and reverse primers (2 µM);
- 0.1 µl of Taq polimerase (5 PRIME–HotMaster Taq DNA Polymerase, 5 U / µl);
- 2 µl of DNA (10 ng / µl);
- 4.1 µl of milliQ water.

For 38 SSRs from Cipriani et al. (2008) the protocol was: one cycle at 95 °C for 2 minutes; 10 touch down cycles at 94 °C for 20 seconds, 55 °C (decreasing 0.5 °C per cycle) for 20 seconds, 65 °C for 40 seconds; 15 cycles at 94 °C for 20 seconds, 50 °C for 20 seconds, 65 °C for 40 seconds, and a final step of 1 hour at 65 °C.

Markers related to resistance loci were utilized to screen 'Lasta', 'Lyana', 'Muscat de St. Vallier', 'Picolit', selected individuals of Vc554 and the three progenies Vc113, Vc114 and Vc315, and are shown in the table below (Table 4).

| Locus | SSR marker | Sequence (5'–3') | Length | References |
|---|---|---|---|---|
| Rpv1 / Run1 | Sc34_8_F | [6FAM]TGGTTGCATCCCACTAAACA | 20 | Di Gaspero, personal communication |
| | Sc34_8_R | ACTTTGGGTTCTTGGGCTTT | 20 | |
| | Sc35_2_F | [HEX]CCACACCAAAATTCCCAGAT | 20 | |
| | Sc35_2_R | CAATCATCCATTGCCACCTT | 20 | |
| Ren1 | Sc47_6_F | [6FAM]GATCAGCCACCAAAAGAGGA | 20 | Di Gaspero, personal communication |
| | Sc47_6_R | GCTTTGTAGTCTGCCTACTTGATG | 24 | |
| | Sc47_20_F | [HEX]TGGATCAAATTAGTGCCTGGA | 21 | |
| | Sc47_20_R | GCCGCTTCAACTCAAAAATC | 20 | |
| Rpv3 | UDV305_F | [6FAM]TGGTGCAATGGTCATAATTT | 20 | Di Gaspero et al., 2012 |
| | UDV305_R | GAGGAAAAGAGAAAGCAAAGA | 21 | |
| | UDV737_F | [HEX]TTTGCATGCGATACCTGAAG | 20 | |
| | UDV737_R | TCCTGCAGCTGTTGACGATA | 20 | |
| | UDV730_F | [6FAM]CAGCAACTACCACTGGCTCA | 20 | |
| | UDV730_R | CAACTGAGGAAGAGCCCAAA | 20 | |
| | UDV732_F | [6FAM]TTTGACAATGGGTCCACCTT | 20 | |
| | UDV732_R | ACCCCCTTTCTTGGGTGAT | 19 | |
| | UDV734_F | [6FAM]TGTGTAATGCAAGGCCAACT | 20 | |
| | UDV734_R | AAGACGTTCAATACCCACATGA | 22 | |
| | UDV736_F | [6FAM]GGATTCAGAACCACCGTTGA | 20 | |
| | UDV736_R | GAGCAATCGAGGCAGAAAAT | 20 | |
| Rpv10 | GF09-44_F | [6FAM]CATCGTTCCTTTCTTACTCGCT | 22 | Schwander et al., 2012 |
| | GF09-44_R | GCTAATGGAGGGTAGTGCTCAA | 22 | |

| | | | | |
|---|---|---|---|---|
| | GF09-47_F | [HEX]CCACATTCTTCCTGCACATAAA | 22 | |
| | GF09-47_R | CTGTTGTAAGGGCTCCCAATTA | 22 | |
| Rpv12 | Sc36_7_F | [6FAM]TGGTTGAGCACAGTTCTTGG | 20 | Di Gaspero, personal communication |
| | Sc36_7_R | CAGAAAGCCCTGATCTCCTG | 20 | |
| | Sc81_9.1_F | [HEX]TTTTGGGAGTTTCCATGTCC | 20 | |
| | Sc81_9.1_R | AAGACACCTTGGGGGATAAAA | 21 | |
| Ren9 | CenGen6_F | [6FAM]TGGTCAATGATCTCCCCATT | 20 | Van Heerden et al., 2014 |
| | CenGen6_R | TTCCAATCAAGGTCATGCAA | 20 | |
| | GF15-10_F | [HEX]CCCCTATATCCTTTCTCATGGTC | 23 | GF. Schwander, unpublished |
| | GF15-10_R | AGGGTCTTGGTGGGAGACTAA | 21 | |
| Ren 3 | GF15-45_F | [6FAM]CAATTACCGAGCTTCCATCAA | 21 | Zendler et al., 2017 |
| | GF15-45_R | TCTGGGATTCCCCTACTTCC | 20 | |
| | GF15-44_F | [HEX]TGGATTTGTTCTTGGCAGTG | 20 | |
| | GF15-44_R | GTGCCAGCTACACCAACAGA | 20 | |

**Table 4.** SSR markers associated with resistance loci already known from literature and primer sequences, that were used to analyze samples in this work.

The following PCR thermal profile was utilized for SSRs associated with Ren3 and Ren9 loci: one cycle at 94 °C for 2 minutes; 10 touch down cycles at 94 °C for 30 seconds, 60 °C (decreasing 0.5 °C per cycle) for 30 seconds, 65 °C for 30 seconds; then, 20 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, 65 °C for 30 seconds, and finally at 65 °C for 10 minutes.

For all other markers the protocol was the one reported below: one cycle at 95 °C for 2 minutes; 10 touch down cycles at 94 °C for 20 seconds, 55 °C (decreasing 0.5 °C per cycle) for 20 seconds, 65 °C for 30 seconds; 20 cycles at 94 °C for 20 seconds, 50 °C for 30 seconds, 65 °C for 30 seconds, and a final step at 65 °C for 7 minutes.

The PCR products were diluted 1:150 in milliQ water and analyzed by capillary electrophoresis (3730 DNA Analyzer–Applied Biosystem) using GeneScan-500 Liz Size Standard. Electropherograms were processed with GeneMapper 4.0 software (Applied Biosystem) providing alleles size.

## 3.7 Genetic mapping

Linkage analysis was performed using JoinMap 4.0 software (Van Ooijen, 2006) on a matrix of data of 1602 markers that were analyzed for 93 individuals of a CP type population. CP populations are those that result from a cross between two heterogeneously heterozygous and homozygous diploid

parents, whose linkage phases were unknown (Van Ooijen, 2006). Markers that were utilized for the construction of the genetic linkage map of 'Lasta' have one of four segregation phases typical of this type of population: <abxcd>, <efxeg>, <hkxhk>, and <nnxnp>. The maximum likelihood mapping algorithm were used and a LOD threshold of 3 was set to determine the linkage groups, which were numbered according to grapevine reference genetic maps (Doligez et al., 2006; Di Gaspero et al., 2007).

## 3.8 QTL analysis

QTL analysis was carried out using MapQTL 5.0 (Van Ooijen, 2004) through both non–parametric Kruskal–Wallis test and interval mapping algorithm. The analysis was based on the parental map obtained in the previous step in combination with the phenotypic evaluations. Phenotypic data of each year were considered separately, as also leaf discs and whole leaf tests. The robustness of each QTL analysis performed was evaluated through a significant LOD threshold, which was calculated by permutation test as 1.9 at the 95% confidence level.

# 4. Results and discussion

## 4.1 Molecular characterization of downy mildew resistance in ‘Lasta’

### 4.1.1 Genotyping

Field evaluations showed that ‘Lasta’ has downy mildew resistance, which is not genetically characterized. For this reason, the cultivar and its parents ‘Lyana’ and ‘Muscat de St. Vallier’ were analyzed using all markers related to resistance loci, in order to better understand the origin and the genetic source of this resistance.

| | **Sc34_8** | | **Sc35_2** | | **GF09-44** | | **GF09-47** | | **Sc81_9.1** | | **Sc36_7** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lasta | - | - | 244 | 248 | 236 | 242 | 286 | 293 | 305 | 322 | 188 | 205 |

**Table 5.** Allelic profile of ‘Lasta’ for molecular markers related to Rpv1 (Sc34_8 and Sc35_2), Rpv10 (GF09-44 and GF09-47), and Rpv12 (Sc81_9.1 and Sc36_7). “-“ means that the allele could not be called.

As shown in Table 5, none of the resistant alleles related to the molecular markers seemed to be present in the cultivar but the most interesting allelic profile was found in Rpv3 locus. Screening with UDV305 and UDV737 markers permitted to identify the presence of 299–279 haplotype of the locus in both parental breds ‘Lyana’ and ‘Muscat de St. Vallier’. By contrast, ‘Lasta’ have inherited the allele related to resistance from UDV737 but not from UDV305, as shown in the table below. Other analyses were performed using UDV markers (Di Gaspero et al., 2012) that are more internal in the locus, but no other haplotype was detected (Table 6).

The physical distance between UDV305 and UDV737 is about of 1,2 Mb and the results obtained from microsatellites analysis may provide the hypothesis that recombination events had occurred in this region.

| | **UDV305** | | **UDV730** | | **UDV732** | | **UDV734** | | **UDV736** | | **UDV737** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lasta | 342 | 360 | 196 | 196 | 152 | 160 | 227 | 246 | 275 | 275 | **279** | 285 |
| Lyana | **299** | 360 | 196 | 208 | 156 | 160 | 227 | 233 | - | - | **279** | 295 |
| Muscat de St. Vallier | **299** | 342 | 196 | 208 | 152 | 159 | 233 | 246 | 266 | 275 | **279** | 285 |

**Table 6.** Allelic profile of ‘Lasta’, ‘Lyana’ and ‘Muscat de St. Vallier’ for UDV markers that characterized Rpv3 locus. The alleles related to resistance of UDV305 and UDV737 are in bold. “-“ means that the allele could not be called.

Considering that the data obtained using the markers in linkage with known resistance genes did not give a clear information on the locus involved in the resistance to downy mildew in the cultivar 'Lasta', a mapping approach of the locus have been established. Starting from this situation, 94 individuals of the mapping population were selected on the basis of purity and integrity of their DNA and were analyzed using ddRADseq technique together with 'Lasta' and 'Picolit'. This allowed the detection of 8,220 SNPs spread all over the nineteen linkage groups. These data were trimmed in order to have better results for mapping. The first step consisted in finding the individuals that had less data available. Only Vc554_126 was discarded because it was the only sample that had more than 80 % of missing data. Then, also SNPs markers were ordered on the basis of the percentage of missing data and we took in consideration the ones that presented less than 2 %. Finally, markers were splitted according to the type of segregation (<abxcd>; <efxeg>; <hkxhk>; <nnxnp>) and 1,588 SNPs were selected for the construction of the map in 'Lasta'.

Microsatellites were also used to be sure that the chromosome calling during the SNP detection was correct. 38 SSRs (Cipriani et al., 2008) that are already known and used in other mapping populations were tested on 'Lasta' and 'Picolit'. Then, those that showed segregation on the parents were checked on the progeny and among these, four markers were selected for mapping: c_35125_1 on chromosome 1; c_34_1 on chromosome 5; s_104_1 on chromosome 9; and c_11660_1 on chromosome 19.

Microsatellites related to known resistance loci (Materials and Methods, Table 4) were also used to analyze Vc554 offspring and ten markers have been chosen because their type of segregation permitted to map 'Lasta' (<abxcd>; <efxeg>; <hkxhk>; <nnxnp>): UDV305 and UDV737 (Rpv3 locus); Sc36_7 and Sc81_9.1 (Rpv12 locus); Sc35_2 (Run1 locus); GF15-10 (Ren9 locus); GF15-45 and GF15-44 (Ren3 locus); Sc47_6 (Ren1 locus); and GF09-47 (Rpv10 locus).

### 4.1.2 Map costruction

After these analyses, a data matrix of 93 individuals and 1,602 markers were utilized as input for JoinMap 4.0 software (Van Ooijen, 2006) to produce the linkage map of 'Lasta'. The maximum likelihood (ML) mapping algorithm was used and the independence logarithm of odds (LOD) score was set to 3. Once the two parental population nodes had been created, a total of 969 SNPs were discarded because they showed a similarity value equal to 1.00; this permits to exclude identical markers from the analysis.

Finally, the resulted map covers a total of 1571.1 cM in length with 580 markers mapped on 19 linkage groups, as shown in Supplementary material 7.1. The specific number of markers that were mapped and the genetic length of each linkage group were reported below in Table 7.

| Linkage Group | Mapped markers | Genetic length (cM) |
|---|---|---|
| 1 | 39 | 70.8 |
| 2 | 21 | 53.2 |
| 3 | 18 | 67.8 |
| 4 | 29 | 49.5 |
| 5 | 38 | 79.1 |
| 6 | 30 | 84.9 |
| 7 | 29 | 79.4 |
| 8 | 38 | 71.2 |
| 9 | 25 | 183.7 |
| 10 | 30 | 99.5 |
| 11 | 19 | 87.3 |
| 12 | 30 | 58.8 |
| 13 | 24 | 80.8 |
| 14 | 45 | 103.4 |
| 15 | 50 | 137.8 |
| 16 | 26 | 76.8 |
| 17 | 10 | 27.3 |
| 18 | 54 | 102.7 |
| 19 | 25 | 57.1 |

**Table 7.** Number of mapped markers and genetic length in centiMorgan for each linkage group.

Gaps between the markers are usually smaller than 20 cM, except for five gaps that are located on linkage groups 9, 11, 13 and 15. The largest gap, consisting of 122.2 cM, was found in the chromosome 9, between the coordinate positions 77,616 and 8,781,040; this genetic distance seems to be too high, suggesting that in this region there were probably some errors related to markers positioning. Due to this huge gap, LG9 is also the largest group in terms of genetic distance, consisting of 25 markers covering 183.7 cM. The smallest group, also in terms of marker coverage, is LG17 with 10 mapped markers covering 27.3 cM. LG 18 has the highest coverage with 54 mapped markers.

Among all the markers that were inserted in the map, five SNPs that had been physically located on a particular chromosome, are now mapped in a different linkage group: chr5_13856867 is now on LG1; chr15_9163827 on LG3; chr9_11863746 on LG13; chr12_12228932 on LG14; and chr11_7803589 on LG18. Similarly, 21 markers that did not have a specific position on the physical map, are now mapped in one of the nineteen linkage groups: chrUn_40206232, chrUn_12200252 and chrUn_33259882 are mapped on LG2; chrUn_10843736 on LG6; chrUn_15151874,

chrUn_7033036, chrUn_9818061 and chrUn_7425659 on LG7; chrUn_17641997 on LG8; chrUn_10732201, chrUn_2009781, chrUn_22144390, chrUn_42007004, chrUn_29850233, chrUn_37578556 and chrUn_9013506 on LG10; chrUn_2484784 and chrUn_23071860 on LG13; chrUn_25889409 on LG17; chrUn_34826306 on LG18; and chrUn_16674634 on LG19. Markers positioning in the genetic linkage map seems to be more reliable compared to the one in the physical map.

As it can be seen from the genetic map (Supplementary material 7.1), the four SSRs tested on the population from Cipriani et al. (2008) (c_35125_1; c_34_1; s_104_1; and c_11660_1) and the ones related to Rpv3 genes were supposed to be mapped but they would not be included probably because of more than 20 % of missing data that caused their distorted segregations. We try to map only LG18 with regression mapping algorithm in order to evaluate if Rpv3–related markers would be included, and in particular UDV737, which resistance allele resulted to be present in 'Lasta'. It was mapped using a LOD score of 3, but its position in the upper part of the chromosome was presumably not correct according to the literature (Di Gaspero et al., 2012); for this reason, and also because the generated map resulted more distorted with many big gaps, it would be excluded. All other microsatellite markers that had been given as input to the software were excluded. This was not surprising for some of them because the previous screening already showed that the resistance loci to which they are related are not present in 'Lasta'.

Unexpectedly, GF15-10 and GF15-44, SSRs respectively related to Ren9 and Ren3, were included in the map on the lower part of linkage group 15, at around 30 cM from the closest mapped marker. The localization of the microsatellites is different from what it was found in 'Regent' by Zendler et al. (2017), so we are not sure about the real presence in the cultivar of the resistance loci, also because a phenotypical evaluation on powdery mildew resistance was not performed to support these data. If this assumption was true, this source of resistance to powdery mildew could originate from different genotypes of American *Vitis* species that are present in the pedigree of 'Lasta', according to the "Vitis International Variety Catalogue" (http://www.vivc.de): *V. rupestris* Ganzin, *V. rupestris* Scheele, *V. riparia* Michaux, *V. berlandieri* and *V. labrusca*.

Genetic maps recently obtained for the identification of resistant loci in different *Vitis* accessions are essentially based on microsatellites markers (e.g. Schwander et al., 2012; Blasi et al., 2011; Blanc et al., 2012). This type of molecular markers shows many advantages, as co-dominant inheritance, reproducibility and locus specificity, but the experiments needed are time– and cost–consuming and the number of SSRs is usually limited for map construction. For example, the linkage map of *V. amurensis* obtained by Blasi et al. in 2011 to map Rpv8 locus covers a total length of 975 cM with 132 markers, of which 126 are SSRs and 6 RGAs (resistant gene analogues). The average

distance between markers is 7.39 cM, whereas in linkage map of 'Lasta' previously presented it corresponds to 2.71 cM. So, the map obtained in this work shows a higher coverage compared to SSRs based maps, thanks to the high number of SNPs markers detected through ddRADseq technique and then utilized to construct the map. About this, the continuously decreasing cost of NGS has resulted in the rapid development of several NGS–based methods for the identification of SNPs, which have become the most widely used markers in genetic studies (Zhu et al., 2018). ddRADseq, like other techniques as RADseq, GBS, Illumina chips, SLAF (Specific Length Amplified Fragment)–seq, etc., is and will be always much more used as a promising rapid cost–effective strategy for the construction of high–density genetic maps (Wang et al., 2017) to facilitate the identification of genomic regions with characteristics of desirable agronomic and resistant traits (Troggio et al., 2008). For example, RAD sequencing were used by Wang et al. in 2012 to construct a genetic map with 1,646 SNPs and a length of 1,917.13 cM. In 2014, Barba et al. constructed maps based on GBS for *V. rupestris* 'B38' (1,146 SNPs) and 'Chardonnay' (1,215 SNPs), spanning 1,645 cM and 1,967 cM, respectively. Moreover, Guo et al. in 2015 obtained a genetic map of 1,929.13 cM containing 7,199 markers using SLAF sequencing (Wang et al., 2017).

### 4.1.3 Phenotyping of the resistance to downy mildew

Phenotyping was performed for two consecutive years to evaluate the resistance against downy mildew using parameters from OIV452 (OIV, 2009) and Materials and methods (Table 3).

The first evaluation was carried out in 2017 by testing all 93 individuals, of the population and the two parental lines through leaf discs bioassay, and the results showed that 63 % of the progeny has been scored as 7 and 9, whereas the remaining 37 % as 1,3, and 5. This means that most of the individuals appears to be completely or quite resistant; only 9 are totally susceptible to the disease by showing a huge sporulation on the leaf discs. As it can be noticed from Figure 9 below, the distribution of the scores followed a trend that was unexpected for a character with a Mendelian control of one or few genes.

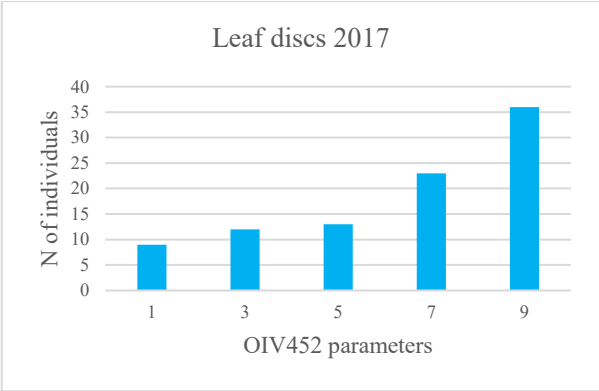


**Figure 9.** Distribution of the scores (1: sensitive; 9: resistant) from OIV452 parameters regarding leaf discs bioassay infected with *P.viticola* in 2017.

The high number of 9 scores (completely resistant) could be an overestimation of the real resistant plantlets because we noticed that many discs of some individuals became brown only three or four days after pathogen inoculation. So it was quite difficult to discriminate if they did not show sporulation because they are resistant or for the unfavorable conditions of the discs.

Another leaf disc bioassay was performed in 2018 on 48 individuals of the populations, the parental lines and some controls. The reduced number of individuals is due to the high death rate that affected the progeny. The scores followed a normal distribution (Figure 10); only one individual was scored 1 (completely susceptible) and seven were evaluated as 9 (totally resistant). 35 % of the individuals were scored as 7 or 9, while 65 % as 1, 3 or 5.

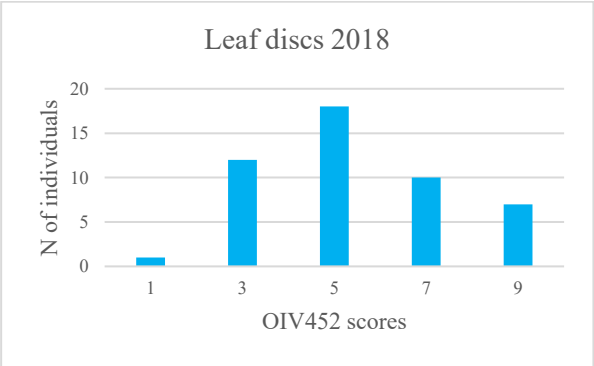


**Figure 10.** Distribution of the scores (1: sensitive; 9: resistant) from OIV452 parameters regarding leaf discs bioassay infected with *P. viticola* in 2018.

In 2018, an *in vitro* inoculation was also carried out on whole leaves, and the results of the evaluation follow a normal distribution with center on score 5 (Figure 11), as the previous one. 27 %

of the analyzed individuals were scored as 7 or 9, whereas 73 % as 1, 3 or 5. In this case, five samples were scored 1, and five were scored 9: the number of totally resistant and susceptible is the same.

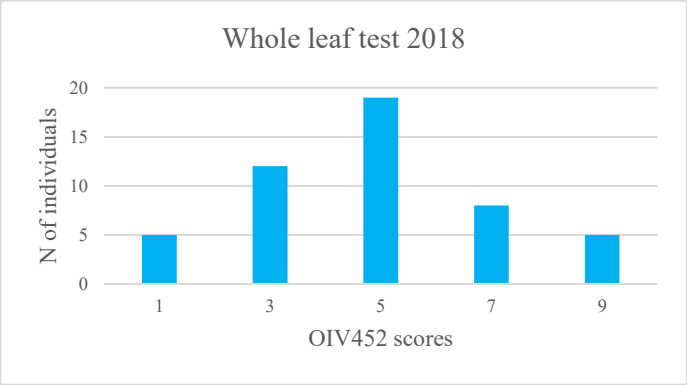


**Figure 11.** Distribution of the scores (1: sensitive; 9: resistant) from OIV452 parameters regarding whole leaf *in vitro* inoculation of *P. viticola* in 2018.

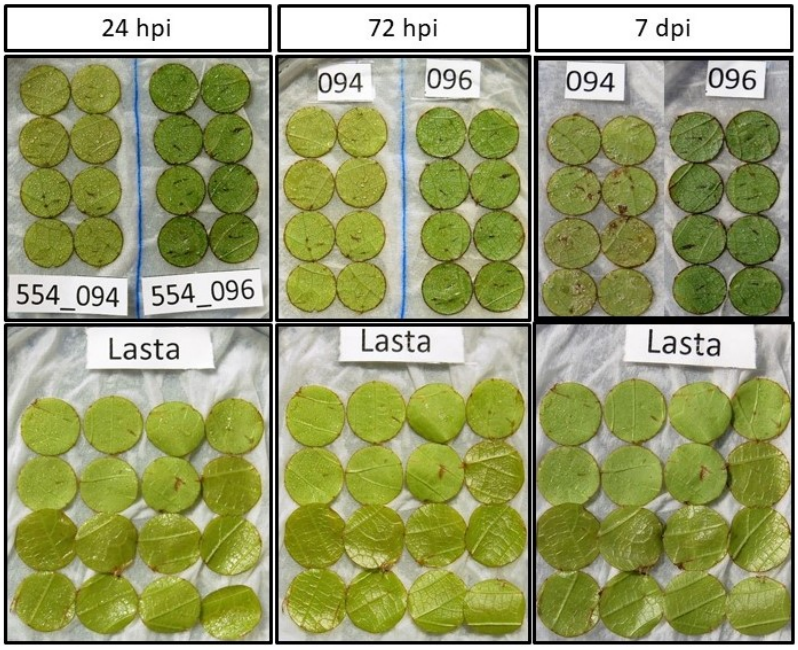


**Figure 12.** Example of two individuals of the mapping population Vc554 and 'Lasta' that were tested through leaf discs bioassay in 2018. The pictures were taken at 24 and 72 hours, and 7 days post infection. 554_094 was scored as 3, while 554_096 and 'Lasta' as 9.

### 4.1.4 QTL analysis

QTL analysis was performed using MapQTL 5.0 software (Van Ooijen, 2004) with the map of 'Lasta' previously obtained and the phenotypic data, that were considered first separately and then, also the mean between the leaf discs evaluations of the two years was taken into account for the

analysis. Both Kruskal–Wallis non parametric test, and interval mapping (only with normal distributed data) were utilized for the detection.

All the analyses showed that the linkage group 18 was the one in which we always observe a significative QTL peak. We decided to perform further analysis only on this linkage group and several cycles of permutation test were conducted on these restricted set of data to establish a LOD threshold of 1.9. The value of LOD related to the detected QTL was just above the threshold (Figure 13) and it could be due to the quality of phenotypic data with many missing values. In the figure below, the QTL peak was obtained using phenotypic data from leaf discs bioassay of 2018.

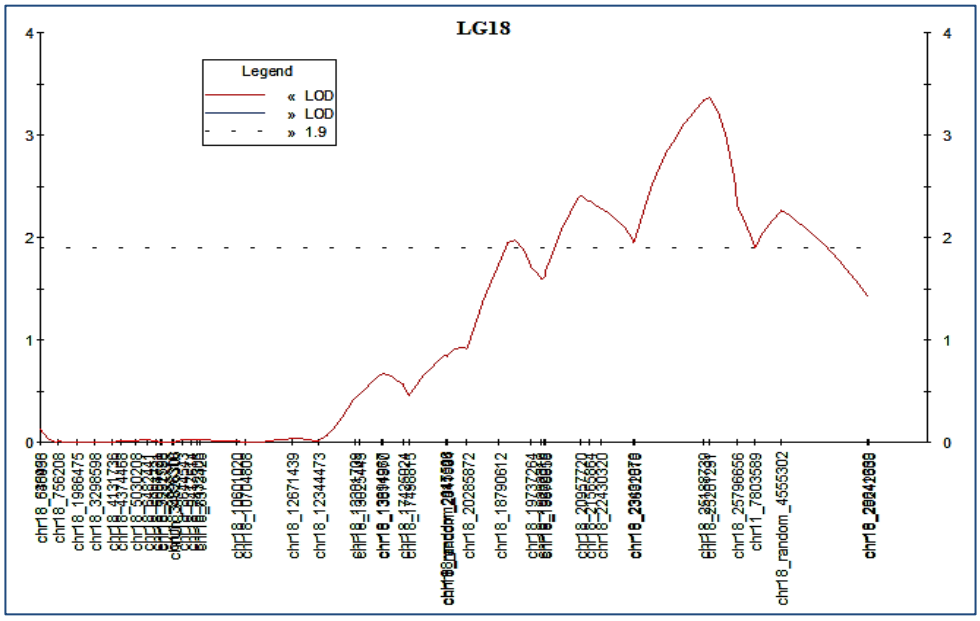


**Figure 13.** QTL plot for resistance to *Plasmopora viticola* localized on LG18 and obtained by Interval Mapping, setting the independence LOD score at 3. The value of LOD threshold was 1.9. Phenotypic data from second year leaf discs bioassay were used.

The presence of the QTL on chromosome 18 should be consistent with the presence of the resistance allele of UDV737 related to Rpv3 locus; in fact, it is located within the markers chr18_23431076 and chr11_7803589, as shown in Figure14. Between these two markers it could be possible to find the Rpv3 locus; on the basis of a comparison with the reference genome PN40024 recently made (Foria, unpublished), the locus should be located between the positions 25,256,951 and 26,356,274, according to the QTL peak found in our analyses. The same study revealed that a cluster of TIR–NBS–LRR genes and a LRR–kinase would be present in this resistant haplotype; both genes encode for proteins that are involved in plant resistant immunity system.

This observation could support also the hypothesis of a possible recombination in this region, that could be investigated by finding other markers that can restrict the locus or by resequencing it.

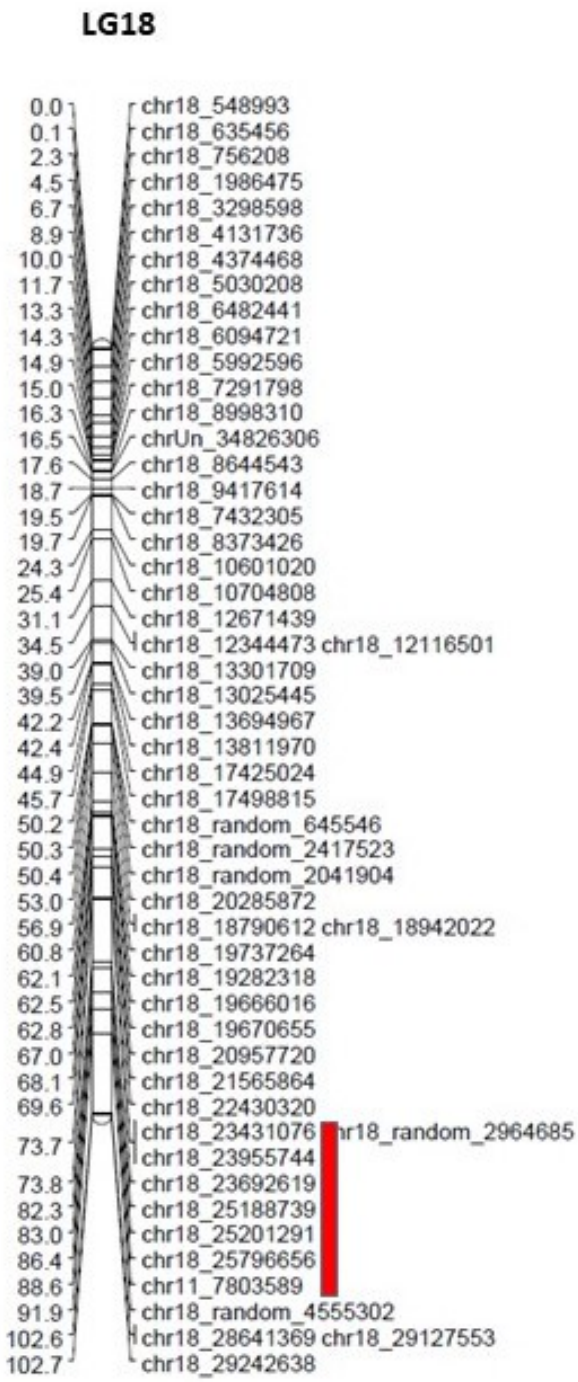


**Figure 14.** Linkage map of LG18. Red line indicates the interval of markers (from chr18_23431076 to chr11_7803589) in which possible QTL responsible for downy mildew resistance was detected.

Maybe, there could be also other minor QTL responsible for downy mildew resistance in 'Lasta' that are difficult to discriminate. In fact, we found also other peaks on LG11 and 13 but the LOD threshold excluded them from the analysis, probably due always to the quality of phenotyping. Also if we have utilized only data from individuals that were phenotypically scored for two years, the signal of these minor peaks did not significantly increase in order to better discriminate minor QTLs.

## 4.2 Pomological evaluation of table grape crossing populations

All 54 individuals of the three populations Vc113, Vc114 and Vc315 and their parental lines had been genotypically analyzed using microsatellites markers related to resistance loci (Table 2, Materials and methods). These tests permitted to investigate if and how the progeny have inherited resistance to powdery and / or downy mildews.

The data related to each agronomical trait collected in the two years were not considered separately but integrated. We decided to use this approach in order to have as much data as possible for each individual and for some of them there are many missing data because there were no production in one year.

Firstly, a descriptive statistical analysis was performed on the three progenies: data were averaged for each individual and a mean value for the whole family was calculated. Standard deviation within the individual determines the standard error of the measurements. Correlation between the traits were evaluated using Pearson's correlation coefficients. Then, we used these data to compare Vc113 and Vc114 trying to understand if the use of 'Italia' and 'Matilde' respectively as male parent could have different effects on pomological traits. Finally, we selected different individuals from each population on the basis of the resistant genotypic profile and some peculiar pomological characteristics, which made them candidates for further investigations or breeding programs.

Given that seedlessness is a favorable property for table grape consumers, we evaluated the presence or absence of seeds in all individuals of each progeny but none of the sample show this characteristic. For this reason, we decided not to consider this trait for the selection.

### 4.2.1 Vc113

Vc113 progeny was obtained by crossing '20/3' and 'Italia', two white table grapes. '20/3' shows resistance to *P. viticola* and molecular analyses confirm the presence of Rpv3 and Rpv12 loci. 'Italia' is a commercial variety that is commonly sold in our country.

This crossing population consists of 29 individuals that has been genotypically characterized: among them, ten carry only Rpv12 locus, one only Rpv3, and 11 both resistance loci.

#### *4.2.1.1 Bunch weight*

The values related to mean weight of the clusters ranges from (19.67 ± 7.76) g of 113_090 to (774.00 ± 168.11) g of the parental line 'Italia'. This last value is extremely higher compared to the rest of the population, which has an average weight of (188.64 ± 81.66) g. Mean weight of '20/3'

measures (218.00 ± 19.26) g, so half of the individuals of the progeny has lower values than the female parent.

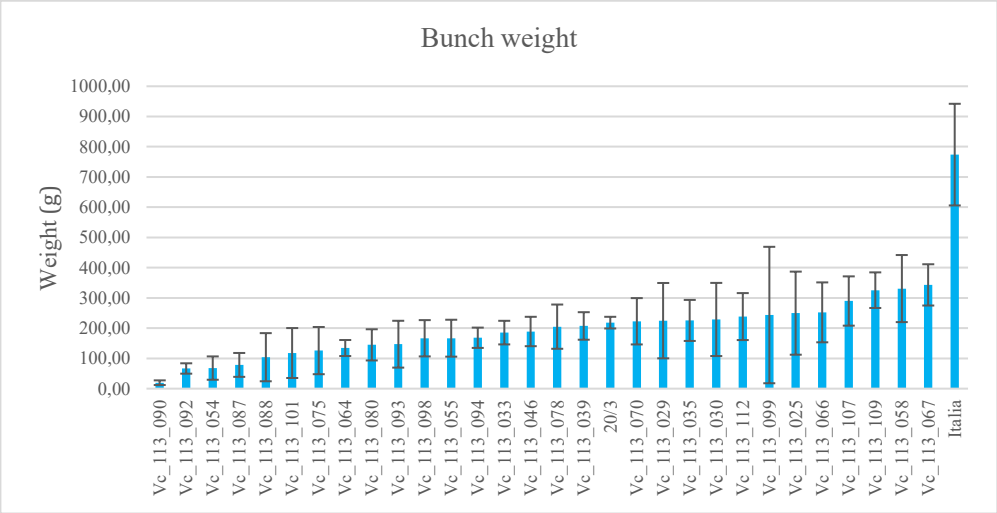


**Figure 15.** Ordered distribution of the mean bunch weight for Vc113 individuals and the parents '20/3' and 'Italia'. The error bars represent the standard deviation calculated within the individual.

*4.2.1.2 Ten berries weight*

Almost all the average weights of ten grapes of each individual are included between the values of the two parents '20/3', with (17.27 ± 0.52) g, and 'Italia', with (86.77 ± 9.85) g. The samples are well distributed within this range and the mean value of the progeny is (32.24 ± 7.96) g. Only Vc113_064 stays out of this range with a lower weight of (14.16 ± 0.66) g. As for cluster weight, 'Italia' has a very high measure respect to the other individuals.

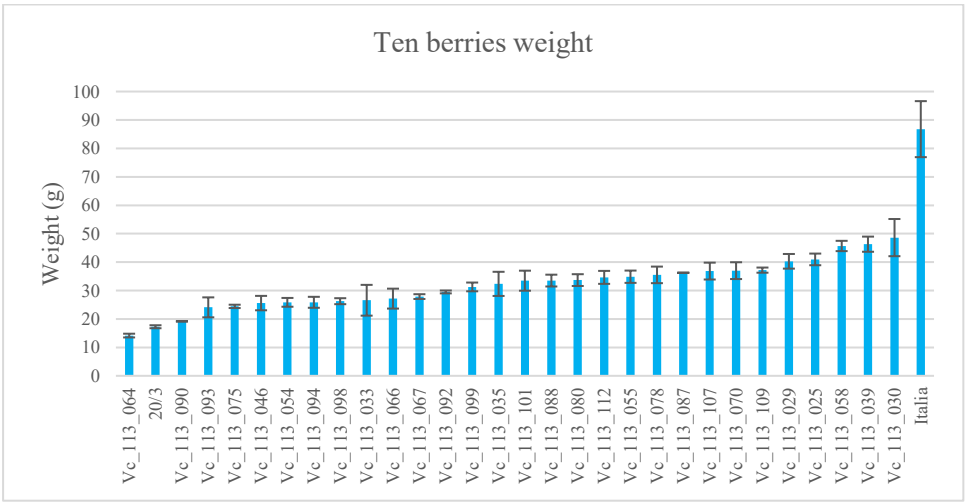


**Figure 16.** Ordered distribution of the mean berries weight for Vc113 individuals and the parents '20/3' and 'Italia'. The error bars represent the standard deviation calculated within the individual.

*4.2.1.3 Size and shape of fruits*

The measures of three dimensions of each berry were considered together as the ratio between the length and the mean width, in order to evaluate only one parameter, the shape of the fruits. If the ratio is equal to 1, the grape has a round shape; if it is lower, the fruit is more flattened, and if it is higher than 1, grapes are elongated, oval. The mean value for Vc113 progeny is 1.16 ± 0.09 and all the individuals have ratios higher than 1, that range between 1.04 ± 0.02 of 113_039 and 1.48 ± 0.07 of 113_054. '20/3' (1.08 ± 0.07) and 'Italia' (1.19 ± 0.07) are included in this range and most of the progeny in included between the values of their ratios.

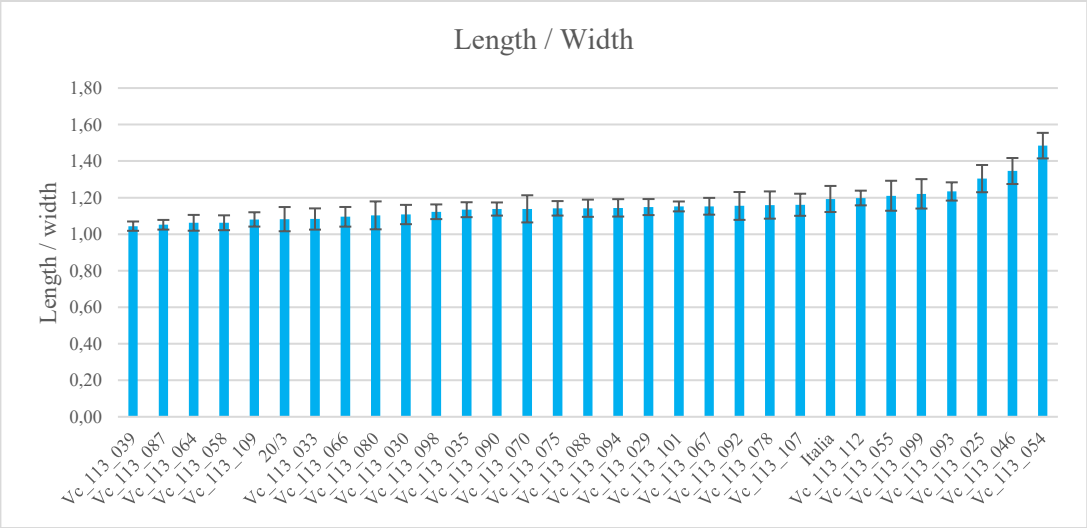


**Figure 17.** Ordered distribution of the average ratio between length and mean width of berries for Vc113 individuals and the parents '20/3' and 'Italia'. The error bars represent the standard deviation calculated within the individual.

*4.2.1.4 Solid soluble content (SSC)*

The mean value of sugar content of the population is (19.14 ± 1.98) °Brix; '20/3' does not move away from this value, whereas 'Italia' has a lower content of (14.99 ± 2.74) °Brix. The highest and the lowest values of SSC belong respectively to 113_092 with (21.84 ± 0.60) °Brix and to 113_075 with (14.13 ± 3.79) °Brix. Half of the progeny shows higher sugar content than both parental lines, as it can be noticed from the Figure 18.

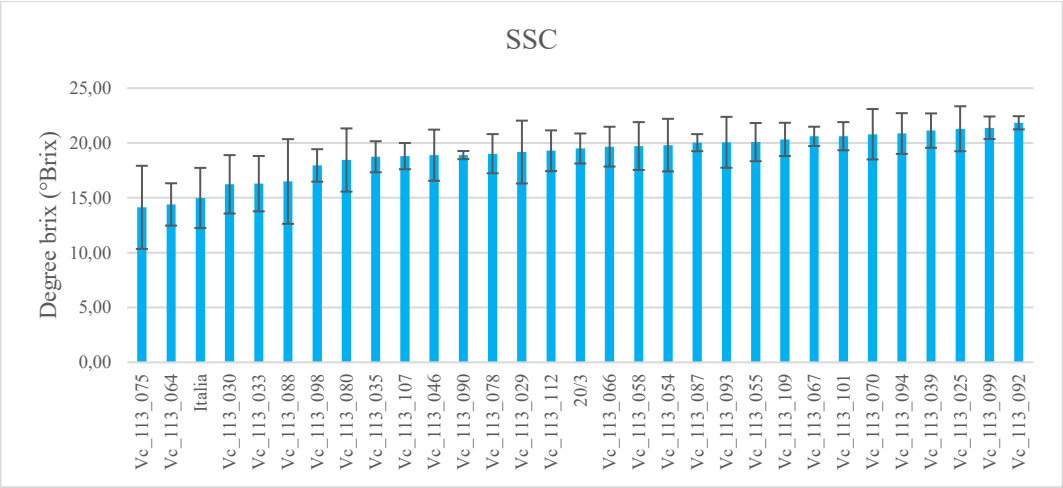


**Figure 18.** Ordered distribution of the mean soluble solid content (SSC) for Vc113 individuals and the parents '20/3' and 'Italia'. The error bars represent the standard deviation calculated within the individual.

*4.2.1.5 Firmness*

Values of firmness of the grapes range from (12.10 ± 2.52) kg / $cm^2$ of 113_092 to (28.92 ± 5.85) kg / $cm^2$ of 113_075, and the mean value of the population is (19.68 ± 3.88) kg / $cm^2$. '20/3' has the second lowest value, while the average firmness of 'Italia' is (22.27 ± 6.65) kg / $cm^2$; most of the progeny has values comprised between the parental ones, except for six other individuals that are more firm than 'Italia'.

113_064 is the only individual from which it was not possible to collect any data during both years, maybe because fruits were too mature when they had been harvested, so they collapsed during the analysis.

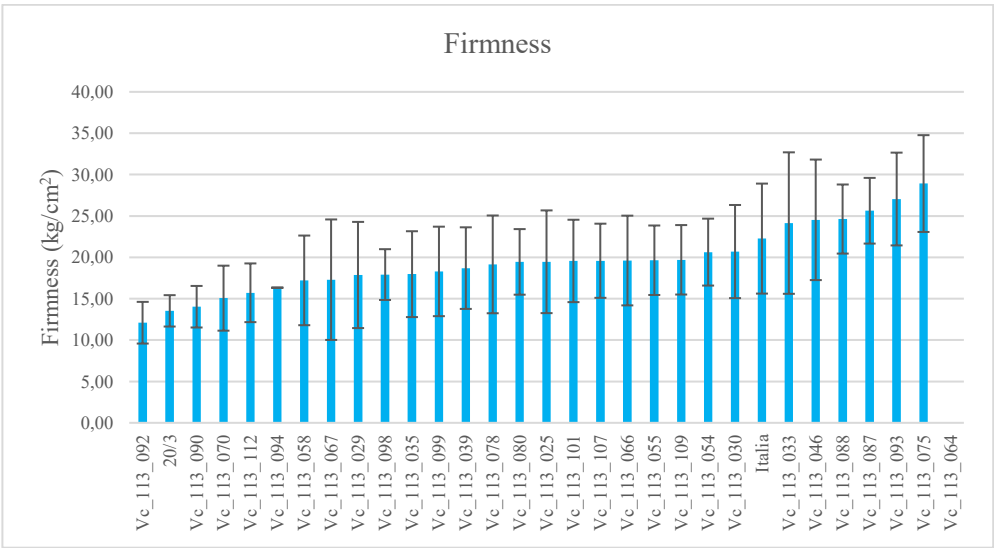


**Figure 19.** Ordered distribution of average firmness for Vc113 individuals and the parents '20/3' and 'Italia'. The error bars represent the standard deviation calculated within the individual.

### 4.2.2 Vc114

This is a cross between '20/3' and 'Matilde'; female parent is the same of the population Vc113 whereas male parent is another well-known commercial table grape variety.

The progeny has 14 individuals; molecular analyses shows that four have inherited only Rpv3, three only Rpv12 and six carry both genes.

#### *4.2.2.1 Bunch weight*

The average bunch weight of Vc114 progeny is (265.93 ± 107.42) kg; the high standard error calculated on the measures is probably due to the wide range in which the values are included: from (89.00 ± 15.77) kg of 114_011 to (588.33 ± 110.13) kg of 'Matilde'. The mean weight of the individuals is well distributed within the interval and the female parent '20/3' stays just under the average value with (218.33 ± 19.26) kg.

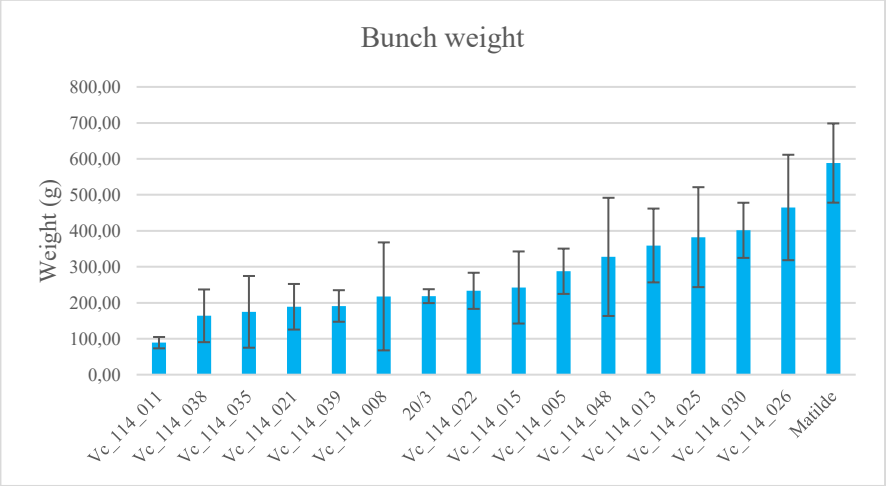


**Figure 20.** Ordered distribution of the mean bunch weight for Vc114 individuals and the parents '20/3' and 'Matilde'. The error bars represent the standard deviation calculated within the individual.

#### *4.2.2.2 Ten berries weight*

The distribution among the progeny of the average weight of ten fruits is quite homogeneous between the (25.31 ± 1.50) kg of 114_021 and (44.91 ± 2.93) kg of 114_022; the mean value is (34.86 ± 6.27) kg. Weights of the parents are the extreme and outer limits of the distribution: the lighter fruits belong to '20/3' with (17.27 ± 0.52) kg, while the heaviest ones belong to 'Matilde' with (73.29 ± 3.77) kg.

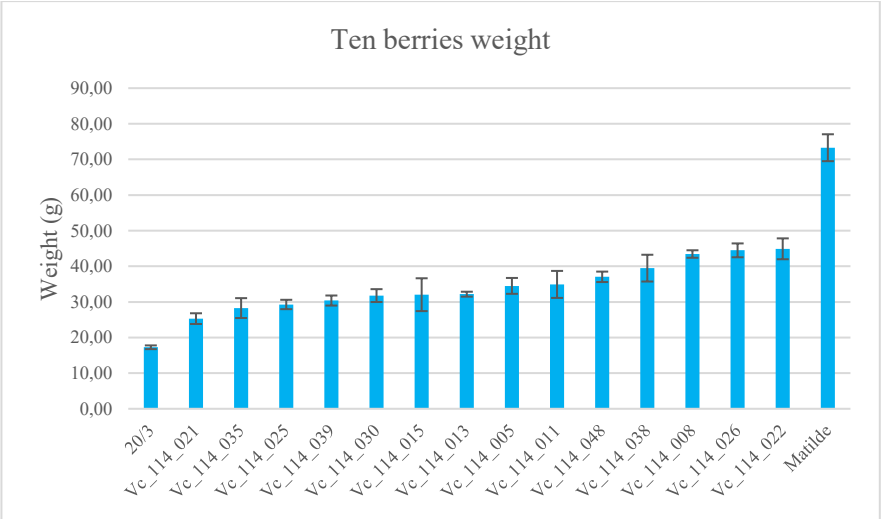


**Figure 21.** Ordered distribution of the mean berries weight for Vc114 individuals and the parents '20/3' and 'Matilde'. The error bars represent the standard deviation calculated within the individual.

*4.2.2.3 Size and shape of fruits*

The ratio between length and mean width of all individuals analyzed exceeds 1, which means that the berries have all round / oval shape. The distribution is so homogeneous: '20/3' has the lower value of 1.08 ± 0.07, while the higher ratio of 1.32 ± 0.07 belongs to 114_030. The male parent 'Matilde' stays just in correspondence with the average value of the progeny, that is equivalent to 1.19 ± 0.06.

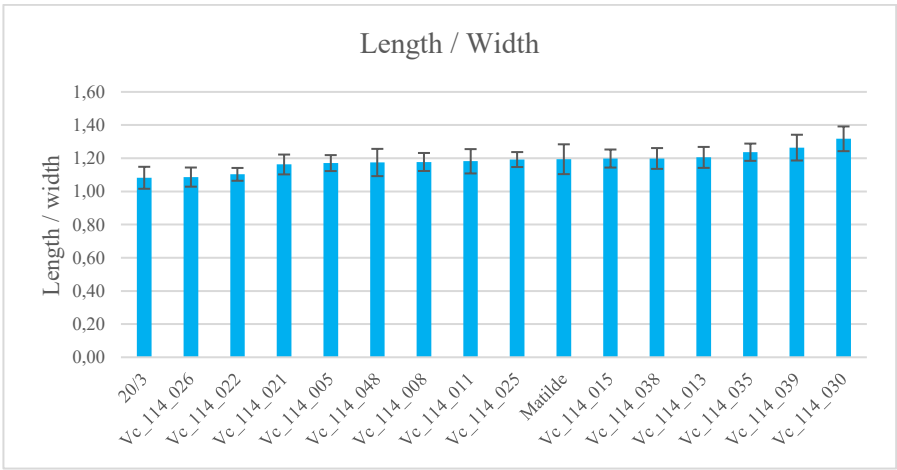


**Figure 22.** Ordered distribution of the average ratio between length and mean width of berries for Vc114 individuals and the parents '20/3' and 'Matilde'. The error bars represent the standard deviation calculated within the individual.

*4.2.2.4 Solid soluble content (SSC)*

SSC mean values are comprised between (15.24 ± 2.50) °Brix of 114_015 and (19.96 ± 1.58) °Brix of 114_035; the average value is (18.23 ± 1.45) °Brix and also for this trait the distribution is quite uniform. 'Matilde' is below this value with (17.11 ± 2.18) °Brix, whereas '20/3' stays above with (19.50 ± 1.37) °Brix.

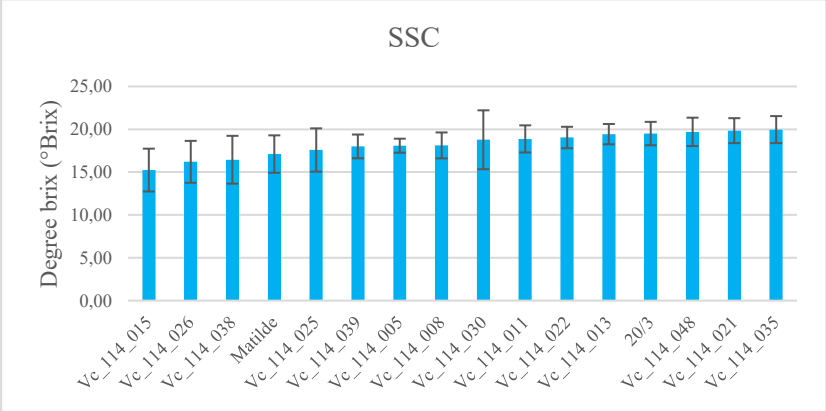


**Figure 23.** Ordered distribution of the mean soluble solid content (SSC) for Vc114 individuals and the parents '20/3' and 'Matilde'. The error bars represent the standard deviation calculated within the individual.

*4.2.2.5 Firmness*

The average value of firmness for the entire population is (20.50 ± 4.12) kg / cm$^2$. The lower value of the distribution belongs to 114_039 with (13.23 ± 4.99) kg / cm$^2$, and the highest one to 114_015 with (29.06 ± 4.53) kg / cm$^2$. '20/3' and 'Matilde' stays below the average firmness with respectively (13.53 ± 1.89) kg / cm$^2$ and (20.40 ± 5.98) kg / cm$^2$. Eight individuals show values higher than the average one, suggesting that most of the progeny produces berries that are firmer compared to those produced from the parents.

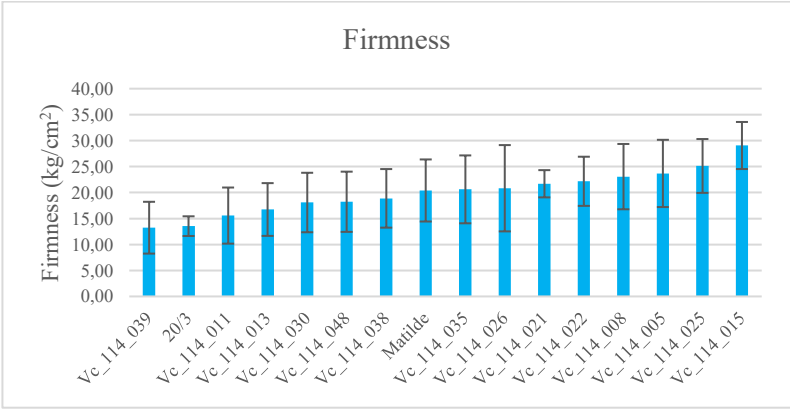


**Figure 24.** Ordered distribution of the mean firmnes for Vc114 individuals and the parents '20/3' and 'Matilde'. The error bars represent the standard deviation calculated within the individual.

### 4.2.3 Vc315

This population is composed by 11 individuals and was obtained by crossing ‘Lasta’ and ‘Kishmish vatkana’. This cross could be an example of the utilization of ‘Lasta’ as source of resistance for breeding purpose. ‘Kishmish vatkana’ is a black table grape variety that originated in Uzbekistan and in which Ren1 locus responsible for powdery mildew resistance was found.

The progeny was analyzed using UDV305 and UDV737 markers for Rpv3 locus, so the haplotype that results in the progeny is the same of ‘Lasta’ and nine individuals have inherited the resistance–linked allele 279 of UDV737. By contrast, Ren1 locus of ‘Kishmish vatkana’ was found in four individuals.

#### *4.2.3.1 Bunch weight*

The average weight of the clusters within the population is (361.33 ± 162.74) g and the parental lines ‘Lasta’ and ‘Kishmish vatkana’ have higher values, respectively of (378.00 ± 90.73) g and (437.67 ± 218.70) g. The extreme values of the distribution belong to 315_005 with (159.67 ± 132.76) g and to 315_008 with (727.50 ± 280.69) g. It can be noticed that the standard deviation of the measures of 315_019 is the most elevated because the productions of the two years differ a lot and bunch weigh was intensively influenced.

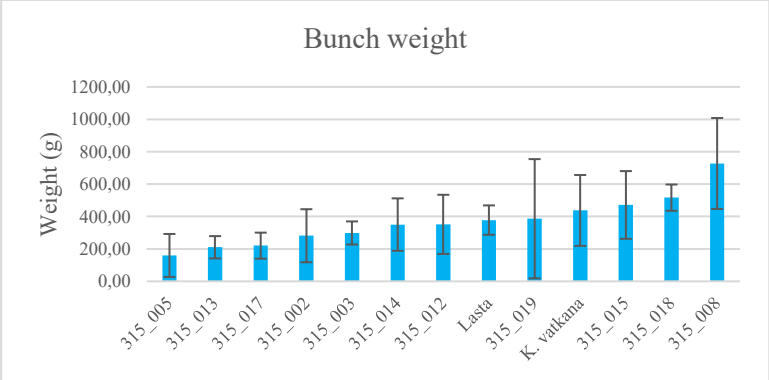


**Figure 25.** Ordered distribution of the mean bunch weight for Vc315 individuals and the parents ‘Lasta’ and ‘Kishmish’. The error bars represent the standard deviation calculated within the individual.

#### *4.2.3.2 Ten berries weight*

The distribution of ten berries mean weight ranges between (24.78 ± 0.61) g of 315_012 and (65.52 ± 3.29) g of 315_008. As for the cluster weight, most of the individuals of the progeny have grapes that weight less than those of the parents, whose values are (55.50 ± 2.53) g for ‘Lasta’ and

(65.46 ± 2.66) g for ‘Kishmish vatkana’. The average value for the progeny is equal to (49.54 ± 14.63) g.

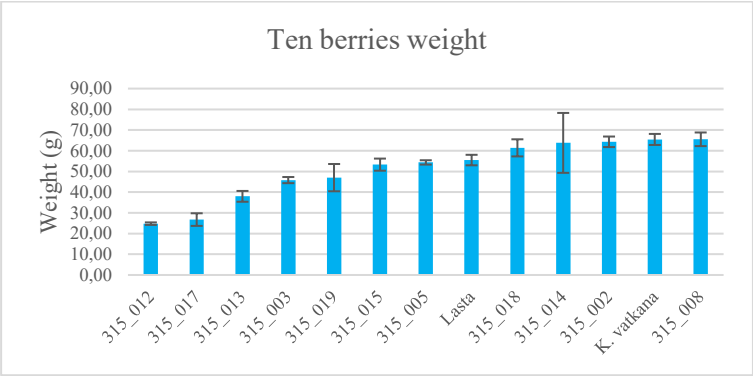


**Figure 26.** Ordered distribution of the mean berries weight for Vc315 individuals and the parents ‘Lasta’ and ‘Kishmish’. The error bars represent the standard deviation calculated within the individual.

*4.2.3.3 Size and shape of fruits*

Vc315 family and its parental lines has length / width ratio values that are higher than 1, indicating that the shape of the berries is quite round and elongated. The most rounded fruits belong to 315_019 with a ratio of 1.11 ± 0.04, while the most elongated are those of 315_014 with 1.49 ± 0.18. The values of ‘Lasta’ (1.28 ± 0.05) and ‘K. vatkana’ (1.29 ± 0.04) are very similar and quite all the individuals have lower ratio than parents ones. The mean value of the progeny is 1.23 ± 0.11.

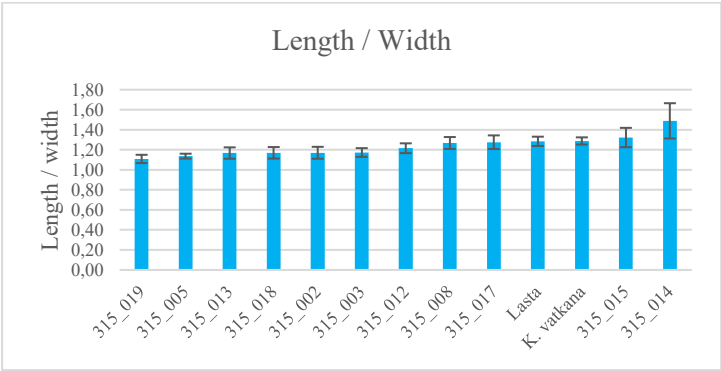


**Figure 27.** Ordered distribution of the average ratio between length and mean width of berries for Vc315 individuals and the parents ‘Lasta’ and ‘Kishmish’. The error bars represent the standard deviation calculated within the individual.

*4.2.3.4 Solid soluble content (SSC)*

Values of SSC of the two parents are almost the same with (17.21 ± 1.58) °Brix for ‘K. vatkana’ and (17.52 ± 1.59) °Brix for ‘Lasta’. They stay just above the average value of the progeny, that is equal to (16.98 ± 2.28) °Brix. The individuals of the population have mean values that are well distributed and ranges from (13.27 ± 2.00) °Brix of 315_012 to (21.47 ± 0.70) °Brix of 315_005. As

it can be seen from the Figure 28, 315_014 has a higher standard error compared to the other individuals.

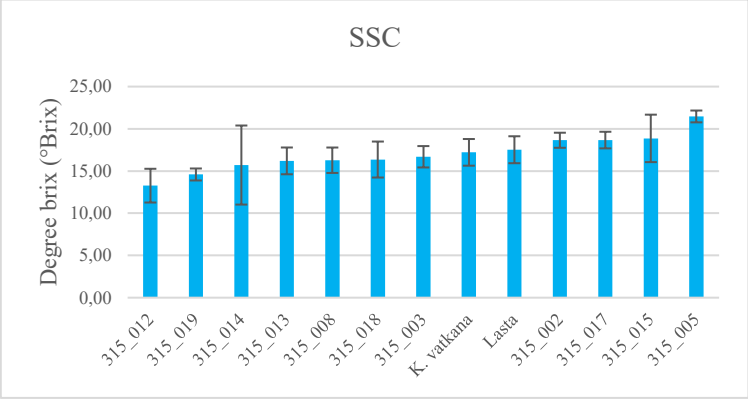


**Figure 28.** Ordered distribution of the mean soluble solid content (SSC) for Vc315 individuals and the parents 'Lasta' and 'Kishmish'. The error bars represent the standard deviation calculated within the individual.

*4.2.3.5 Firmness*

The value of 'Kishmish vatkana' is lower than the average firmness of the progeny of (16.63 ± 3.64) kg / cm$^2$, whereas 'Lasta' has the firmest berries with (21.67 ± 2.93) kg / cm$^2$. Most of the individuals of the population are comprised between the values of the parental lines; only four individuals have lower values compared to the male parent and 315_002 is the lower limit of this distribution with (13.68 ± 3.40) kg / cm$^2$.

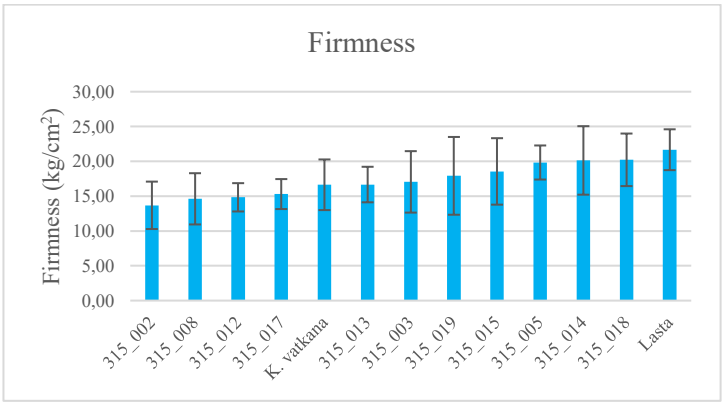


**Figure 29.** Ordered distribution of the mean firmness for Vc315 individuals and the parents 'Lasta' and 'Kishmish'. The error bars represent the standard deviation calculated within the individual.

### 4.2.4 Correlation between pomological traits

To evaluate if there is positive or negative correlation between the traits that were considered for the analyses we utilize Pearson's correlation coefficient (*p*). The estimation was performed

considering data of each progeny separately, as shown in Figure 30; furthermore, we used values of length and width of berries instead of their ratio.

| Vc113 | Bunch weight | Ten berries weight | Berry length | Mean berry width | SSC | Firmness |
|---|---|---|---|---|---|---|
| Bunch weight | 1 | 0.480 | 0.371 | 0.504 | 0.197 | -0.159 |
| Ten berries weight | | 1 | 0.775 | 0.948 | 0.305 | -0,141 |
| Berry length | | | 1 | 0.718 | 0.476 | -0.124 |
| Mean berry width | | | | 1 | 0.316 | -0.187 |
| SSC | | | | | 1 | -0.557 |
| Firmness | | | | | | 1 |

| Vc114 | Bunch weight | Ten berries weight | Berry length | Mean berry width | SSC | Firmness |
|---|---|---|---|---|---|---|
| Bunch weight | 1 | 0.142 | -0.040 | 0.042 | -0.175 | 0.166 |
| Ten berries weight | | 1 | 0.775 | 0.981 | -0.306 | 0.048 |
| Berry length | | | 1 | 0.738 | -0.312 | -0.138 |
| Mean berry width | | | | 1 | -0.341 | 0.105 |
| SSC | | | | | 1 | -0.443 |
| Firmness | | | | | | 1 |

| Vc315 | Bunch weight | Ten berries weight | Berry length | Mean berry width | SSC | Firmness |
|---|---|---|---|---|---|---|
| Bunch weight | 1 | 0.469 | 0.508 | 0.344 | -0.349 | -0.055 |
| Ten berries weight | | 1 | 0.955 | 0.853 | 0.259 | 0.316 |
| Berry length | | | 1 | 0.751 | 0.303 | 0.272 |
| Mean berry width | | | | 1 | 0.371 | 0.168 |
| SSC | | | | | 1 | 0.169 |
| Firmness | | | | | | 1 |

**Figure 30.** Pearson's correlation coefficient (*p*) for the six traits considered for the analyses, separated on the basis of the progeny: Vc113 (above), Vc114 (centre) and Vc315 (below).

Traits that were positively correlated to a larger extend were ten berries weight and berry's dimension (length and width) (Viana et al., 2013) in all three analyses, with *p* between 0.775 and 0.981. Bunch weight do not always show a significant correlation with traits related to weight and dimension of berries, and an explanation could be the presence of different number of fruits for each cluster. In fact, Fanizza et al. in 2005 observed that cluster weight was positively correlated with the

number of berries per cluster, suggesting that the number of berries is the most important characteristic that determines cluster weight and yield (Viana et al., 2013). Solid sugar content (SSC) and berry weight should be negatively correlated (Coelho et al., 2011), but only for Vc114 analysis this assumption is valid. SSC content and firmness are characterized by a negative correlation in most cases.

### 4.2.5 Comparison between Vc113 and Vc114

Vc113 and Vc114 progenies have female parent in common ('20/3'), so it could be interesting to compare the average values of the data collected to start investigating if the other parent used in the cross have positive effects on pomological characteristics of the individuals of the population. We can not utilize the parents–offspring regression to study the heritability of the trait within the generation because there are only two population that had been analyzed. So a *t*–test was used to investigate if the differences between the mean values of each progeny for each traits are statistically relevant.

#### *4.2.5.1 Bunch weight*

Average bunch weight of the parents and the two progenies were compared and it can be noticed that the mean value of male parent 'Italia' is the highest, followed by 'Matilde'. Mean value of Vc114 population is comprised between the parental ones, while average weight of Vc113 is the lowest one.

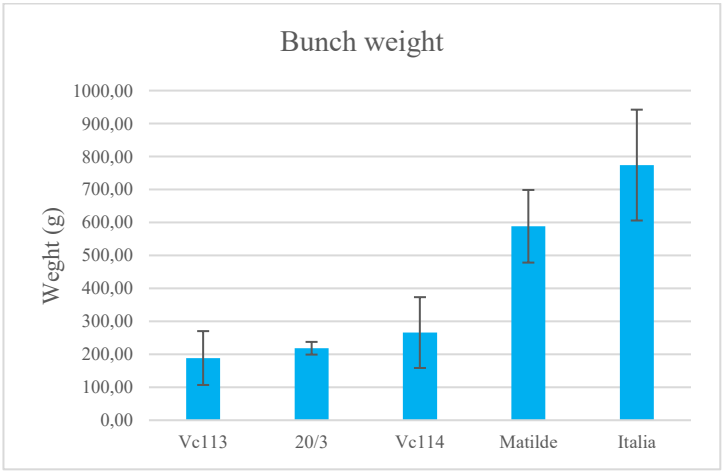


**Figure 31.** Distribution of mean values of bunch weight related to the three parents '20/3', 'Italia' and 'Matilde', and Vc113 and Vc114 progenies. The error bars represent the standard deviation calculated within the individual.

*4.2.5.2 Ten berries weight*

The mean weights of ten berries are (32.24 ± 7,6) g for Vc113 and (34.86 ± 6,23) g for Vc114, and both are comprised between the values of the respective parents. '20/3' has the lowest weights, whereas 'Italia' the highest.

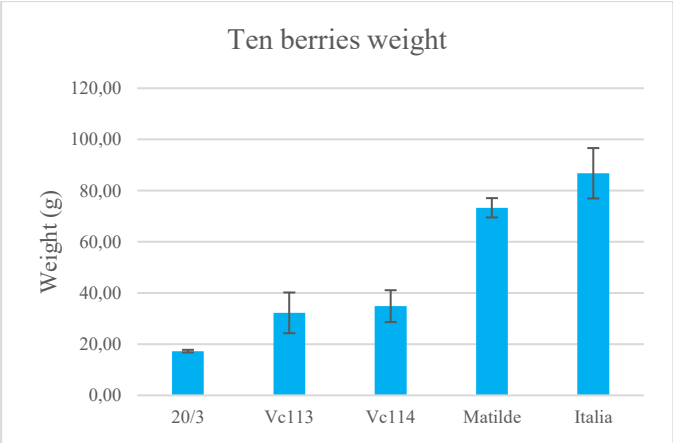


**Figure 32.** Distribution of mean values of berries weight related to the three parents '20/3', 'Italia' and 'Matilde', and Vc113 and Vc114 progenies. The error bars represent the standard deviation calculated within the individual.

*4.2.5.3 Size and shape of fruits*

This trait do not show any significative differences between the mean values of the parents and the progenies. 'Matilde', 'Italia' and Vc114 has the same value for the ratio, equal to 1.19; Vc113 average ratio is 1.16 and '20/3' has the lowest value of 1.08.

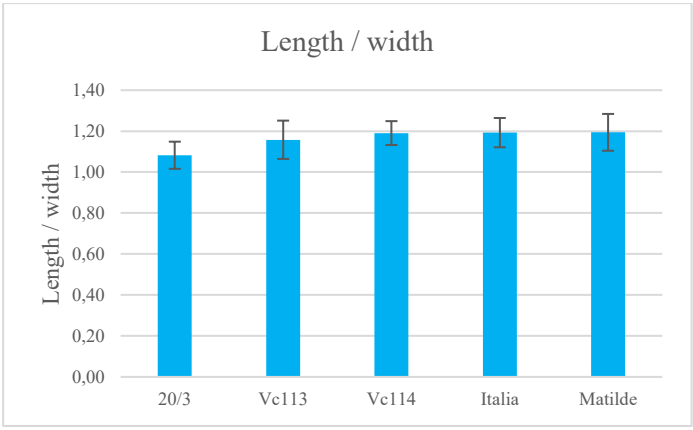


**Figure 33.** Distribution of mean values of the ratio between length and mean width of berries related to the three parents '20/3', 'Italia' and 'Matilde', and Vc113 and Vc114 progenies. The error bars represent the standard deviation calculated within the individual.

*4.2.5.4 Soluble solid content (SSC)*

Also for soluble solid content the mean values of the progenies are comprised between those of the parents. In this case, '20/3' has the highest value, whereas 'Italia' the lowest; the variation of these average sugar content ranges from 14.99 °Brix to 19.50 °Brix.

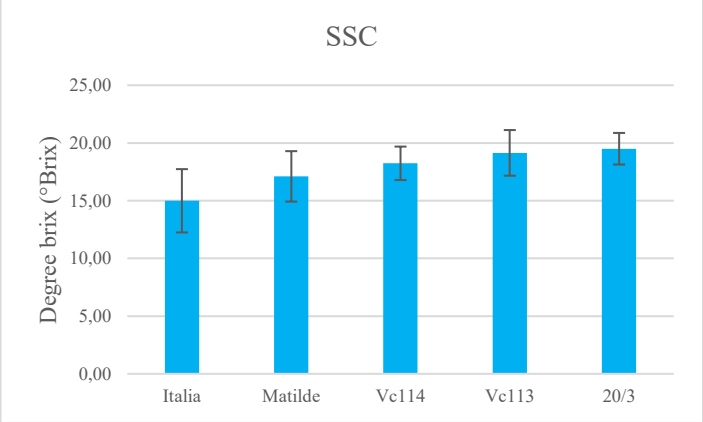


**Figure 34.** Distribution of mean values of soluble solid content (SSC) related to the three parents '20/3', 'Italia' and 'Matilde', and Vc113 and Vc114 progenies. The error bars represent the standard deviation calculated within the individual.

*4.2.5.5 Firmness*

The distribution of mean values for this trait shows that average firmness of Vc113 population is comprised between parental ones, whereas Vc114 mean values are higher compared to 'Matilde', that stays just below, and '20/3'. We can suppose that 'Matilde' have a stronger effect on the progeny for this characteristic.

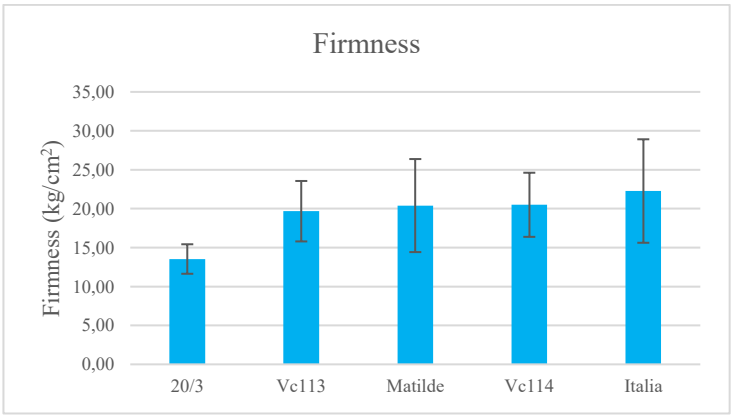


**Figure 35.** Distribution of mean values of firmness related to the three parents '20/3', 'Italia' and 'Matilde', and Vc113 and Vc114 progenies. The error bars represent the standard deviation calculated within the individual.

*4.2.5.6 Student's t-test*

Using Microsoft Office Excel software, Student's *t*–test was applied to verify if the differences between the mean values of the two progenies for each trait are statistically significative, which means if they are affected from the male parental used in the cross, or not. The values of *t* that have been calculated are shown in Table 8.

| Trait | *t* value |
|---|---|
| Bunch weight | 0.019 |
| Berries weight | 0.199 |
| Length / width | 0.086 |
| SSC | 0.059 |
| Firmness | 0.540 |

**Table 8.** *t* values related to each agronomical trait, that was obtained from the mean values of the individuals of Vc113 and Vc114 populations.

Considering 41 as the number of degrees of freedom, because the two sets consists of 29 and 14 individuals, we compared the *t* values obtained with the critical value related to these degrees of freedom and they all resulted below this threshold, suggesting that the differences are not significative and cannot be ascribed to the different parent. This is not unusual in the analysis of pomological traits, and in this case it is important to precise that the mean values can be affected also by the conditions of cultivation which cannot be controlled and guarantee to be the same for the two years, for example due to the climate conditions of the different growing seasons.

### 4.2.6 Selection of individuals

Among all the individuals of the three populations, 18 individuals were firstly selected because they have inherited both resistant loci from their parents: ten individuals from Vc113 and five of Vc114 that carry Rpv3 and Rpv12 loci; three individuals from Vc315 that show Ren1 and the resistant allele associated to UDV737 marker of Rpv3 locus. Then, we evaluated each pomological characteristic of these selected individuals, as shown in Table 9.

| | Bunch weight (g) | Ten berries weight (g) | Length/width ratio | SSC (° Brix) | Firmness (kg/cm$^2$) |
|---|---|---|---|---|---|
| 113_033 | 185.33 | 26.58 | 1.08 | 16.29 | 24.14 |
| 113_035 | 225.60 | 32.34 | 1.13 | 18.75 | 17.97 |
| 113_054 | 68.00 | 25.85 | 1.48 | 19.81 | 20.63 |
| 113_055 | 166.83 | 34.86 | 1.21 | 20.08 | 19.65 |
| 113_058 | 331.00 | 45.68 | 1.06 | 19.72 | 17.22 |
| 113_080 | 144.83 | 33.67 | 1.10 | 18.45 | 19.45 |
| 113_093 | 147.00 | 24.09 | 1.23 | 20.07 | 27.05 |
| 113_099 | 243.50 | 31.27 | 1.22 | 21.39 | 18.30 |
| 113_101 | 117.83 | 33.46 | 1.15 | 20.62 | 19.57 |
| 113_109 | 325.67 | 37.18 | 1.08 | 20.33 | 19.70 |
| 114_005 | 287.50 | 34.50 | 1.17 | 18.08 | 23.68 |
| 114_008 | 217.67 | 43.45 | 1.18 | 18.12 | 23.07 |
| 114_022 | 233.17 | 44.91 | 1.10 | 19.04 | 22.17 |
| 114_030 | 401.33 | 31.78 | 1.32 | 18.77 | 18.08 |
| 114_035 | 174.67 | 28.27 | 1.24 | 19.96 | 20.62 |
| 315_002 | 281.50 | 64.32 | 1.22 | 18.64 | 13.68 |
| 315_018 | 516.67 | 61.39 | 1.17 | 16.36 | 20.22 |
| 315_019 | 386.50 | 47.00 | 1.11 | 14.59 | 17.92 |

**Table 9.** Individuals of Vc113, Vc114 and Vc315 progenies that were selected because they carry two resistant loci from their parents. For each, the values related to bunch weight, ten berries weight, length / width ratio, SSC and firmness were reported.

Jayasena and Cameron in 2008 affirmed that consumer acceptance increased with the increase in °Brix from 16 to 20, whereas berries with °Brix values higher than 20 could not get a better consumer acceptance than those with 20 °Brix. For this reason, only Vc315_019 should be discarded because its SSC value is lower than 16; all other individuals have berries with °Brix acceptable for the consumers. Measures of firmness can not be a parameter for the selection, because use of penetrometer was an adaptation that we made and data obtained are not accurately comparable with most of the analyses that are made through other techniques (Sato et al., 1997; Rolle et al., 2011; Rolle et al., 2012). A more efficient selection can be made on the basis of the ten berries weight, according to which we can select only individuals with values higher than 40 g (about 4g for one berry) because the consumers usually prefer table grapes with bigger berries. Finally, the individuals that could be used in breeding programs or have a potential value on table grape market are: Vc113_058, Vc114_008, Vc114_022, Vc315_002 and Vc315_018.

# 5. Conclusions

The aim of the first part of the thesis was to study the resistance to downy mildew in table grape cultivar ‘Lasta’, that resulted to be still unclear on the basis of genotypical analyses previously done. The screening performed on the cultivar and its parents ‘Muscat de St. Vallier’ and ‘Lyana’ using known microsatellite markers suggested that the resistance locus resides on chromosome 18 because the resistant–related allele from UDV737 was inherited, but not the one from UDV305; both markers characterized the Rpv3 locus and are located downstream and upstream of the putative gene. From this evidence, it was supposed that a recombination event had occurred upstream of the locus and it did not affect the functionality of the gene.

The population obtained from ‘Picolit’ and ‘Lasta’ was utilized to produce a genetic linkage map, which is almost totally constituted from SNPs detected by ddRADseq technique. We tried to integrate in the map also SSRs markers in order to be sure of the correct calling of the chromosome, but almost all were excluded from the software because of the distortion of their segregation. Probably, this is due to large number of individuals with missing data that were not well genotyped. The genetic map of ‘Lasta’ shows that the linkage group with the highest number of included markers (54) is LG18.

Phenotypic data collected in two years on the resistance level of each sample utilized for map construction allowed the identification of a possible QTL responsible for the resistance on LG18, in correspondence of the region between the markers chr18_23431076 and chr11_7803589. According to previous studies, TIR–NBS–LRR genes and a LRR–kinase should be present in this region. This result could confirm what it was observed after genotypic analyses with microsatellites. LOD score value of the QTL is just above the calculated LOD threshold (1.9). These low values made the clear identification of possible minor QTLs influencing the resistance so difficult. This is probably due to quality of the phenotypic data, and to the fact that in the second year the high death rate have reduced the number of individuals of mapping population from 93 to 48.

The near future perspectives could interest the improvement of the QTL analysis, taking even more attention during phenotyping, in particular in the evaluation of the symptoms on leaf discs, in order to have data of better quality. Then, resequencing of the region where the QTL is located could be a good strategy to verify if the recombination had really occurred, and also where is exactly located.

The second part of the work deal with the pomological evaluation of three cross progenies that could be interesting for breeding purpose because they carry resistance to powdery and / or

downy mildew. In general, the analyses revealed that for some traits, like bunch and berries weight and firmness, the variation within the individuals of the same population is high, while for the ratio between length and width of berries the values do not vary a lot within the progeny. We also selected some individuals that could have some potentialities on table grape market for the combination of resistant genotypic profile and good pomological characteristics.

The comparison between Vc113 and Vc114 populations, that have the female parent in common, the resistant genotype '20/3', allows to understand that the effect of the male parent on the characteristics of the progeny is not significative, according to Student's *t* test. This could be an initial step of evaluations of these progenies, trying to better understand with more data of each trait the characteristics of the production.

Another approach could be to extend the evaluation to other progenies that have the same female parent '20/3' in order to consider also the heritability of each trait using parents–offspring regression, which was not possible with only two sets of data.

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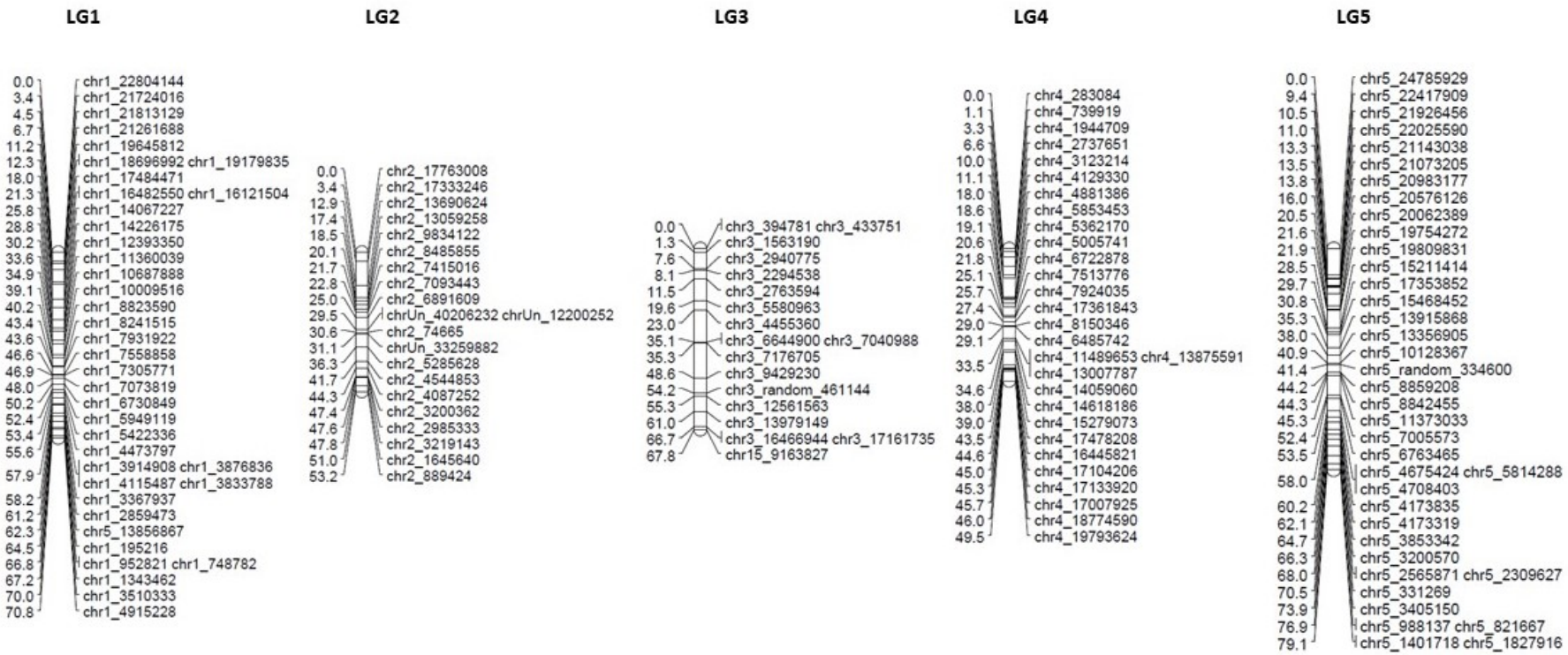
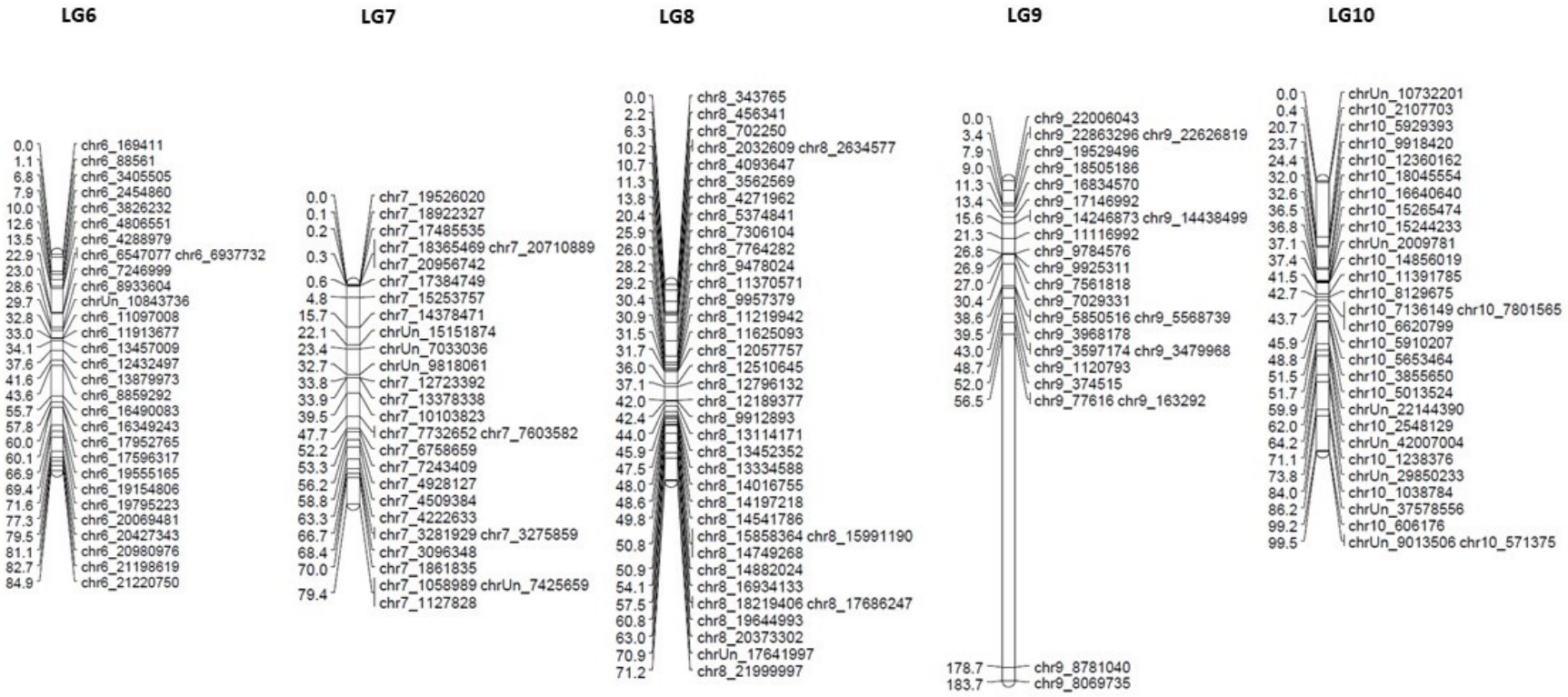
# 7. Supplementary material

## 7.1 Genetic linkage map of ‘Lasta’

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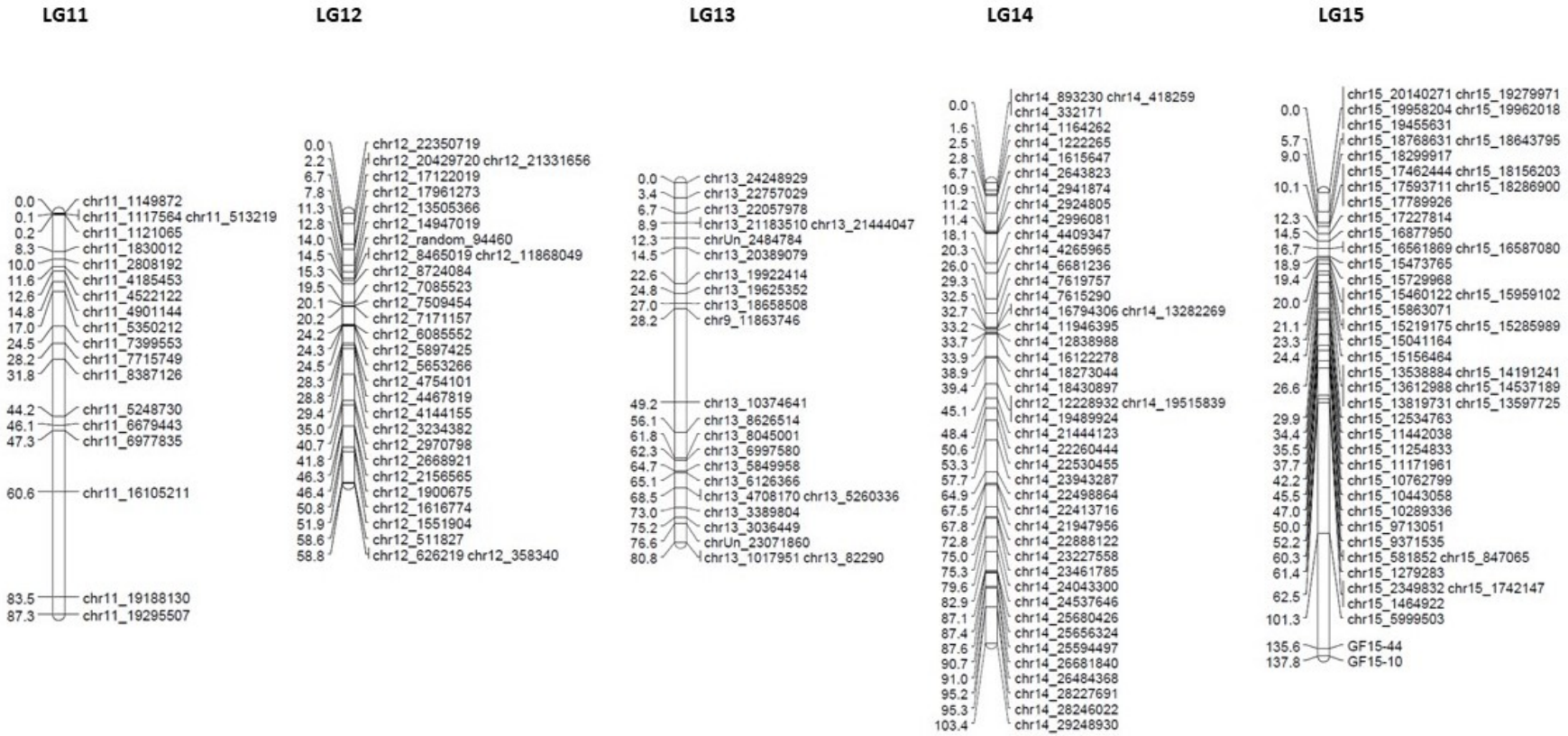
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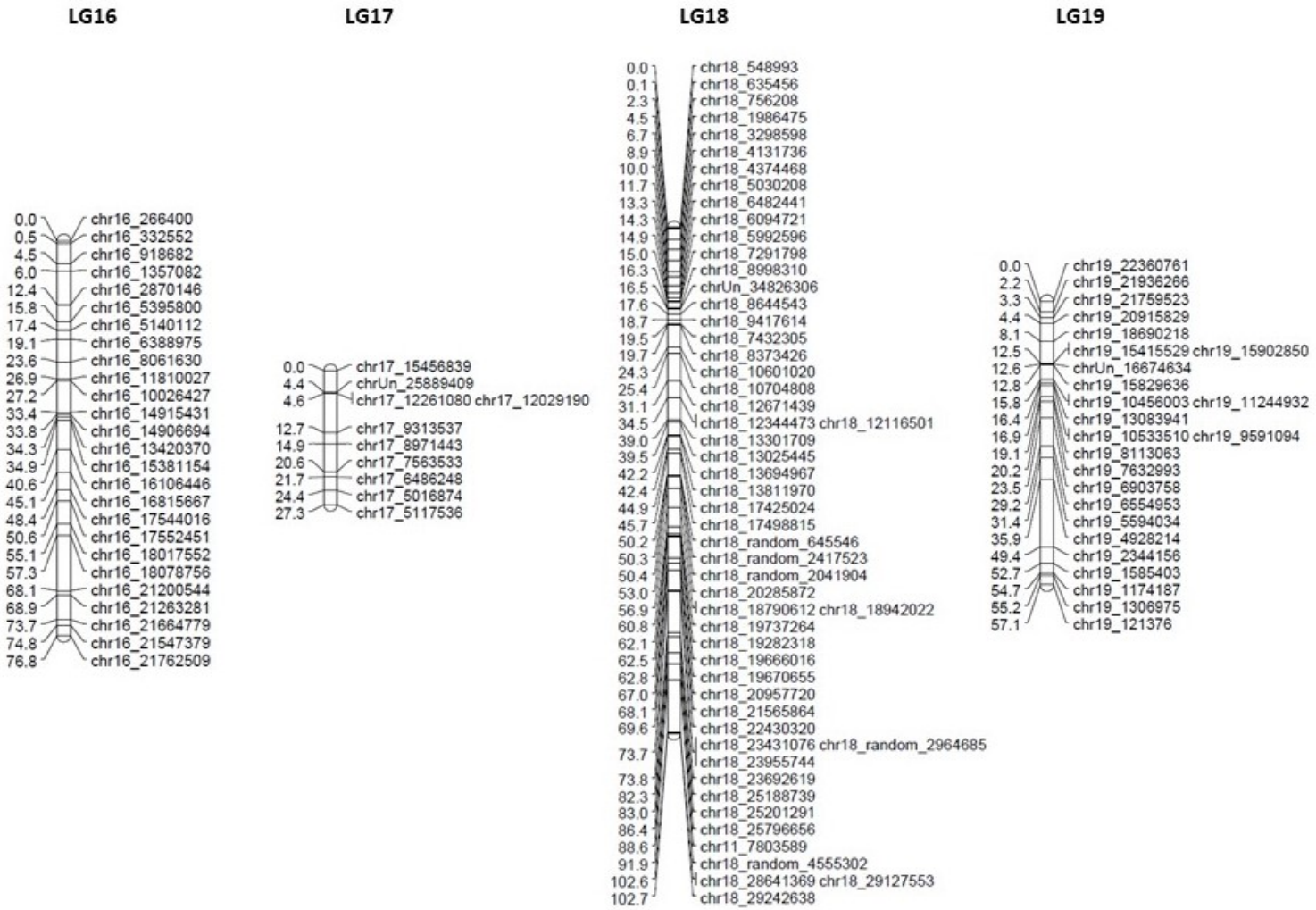
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