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Genetic Analysis of Resistance to Pythium ultimum a Major Component of Replant Disease in Apple Rootstocks

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Abstract

Apple rootstocks from the Geneva® breeding program tolerated apple replant disease in experimental and commercial plantings in North and South America, Europe and Africa. Apple replant disease (ARD) is biological in nature and composed of several fungal, oomycete and nematode actors that when combined can stunt or even kill young roots. A major contributor to the ARD syndrome is the necrotrophic soilborne oomycete *Pythium ultimum*, which can individually overwhelm young roots and root hairs causing them to decline. Genetic resistance to ARD and its components has been incorporated into apple rootstocks from a wild apple species *Malus x robusta* 'Robusta 5'. This research was aimed at increasing our understanding of the genetic complexity of the resistance to *P. ultimum* in progeny of 'Robusta 5'. In a replicated experiment we phenotyped 48 individual progeny (breeding lines) belonging to a larger population derived from a cross between replant susceptible apple rootstock 'Ottawa 3' and resistant 'Robusta 5'. We also leveraged existing genomic infrastructure in the form of high-density genetic maps composed of microsatellite and single nucleotide polymorphic markers segregating in the same cross. When combined with the genotypic means of the 48 progeny in Quantitative Trait Locus (QTL) analysis, candidate genomic locations were identified on chromosomes 2, 5, 13, 16 and 17 that were associated with relative susceptibility of those breeding lines to *P. ultimum* infection. The allelic effects of the loci were measured using a generalized linear model and their combinatorial interactions were studied. Of the resistance allelic effects examined all but one were derived from 'Robusta 5'. The ultimate goal of this work is to develop genetic markers that can aid in the selection of *P. ultimum* resistant rootstocks. However, the multi-locus nature of this resistance trait may necessitate that only loci with larger effects (on chromosome 5, 17 and 13) be targeted for further development.

Apple is one of the most valuable fruit crops in the United States. The 2021 apple crop was valued at nearly $3.2 billion grown on 382,000 acres of land (www.USAPPLE.org). Every state in the United States grows apples, and 29 states raise apples commercially. Washington State is responsible for approximately 60 percent of the total U.S. apple production. Other leading states include Michigan, New York, Pennsylvania, California and Virginia. An increasing share of orchard land is becoming certified for organic apple production with Washington State accounting for about 70 percent of the nation's certified organic apple acres, followed by California. The development of apple cultivars for new and traditional markets has contributed to much of the industry's growth and economic viability. Thus, it is important for the U.S. apple industry to continue the rapid deployment of new, viable apple cultivars. However, due to the encroachment of urban development there is a paucity of sites suitable for apple production that have not been previously planted with pome fruit, resulting in the need to plant new orchards on the same plot of land. The need to establish new orchards on old orchard ground

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increases the risk of exposing the young trees to replant disease, a complex syndrome that affects apple root growth and development.

Chronic root health issues are common in perennial crop production systems, and primarily arise due to the activity of soil-borne pathogens and parasites. Many individual pathogens, as well as complexes of soil borne pathogens, can negatively affect root health, plant growth and productivity. The buildup in pathogen densities over time in perennial cropping systems has been documented in major apple production areas in multiple countries (Mazzola, 1998; Mazzola and Manici, 2012; Rufato et al., 2021) and may play a part in reduced productivity over the lifespan of the orchard. This increase in pathogen densities was shown to contribute to the general difficulty in replanting of sites with an economically viable crop of the same or similar species (Rumberger et al., 2007).

Apple replant disease has generally been attributed to biotic factors, although the identity and consistency of the complex inciting this disease have been arguable (Kviklys et al., 2016; Reim et al., 2022; Yim et al., 2015). Discrepancies as to the nature of this disease can be ascribed to numerous factors, including an insufficient depth of analysis conducted within investigations of the subject. Meaningful studies concerning the etiology of replant disease have utilized a multiphasic approach, incorporating a diversity of methods to discern the causal biology. The principal elements identified as causal agents of apple replant disease include members of the fungal/oomycete genera *Ilyonectria*, *Phytophthora, Pythium*, and *Rhizoctonia* spp., along with the endoparasitic nematode *Pratylenchus penetrans* with different species dominating at any specific replant orchard site. *Pythium ultimum* is among the most virulent species of *Pythium* affecting apple (Mazzola et al., 2002; Zhu et al., 2017; Zhu and Saltzgiver, 2020), and functions as causal agent of apple replant disease on a global basis (Fernanda Ruiz-Cisneros et al., 2017; Grigel et al., 2019; Jeffers et al., 1982; Mazzola, 1998). In the absence of soil fumigation, there are few economically effective and ecologically desirable choices for management of tree fruit replant diseases. One option is to establish new plantings on sites not previously used with the respective crop; however, the availability of such land in the primary production regions ranges from limited to non-existent. Certain cultural practices, such as fallowing for extended periods have been reported to provide partial control of the peach replant problem (Leinfelder and Merwin, 2006; Leinfelder et al., 2004). In contrast a fallow period of up to three years provided no detectable benefit to growth and yield of apple on replant orchard ground (Mazzola and Mullinix, 2005). As is the case for a preponderance of crop species, host tolerance/resistance, in this case apple rootstocks, is an economically attractive mean to employ for the management of diseases in tree fruit production ecosystems. Tolerance to replant disease, and correspondingly individual components of the pathogen complex, has been detected in apple germplasm (Isutsa and Merwin, 2000; Leinfelder et al., 2004; Rumberger et al., 2004) and seems to be the best and more reliable long-term option for curbing the effects of this disease. However, even tolerant rootstocks exhibit increased growth and yield in response to soil fumigation thus indicating incomplete resistance to the causal pathogen complex among the commercially available apple rootstock germplasm (Auvil et al., 2011; Mazzola et al., 2015, Macedo et al., 2019; Wang and Mazzola, 2019; Spornberger et al., 2020). The initial basis for selecting apple rootstocks from the Geneva® breeding program, subsequently identified as tolerant to apple replant disease, centered on scion vigor, dwarfing, precocity, resistance to fire blight and to phytophthora crown and root rot (Cummins and Aldwinckle, 1983; Gardner et al., 1980). Resistance to some components of the pathogen complex that incites apple replant disease was identified in germplasm developed by the Geneva® breeding program (Reim et al.,

2020; Reim et al., 2022; Zhu and Saltzgiver, 2020). Multiple modes of action may contribute to the resistance in rootstock germplasm and may include rhizodeposition of substances promoting beneficial communities in the rhizosphere and endophytic biome (Leisso et al., 2017; Rumberger et al., 2004; Van Horn et al., 2021), morphological changes in roots and/or a higher level of activation of the defense response to pathogenic components like *Pythium* species as identified in the laccase dependent lignification response found in G.935 apple rootstock (Zhu et al., 2021). Apple rootstocks demonstrate significant variation in susceptibility/tolerance to this pathogen (Mazzola et al., 2009), however the genetic basis of this tolerance is not known and thus of limited value in breeding efforts which seek to develop rootstock resistance to replant disease. The genetic contributor to the replant tolerance and resistance to *Pythium* species is *Malus* × *robusta* 'Robusta 5' (R.5) which is a parent to 12 Geneva® apple rootstocks that have demonstrated tolerance to the replant complex and resistance to some of its individual components (Isutsa and Merwin, 2000; Mazzola et al., 2009; Reim et al., 2022; Utkhede, 1985; Zhu and Saltzgiver, 2020). Genetic mapping of quantitative and qualitative traits has been accomplished in several instances with progeny of 'Robusta 5', more specifically with fire blight caused by Erwinia amylovora (Gardiner et al., 2012), powdery mildew caused by *Podosphaera leucotricha* (Wan and Fazio, 2011), gene expression (Jensen et al., 2014), and in the discovery of genetic factors associated with dwarfing (Fazio et al., 2014). Progeny belonging to the same apple rootstock breeding population was screened for tolerance to *P. ultimum* to discover the nature of inheritance and as a means to develop a marker assisted selection program.

Materials and Methods

Germplasm. The 48 breeding lines used for this investigation are part of a larger progeny of a cross between 'Ottawa 3' (O.3) and 'Robusta 5' (R.5) which has been used to construct genetic maps and infer genetic inheritance of many traits including dwarfing (Fazio et al., 2014), powdery mildew resistance (Wan and Fazio, 2011), nutrient uptake (Fazio et al., 2013) and rootstock gene expression (Jensen et al., 2014).

Inoculation with Pythium ultimum, data collection and analysis. *Pythium ultimum* oospore inoculum was prepared using isolate 60-1198 which was recovered from roots of Gala/M9 apple growing at an orchard located in North central Washington State (Mazzola et al., 2002). Inoculum was prepared by inoculating 30 ml of potato carrot broth supplemented with two drops of wheat germ oil per L and 100 $\mu g \cdot ml^{-1}$ ampicillin in Petri dishes with a 5 mm-diameter agar disks cut with a cork borer from the edge of an actively growing colony of *P. ultimum*. Plates were incubated at 22 °C for approximately 1 month until abundant oospore production is observed. *P. ultimum* mycelia and oospores were collected by filtering the liquid medium through a double layer of cheese cloth and comminuted by blending for 2 min in 100 ml of water. The suspension was applied to pasteurized soil as a mist to obtain an initial density of approximately 300 propagules per gram of soil, which is within the propagule density commonly detected in apple orchard soils (Mazzola et al., 2002; Mazzola et al., 2009).

Each rootstock breeding line was represented by eight individual plants and was planted into separate pots containing *P. ultimum* infested soil. Plants were grown in the spring of 2014 in the USDA ARS Wenatchee greenhouse and roots were harvested after three weeks. For these assays, the percentage of *P. ultimum* infected root segments was determined by plating 10 randomly selected segments (0.5-1.0 cm) per plant onto PSSM agar (Mazzola et al., 2001). Hyphal growth from root segments was examined with a compound light microscope (100× magnification) after 24, 48 h and 72 h of incubation at room temperature.

Genetic mapping, quantitative trait locus analysis and effect modeling. The genetic map used for this research was updated from the consensus maps constructed used to identify the dwarfing loci composed mostly of microsatellite loci (Fazio et al., 2014; Liebhard et al., 2002; N'Diaye et al., 2008; Silfverberg-Dilworth et al., 2006) with the addition of about 3,000 additional single nucleotide polymorphic (SNP) loci from the 20K Illumina Infinium SNP chip array (Bianco et al., 2014) using Joinmap 5 genetic mapping software (Van Ooijen, 2018), such map validated against several apple genome assemblies and multiple progenies (Peace et al., 2019; Vanderzande et al., 2019). The breeding line phenotypic means were used as an input in the MapQTL 6 Software for QTL analysis (Van Ooijen, 2009). The Kruskal-Wallis analysis was used to identify peak marker loci depicted in Fig. 1 using SAS JMP PRO 16 (SAS Institute Inc., Cary, North Carolina). Further QTL analysis used the restricted Multiple QTL Modeling (rMQM) in MapQTL6 where known markers associated with the QTL are used as cofactors in the approximate multiple-QTL model with additive and dominant gene actions only. The locus interaction model was initially constructed as a full factorial using the standard least squares methods in Minitab software with all five loci and then scaled down to display significant effects. The main effects and interaction plots were produced using Minitab software.

Results and Discussion

The inoculation with *P. ultimum* spores resulted in differential (according to genotype) successful colonization of susceptible rootstock genotypes. The distribution of the genotypic means (Fig. 1) for the trait was quasi

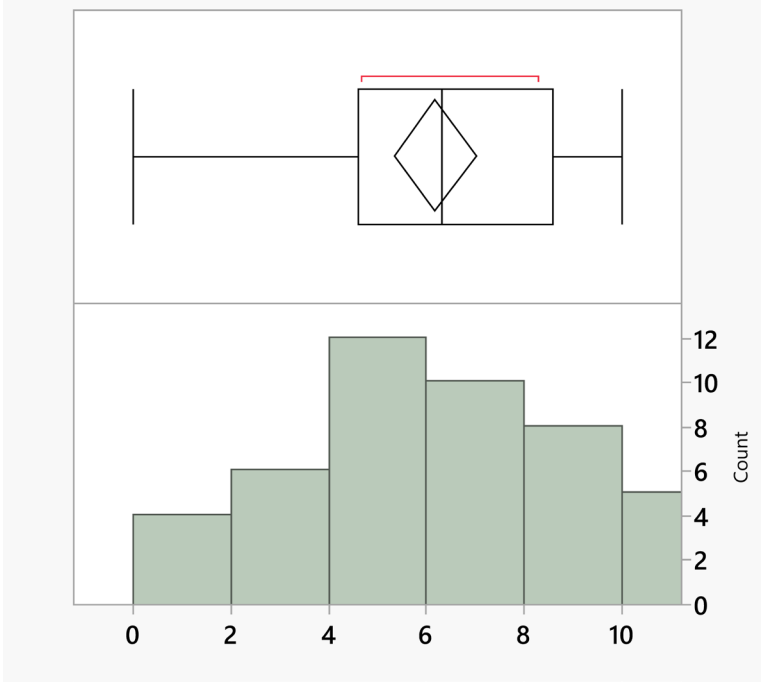

Figure 1. Distribution of genotypic means for Pythium Score of 48 apple rootstocks progenies of the 'Ottawa 3' × 'Robusta 5' cross where 0 represents more resistant and 10 more susceptible plants.

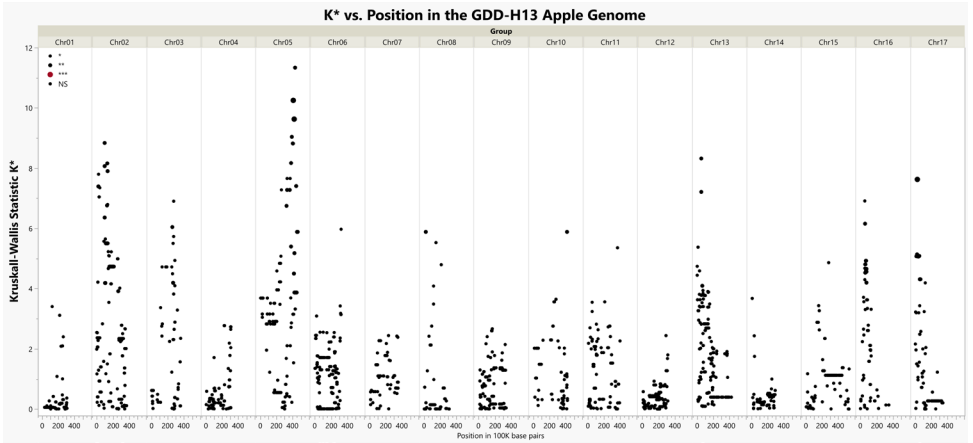

Figure 2. Marker locus significance plot of the Kruskall-Wallis non-parametric statistic K* aligned with the GDD-H13 apple genome.

normal with a mean score of 6.19, a standard deviation of 2.74 with the upper and lower 95% boundaries of 7.02 and 5.37 respectively. Rootstocks with a mean score below 3 were considered resistant/tolerant, scores between 3 and 7 intermediate and with scores above 7 susceptible. This type of distribution of genetic means is typical for complex traits involving more than one segregating factor. The Kruskall-Wallis (KW) statistical analysis is regarded as the non-parametric equivalent of the one-way analysis of variance in MapQTL 6 where a segregating QTL with strong effects linked closely to the tested marker will result in large differences in the average rank of marker genotypes. This analysis is used to glance at the whole genome effects on the studied trait and for *Pythium* Score it yielded two peaks with a P-value of at least 0.005 on chromosomes 5 and 17 and additional peaks with P-value of at least 0.05 on chromosomes 2, 16 and 13 (Fig. 2, Table 1). The allelic contribution of the 'Robusta 5' parent can be surmised by the marker classes represented in the results: classes nn and np represent markers that are heterozygous in 'Robusta 5' and homozygous in 'Ottawa 3' such that segregation of the 'Robusta 5' alleles can be surmised, whereas classes ac, ad, bc, and bd represent the combination in the progeny of all available alleles at a locus (a and b inherited from 'Ottawa 3' and c and d inherited from 'Robusta 5'). Further analysis with restricted Multiple QTL Modeling confirmed the significant QTLs where the corresponding markers selected as co-factors, explained 65% of the observed variation. Only markers representing chromosomes 2, 5, 13, and 17 resulted as significant ($P< 0.05$) in the general linear model test (Table 2 ANOVA). Markers representing chromosome 16 did not show effects strong enough to be considered significant. Similarly, all interactions in the full factorial were not significant at $P< 0.05$ level. This is likely due to the low number of individuals tested making less degrees of freedom available for all tests. The number of marker classes represented within a locus could also be a factor where markers having only two classes (nn, np) use less degrees of freedom than markers with four classes (ac, ad, bc, bd). The type of interactions among loci as observed in Fig. 4 may also have contributed where in some of the pairwise interactions only one class of markers seems to ensue changes in the mean. Nevertheless, we produced the interaction graphic on Fig. 4 to illustrate that

Table 1. Marker loci showing significant effects on Pythium susceptibility based on the K* statistic of the Kruskall-Wallis analysis in MapQTL6 software.

Marker Name	Significance of the K* Statistic	Short Designation	Chromosome and Position on GDD-H13 Genome	Segregation type[a]
RosBREEDSNP_SNP_GA_602526_Lg5_00737_MAF50_1623827_exon1	0.005	Pyt_Chr05	5	nn, np
RosBREEDSNP_SNP_CT_3132636_Lg17_01584_MAF50_MDP0000810883_exon15	0.005	Pyt_Chr17	17	nn, np
RosBREEDSNP_SNP_CT_14500796_Lg2_00002_MAF50_1621685_exon5	0.05	Pyt_Chr02	2	nn, np
RosBREED_SNP_TC_3303558_Lg16	0.05	Pyt_Chr16	16	nn, np
RosBREEDSNP_SNP_TC_4948282_Lg13_02336_MAF40_522995_exon1	0.05	Pyt_Chr13	13	ac, bc, ad, bd

[a] Segregation type according to MapQTL6 format where nn and np correspond to alleles originating from parent 2 of the cross.

Table 2. ANOVA for the four significant markers in the General Linear Model analysis.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Pyt_Chr02	1	20.47	20.468	4.20	0.04
Pyt_Chr05	1	22.84	22.837	4.68	0.03
Pyt_Chr13	3	44.10	14.699	3.01	0.04
Pyt_Chr17	3	40.76	13.585	2.78	0.05
Error	36	175.61	4.878		
Lack-of-Fit	24	126.47	5.270	1.29	0.332
Pure Error	12	49.14	4.095		
Total	44	331.77			

not all the allele contributions by 'Robusta 5' have the same effect and that the lowest score of susceptibility may only be obtained by one combination of alleles. One very interesting phenomenon is the effect on chromosome 13 where only one allelic combination (allele a from O.3 and allele d from R.5) resulted in a susceptibility score that is well below the overall mean. Both 'Robusta 5' and 'Ottawa 3' are interspecific hybrids (R.5 = *Malus prunifolia* × *Malus baccata* and O.3 = Malus domestica 'Malling 9' × unknown crabapple) (Wan and Fazio, 2011; Wertheim, 1998) and it is possible that the intra-locus interaction might be a result of resistant alleles coming from different wild species. We are in the process of determining the origin of the resistant allele coming from O.3. When the most resistant alleles are combined in a group of individuals the mean susceptibility score can be as low as 2.6, whereas when the opposite are combined the score can be as high as 9.5 (Fig. 5). In the KW analysis, the locus with the highest K* statistic was near a previously published putative location of mi397a microRNA which is activated shortly after inocula-

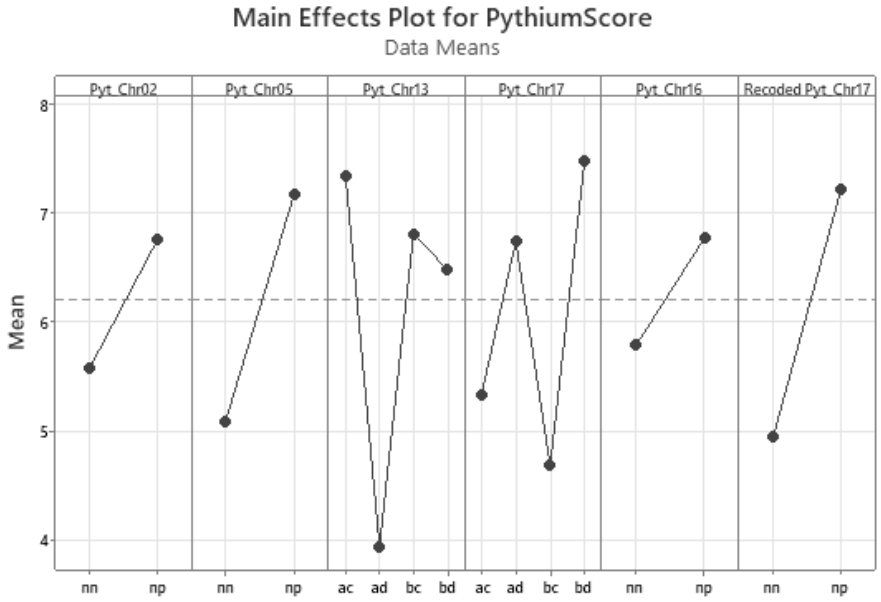

Figure 3. Main effects plot for *Pythium* Score the four significant markers displayed in Table 1. In the panel on the right Pyt_Chr17 was recoded to reflect only the inheritance of the "R5" alleles.

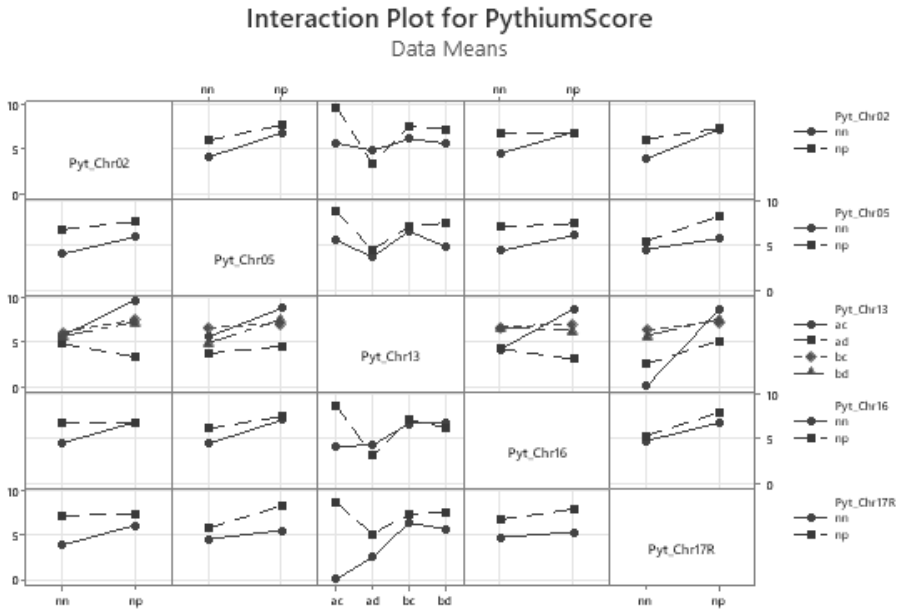

Figure 4. Interaction plot showing all the pairwise allele combination means for all markers.

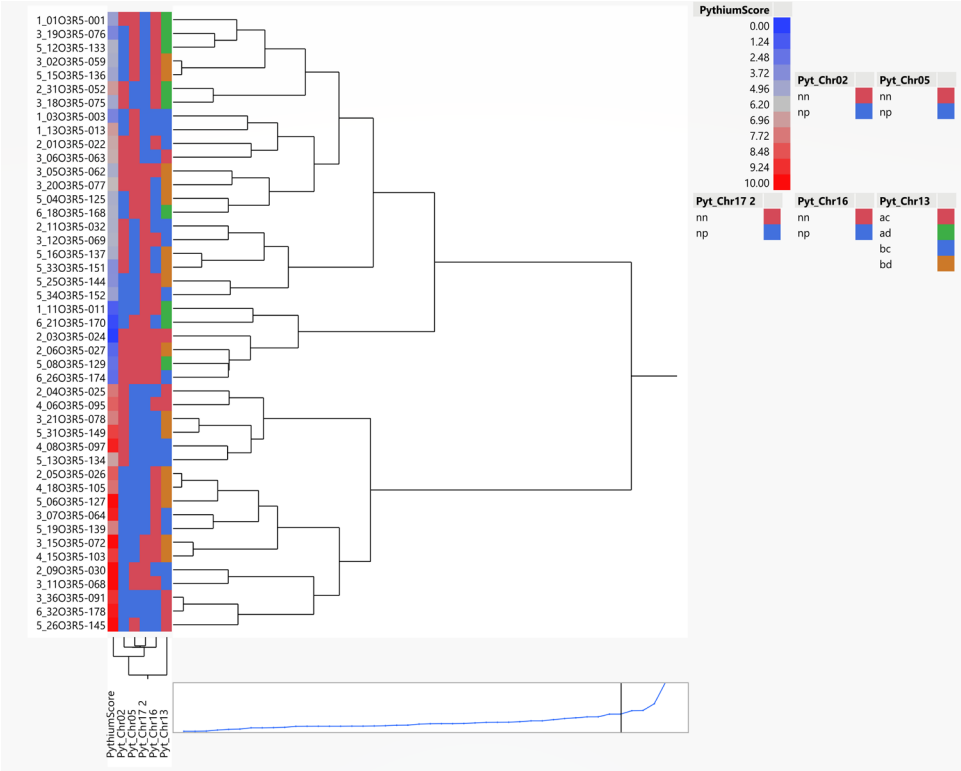

Figure 5. Dual clustering dendrogram displaying a grouping according to marker locus classes and susceptibility score. The cluster containing individuals 170, 024, 027, 129, and 174 represents the most resistant individuals and the best combination of marker alleles for resistance.

tion with *P. ultimum* and has major effects on a laccase enzyme involved in lignification. We are in the process of developing markers specific to the mi397 locus to monitor its correspondence to the resistance in breeding populations. The phenotypic characterization of resistance to *P. ultimum* in apple rootstocks is a very labor-intensive endeavor, hence the need to develop markers that can aid by reducing the pool of breeding lines slated to undergo phenotypic selection. We are in the process of characterizing additional breeding lines in the same cross in order to validate the results of this research. To the best of our knowledge this report represents the first description the genetic components of resistance to *P. ultimum* in Malus species.

Conclusion

Based on the results of the QTL analysis, resistance to *P. ultimum* derived from wild apple species *M. x robusta* 'Robusta 5' is very complex in nature consisting of larger effect loci on chromosome 5, 13, and 17 and minor effects on chromosome 2 and 16. Being able to select for the major resistance loci would be a boon to breeding new apple rootstock with resistance to components of the replant disease. Combining these loci with other essential apple rootstock loci that influence dwarfing, precocity, resistance to fire blight and nutrient absorption will be a difficult combinatorial challenge that will require large breeding populations and keen ability to follow relevant alleles with robust genetic markers.

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About the Cover:

'Brewster' lychee. *Litchi chinensis* is the only member of the genus *Litchi* in the Sapindaceae family. Lychee is a sub-tropical fruit native to south China where it has been cultivated for more than 1000 years. The flesh is white or pinkish, the taste is sub-acid, with a consistency like grape, but sweeter. The hardwood trees can grow 8 to 16 m tall, and the dense evergreen canopy is dome shaped. Lychee is grown commercially in south and coastal Florida where there is some chilling with no risk of hard freezes. There are 200 cultivars, and 'Brewster', the second most important commercial cultivar in south Florida, was brought from China to the U.S. by Rev. William N. Brewster. The fruit is medium to large, sweet, and juicy, and in taste tests it consistently outranks most other cultivars. It has relatively large seeds, but more flesh than many cultivars. 'Brewster' has bright purplish red skin and is resistant to anthracnose. In Mexico 'Brewster' ripens in April and the season extends to mid-July in central Florida. Lychee pulp is high in vitamin C, with smaller amounts of B vitamins and has moderate amounts of polyphenols and anthocyanins. *Photo by Johnathan Crane.*