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**Project Title:** Genetic mapping of *Xylella fastidiosa* resistance gene(s) in grape germplasm from the southern United States.

**Principal Investigator:**

Andrew Walker (PI)  
Department of Viticulture and Enology,  
University of California  
Davis, CA 95616  
awalker@ucdavis.edu

**Cooperating Staff:**

Summaira Riaz  
Department of Viticulture and Enology,  
University of California  
Davis, CA 95616  
snriaz@ucdavis.edu

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**ABSTRACT**

This project provides molecular support to the PD resistant grape breeding program by characterizing new forms of PD resistance, tagging genomic regions and identifying tightly linked markers that can be used for marker-assisted selection (MAS). We completed screening and genotyping of over 200 accessions that were acquired from States along the Gulf of Mexico, by utilizing 22 SSR and 14 chloroplast markers to develop fingerprint profiles for them. Population structure analysis allowed us to identify different groups that are distinct by DNA profiles, phenotypically and that were collected from different regions. The *V. arizonica* like group was composed of several species with distinct maternal and paternal inheritance. The species within this group are also very distinct from southeastern PD resistant species, once thought to be the only source of PD resistance. Greenhouse screening was completed on a subset of genotypes, and crosses with 8 new resistant lines were made in 2012 and 2013 and 2014. This germplasm screening provides opportunities to explore and identify resistance loci that may provide different resistance mechanisms allowing us to expand the genetic base of the PD resistance-breeding program. Progress was made with b43-17 and b40-14 both of which carry a major locus on chromosome 14, as well as minor QTLs on different chromosomes. For *V. arizonica/candicans* b43-17, a minor QTL has identified on chromosome 19 (*PdR2*) and for *V. arizonica* b40-14, a minor QTL was identified on chromosome 5. Mapping of a multigenic source of PD resistance from *V. arizonica/girdiana* b42-26 continues. This project provides the genetic markers critical to the successful classical breeding of PD resistant wine, table and raisin grapes. Identification of markers for *PdR1* allowed us to reduce the seed-to-seed cycle to 2 years and produce selections that are PD resistant and 97% *vinifera*. We also completed the physical map of *PdR1* locus and are currently analyzing the data and comparing it with susceptible sequence of PN40024. We have identified multiple candidate genes and now have sequence available for the native promoter cloning and testing. These efforts will help us better understand how these genes function and could also lead to PD resistance genes from grape that would be available to genetically engineer PD resistance in *V. vinifera* cultivars.

**LAYPERSON SUMMARY**

A major focus of this project is to broaden the genetic base of PD resistance by searching for and characterizing new forms of PD resistance. We have made rapid progress breeding PD resistant winegrapes that are now approaching release. This progress could not have been made without the development and use of DNA markers for PD resistance and the discovery of strong single gene resistance in forms of *Vitis arizonica*. The next phase of the breeding program is now underway – combining multiple PD resistance sources into one background. Although single gene resistance is easy to breed with, it is often overcome by aggressive pathogens and pests. With this in mind, our PD breeding/genetics program is now characterizing resistance from other backgrounds and developing DNA markers so that we can combine these resistances into a single individual. Combining these genes together will require good markers since the resistant progeny resulting from efforts will appear the same – resistant. We will need the markers to the multiple sources to verify different genes have been combined. We have discovered more sources of strong resistance and are now mapping and developing markers to determine if these new genes control different types or forms of resistance.

We plan to combine these multiple resistance sources in our breeding program to ensure broad and

urable PD resistance. This project provides the genetic markers critical to the successful classical breeding of PD resistant wine, table and raisin grapes. Identification of markers for *PdR1* has allowed us to reduce the seed-to-seed cycle to 2 years and produce selections that are PD resistant and 97% *vinifera*. These markers have also led to the identification of 6 genetic sequences that may house the PD resistance gene, and which are being tested to verify their function. These efforts will help us better understand how these genes function and could also lead to PD resistance

## INTRODUCTION

Identification, understanding and manipulation of novel sources of resistance are the foundation of a successful breeding program. This project has multiple dimensions: exploring multiple grape species backgrounds for Pierce's Disease (PD) resistance; developing and screening breeding populations via a greenhouse testing; genetically mapping segregating populations to identify genomic regions that carry disease resistance genes; developing markers to expedite grape breeding by Marker Assisted Selection (MAS); and physically mapping resistance regions to identify and characterize PD resistance genes. This project provides the genetic support to molecular breeding efforts (see companion Pierce's Disease breeding project). To date, we have completed mapping a major PD resistance locus originating from *V. arizonica/candicans* b43-17, which is the foundation of our PD breeding efforts. We completed mapping in two other resistant *V. arizonica* forms: b42-26 *V. arizonica/girdiana* from Loreto, Baja California; and b40-14 *V. arizonica* from Chihuahua, Sonora. These accessions are morphologically and genetically (both paternally and maternally) different than b43-17, and both possess strong resistance to PD and greatly suppress *X. fastidiosa* (Xf) levels in stem tissue after greenhouse screening. We have identified a major locus for PD resistance on chromosome 14 (*PdR1c*) and one minor QTL on chromosome 5 in b40-14 and identified multiple QTLs in the b42-26 resistant line. In response to recommendations from the CDFA-PD board and reviewer recommendations to broaden resistance, we have expanded the search for additional resistance sources by screening germplasm collected from different parts of US and Mexico. We screened over 200 accessions collected from the southeast and southwest US to identify new and unique sources of resistance. Both nuclear and chloroplast markers were used to identify groups and accessions that are unique both maternally and paternally. Breeding populations were developed with eight newly identified PD resistant accessions. The breeding part of the program produces and greenhouse screens the seedling populations. While the tightly linked genetic markers generated in this project are being used to optimize and accelerate the PD breeding program. These markers are essential to the successful introgression of resistance from multiple sources, with the goal of more durably resistant grapevines. Preliminary greenhouse screen results indicated that we have 20 more accessions that possess strong PD resistance and further work is required to fully understand the resistance mechanisms in these lines. The results of molecular marker work points out that the southeastern resistant material is genetically distinct from the species in Mexico and that the Sierra Madre Mountains in Mexico act as a physical barrier delineating and separating the Mexican grape species we tested. The results of this study feeds into project "Molecular-functional approach to facilitate the discovery of novel *Xylella fastidiosa* resistance gene(s) and markers in Native American species" that is continuation of this work and funded by the CDFA PD/GWSS from 2014 - 2017.

## OBJECTIVES

1. Fine scale mapping of additional QTL for PD resistance in the 04191 ((F2-7 x F8909-17) population (*Completed*)
2. Greenhouse screen and genetically map PD resistance from other forms of *V. arizonica*: b42-26 (*V. arizonica/girdiana*) and b40-14 (*V. arizonica*).
3. Evaluate *V.* germplasm collected from across the southwestern US to identify accessions with unique forms of PD resistance for grape breeding. Determine the inheritance of PD resistance from *Muscadinia rotundifolia*, develop new and exploit existing breeding populations to genetically map this resistance.
4. Complete the physical mapping of *PdR1a* and *PdR1b* and initiate the sequencing of BAC clones that carry *PdR1a* gene candidates.

## RESULTS

**Objective 1. Fine scale mapping of additional QTL for PD resistance in the 04191 ((F2-7 x F8909-17) population.**

A framework genetic map of the 04191 population (*V. vinifera* F2-7 x F8909-17) was developed to identify minor quantitative trait loci (QTL), other than the *PdR1a* locus, that are contributing to PD resistance. A total of 139 SSR markers representing all 19 chromosomes were added to a set of 150 progeny. QTL analysis confirmed a major locus *PdR1a* on chromosome 14, and identified a minor QTL (*PdR2*) on chromosome 19 (Fig.1). *PdR2*'s LOD score was 2.3 and it explains 7% of the phenotypic variation and peaks at marker CB918037. This QTL is within a 10 cM interval – a distance that makes MAS for this locus ineffective for MAS. To shorten the genetic distance between the markers, 7 additional SSR primers were developed in this region. Three of the seven tested primers resulted in clean amplifications and were polymorphic for F8909-17, the PD resistant parent. These markers were added to the population of 150 seedlings. Identification of this minor QTL is important to further our understanding of how PD resistance works and it will help us understand genetic interactions among the major and minor resistance genes. The 04191 population segregates for both major and minor QTL. In order to study the impact of only the minor QTL, we made two crosses with 04373-02 and 04373-22 and Pinot blanc. The goal is to discard all those plants that carry the *PdR1a* locus, greenhouse screen all other plants to test their level of resistance to PD, and use these populations to study and verify the *PdR2* region without interactions with the *PdR1* locus. A total of 100 plants were screened with SSR markers and 43 plants were planted in the field in Spring 2012. These plants were greenhouse screened and ELISA results are scheduled for Spring 2014. Cane lignification index and leaf scorch symptoms indicated 11 of the seedlings were as resistant as U0505-01 (moderate to strong resistance) and 17 seedlings were susceptible. ELISA results are in process.

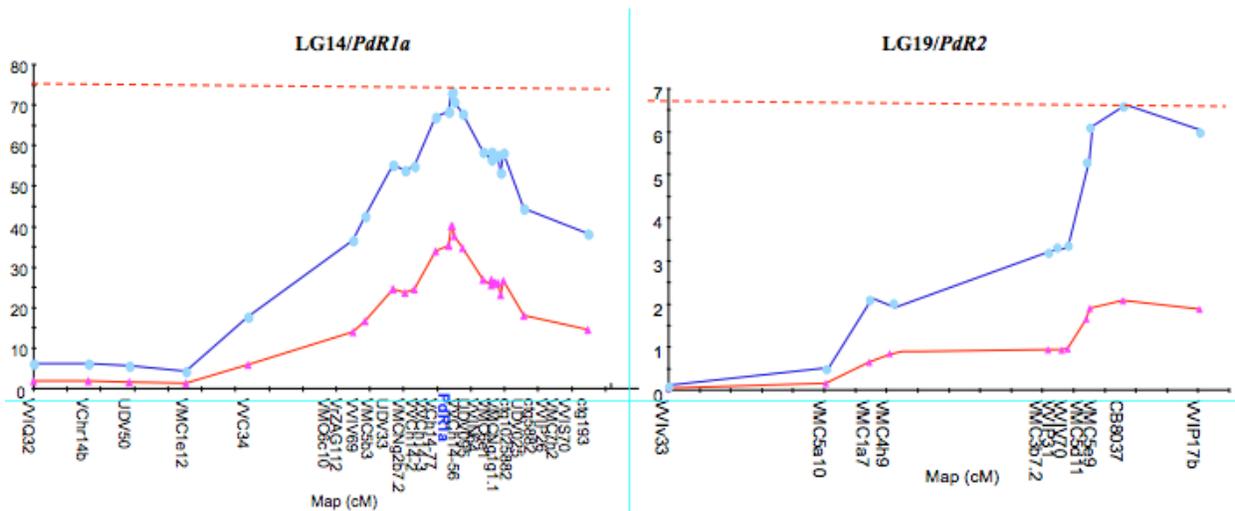


Fig. 1. Interval mapping analysis of the 04191 population. We verified the *PdR1a* locus on LG 14 and identified a minor QTL (*PdR2*) on LG 19. Blue line display the phenotypic variation explained by the loci.

It was clear that both loci work independently of each other and do not have an additive impact. The mean ELISA values of resistant and susceptible plants with the *PdR1a* locus were very different, however, the mean values of resistant and susceptible for *PdR2* locus were higher for the resistant plants.

**Objective 2. Greenhouse screen and genetically map PD resistance from two other forms of *V. arizonica*: b42-26 (*V. arizonica* /*girdiana*) and b40-14 (*V. arizonica*).**

The accession b40-14, a pure form of *V. arizonica*, is homozygous resistant to PD, and all seedlings from the tested F1 progeny were resistant to PD. Two resistant siblings of this population were used to develop the 07388 (R8918-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*) populations. In the previous report, we described the preliminary results with 07744 and genetic mapping with 152 markers. From March to July, we tested a total of 606 SSR markers and 224 polymorphic markers were added on the entire set of 122 plants (Table 1). A total of 216 markers were polymorphic for the female resistant parent – R8918-05.

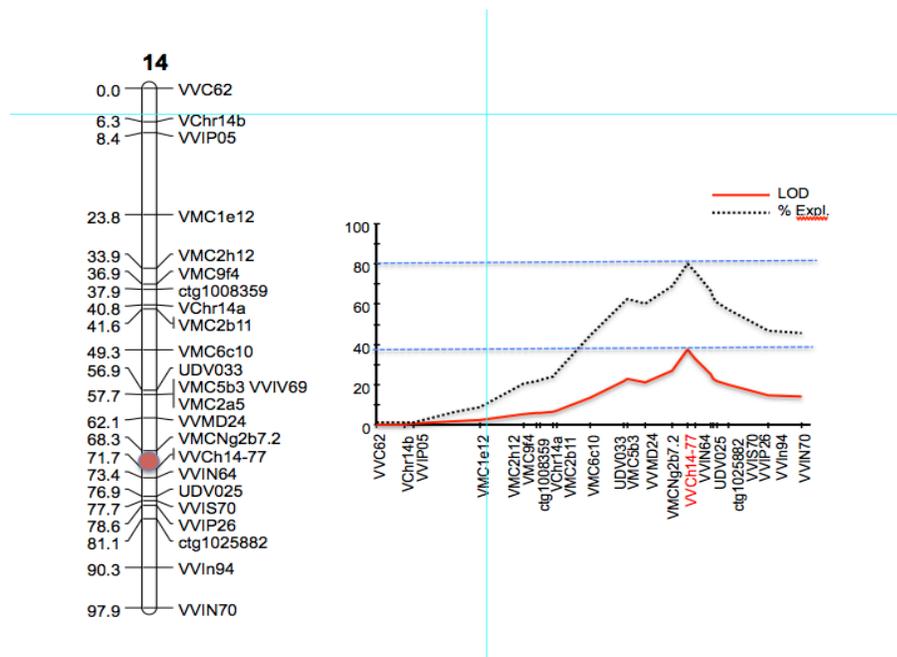
**Table 1.** List of markers tested and completed for the 07744 population derived from the b40-14 background

Marker series	Tested	Amplified	Polymorphic	Completed
VMC, VMCNg	271	161	133	106
VVI	93	84	56	50
UDV	55	54	35	26
VChr	3	3	3	3
VVMS, VVMD, VrZAG	35	34	25	22
Other unpublished	4	4	2	2
EST-SSR (SCU, VVC, CTG)	145	108	68	15
Total	606	448	322	224

**Table 2.** Characteristics of the framework map of R8918-05, a PD resistant selection used as the maternal parent in the 07744 population.

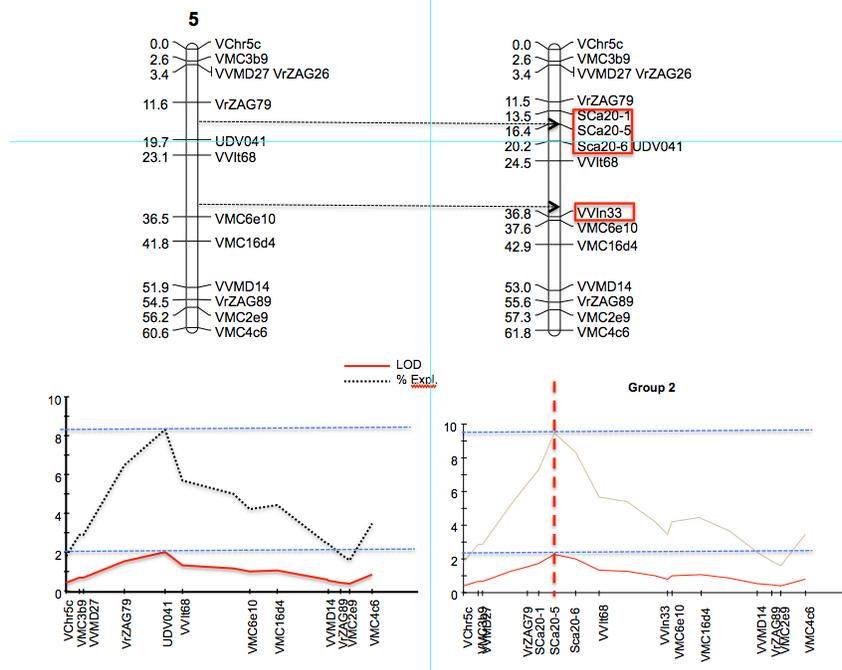
Chromosome	Mapped Markers	Length (cM)
Chr1	15	72.7
Chr2	4	59.6
Chr3	6	37.9
Chr4	11	98.3
Chr5	13	60.6
Chr6	11	40.8
Chr7	12	88.0
Chr8	11	54.7
Chr9	10	87.7
Chr10	10	74.5
Chr11	9	79.7
Chr12	8	52.5
Chr13	11	71.9
Chr14	26	97.9
Chr15	8	35.9
Chr16	9	67.5
Chr17	12	56.2
Chr18	13	136.2
Chr19	13	55.8
Total	212	1328.4
Ave marker distance (cM)	6.3 cM	
Number of gaps > 20 cM	14	

A framework genetic map of R8918-05 was produced with JoinMap (4.0). A total of 212 markers mapped to 19 grape chromosomes with average distance of 6.3 cM between markers. The updated map did not have fragmented groups and provided adequate genome coverage when comparisons were made to the previously published integrated *Vitis* genetic maps. QTL analysis was carried out with MapQTL. A major locus for PD resistance was identified on chromosome 14. PD resistance from b40-14 (which we have named *PdR1c*) maps in the same general region as *PdR1a* and *PdR1b* between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM (Fig. 2). The LOD threshold for the presence of this QTL was 39 and this major locus explained 80% of the phenotypic variation.



**Fig. 2.** Interval mapping of *PdR1* indicating a peak at LOD 34.0 with a 95% confidence interval. The X-axis indicates the position of the markers; LOD values are plotted on the Y-axis.

Using the updated genetic map, we also identified a minor QTL with a LOD score of 2.0 on chromosome 5 that explained 8.3% phenotypic variation for resistance (Fig. 3). We did not find evidence for any other QTL on the remaining 17 chromosomes. Together the two QTLs explained 88% phenotypic variation for resistance within the b40-14 background. We designed more primers to reduce the gap between markers on chromosome 5 by utilizing the Pinot noir genome sequence (Fig. 3). A total of 275 seedlings from five different crosses were also tested with markers that are in linkage with the major locus on chromosome 14; seven of recombinant individuals were saved and planted in the field in Spring of 2013. These recombinants are scheduled for greenhouse testing and the addition of other flanking markers. These recombinants will help reduce the gap between the markers on chromosome 14. The updated data will be used for a manuscript describing the genetic map and QTL identification from the b40-14 background (manuscript in progress).



**Fig. 3.** Interval mapping of QTL on chromosome 5 from the b40-14 background. More markers were developed using the PN40024 sequence. The X-axis indicates the position of the markers; LOD values are plotted on the Y-axis.

The F1 population 05347 (F2-35 x b42-26) has resistance from *V. arizonica/girdiana* b42-26, a third resistant accession that was collected in Loreto, Baja California. A total of 918 SSR primers were tested, 763 amplified b42-26 DNA successfully, and 185 markers were polymorphic. The percentage of polymorphic markers was relatively low. We have not observed levels this low in any other grape genotype, which was likely the result of this plant being from an isolated and now inbred population. A framework genetic map was developed for 199 seedlings with 185 markers. A large number of markers showed segregation distortion. There were only four chromosomes with relatively low marker coverage; all other chromosomes have evenly distributed markers. We have repeated and completed the greenhouse screen on 199 seedlings that rooted successfully. Thirty-five of the seedlings were tested 3 times, 77 tested twice and 87 were tested once. An ANOVA on the 35 genotypes tested in all three trials indicated that only genotype matters and there were no significant interactions. The same was true for the 77 genotypes tested twice when compared pairwise. The updated results were used for QTL analysis. One-way ANOVA and interval mapping revealed QTLs on chromosome 8, 12 and 14 that explained over 25% of the phenotypic variation. Currently, we are refining the maps of these three chromosomes with more markers and establishing the association of markers that are in linkage with the resistance for potential use in marker-assisted screening.

**Objective 3.** Evaluate *Vitis* germplasm collected from across the southwestern US to identify accessions with unique forms of PD resistance for grape breeding.

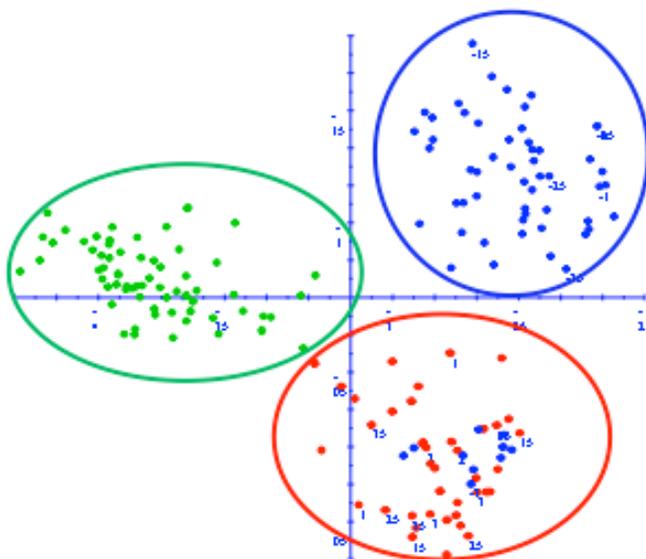
Many *Vitis* species growing in the southeastern US are resistant to PD, which led to the hypothesis that southeastern germplasm co-evolved with Xf and developed resistance to this disease. However, recent comparative sequencing work with Xf indicates that it originated in South America. Thus, it is important to determine whether resistance derived from southwestern US and Mexican species differs from that from the southeastern US. Previously, our focus has been on three accessions of *Vitis* species that Olmo collected in northern Mexico in 1960. These three accessions are complexes of multiple *Vitis* species and it is not known which particular species is controlling PD resistance. This point is confounded by the complete fertility among the *Vitis* species and the great number of hybrids that occur in the wild. It is extremely important for a breeding program to incorporate multiple unique resistance mechanisms, understand genetic diversity, and the mode of inheritance to facilitate decision making for resistance breeding. We have made strong progress assessing genetic diversity and population structure of southwestern US accessions. We examined a collection of 219 accessions from the southern US and Mexico including Olmo's Mexico collections (which he collected across northern and central Mexico from the west to the east). DNA was collected from those genotypes and six *V. vinifera* accessions were added as outliers. A total of 22 SSR markers were selected for their polymorphism and coverage of all 19 grape chromosomes. Six markers that span the *PdR1* locus were added to scan for alleles linked to *PdR1* (Table 3). All amplified products were run on the ABI 3500 genetic analyzer and analyzed with the Gene Mapper program to obtain fingerprint profiles. Hierarchical clustering (Ward method) and Principal Coordinate Analysis (PCA) were carried out with DARWIN software (version 5.0.158) to determine the number of groups. STRUCTURE V2.3.1 was used to infer the number of pseudo-populations or clusters with 22 markers.

Table 3. List of SSR markers used for fingerprint analysis of 219 accessions.

Marker name	Label	Chromosome assignment	Amplified product size
VVMD31	6-FAM	7	200
VVIv37	6-FAM	10	140-180
VVIv67	HEX	15	350-400
VVIp60	HEX	1	>300
VMC1b11	HEX	8	150-190
VVMD7	NED	7	220-270
VrZAG62	HEX	7	175-215
VVMD28	6-FAM	3	216-270
VVMD32	NED	4	240-280
UDV108	HEX	18	200-276

UDV124	6-FAM	13	170-230
VVIn73	6-FAM	17	240-270
VrZAG93	NED	2	180-240
VVIp31	6-FAM	19	150-220
VVS02	6-FAM	11	120-170
VMC4f3.1	HEX	12	160-290
VVMD5	6-FAM	16	210-290
VVMD27	NED	5	170-220
VrZAG79	6-FAM	5	230-280
VVIP26	HEX	14	120-180
VVIb23	hex	2	250-320
VVIq52	6-FAM	9	70-90
VVMD21	6-FAM	6	240-260
ctg5882	tet	14	96-116
VVIs70	hex	14	180-210
VMC1e12	Hex	14	210-300
VVip22	6-FAM	14	350-400
VVIn64	tet	14	70-90
VMC5b3	tet	14	170-220

After exclusion of those accessions that did not have enough representation in the study set, analysis was carried out on a set of 180 accessions with three methods. All three methods, hierarchical clustering (Ward method), PCA and a model-based clustering method implemented in the program STRUCTURE revealed three main groups. Figure 4 presents the results of the PCA with three distinct groups. Most of the accessions from the Mexican species collections appear to be introgressive hybrids among *V. arizonica*, *V. berlandieri*, *V. candicans* (*V. mustangensis*), *V. cinerea* var. *blancoii*, *V. girdiana*, and *V. monticola*. Strong resistance to PD occurs in *V. arizonica/candicans*, *V. arizonica/girdiana*, *V. arizonica/monticola* forms.



**Fig. 4.** Principal Coordinate Analysis constructed with genotypic data from 22 SSR markers on 159 accessions using DARWIN software. Blue represents the *V. cinerea*-like accessions; red the *V. aestivalis*-like accessions; and green the *V. arizonica*-like accessions. The axis 1 and 2 presents 9.13 and 5.74 percent of the variation, respectively.

All accessions that are part of this study were greenhouse-tested Fall 2013/Spring 2014, and results will be available by Fall 2014. The goal of this study was to investigate the phylogeographic diversity of plant material collected from Gulf coast states and the southern US, and determine the relationships between species, PD resistance and the genetic control of that resistance. This will allow us to better understand

the evolution of resistance, and the range of resistance mechanisms and their control. With this information we can more effectively combine different resistance sources to achieve more durably resistant plant material.

To determine the inheritance and nature of resistance of the best forms, we have made crosses since 2012 to develop breeding lines with the most resistant accessions. Small breeding populations were planted in Spring 2013. In 2013, we made additional crosses to expand the existing populations as well as used four new PD resistant accessions to develop breeding populations (Table 4). Seedlings that were generated from the 2012 crosses were tested with markers and true-to-cross seedlings were transferred to the field. Currently these small populations are scheduled for greenhouse testing. All crosses made in 2013 will be evaluated in 2014.

**Table 4.** Crosses made in 2013 to develop genetic maps in new accessions from the southern US and Mexico. Crosses 08-319-29 and 08326-61 are female flowered selfed progeny of Zinfandel and Cabernet franc, respectively. F2-35 is also female and a cross of Cabernet Sauvignon x Carignane.

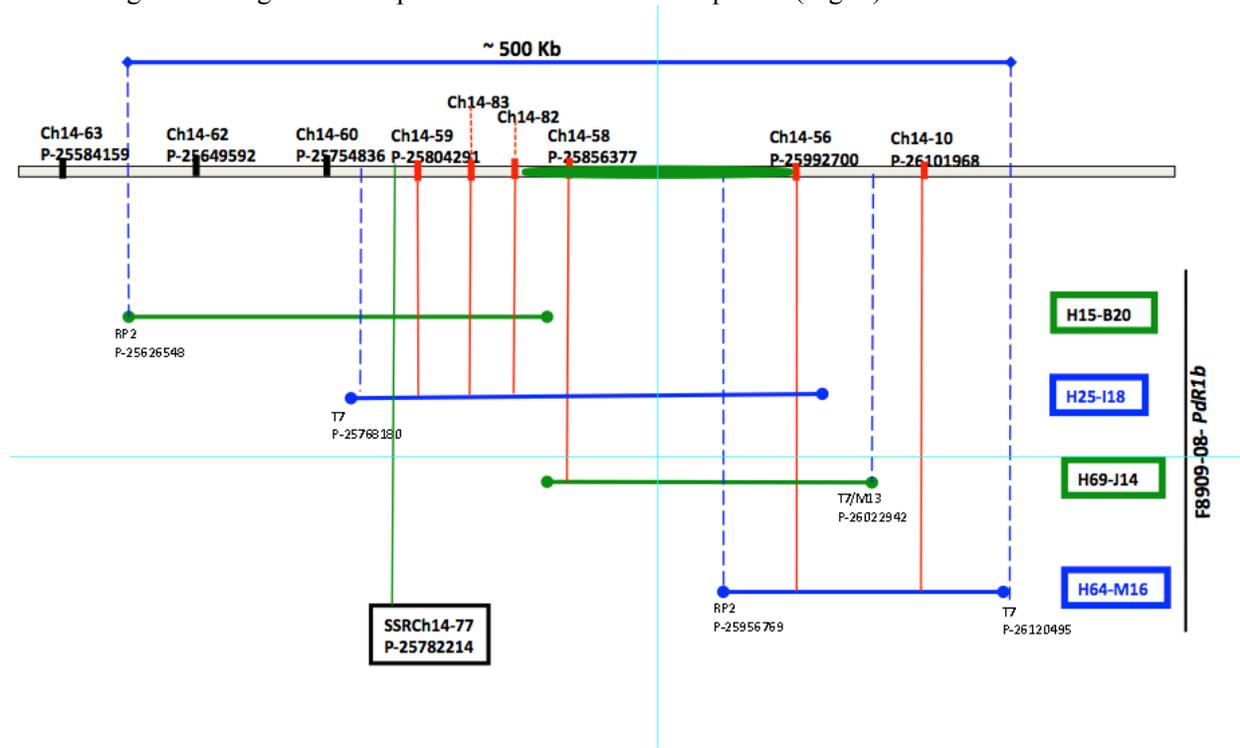
Resistant Source/ new or existing	Geographic Origin - Appearance Phenotype	Pure <i>Vinifera</i> Types used in 2013 crosses	Estimated # of Seed
ANU5	Littlefield, AZ	Alicante Bouschet	250
expands existing	<i>V. girdiana</i>		
b40-29	Chihuahua, MX	F2-35	1250
expands existing	<i>V. arizonica</i>	08319-29	2000
b41-13	Ciudad Mante, MX	F2-35	750
new	<i>V. arizonica- mustangensis-champinii</i>		
b43-57	Guadalupe, MX	Malaga Rosada	1000
new	<i>V. arizonica- mustangensis-champinii</i>	Rosa Minna	900
b46-43	Big Bend, TX	08326-61	850
expands existing	<i>V. arizonica glabra- monticola</i>		
b47-32	Big Bend, TX	F2-35	1950
expands existing	<i>V. arizonica glabra- monticola</i>	08326-61	70
SC36	San Diego, CA	Palomino	350
new	<i>V. girdiana</i>	Grenache	600
T03-16	Lahitas, TX	Palomino	175
new	<i>V. arizonica</i>	Grenache	20

**Objective 4.** Complete the physical mapping of *PdR1a* and *PdR1b* and initiate the sequencing of BAC clones that carry *PdR1a* gene candidates (in cooperation with our companion resistance gene characterization project).

The genomic region of *PdR1* locus is complex with long arrays of tandem repeats of retrotransposon elements and presents a challenge to the correct assembly of this region. We initiated the physical map project without any previous knowledge of the sequence and opted for 454 pyro sequencing, which did not result in a clear assembly. After that, we used Sanger reads developed from H69J14 BAC clone shotgun and fosmid library that carries the PD resistance gene(s). This assembly generated big enough contigs to search for the resistance genes and identified 6 putative candidate genes ranging from 2Kb to 3.1Kb in the resistance region. Copies 1 – 4 are 97-99% similar and differ in size (potentially tandem repeats of one gene); they were up to 78% similar to the four copies of genes from the Pinot noir (PN40024) sequence. This information enabled us to examine their function by transforming susceptible Chardonnay and tobacco with the gene candidates in our companion project. However, we need to determine the organizational structure of the genes to decipher how they evolved. Moreover, complete assembly is required to carry out the cloning of the corresponding promoter region of the resistant genes. A detailed comprehensive comparison of the H69J14 clone sequence to the PN40024 sequence is also not possible due to major re-arrangement of repetitive elements between the two genomes, and the presence

of gaps in the contigs of the H69J14 BAC clone. In December 2013, we have used PacBio RS II system sequencing to produce very long reads with average lengths of 4,200 to 8,500 bp and the longest reads of over 30,000 base pairs. As no amplification is required, the read accuracy is very high and de novo assembly of the genome can be performed with up to 99% accuracy.

We have isolated four BAC clones that overlap with each other and provide an approximately 500Kb long stretch of genomic region in comparison to the PN40024 sequence (Fig. 5).



**Fig. 5.** The position of four BAC clones relative to each other and to the PN40024 sequence. Orange lines are markers that were used to screen the BAC library. All four clones represent the *PdR1b* haplotype.

To date, we completed the sequencing and assembly of all four BAC clones. The assembly generated 604 Kb long sequence with out any gaps; the entire region is 126 Kb more than corresponding PN40024 sequence, which is 491.2 Kb. The expansion of this region in the resistant accession is due to transposable elements (both type I and II). We identified multiple open reading frames of the Leucine-rich repeat Receptor Kinase gene family that regulates a wide variety of functions in plants including stem cell maintenance to hormone perception, and defense and wounding response for both host as well as non-host specific defense. The next step is to fully annotate the sequence, carry out comparative sequence analysis (manuscript in progress) and proceed to promoter isolation and characterization of the resistant genes. The results of this work will feed into the project “Molecular-functional approach to facilitate the discovery of noval *Xylella fastidiosa* resistance gene(s) and markers in Native American species” that is continuation of this work and funded by the CDFA PD/GWSS from 2014 - 2017.

**Funding for this project was received from the CDFA PD/GWSS Board.**