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Development and characterization of a large set of microsatellite markers for grape phylloxera (*Daktulosphaira vitifoliae* Fitch)

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Summary

This study describes novel simple sequence repeat (SSR) primers from a genomic DNA sequence of the grape phylloxera. A total of 130 SSR primers were designed from 145 unique sequences with di, tri, tetra and penta simple sequence repeats. The SSR primers were tested on DNA from 10 grape phylloxera strains chosen for their behavioral and geographic diversity. Eighty-nine primers generated easy to score alleles with standardized conditions of amplification. Twenty-eight new and four previously published markers were selected to genotype 32 root and leaf phylloxera samples in order to identify reliable markers for future genetic diversity and phylloxera population studies. SSR data from these samples was also used to determine the frequency of null alleles, and locus specific estimates of population differentiation and clustering. Up to six alleles were detected with a mean expected heterozygosity (He) of 0.51. The observed heterozygosity (Ho) was 0.73 and the majority of markers had higher Ho values. Null alleles for four markers were considered to be the result of homozygous genotypes. The 89 SSR loci developed in this study represent a new and informative set of markers that are easy to combine for multi-loading and suitable for large-scale genetic analyses of population structure, genetic diversity, and the origin of host specific strains in grape phylloxera.

K e y w o r d s : *Daktulosphaira vitifoliae*, *Vitis*, SSR markers, population dynamics, microsatellite markers, phylloxera, grape, host parasite interactions.

Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch) are North American aphid-like insects that feed exclusively on the leaves and roots of grape species. They have a two-stage life cycle with parthenogenic phases on the roots and foliage, and a rarely observed sexual cycle. Their feeding forms pocket-like galls on leaves and hooked galls (nodosities) on young root tips. They also form swollen galls on mature roots (tuberosities) on the highly susceptible European grape, *Vitis vinifera* L. These galls split and crack, which allows entry of soil-borne fungi that decay the roots and eventually kill the infested vine (Granett *et al.* 2001). This insect was inadvertently introduced into Europe in the 1860s and eventually destroyed most of the *V. vinifera* vineyards. Over 100 years ago, breeders began producing phylloxera resistant rootstocks using North American *Vitis* species, which co-evolved with phylloxera and developed resistance. Concerns about the durability and breadth of resistance in rootstocks have stimulated multiple studies of grape phylloxera's genetic diversity, population structure, their feeding behavior and adaptation to different grape hosts. Initial studies used genomic DNA-based molecular markers like AFLP, RAPD, and mitochondrial DNA sequences to examine genetic variation and pest population structure and dynamics over time and space (Fong *et al.* 1995, Forneck *et al.* 2000, Downie 2002). Subsequently, a limited number of co-dominant SSR markers were developed and used to study the mode of reproduction and population structure within vineyards in Australia (Corrie *et al.* 2002, Corrie and Hoffmann 2004), Europe (Vorwerk and Forneck 2006) and California (Lin *et al.* 2006).

Simple Sequence Repeat (SSR) markers are versatile genetic tools that provide accurate and reproducible data, and provide insight into mutation rates. The number of repeats at the analyzed locus normally characterizes allele sizes of SSR markers, with an accuracy of up to 1 base pair. These markers are co-dominant and easily optimized for high throughput screening. SSR markers have been used for population genetic studies across a wide range of organisms and have been used to study genetic diversity, population genetics and modes of reproduction. To date, 12 SSR primers have been developed from grape phylloxera genomic DNA. They have been used to study genetic diversity and population structure (Corrie *et al.* 2003, Vorwerk and Forneck 2006, Lin *et al.* 2006). However, the number of markers that are polymorphic is limited and most generate only 2 to 4 alleles per primer pair. This low level of polymorphism limits studies on genetic diversity, migration, reproductive mode and adaptation of grape phylloxera strains to different rootstock hosts.

This study characterizes 89 new SSR primers generated from a phylloxera genome sequence developed by Lin *et al.* (2012). A set of 32 phylloxera samples collected from the University of California, Davis vineyards was used to evaluate the effectiveness of 28 select primers. These primers were chosen because they generated clean amplifications with three or more alleles in a test set of 10 diverse

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phylloxera strains collected from multiple rootstock hosts and California locations. The new markers were compared to the four previously published markers to determine heterozygosity, the occurrence of null alleles, genetic diversity and population structure so that the best markers could be used for future genetic and host adaptation studies.

Material and Methods

Phylloxera DNA for sequencing: Adult grape phylloxera and eggs from four strains (two type A strains that feed primarily on *V. vinifera* roots, and two type B strains that feed primarily on AXR1 rootstock (Granett *et al.* 2001)) were pooled. DNA extractions were carried out using the protocol reported by Lin and Walker (1996).

454 pyrosequencing was carried out with a Roche GS-FLX sequencer according to the manufacturer's protocols (Roche, Branford, CT, USA). Sequencing data was assembled with Newbler version 2.0 (Roche). Tandem Repeats Finder software was used to identify microsatellite regions with different motifs (Benson 1999). A stand-alone BLASTn analysis was performed to remove redundant sequences. A total of 145 unique sequences with di, tri, tetra and penta simple sequence repeats and enough flanking sequence on each side to design primers was selected.

SSR primer design and testing: Primers were designed for 130 of the sequences with the web-based software Primer3 using the following criteria: 35-60 % GC content, 22-26 base pair length and optimum melting temperature of 60 °C (Rozen and Skaletsky 2000). All primer pairs were tested on a set of 10 grape phylloxera samples that included both type A and B, other California rootstock strains, and two leaf gall samples from the eastern United States to check for successful amplification, clarity of amplified product and level of polymorphism. The PCR amplifications were performed in 10 µl reactions consisting of 10 ng template DNA, 5 pmoles of each primer, 2.5 mM of each NTP, 1µl 10x gold PCR buffer (Perkin Elmer, Waltham, Massachusetts), 0.05 unit AmpliTaq Gold DNA polymerase (Perkin Elmer) and 2 mM MgCl2 solution. All SSR primers were amplified at a 56 °C annealing temperature, keeping all other conditions of the protocol constant: 10 min at 95 °C; 35 cycles of 45 sec at 92 °C, 45 s at 56 °C, 1 min at 72 °C; with a final extension of 10 min at 72 °C. Amplification products were separated on denaturing 5 % polyacrylamide sequencing gels and visualized by silver staining with a commercial kit (Promega, Madison, Wisconsin).

Evaluating phylloxera population diversity: Twenty-eight of the new and 4 previously published polymorphic SSR markers (DV3, Dvit1, Dvit2, and Dvit6), were used to examine phylloxera samples collected from the University of California, Davis (UCD) vineyards (Tab 1). Twenty-six phylloxera samples were collected from three different blocks in the UCD vineyards. One to three individual adults, 5 to 10 crawlers, or 10 to 15 eggs were isolated from root samples and placed in 1.5 mL test tubes using sterilized equipment. Tubes were stored at -20 °C until DNA extraction. Two samples of foliar phylloxera consisting of one adult and 10 eggs were collected from leaf galls on infested St. George rootstock. Six strains from the test set of 10 samples used to test the amplification success rate and clarity of bands of new primers were added as references (Tab. 1).

Fluorescently labeled primers (6-FAM, HEX or VIC, and NED) were used to amplify the phylloxera genomic DNA as described above. Amplifications for each primer were carried out separately. After a 1:3 dilution of the PCR product, up to four primers were mixed, taking into account the size of the amplified fragments and/or the fluorescent label of the primers. PCR products were combined with mix of HD-formamide and GeneScan 600LIZ® as the internal size standard. Microsatellite fragments were resolved on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) and alleles were identified using Gene Mapper v.4.1 (Applied Biosystems, Foster City, CA).

Ranking the SSR markers: A list ranking the utility of the 32 SSR markers was generated by comparing the quality of their signals and assigning them a value of 1 through 3 (1 good; 2 medium; and 3 poor).

Data analysis: The microsatellite tool kit software (Park 2001) was used to calculate expected heterozygosity (He), allele frequencies (AF), and polymorphic information content (PIC) which measures how informative the markers were in regard to expected heterozygosity and the number of identical samples for the 32 markers. Observed heterozygosity (Ho) was calculated as the ratio between heterozygous genotypes and the total number of genotypes analyzed for each marker. Micro-Checker V2.2.3 software was used to determine the occurrence of null alleles, with a 95 % confidence interval with four different methods (van Oosterhout *et al.* 2004).

Pairwise similarity between the multi-locus genotypes was estimated by using the "proportion of shared alleles" (ps) as described by Bowcock *et al.* (1994). The -ln (ps) option of MICROSAT version 2.0 (Minch *et al.* 1997) was used to calculate the genetic distance between all pairwise combinations of genotypes. Pairwise similarity estimates and genetic distance comparisons were calculated by using only the four previously published markers and these results were compared with the data set from the 28 new markers. A dendrogram based on genetic distance was constructed with the unweighted pair-group method using arithmetic means (UPGMA) algorithm with PHYLIP software version 3.6. Treeview (Page 1996) was used to display the dendrogram.

Results

SSR primer development and testing: A total of 130 primers were developed and tested on the set of 10 grape phylloxera strains. Six primers failed to amplify genomic DNA and 35 primers generated multiple bands indicating that either the primer sequences had multiple priming sites due to a lack of sequence specificity,

Table 1

Grapevine host and location of the phylloxera samples collected from the University of California, Davis vineyards. The last six samples (bold) were used as a reference and five of them were used to test amplification success rate of new SSR markers. Four italicized samples had more than two alleles due to the mixing two different kinds of phylloxera in the same tube

Sample ID	Host cultivar or selection	Type	Location
30103	St. George	Foliar	Indexing, Row 4
30202	St. George	Foliar	Indexing, Row 4
10101	Chardonnay	Root	II81:10
10201	Unknown	Root	G block
30302	05024-05	Root	G30:03
30201	05026-35	Root	G30:53
30101	05025-028	Root	G31:01
30503	05025-78	Root	G31:55
30401	05025-080	Root	G32:01
31001	AT0023-116	Root	J03:01
30902	OP0540-153	Root	J12:01
32303	06354-002	Root	J15:01
30703	06348-025	Root	J15:58
30603	06348-27	Root	J16:01
31403	06348-027	Root	J16:01
31203	06353-040	Root	J16:58
31503	06353-041	Root	J17:01
31302	06384-069	Root	J17:58
32001	06384-070	Root	J18:01
32101	06718-050	Root	J18:58
30801	U0502-10	Root	M10:26
31102	09331-108	Root	M12:26
31601	08343-01	Root	M21:01
32401	08381-40	Root	M22:01
32501	08379-26	Root	M23:65
32201	09345C-07	Root	M27:01
AXR-R1	AXR#1	Root	Biotype B, Willits, Mendocino County, CA
Vin-R1	Chardonnay	Root	Biotype A, Davis, Yolo County, CA
Fre-R1	Freedom	Root	Oakville, Napa County, CA
Fre-R2	Freedom	Root	St. Helena, Napa County, CA
101-R2	101-14 Mgt	Root	Geyserville, Sonoma County, CA
WEO4802	St. George	Foliar	National Clonal Germplasm Repository, Winters, CA

or that genomic regions represented by these primers were duplicated in the grape phylloxera genome, thus resulting in multiple bands. Eighty-nine primers produced clean bands: 28 were mono-morphic (one allele), 27 produced two alleles, and 34 primers identified 3 to 7 unique alleles per locus for set of 10 phylloxera samples used to test the primers (Tab. 2).

Genetic diversity: The 28 polymorphic markers that generated three or more alleles for the test set, and the four previously published SSR primers were used to evaluate the population structure and genetic diversity of 32 phylloxera samples, 27 of which were from the UCD vineyards. The initial genotypic analysis indicated that four of these samples had more than two alleles due to the presence of more than one genotype in the DNA sample (Tab. 1). Both possible diploid combinations were kept. Only two samples from M block were duplicates and had the same allelic profile for all of the 32 tested markers. Tab. 3 presents the results of Ho, He, number of alleles, and the PIC content for the 32 markers. Among the new 28 markers, only one marker was monomorphic, the 27 others produced two to five unique alleles. Twenty-six markers had high levels of Ho, five markers had lower Ho than He, and only one marker (Dvit6) had the same value for Ho and He. Null alleles were detected for four of the new markers; three markers (Phy_II_13, Phy_III_42, and Phy_III_49) had an excess of homozygous genotypes; and Phy_III_65 produced no data for the majority of the samples (Tab. 3).

Clustering by genetic distance: The dendrogram constructed with UPGMA divided the phylloxera samples into three major clusters (Figure). Cluster A consisted of phylloxera samples from leaf galls and it was separate from the root samples. Cluster B consisted of root phylloxera collected from M block and the type A control. Two samples in this group were identical at all loci, even though they were collected from the roots of two different

Table 2

Features of 89 SSR primers derived from the genomic DNA sequence of grape phylloxera. Number of unique alleles was detected from a set of 10 samples root and foliar samples

Marker name	Genebank accession no.	Probe DB PUID	Amplified product size	Total no. of unique alleles observed	Marker name	Genebank accession no.	Probe DB PUID	Amplified product size	Total no. of unique alleles observed
Phy_II_6	GF111388	1242485	125	4	Phy_III_45	GF111364	1242461	144	1
Phy_II_7	GF111301	1242398	143	1	Phy_III_46	GF111329	1242426	112	3
Phy_II_8	GF111350	1242447	121	4	Phy_III_47	GF111330	1242427	140	1
Phy_II_10	GF111351	1242448	146	5	Phy_III_49	GF111331	1242428	128	4
Phy_II_11	GF111389	1242486	126	1	Phy_III_51	GF111332	1242429	118	1
Phy_II_12	GF111302	1242399	133	1	Phy_III_52	GF111365	1242462	250	1
Phy_II_13	GF111352	1242449	147	6	Phy_III_53	GF111333	1242430	245	3
Phy_II_16	GF111353	1242450	120	6	Phy_III_54	GF111366	1242463	132	1
Phy_II_20	GF111303	1242400	180	1	Phy_III_55	GF111367	1242464	137	6
Phy_II_23	GF111304	1242401	116	5	Phy_III_61	GF111334	1242431	128	4
Phy_II_24	GF111305	1242402	200	4	Phy_III_62	GF111335	1242432	150	1
Phy_II_25	GF111306	1242403	142	1	Phy_III_63	GF111336	1242433	143	7
Phy_II_26	GF111368	1242465	124	3	Phy_III_64	GF111337	1242434	144	1
Phy_II_27	GF111354	1242451	149	1	Phy_III_65	GF111369	1242466	112	3
Phy_II_28	GF111307	1242404	147	5	Phy_III_68	GF111338	1242435	125	1
Phy_II_29	GF111308	1242405	135	5	Phy_III_69	GF111370	1242467	137	4
Phy_II_30	GF111309	1242406	146	3	Phy_III_71	GF111371	1242468	148	2
Phy_II_31	GF111310	1242407	115	6	Phy_III_86	GF111339	1242436	143	3
Phy_II_32	GF111311	1242408	139	6	Phy_III_87	GF111340	1242437	149	3
Phy_II_34	GF111312	1242409	125	5	Phy_IV_1	GF111341	1242438	249	2
Phy_II_35	GF111313	1242410	123	7	Phy_IV_2	GF111372	1242469	235	1
Phy_II_36	GF111314	1242411	130	6	Phy_IV_4	GF111373	1242470	248	4
Phy_III_5	GF111355	1242452	149	2	Phy_IV_6	GF111374	1242471	151	1
Phy_III_7	GF111315	1242412	149	2	Phy_IV_7	GF111342	1242439	145	1
Phy_III_11	GF111356	1242453	148	1	Phy_IV_8	GF111375	1242472	221	2
Phy_III_12	GF111357	1242454	169	1	Phy_IV_10	GF111376	1242473	140	2
Phy_III_15	GF111316	1242413	139	3	Phy_IV_13	GF111377	1242474	197	2
Phy_III_17	GF111358	1242455	144	2	Phy_IV_14	GF111378	1242475	171	1
Phy_III_19	GF111359	1242456	106	6	Phy_IV_18	GF111343	1242440	147	1
Phy_III_20	GF111317	1242414	226	2	Phy_IV_21	GF111344	1242441	164	2
Phy_III_22	GF111360	1242457	138	1	Phy_IV_25	GF111345	1242442	161	6
Phy_III_27	GF111318	1242415	101	2	Phy_IV_26	GF111346	1242443	219	2
Phy_III_28	GF111319	1242416	169	2	Phy_V_2	GF111379	1242476	161	2
Phy_III_29	GF111320	1242417	233	2	Phy_V_3	GF111380	1242477	287	2
Phy_III_30	GF111321	1242418	141	4	Phy_V_7	GF111381	1242478	172	2
Phy_III_31	GF111361	1242458	215	2	Phy_V_8	GF111382	1242479	155	1
Phy_III_32	GF111322	1242419	187	1	Phy_V_9	GF111383	1242480	178	1
Phy_III_33	GF111362	1242459	126	2	Phy_V_10	GF111384	1242481	165	1
Phy_III_34	GF111323	1242420	184	2	Phy_V_11	GF111385	1242482	170	2
Phy_III_35	GF111324	1242421	147	2	Phy_V_12	GF111347	1242444	299	2
Phy_III_36	GF111325	1242422	199	4	Phy_V_13	GF111386	1242483	169	2
Phy_III_38	GF111326	1242423	149	3	Phy_V_16	GF111387	1242484	213	2
Phy_III_40	GF111327	1242424	131	2	Phy_V_18	GF111348	1242445	141	1
Phy_III_42	GF111328	1242425	150	6	Phy_V_19	GF111349	1242446	180	1
Phy_III_44	GF111363	1242460	112	2					

hosts that were four rows apart from each other (Tab. 1). Cluster C was more diverse; most of the samples came from J and G blocks, which are adjacent and only separated by a 15-meter dirt road. Two groups of three samples in this cluster were identical, although data was missing for up to three markers. Analysis with only the four previously published markers also separated the leaf phylloxera from the root phylloxera. However, the root samples clustered together and refined grouping of samples from different blocks was not possible (data not shown). These results were expected, and confirm that better distinction among groups of samples requires large set of polymorphic SSR markers for predominantly clonally reproducing phylloxera.

Table 3

Ranking of 32 tested markers, number of alleles observed, expected heterozygosity (He), observed heterzygosities (Ho), polymorphic information content (PIC)

Marker name	Rating*	Null allele	No. of alleles	He	Ho	PIC
Phy_II_6	1	no	2	0.50	0.85	0.37
Phy_II_10	1	no	1	0.00	0.00	0.00
Phy_II_13	1	yes	3	0.52	0.09	0.46
Phy_II_16	2	no	4	0.65	0.97	0.56
Phy_II_23	1	no	3	0.59	0.91	0.49
Phy_II_26	3	no	3	0.57	1.00	0.47
Phy_II_28	3	no	5	0.79	0.97	0.75
Phy_II-29	3	no	2	0.63	0.97	0.54
Phy_II_31	2	no	4	0.59	0.97	0.49
Phy_II_34	2	no	4	0.64	1.00	0.56
Phy_II_36	1	no	4	0.50	0.90	0.37
Phy_III_15	1	no	3	0.58	0.97	0.47
Phy_III_19	1	no	4	0.63	1.00	0.55
Phy_III_30	1	no	4	0.65	0.85	0.58
Phy_III_36	1	no	3	0.50	0.82	0.39
Phy_III_38	3	no	3	0.54	0.72	0.42
Phy_III_42	1	yes	3	0.36	0.12	0.32
Phy_III_46	2	no	2	0.51	0.97	0.37
Phy_III_49	2	yes	3	0.14	0.03	0.14
Phy_III_53	2	no	2	0.17	0.13	0.16
Phy_III_55	1	no	5	0.69	1.00	0.63
Phy_III_61	2	no	3	0.56	0.91	0.46
Phy_III_63	1	no	3	0.57	0.97	0.47
Phy_III_65	2	yes	2	0.21	0.00	0.19
Phy_III_69	1	no	3	0.55	0.93	0.44
Phy_III_86	1	no	3	0.30	0.34	0.28
Phy_III_87	1	no	4	0.66	1.00	0.58
Phy_IV_4	2	no	2	0.51	0.70	0.38
DV3	3	no	4	0.65	0.93	0.57
Dvit1	2	no	4	0.64	1.00	0.56
Dvit2	2	no	6	0.67	0.90	0.60
Dvit6	1	no	3	0.36	0.36	0.33

* Marker quality rating: 1 = good, 2 = medium, 3 = poor.

Discussion

This paper presents the development and characterization of 89 SSR markers for grape phylloxera. A limited number of SSR markers have been developed in grape phylloxera using genomic libraries (Corrie *et al.* 2002, Lin *et al.* 2006). Recent advances in sequencing technology have made it possible to generate large amounts of sequence data that can be scanned for simple sequence repeats, allowing the development of markers at relatively low cost compared to the use of repeat rich genomic libraries.

Information regarding phylloxera's genome organization and mode of reproduction is limited (Forneck and Huber 2009). SSR markers have been used to study the clonal reproduction and population structure of phylloxera in Europe and the USA (Corrie and Hoffmann 2004, Lin *et al.* 2006, Vorwerk and Forneck 2006). However, the small number of available markers limited the ability of these studies to fully evaluate modes of reproduction and clearly distinguish the adaptation of strains to rootstocks. This issue becomes even more important when one considers several problems associated with SSR markers including large allele dropouts, stutter due to slip strand mispairing during polymerase chain reactions, null alleles (caused by mutations in priming sites) and homoplasy, where electromorphs have identical size, but are not necessarily identical by descent due to convergent mutations. These drawbacks can lead to genotyping errors that impact the ability to draw sound conclusions from SSR marker data (Bonin *et al.* 2004). Sixty-eight percent of the primers tested in this study generated clean amplified products on the set of 10 phylloxera strains. This test set included the well-studied A and B types (Granett *et al.* 2001), six rootstock specific isolates being studied in the Walker lab, and two isolates collected from *V. vulpina* L. leaf galls from the eastern United States. The samples obtained from *V. vulpina* were very different from the eight California samples, and the type A and type B isolates were also easily separated from the other California isolates (data not presented). These results also demonstrated that the new SSR primers could detect differences among rootstock specific phylloxera types. Twenty-eight of the newly developed markers were further tested on a set of 32 phylloxera samples: 27 from

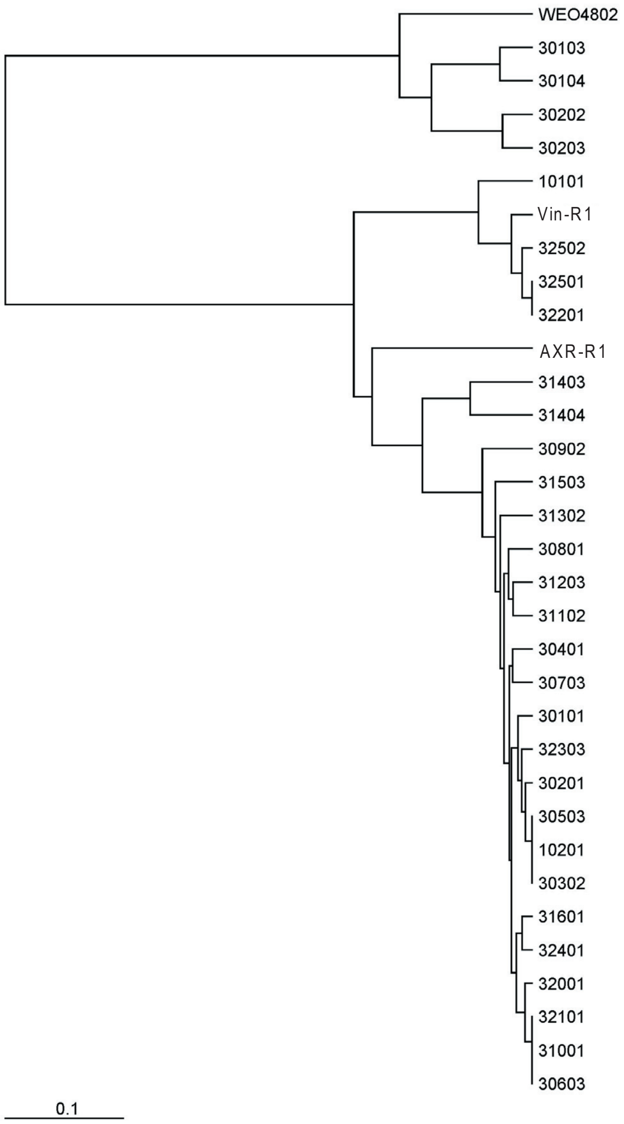

Figure: Dendrogram of grape root and leaf phylloxera samples based on cluster analysis (UPGMA) of genetic dissimilarity estimated using the (-ln(ps) transformation of the proportion of the shared alleles (ps). Analysis was carried out with 32 SSR markers.

the UCD vineyards and five others from the test set. This data was used to select an optimal set of SSR markers that were polymorphic, generated reproducible amplifications, were easy to score, had low levels of allele dropouts, and lacked null alleles. Analysis of the genotypic data indicated high levels of Ho for majority of the markers. Four markers resulted in null alleles, most likely due to an excess of homozygotes (Tab. 3). It is preferable to use only those microsatellite markers that are not prone to null alleles to generate less ambiguous data. Twenty-four of the new SSR markers were ranked 1 or 2 with very good to medium quality and are being used to study nation-wide and regional phylloxera population dynamics.

We used UPGMA cluster analysis to generate a dendrogram based on the calculated genetic distances (Figure). Analysis with 28 of the new SSR markers separated the foliar and root phylloxera and further divided the root samples into two sub groups mostly based on the collection block. Analysis with the four previously published markers also separated the leaf phylloxera from the root phylloxera, but refined grouping of samples from different blocks did not occur (data not presented). The vineyard blocks chosen for this study contained breeding populations from diverse genetic backgrounds; primarily hybrids among *V. vinifera* cultivars and North American *Vitis* species. The relatively small number of isolates tested in this study makes it difficult to infer much about the adaptation of phylloxera populations to different *Vitis* species backgrounds or specific reproductive modes. It would be interesting to intensively sample the entire vineyard while focusing on diverse *Vitis* backgrounds to determine whether the new markers can distinguish any such trend.

The new SSR primers described in this study will prove to be very useful tools for examining the population structure of grape phylloxera. Studies are underway to evaluate the genetic diversity of leaf gall phylloxera collected from across their native range; to compare overall diversity and population structure of California phylloxera with the above data set; and to determine the main mode of reproduction in foliar and root forms of phylloxera and its impact on genetic variation.

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