Molecular Marker Based Diagnostics for Almond Non-Infectious Bud-Failure

Project No.: **13-HORT7-Gradziel**

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Overarching Project Objectives:

A. Consolidate historical and recent data from almond x almond as well as almond x peach breeding populations for evaluation of possible inheritance patterns of non-infectious bud-failure (BF) in progeny.

B. Develop genetic/epigenetic model(s) based on compiled progeny segregation and development patterns and current research on similar genetic/epigenetic afflictions.

C. Initiate a preliminary assessment currently available molecular-based diagnostics for discriminating between high and low-BF expression.

D. Publish results from BF-heritability studies as a basis for a subsequent outside funding proposal (e.g.,USDA) targeting molecular-based BF predictors.

Current Project Objectives:

A. Obtain DNA methylation profiles from a set of clonal sources of different ages and BF-potentials.

B. Correlate DNA methylation profiles with differing clone ages as well as differing BF-potential within clones of the same almond variety as well among different varieties for BF-potential.

C. Identify genomic regions associated with advancing clone age and possibly BF expression as a basis for future studies to develop more specific and accurate molecular markers flanking these regions.

D. Begin to develop almond BF as a model system for epigenetic/genetic disorders in plants as a foundation for more extensive outside research funding of basic mechanisms as well as applied predictors and manipulations (including the possible remission of BF and other genetic disorders such as Cherry-Crinkle).

Interpretive Summary:

This research is a continuation of a project jointly funded by the Almond Board of California (ABC) and the California nursery industry (California Fruit Tree, Nut Tree, and Grapevine Improvement Advisory Board [IAB]). It advances previous UC Davis (UCD) studies which have led to an understanding of the pattern of non-infectious bud-failure (BF) development within propagation sources (clones) of commercially important almond cultivars including Nonpareil and Carmel, which allow effective selection of clonal sources with lower probabilities of expressing BF during the crucial early years of orchard growth. Attempts to develop molecular markers as indicators of BF-potential have proven unsuccessful, presumably because BF genetic deterioration is not associated with changes in the marker-targeted DNA sequence of the gene(s) involved, but rather involves suppression of gene activity through still poorly understood epigenetic mechanisms. This project is thus pursuing epigenetic markers based on the methylation patterns for individual genes from clones of Nonpareil and other important almond cultivars which differ in the level of BF expression and/or the clone age (since it is known that the potential for BF-expression increases with age of susceptible cultivars). We have now identified a number of methylation-markers associated with the level of BF expression, as well as with the age of the clone. Because of the large number of potential markers and the inherent difficulties in accurately scoring both BF-potential and clone age, we are now analyzing the data through both large-scale statistical analysis and individual assessment of putative candidate gene function to identify epigenetic markers associated with BF expression. A strong association might then be used as predictor of the ultimate level of BF expression in vegetative progeny from different nursery source trees and, if highly correlated, may help identify the gene(s) controlling this disorder, which in turn might lead to a better understanding of BF development as well as its control.

Introduction:

Non-infectious bud-failure (BF) remains a major threat to almond production in California, particularly with the recent rapid expansion of acreage on inherently more water and heat stressed regions. It is a particularly serious problem for the commercially important cultivars Nonpareil and Carmel, which together make up approximately 50% of total plantings. Clonal selection of low BF sources has allowed continued plantings of both Nonpareil and Carmel after BF first became a problem in these cultivars. However, BF-potential (which is related to the age and propagation history of the cultivar) in even the best clonal sources of Carmel may not be sufficiently low to ensure continued commercial use. Careful selection of low-BF Nonpareil clones in the 1970s, 80s

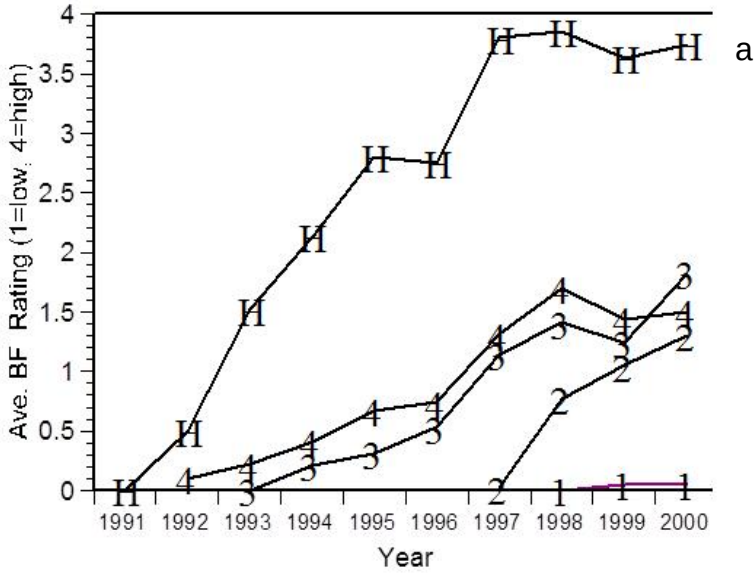


Figure 1. Development of BF- expression in vegetative progeny of different clonal sources of Carmel. (1-original Carmel seedling tree, 2-standard low-BF FPS 1 source, 3-medium-BF FPS 2 source). (Line 4 and Line H are other Carmel clones that are not covered in this report.)

and 90s has allowed continued plantings of this dominant variety, though recent BF expression in some Nonpareil sources caution that they may also be progressing towards a new round of BF expression. High BF expression was also a major contributor to the early abandonment of otherwise very promising cultivars such as Merced, and will likely be found in some of the recently released California varieties, particularly those which have the BF-susceptible cultivar Nonpareil as a parent (which includes virtually all currently commercially important cultivars).

BF-like symptoms have been observed in isolated trees of some recent releases including the cultivar Winters. Molecular marker analysis has verified the Winters identity but the source of the budwood was not virus-free FPS foundation stock but was probably propagated from virus infected wood gathered from the early Delta research block trials. Similarly, BF-like 'crazy- top' shoot growth was also observed in Marcona trees recently planted in the southern San Joaquin valley. ELISA analysis however showed the symptoms to be the result of Prunus Necrotic Ringspot virus infection. While BF has been shown to be inherited in progeny, the genetic control of BF remains elusive.

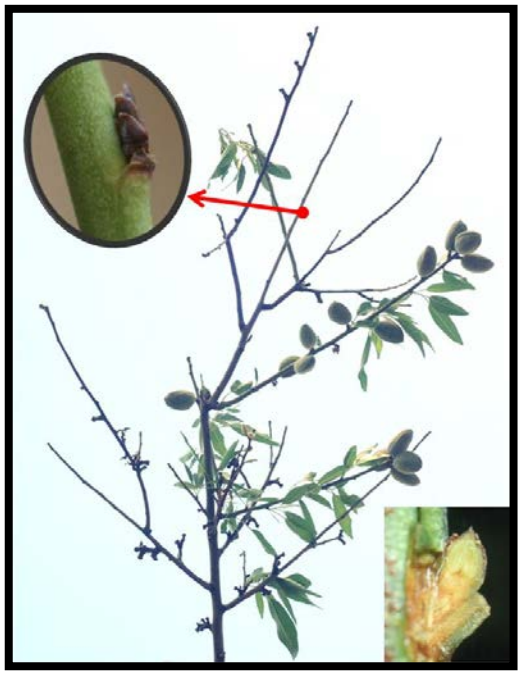


Figure 2. Characteristic shoot development pattern of non-infectious bud-failure resulting from a seasonal pattern of die back and regrowth. Lower inset shows the characteristic die back of buds the previous fall with no further development of buds through the winter and following spring (upper inset).

Populations which should segregate for BF-expression have been developed from crosses of almond selections to high-BF Nonpareil clones (to asses BF-potential among clones of the same variety), and recently from crosses of almond varieties to early-flowering peach genetic-tester lines (to asses latent BF-potential among different varieties). Resultant inheritance data will be used to establish and test different genetic and molecular models for BF.

Results and Discussion:

<u>Bud-failure characterization.</u>

Farm calls over the course of this project have typically identified multiple and distinct causes of shoot bud-failure in almond;

- Nutrient deficiencies/toxicities
- Variety growth habit
- Low winter chilling
- Wind rubbing
- Virus/viroids
- Bacterial (?) bud-drop
- Noninfectious Bud-Failure (BF) (also known as Crazy Top)

Figure 3. 'Rough-bark' trait sometimes observed in severe noninfectious bud failure.

True non-infectious bud-failure is characterized by the death of terminal or sub-terminal shoot buds during the previous Fall, which can be verified by a brown necrosis of the internal bud tissue at that time (see insets in **Figure 2**) as well as failure of subsequent bud swelling and development during the subsequent winter and spring. The disorder becomes evident with the failure of the vegetative buds to grow the following spring resulting in sections of blind or bare shoot-wood and the subsequent pushing of the still-viable basal vegetative buds. Flower buds are not affected and can often developed into fully formed nuts despite the lack of any nearby vegetative leaf growth. A third distinct BF characteristic is that once bud-failure symptoms develop, normal growth is not restored in subsequent seasons but rather the disorder gets worse with each following season (though the extent and rate of symptom development may vary in subsequent years depending upon growth rate, heat stress from the previous summer, etc.). This recurring sequence of terminal shoot-bud failure and pushing of a viable basal buds results in a punctuated and erratic shoot development pattern commonly termed "crazy top" (**Figure 2**). In some severe cases of BF, the bark on young shoots can develop a characteristic splitting or cracking often called ‘rough bark’ (**Figure 3**). BF is ‘noninfectious’ i.e. it cannot be transmitted to other trees by budding, grafting or transferred by feeding insects.

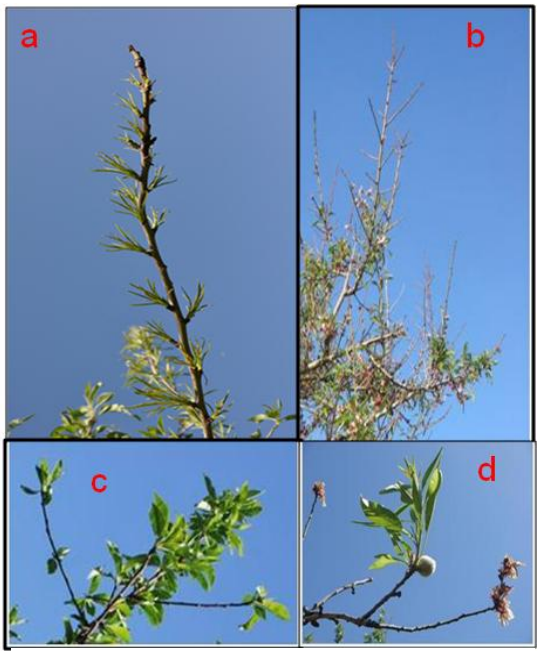


Figure 4. Expression of bud-failure from different biotic and abiotic agents.

In contrast, bud-failure from nutrient deficiencies/toxicities (including some herbicide toxicities) often show some bud development during the winter chilling period and subsequent spring growth, as is the case with zinc-deficiency in **Figure 4a**. Leaf and shoot appearance is often characteristic of the specific toxicity/deficiency. Normal growth can also be restored with the proper nutrient treatment.

Similarly, some varieties such as the late-blooming variety Savanna (**Figure 4b**) show a late leafing-out on terminal shoots that give an early impression of BF. Close examination of shoots, however, typically showed buds are developing although at a delayed rate. This can also be confirmed by revisiting the orchard one to two weeks later when normal shoot development should be observed.

In years with low winter chilling, some varieties, including Carmel, may also show a delay in terminal or subterminal lateral bud development (**Figure 4c**). Again, a close examination of the buds will show some degree of swelling or development from the previous fall, ruling out noninfectious bud failure. As with late blooming varieties,

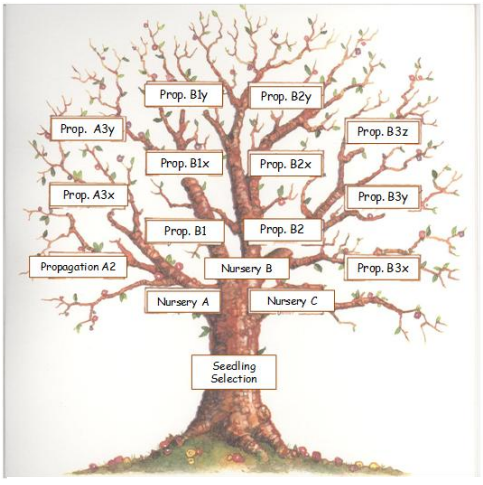


Figure 5. Tree model for the increase in potential for BF appearance either in an orchard tree or (analogously) nursery propagation sources.

buds may continue development at a later date though in some cases they appeared to become dormant or even desiccated. Serious BF-like symptoms were observed in the spring of 2012 and again in 2013 for the cultivar Monterey. While initial symptoms, including normal floral bud development with terminal and sub-terminal axillary vegetative bud failure, appear similar to noninfectious bud-failure, later observations showed that many vegetative buds were still viable and pushed normal looking spring growth, although at a much delayed time (**Figure 6**).

Figure 6. BF-like symptoms in the cultivar Monterey observed in the spring of 2013. While initial symptoms, including normal floral bud development with terminal and sub-terminal axillary vegetative bud failure, appear similar to non-infectious bud-failure, later observations showed that many vegetative buds were still viable and pushed normal looking spring growth, although at a much later time.

A similar appearance is sometimes caused when shoots or branches rubbed together in the wind causing the sloughing of buds. Close examination of the shoots can often identify the physical damage from rubbing as well as the responsible branch.

A form of bud failure often observed on old to very old trees is infectious bud failure, or bud failure caused by virus infection (typically Prunus Necrotic Ringspot Virus or Prunus Dwarf Virus). Where noninfectious bud failure will typically first appear in the rapidly growing shoots at the tops of trees, infectious bud-failure tends to be more prevalent at the slower growing shoots on the trees lower branches. New shoot growth tends to show shortened internodes and be willowy giving a 'mules-tail' appearance (**Figure 4d**). Flowers may or may not be affected depending upon the virus and variety. Diagnosis of infectious bud failure is by graft or bud transmission to a susceptible host, or by ELISA or molecular analysis (see **Appendix A** and **B**).

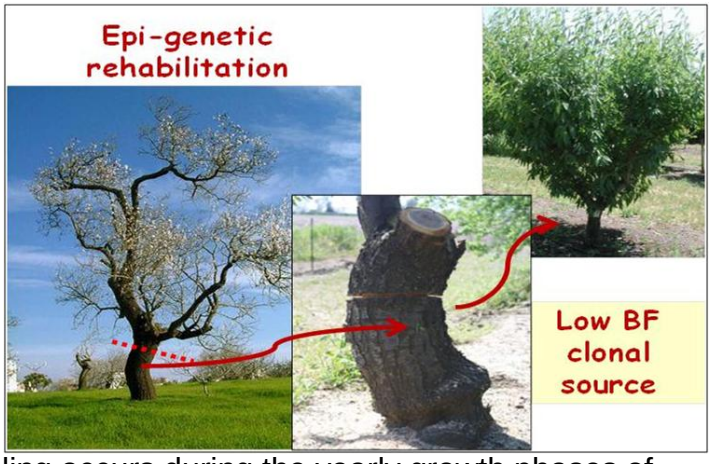


Figure 7. Rehabilitating Nonpareil almond to a lowered BF status by propagating new nursery foundation blocks from BF-dormant basal epicormic buds pushed from 100-year-old trees.

<u>Models for Non-infectious Bud-Failure development.</u>

In our evolving model of BF, the critical fall bud degeneration results from the deterioration in function of gene(s) vital to vegetative bud transition to winter dormancy. This deterioration results from a gradual genetic 'ageing' of a crucial gene complex as a consequence of repeated phase cycling and controlling cells. Such cycling occurs during the yearly growth phases of almond shoots and appears to also occur, and may even be amplified, by vegetative propagation. The typically ramified

propagation history of most vegetatively propagated tree crops is thus analogous to the growth and development of a mature tree (**Figure 5**). Since BF appears to be determined by an 'internal aging' process, the appearance of BF symptoms at their terminus of one branch (clonal propagation source) is a good predictor of imminent BF appearance on other branches (propagation source) all of which arose from a common source. Genetic deterioration also appears to be correlated with environmental stresses, particularly heat, during early-season bud development of summer dormancy (4, 20). Low BF-potential propagation sources have been selected from among clonal lines in which gene ageing is limited owing to their lineage (recent position in line of descent from original cultivar seedling tree) and previous growth environment (including low heat stress and propagation method) (See citation 12). Such vegetative progeny based clonal studies, however, typically require 10 or more years to accurately characterize clonal-source BF-potentials. A well-characterized example of this approach was the selection in the 1990s of Carmel clonal nursery sources which showed lower potential for developing BF symptoms when used as propagation material (**Figure 1** and **Table 1**). Significantly, even the best sources showed symptoms within the first 10 years of tree growth showing that while the BF potential could be reduced dramatically, it would still be a concern even in the most promising propagation sources (particularly since an additional 2 vegetative generations of ageing {i.e., mother block and grower trees plantings and growth} are required prior to commercialization). This clonal-source selection as applied to Carmel was originally applied to Nonpareil when BF symptoms became particularly problematic in the 60s, 70s and 80s. To, in a sense, turn back the internal-aging clock, epicormic buds from the base of old Nonpareil trees initially planted in the early 1900s were pushed to develop shoot growth from which clonal source material was propagated (**Figure 7**). Because the Nonpareil cultivar originated in the 1880s, these basal epicormic (i.e. poorly differentiated) buds from old trees would represent relatively low BF potentials (because they were laid down early in tree growth and remained largely dormant in the intervening years). As such, they would serve as good foundation material for continued Nonpareil propagations. That it took approximately 50 years for Nonpareil to initially show BF-symptoms indicates that the original seedling selection had relatively low initial BF potential. However, while low BF-potential was recovered from trees planted in the early 1900s, their BF-potential would be expected to gradually age (decay) in the ensuing 50 years to the point that BF-expression again became a problem by the 1960s. Nonpareil clone rehabilitation through appropriate epicormic bud selection at that time has allowed Nonpareil to remain relatively free from bud failure, though the passage of an additional 50 years since those initial epicormic selections suggest that BF may again become a problem in this cultivar.

Table 1. Proportion of trees showing BF.-expression in FPS foundation stock and grower trees derived from that stock (but 2 to 3 generations more advanced through nursery propagations).

Updated Data (2013)	DELTA	KERN	FPMS
CARMEL			
3-56-1-90	7%	26%	-
NONPAREIL			
3-8-2-70		11%	-
3-8-6-72		7%	-
3-8-5-72	-		
3-8-8-72	-		-
3-8-16-90			-
3-8-12-72			-
3-8-18-92			

Evidence of such low BF-potential erosion has recently been observed in a Nonpareil-clonal source originally identified for low BF-expression/BF-potential (**Table 1**). While increasing levels of BF-expression are expected in relatively young (20 years) clonal sources of Carmel because of its higher initial (seedling tree) BF potential, BF has not been previously observed in the generally more durable low BF-potential Nonpareil clonal sources selected in the 60s, 70s and 80s. The commercially important IR2 Nonpareil selection (3-8-2-70) was selected at a similar time and from similar material as the other industry important sources, Jeffries and McEnespy. BF expression in Nonpareil trees from this and related lineages has recently been documented with a slight increase in 2013 (**Table 1**). [Data in **Table 1** was developed from 20 plus year-old orchards of these initial clonal sources which are still present in some Sacramento and San Joaquin Valley locations]. Consequently, the BF expression levels serve as an indication of the BF-durability of these different sources. Southern San Joaquin Valley locations (Kern County in **Table 1**) consistently give some of the best assessments of long-term BF-durability (see Reference 12) because of the generally greater heat stress. [Interestingly, the IR2 (3-8-2-70) Nonpareil clone also shows some of the highest levels of cumulative production in recent San Joaquin regional trial studies by Bruce Lampinen et al. (See Project 13-HORT2-Lampinen, Field Evaluation of Almond Varieties and **Appendix C**).

Variety	DELTA	KERN	FPMS	Grower
Aldrich	-	-	-	
Butte	-	-	-	
Chip's	-	?		
Donna	-	-		
Fritz	-	-	-	
Jenette	-	X		
Jiml	-	-		
Johlyn	-	?		
Kahl	-	?		?
Kaperiel	-	-	-	
Livingston	-	-		
Milow	-	-	-	
Mission	-	-	-	
Monterey	-	-	-	
Morley	?	-		
NPU	-	-	-	
Padre	-	-	-	
Peerless	-	-	-	
Plateau	-	-		
Price	-	-	-	
Rosetta	-	-	-	
Ruby	-	-	-	
Sano	?	?		
Savana	?	-		
Sonora	-	-	-	
Wood Colony	-	-		
Yokut	?	X		
Winters	-	-		X
Kester	-	-	-	

Figure 8. Results from 2012-13 BF surveys from the Delta and Kern Regional Variety Trials as well as local grower trials and FPS foundation plotssources.

While careful selection in the 1960s, 70s and 80s of source material based on BF-expression (as determined using both such vegetative progeny tests and the more rapid test-crosses method described below), allowed continued production of low BF Nonpareil trees, even these more elite lines are beginning to again show BF. Reduced BF-expression may be maintained by carefully selection of those propagation lineages remaining free from BF-expression or returning to the original mid-1900s selections (where available). As part of this project, new FPS parent clonal stock were established via such basal epicormic buds rehabilitation (**Figure 7**) for the Nonpareil sessions (3-8-5-72), (3-8-2-70), (3-8-8-72) and (3-8-16-91) and Carmel accession 3-56-1-90.

Figure 9. BF-like symptoms on Winters trees in Fresno County in 2010-11.

Several recent varieties such as Yokut, Kochi and Jenette continued to show evidence of early BF expression in 2013 (**Figure 8**). However, since plantings of these varieties are not expected to be commercially

significant, the evaluation/selection of low BF-potential sources may not be warranted. A single case of potential BF in the more commercially important cultivar *Winters* has been identified in eastern Fresno County (**Figures 8 & 9**). The low number of trees showing symptoms also showed growth habits somewhat inconsistent with the Winters variety. Molecular analysis of leaf samples collected from these trees, however, has verified that they are the cultivar Winters (**Appendix B**). Winters has been known to be vulnerable to BF based on both lineage (it has the BF-affected cultivars Nonpareil, Harriet, and Jordanolo as parents, see **Figure 12**), however, from BF test-crosses [in an earlier Winters x high BF Nonpareil cross, progeny showed a low proportion of bud failure trees indicating a low BF potential]. The low potential for Winters was comparable to Sonora, which gave similar progeny test results and despite its extensive plantings has only shown the occasional BF tree). A more recent and more accurate test of BF potential involves the control crossing with an early flowering peach tester stock (UCD 40A-17) as described below in Genetic/Epigenetic Models. Results (described below) support an existing but low BF potential for the Winters cultivar. In addition, the bud-wood source used to propagate the early Fresno County test block trees where BF was observed was not from the established FPS foundation source, but was traced back to very early test plantings in the Stockton area which were later found to be virus-infected.

Figure 10. Bud-failure in the Marcona almond variety resulting from Prunus Necrotic Ringspot Virus infection.

BF-like symptoms have also been observed in Southern San Joaquin Valley *Marcona* plantings (**Figure 10**). Molecular (ELISA) analysis, however showed the symptoms to be the result of virus induced bud-failure, in this case due to infection of Prunus Necrotic Ringspot Virus (PNRSV in **Appendix A**). The virus was also verified through graft-transmission (work done in cooperation with FPS labs). Extensive source selection/virus testing of different Marcona clones has identified a single tree source which has been shown to be negative for both Prunus Necrotic Ringspot Virus and Prunus Dwarf Virus (**Appendix A**). In 2013, this clonal source material has been transferred to FPS foundation stock orchards after undergoing final trueness-to-type testing and has been included for long-term evaluation in the new RVT trials.

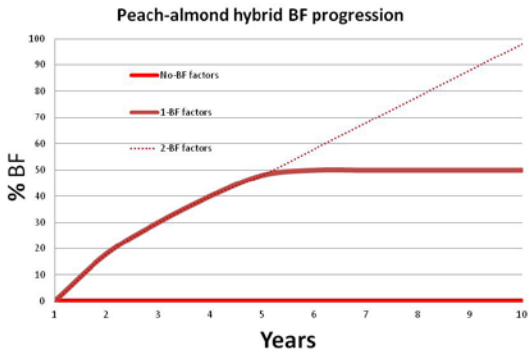


Figure 11. Expected peach by almond progeny performance when the almond parent contains one or two high BF-genes forms.

<u>Genetic/epigenetic models and associated molecular-based diagnostics.</u>

Different genetic control models, including control by 1 to 3 Mendelian-type genes, as well as various epigenetic mechanisms are consistent with observed segregation patterns (**Figure 1 & 11**) when the almond parent contains BF. In almond by almond crosses, the possible interaction between functional and non-functional forms of the BF gene(s) is possible because each parent will contribute a genetic factor and the presence of a functional BF factor may be masked by the presence of a nonfunctional BF-factor. However, previous work with almond by peach interspecies hybrids, (**Figure 11**), has demonstrated that the very early flowering peach tester (UCD40A-17) appears to lack a BF-type gene and so would not act to mask any aberrant BF-gene expression of the almond parent tested. With no homologous BF-functional gene to mask the expression of BF-expressing genes, progeny should show BF-symptoms when BF-forms of the gene are present. Because BF-factors would be inherited entirely from the almond parent, the performance of the peach by almond progeny could be used to precisely determine the almond parent genotype as shown in **Figure 11**. If the almond parent contained no BF-inducing factors/genes then no progeny would show BF (solid basal red line in **Figure 11**). If the almond parent had one BF factor and one normal factor than only half the progeny would be expected to eventually show BF (curved rust line in **Figure 11**). If both factors/genes in the tested almond parent were BF then all progeny would be expected to eventually show BF (dotted line in **Figure 11**). Thus progeny performance can identify the BF-potential of almond parents even when no BF has previously been observed in those parents, though the test requires several years for completion. In addition, data from earlier studies suggest that the strength of BF-potential in the almond source will be correlated with the rate of BF expression in the seedlings and the final level of BF expression in individual seedlings.

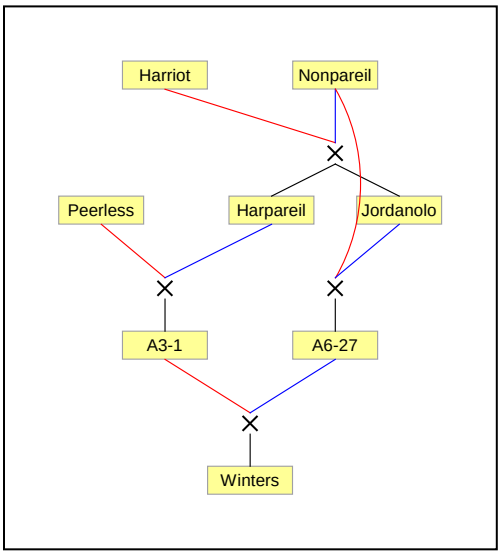


Figure 12. Lineage analysis of the Winter's variety as developed using PediMap software which may allow the correlation of BF-expression with specific molecular markers (if determined by genetic differences) and possibly epigenetic markers (if determined by a change or alteration in gene function/activity).

Thus, while test-cross progeny from an almond x high-BF almond cross in are useful in identifying low-BF sources within the same clone, test-cross progeny from almond x early-flowering peach testers are useful in the early identification of general BF-potential of new breeding selections and varieties such as Winters. We are currently in the fifth year of progeny testing from a Winters by UCD40A-17 test cross. Of 25 individuals in the population, none has shown bud- failure to date though according to the peach-almond gene model, approximately 30% of the individuals should be showing bud failure if Winters was a strong carrier. Similar results have also been obtained with Sonora and other well-established almond cultivars such as Peerless, which have occasionally shown bud-failure symptoms, but only in isolated instances. Because of Winters unique and well-established lineage (**Figure 12**) and it's having both the Nonpareil and Jordanolo as parents, this variety as well as high-and low-BF Nonpareil clones and breeding selections are being further analyzed using high-resolution genetic mapping. Association mapping procedures could then be used to identify certain genetic

combinations in progeny which are always associated with BF expression even if those genes are not causative (i.e., the BF-gene). These genes might then be used as markers (since their

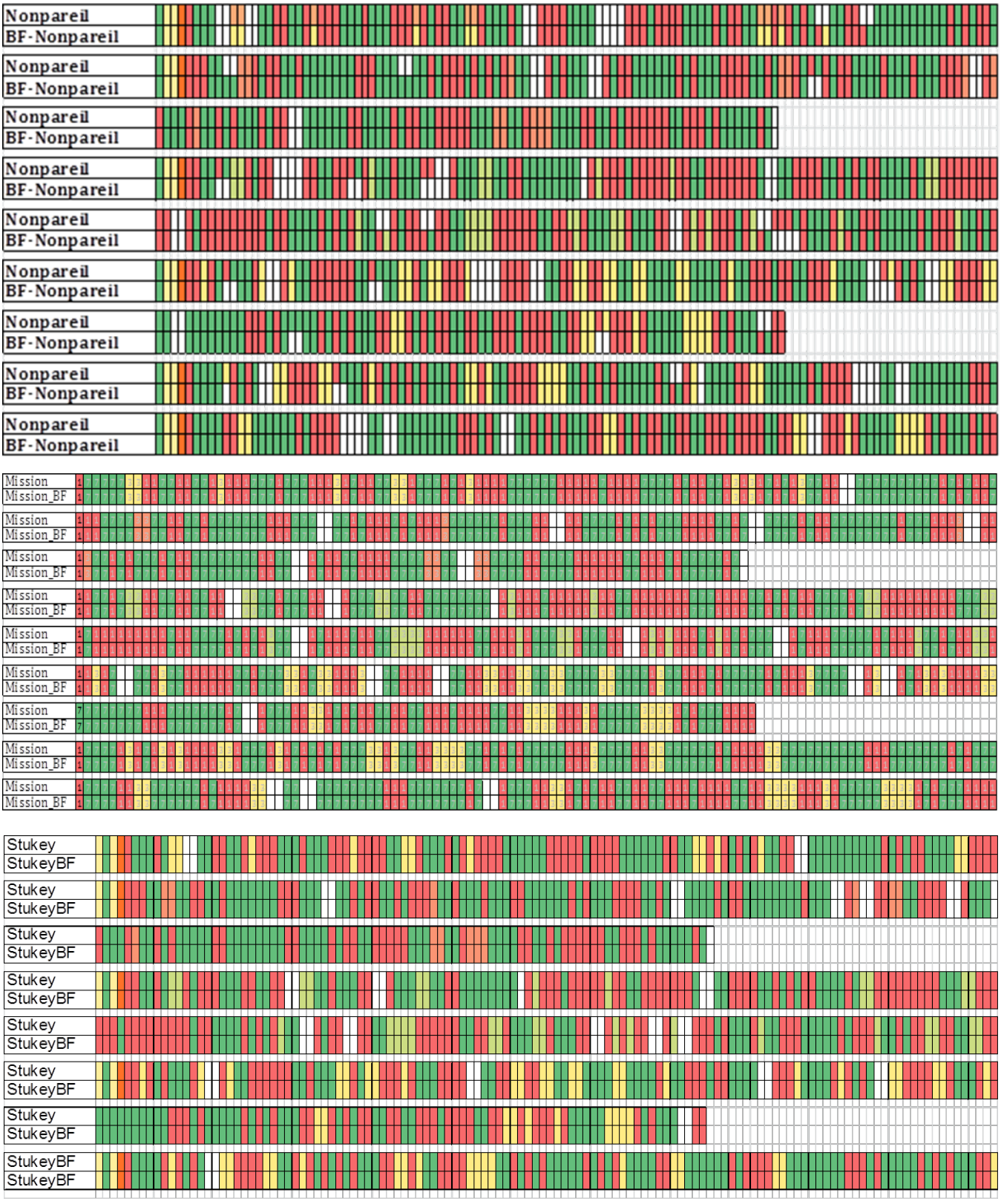


Figure 13. Sampling from final RosBREED analysis of high-BF versus low-BF Clones of *Nonpareil*, *Mission* and Stuckey (a synthetic clone derived from embryo budding) showing no genetic differences in the over 500 markers saturating all eight Prunus chromosomes.

association with that trait indicates they are closely linked to the causative gene) as well as a starting point to identify the specific causative gene. Final results from the 2010-2013 USDA-funded RosBREED project, however, have shown no marker differences between high-BF and low-BF clones of key varieties (**Figure 13**). Results support, but do not prove, the hypothesis that BF is due to a change in function (epigenetic change) of the BF-gene rather than a change to genetic nucleotide structure (mutation) which is required for standard marker assisted selections.

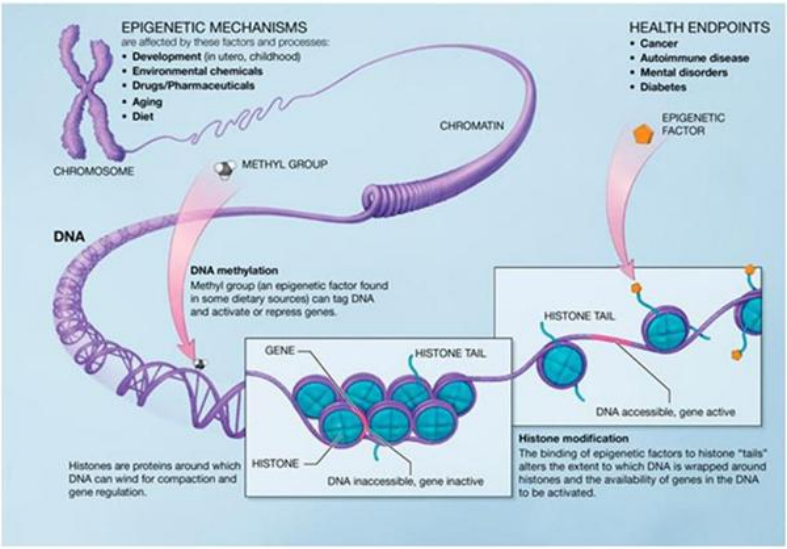


Figure 14. A summary of possible epigenetic mechanisms from more advanced human studies where control of a trait is determined not just by the simple presence or absence of a gene but rather by epigenetic mechanisms which act to enhance or suppress expression of genes.

Because, as previously described, genetic differences may be discernible in certain intraspecific peach by almond hybrids as presence/absence rather than variability in the expression level, several hundred progeny from a high-BF Carmel by UCD40A-17 test cross, in which progeny are expected to strongly segregate for BF (based on previous performance), have been generated in 2011-13 and planted in the spring of 2014. The presence and extent of BF in individual progeny trees will be rated based on criteria developed in literature (See citation 12). Information on the time that BF was first observed in individual progeny trees will also be included in the database. The rate of BF progression in both individual trees as well as in the combined progeny population will be evaluated as a possible predictor of BF-potential of the almond parent variety. Inheritance models supported by this preliminary data will then be evaluated. Previously established genetic relationships (See citations 3, 4, 8) among almond varieties tested will also be considered when evaluating inheritance models.

Epigenetic model.

Standard genetic dogma states that a trait such as BF results from the action of a specific protein controlling a specific plant developmental process. Since the specific protein structure is coded for by a unique sequence of DNA (gene), the definitive marker for that trait is the DNA sequence coding for the controlling protein. This model has proven successful in describing and genetically manipulating numerous processes in plant development and has led to a proliferation of accurate DNA-based molecular markers for many traits (**Appendix D**). Previous findings suggest that BF is a genetic disorder in almond which is expressed as a failure of vegetative bud growth leading ultimately to tree decline. Current data indicates that BF does not fully follow the standard genetic model but rather is due to the failed expression of a gene/gene complex required for normal growth and development. In this case the DNA sequence (gene) is identical in both the normal and BF condition, obviating the value of traditional molecular markers as predictors of this disorder.

Figure 15. 104-year-old Mission (top-left) and Nonpareil (top-right) trees use for clonal age sampling. Sample of the fruiting spur from the top of the trees from which 'Top' leaf samples were collected for testing (Bottom left). Epicormic shoots from the base of the trees from which 'Base' leaf samples were collected (Bottom-right, arrow).

The aberrant nature of such 'epigenetic' conditions have discouraged their research in mainstream genetics with most early studies limited to genetic disorders with dramatic economic consequences, such as almond BF and cherry crinkle and also some disorders and diseases in humans (**Figure 14**). Recent advances in our understanding of organismal genomics has shown that a diversity of epigenetic mechanisms exists which can play important roles in development. This realization has led to a research surge on epigenetic mechanisms, including the development of more accurate molecular-based diagnostics and possible treatments. For models based on standard Mendelian-gene control, a diverse array of molecular-based diagnostics is available (As summarized in literature citations 3, 4, 8, 14, 16, 21). In this case the choice of molecular diagnostic would be made using standard marker assisted selection approaches such as PediMAP/Flex QTL software (see **Figure 13** and citation 8). Initial field data, however, shows non-Mendelian segregation patterns, again supporting epigenetic control. Unlike Mendelian genetic control, where genes/traits are either present/absent, epigenetic mechanisms can vary in their degree of trait suppression resulting in varying levels of BF-phenotype.

Epigenetic analysis.
Objectives:

A. Obtain DNA methylation profiles from a set of clonal sources of different ages and BF-potentials.
B. Correlate DNA methylation profiles with differing clone ages as well as differing BF-potential within clones of the same almond variety as well among different varieties for BF-potential.
C. Identify genomic regions associated with advancing clone age and possibly BF expression as a basis for future studies to develop more specific and so accurate molecular markers flanking these regions.

Materials and Methods:

Thirty-seven different selections showing either differences in BF, differences in clonal age, or both, were selected for testing (**Table 2**). BF- level was determined by vegetative progeny testing as described in Kester et al. (Citation 12). High BF rating indicated that the sample was taken from a tree showing BF expression. Medium- BF ratings were given to trees that showed no BF expression but were known by from previous vegetative progeny testing to express BF in vegetative progeny while to low-BF rating indicated that the sample was taken from trees that showed no BF expression and were known by from previous vegetative progeny testing to not show BF in vegetative progeny.

Samples differing in clonal age were also collected from 104 year old almond trees from a dry-land almond orchard in the Capay Valley near Esparto, California. Leaf samples were collected from the most recent top growth, from mid-level growth, and from epicormic shoots at the base of the tree (**Figure 15**).

The Methylation-sensitive representational difference analysis (MS-RDA) procedure was followed as recommended by Ushijima and Yamashita (Citation 17) and adapted as needed for almond samples (which usually contain high levels of phenolic compounds). MS-RDA is a genome subtraction method that isolates DNA fragments differentially methylated between two genomes.

Table 2. Thirty-seven different selections showing differences in BF, differences in clonal age, or both.

C#	Item	Source	BF potential	Age
1	Carmel	WEO	High	
2	Carmel-Mod-BF	Arb-Marine Rootstock	Medium-Hi	Medium
3	Carmel-OK	FPS19-9	Medium	Medium
4	Drake-Base	Esparto	No-BF	Low
5	Drake-Middle	Esparto	No-BF	Medium
6	Drake-Top	Esparto	No-BF	High
7	Mission-BF	WEO-BF	High	Medium
8	Mission-OK	FPS19-13	No-BF	Medium
9	Nonpareil-Base-OK	Esparto	Low	Low
10	Nonpareil-Top-BF	Esparto	High	High
11	Nonpareil-BF	Arb Nonp Lane	High	Medium
12	Nonpareil-Mod-BF	Arb-Marine Rootstock	Medium-Low	Medium
13	Nonpareil-OK1	FPS21-17	Medium-Low	Medium
14	Nonpareil-OK2	FP21-25	Medium-Low	Medium
15	Nonpareil-OK3	FPSxx-x	Medium-Low	Medium
16	TurkmenTopBF	Repo	High	Medium
17	TurkmenBaseOK	Repo	Low	Medium
18	Peerless-Base	Esparto	No-BF	Low
19	Peerless-Middle	Esparto	No-BF	Medium
20	Peerless-Top	Esparto	No-BF	High
21	Primal 161	2007,12-161	No-BF	Low
22	Primal 164	2007,12-164	No-BF	Low
23	Primal 192	2005,20-192	No-BF	Low
24	Primal 209	2007,12-209	No-BF	Low
25	STU 5-1-OK	F5C, 5-1	No-BF/Lo	Low
26	STU 5-2BF	F5C, 5-2	High	Low
27	STU 6-BF	F5C, 6-7or9	High	Low
28	STU 6-OK	F5C, 6-8	No-BF/Low	Low
29	Winters-OK	FPS2137	No-BF	Medium
30	Winters-BF	DELTA-BF-BROWNE	High	Medium
31	BF Winters-Upper	R11 fr N east	High	Medium
32	BF Winters-Lower	R11 fr N east	High	Medium
33	"Healthy" Winters	Greg Browne	No-BF	Medium
34	Carmel A	Billings-Kern	Mid	Medium
35	Carmel B	Billings-Kern	Mid	Medium
36	MONTEREY-OK	FPS21-13	No-BF	Medium
37	MONTEREY-PeudoBF	Arb-Marine Rootstock	No-BF	Medium

Results and Discussion:

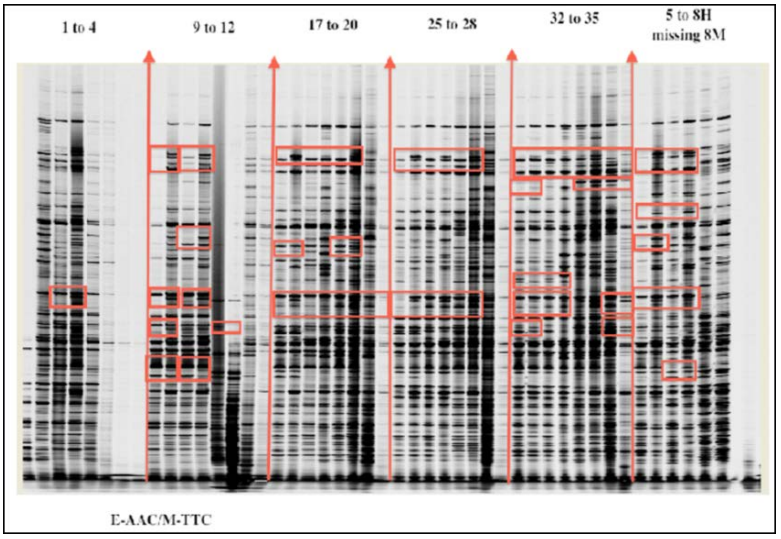


Figure 16. Results from 2013 showing a large number of variable and so potentially useful markers from MS-RDA, which utilizes the methylation-sensitive restriction endonuclease HpaII to recognize the 5'-CCGG-3' 4-bp motif and thereby isolates DNA fragments differentially methylated within clone variants {orange boxes}.

Over 12,000 markers were evaluated for clones of different BF-potential as well as clonal age. Promising markers showing clear differences among the tested samples were identified using the Methylation-Sensitive Representational Difference Analysis (MS-RDA). This technique utilizes the methylation-sensitive restriction endonuclease HpaII to recognize the 5'-CCGG-3' 4-bp motif and thereby isolates DNA fragments differentially methylated within clone variants (orange boxes, **Figure 16**). This test represents one of the first successful applications to Prunus and the first in almond, of epigenetic differentiation via methylation patterns. Because of the huge amount of data generated, the major challenges accurate interpretation of the data. This is typically achieved through powerful statistical packages which can identify significant associations in the data (in this case between certain methylation patterns in the degree of BF and, separately, the clonal age of the cultivar being tested). Within any individual clone, we would also expect to see a correlation between clonal age and potential for showing BF. The difficulty was such statistical analysis is that the results are only as good as the data. While a certain amount of error is to be expected in the development of the marker profiles (for example, see discussion of RosBREED marker errors in the 2012/13 Almond Variety Development report, Project 13-HORT1-Gradziel), the bigger challenge here is an accurate characterization of both clonal age and BF potential as both involve a large degree of estimation. *(Differences in clonal age are given in estimated years from clone divergence from non-BF to BF types).* DNA methylation / demethylation profiles for the pooled data are summarized in **Figure 17**. The graphical display strongly hints at differences between BF and non-BF types that are difficult to determine; however, it is not definitive because of the limited number of clones. **Figure 18** displays results when markers are selected based on Mission BF phenotype and then compared to a more limited number of other genotypes where BF potential is more defined. Interestingly, almost identical patterns are observed between non-BF and BF Mission and non-BF and BF Winters with some additional agreement with other genotypes. Because BF is particularly rare in both Mission and Winters, they were felt to be good initial candidates for inspection. However the extensive nature of these differences as well as their apparent general diffusion throughout the eight chromosomes (data still under evaluation) suggests that the association may be primarily with clonal age and only secondarily with BF (as BF would be expected to be correlated with clonal age).

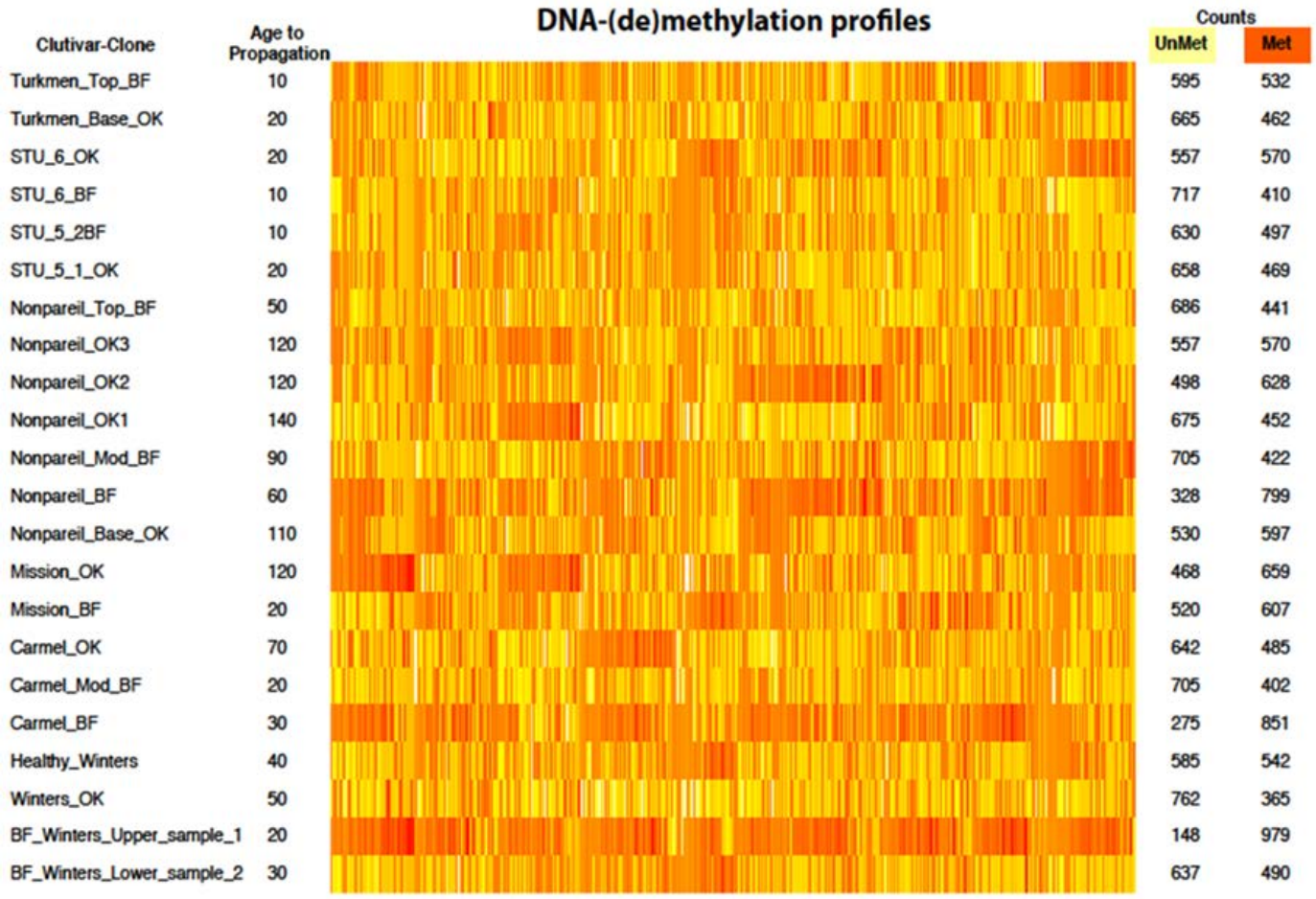


Figure 17. Graphical summary of DNA methylation/demethylation profiles for the evaluated clones. Propagation age is given in estimated years of clone divergence from non-BF to BF types. Counts (right columns) are for the number of unmethylated to methylated markers.

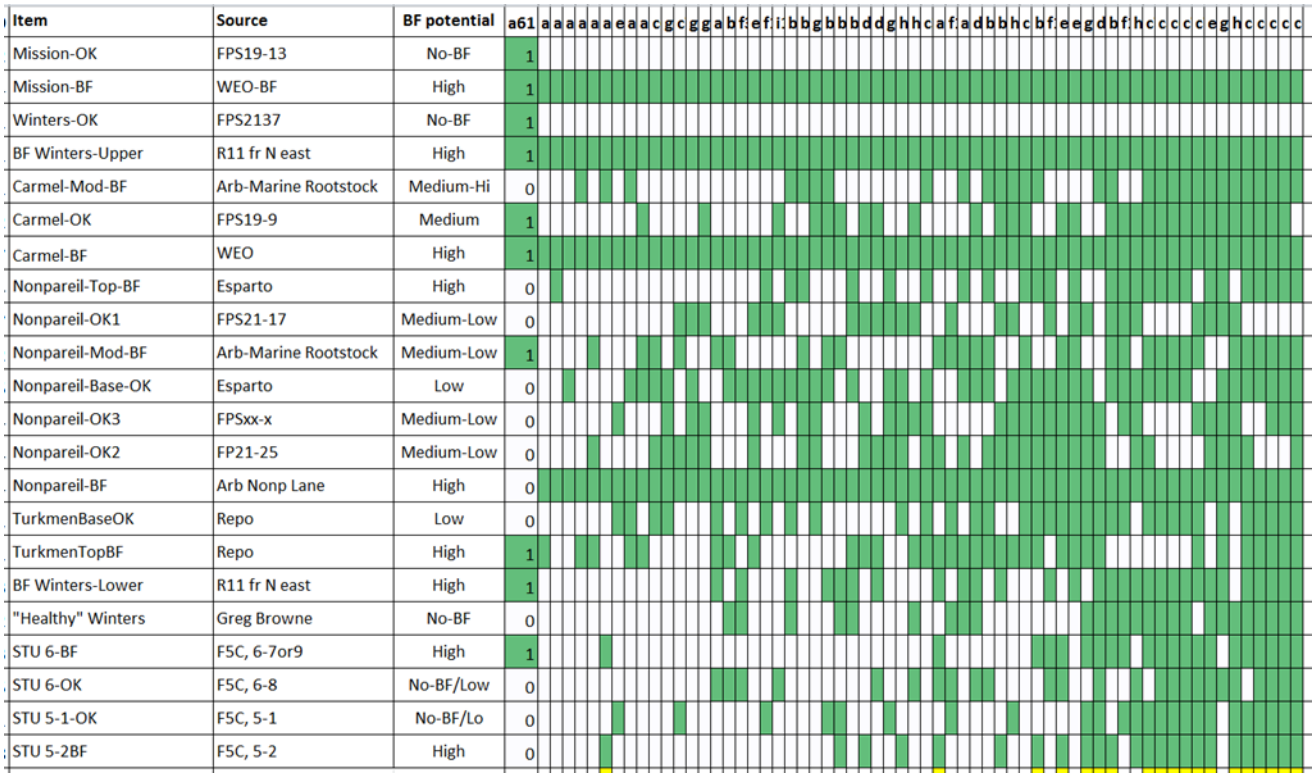
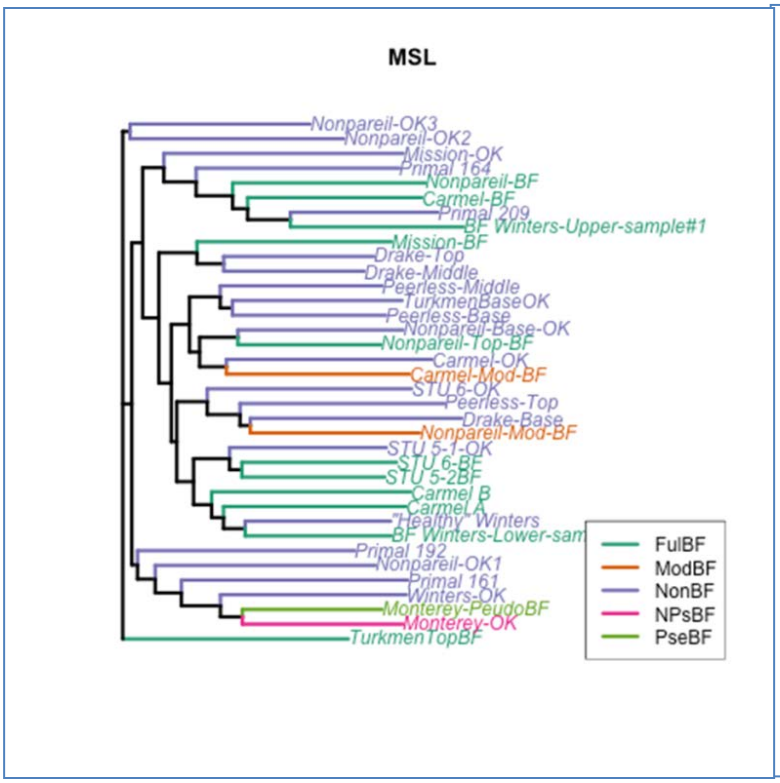


Figure 18. Association patterns for markers selected for differential expression between BF and non-BF expression in the cultivar Mission for BF occurs but is very rare. An almost identical pattern is observed in Winters were BF expression is also very rare, suggesting markers may be more for clonal aging residents specific BF phenotype.

A pooled analysis of all the methylation-sensitive markers with BF expression for different clonal sources using more powerful, but obtuse statistical methods shows greater promise for differentiating between clones of differing levels of BF as well as different 'age' status (**Figure 19**). Several, though not all, profiles suggest the ability to differentiate BF from non-BF clones. The ability to differentiate clones with low BF potential (no BF expression in original tree and vegetative progeny from that tree) versus medium BF-potential (no BF expression in original tree but some levels of BF expression in vegetative and/or sexual progeny) also appears promising for some clones. This ability would be crucial to the application of such markers since the real need by industry is to be able to select among non-symptomatic clones for those sources that will remain nonsymptomatic in vegetative progeny and against those sources which would show BF symptoms (within the first 5 to 10 years) in vegetative progeny. Because of the value of such a marker and its utility even within a single clone such as Nonpareil or Carmel, it would be particularly useful; however, a marker may be inherently more error-prone if it is associated with ('linked' or close by) the gene but not actually involved in the epigenic event.



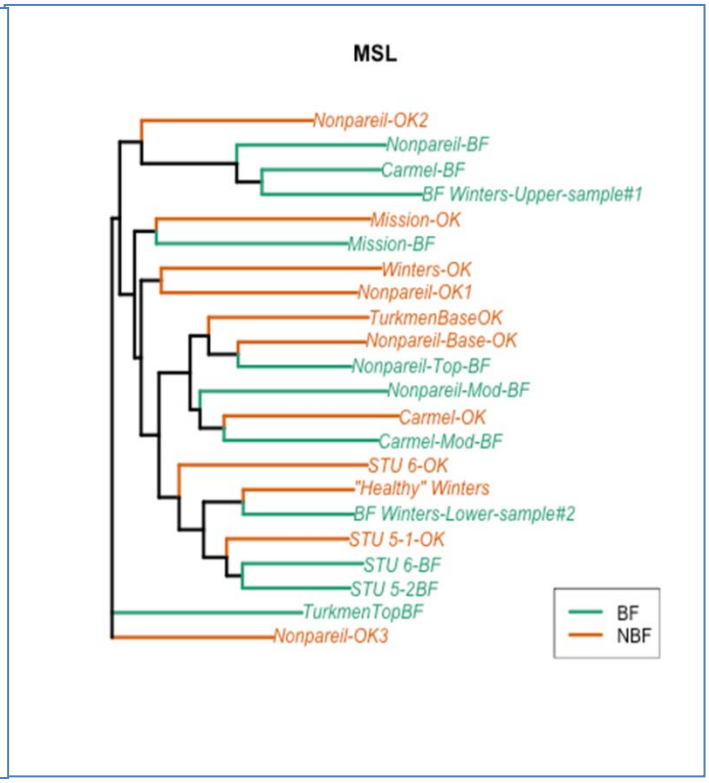


Figure 19. Pooled statistical analysis of all the methylation-sensitive markers with clone age and/or BF expression for different clonal sources shows differing differentiation profiles depending on initial assumptions (estimations) for clone age and specific level of BF-potential. Several, though not all, profiles suggest the ability to differentiate BF from non-BF clones. The ability to differentiate clones with low BF potential (no BF expression in original tree and vegetative progeny from that tree) versus medium BF-potential (no BF expression in original tree but some levels of BF expression in vegetative and/or sexual progeny) also appears promising for some clones are not others.

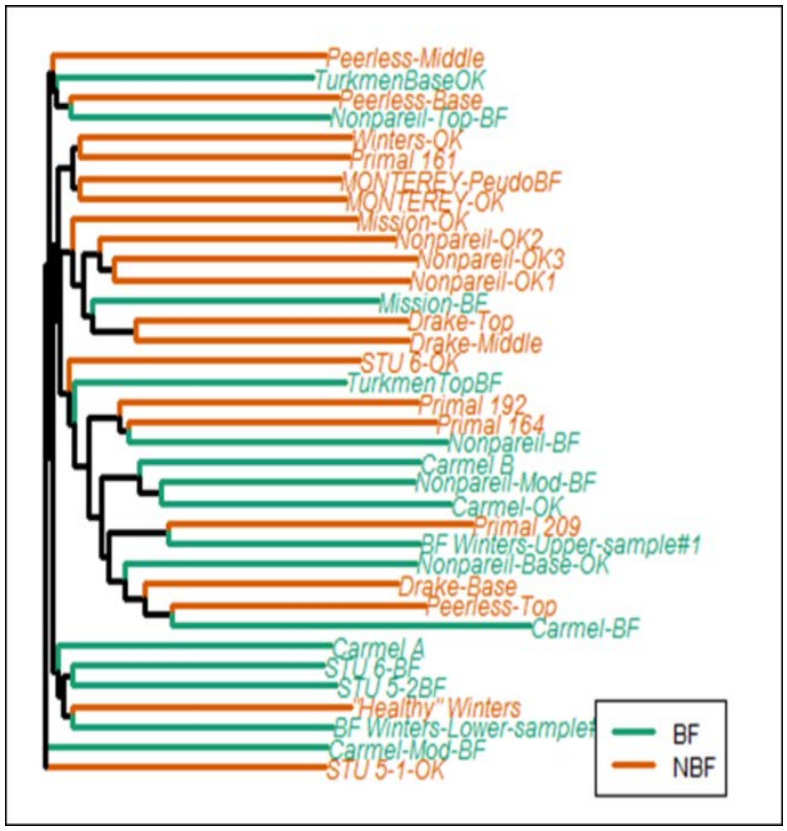


Figure 20. Markers selected for their strong association with BF expression in a subgroup of clones whose BF-potential scores were based on more extensive field testing of BF expression in vegetative progeny, resulting in more accurate BF scoring. Two markers (blue arrows) show good correlation between marker expression (level of methylation) and BF expression, and so represent candidates for more detail genetic analysis.

Thus while preliminary data is very promising, the verification of utility will require further analysis both in focusing in on markers most strongly associated with clonal aging and/or BF expression, and concurrently, more accurately defining what is meant by clonal age in BF expression. Consequently, success will depend on large-scale statistical analysis anchored by an accurate characterization of the processes involved. Thus we are pursuing the analysis in a convergent process where we are using the statistical analysis to identify promising marker candidates and then testing whether those candidates facilitate the development of a specific developmental model as specific genetic targets for further analysis. For example, in **Figure 20**, two markers have been identified which show a very high association with BF expression in a select group of clones were competence in the level of BF-potential is based on an extensive assessment of vegetative progeny populations and so more dependable. We are currently determining whether this association extends to other clones in the analysis and, concurrently working to determine the function of the genes marked. While a successful identification of marker(s) highly correlated with BF expression (and/or BF expression in vegetative progeny without expression in the initial clone) is the final goal of this research, more effort will probably be required than the limited time of this project. As initially stated, however, the goal of this project was to develop a solid foundation upon which to build a more extensive proposal soliciting larger-skill federal funding.

Appendix A. ELISA confirmation that BF-symptoms in Marcona are the result of infection by Prunus Necrotic Ringspot Virus (PNRSV; PDV – Prunus Dwarf Virus).

ELISA Testing for Marcona trees

	PNRSV		PDV
Marcona, tree BL7	positive	4/30/2010	negative
Marcona, tree DRT3	positive	4/30/2010	negative
Marcona, tree DRT4	positive	4/30/2010	negative
Marcona, tree DRT7	positive	4/30/2010	negative
Marcona, tree DRT11	negative	4/30/2010	negative
Marcona, tree DRT14	positive	4/30/2010	negative
Marcona, tree DRT18	positive	4/30/2010	negative

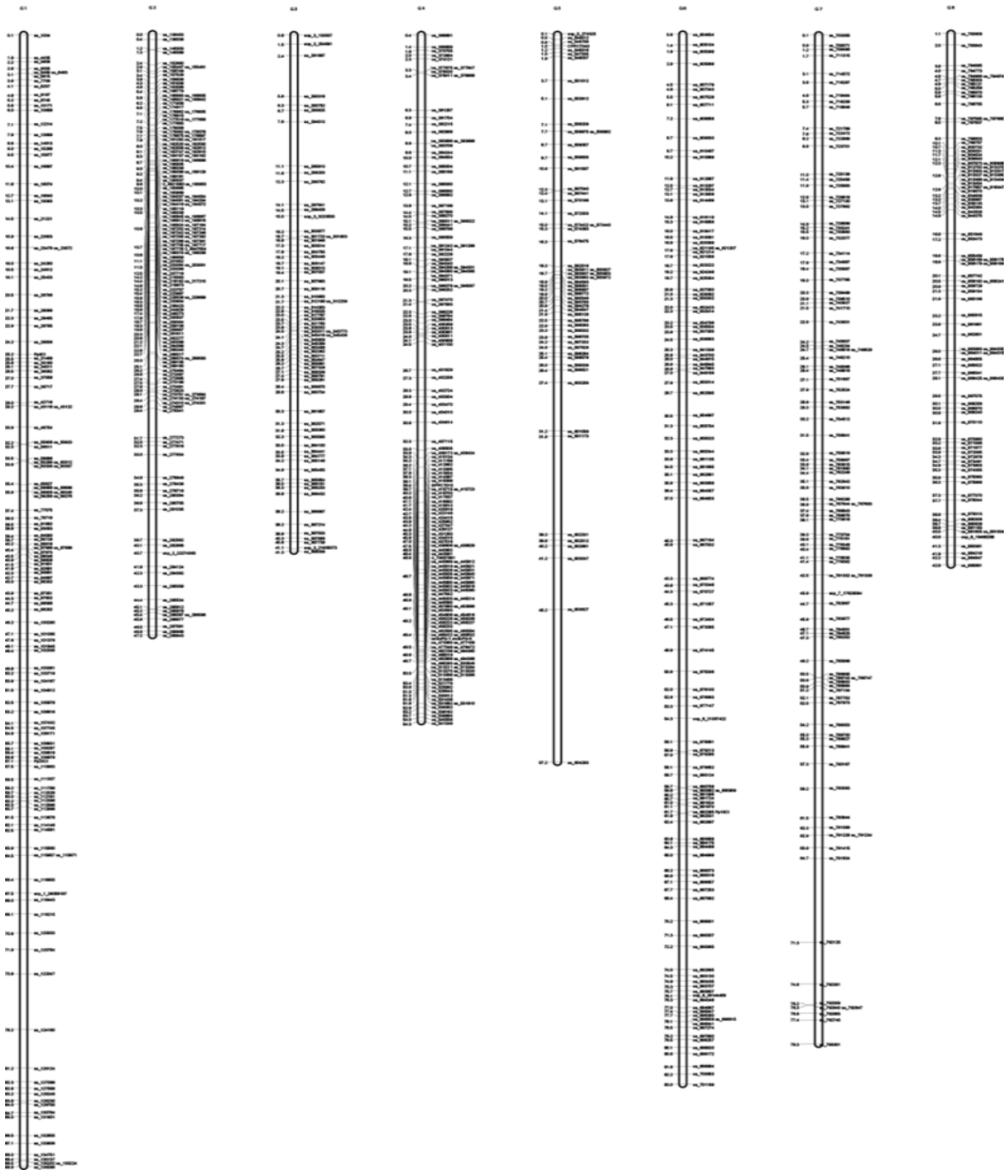
Appendix B. Molecular marker analysis verifying that the affected trees (Brown-Winters) are the Winters variety and not a propagation error.

NONPAREIL	182	194	130	148	142	146	211	233	99	110	212	259	224	236	155	155	148	158	151	167	115	121	134	152
PADRE	182	196	122	180	136	142	199	209	99	108	227	227	236	244	129	147	148	148	143	149	141	145	146	168
PRICE	194	196	146	148	130	148	199	211	110	114	212	227	224	236	129	155	148	148	167	167	121	141	134	166
RUBY	194	196	122	142	136	146	199	211	108	110	227	227	224	236	147	155	148	156	143	167	141	145	134	168
SOLANO	182	194	130	130	130	142	211	233	99	99	212	255	224	236	155	155	138	158						
SONORA	182	194	148	148	130	142	211	233	99	99	255	259	224	236	145	155	138	158	149	151	115	121	134	152
THOMPSON	194	216	122	130	130	142	203	211	99	114	212	227	224	236	147	155	136	148						
WINTERS	182	200	130	136	132	132	233	233	116	116	227	229	236	242	155	155	146	158	143	167	121	145	134	134
Brown-Winters	182	200	130	136	132	132	233	233	116	116	227	229	236	242	155	155	146	158	143	167	121	145	134	134

Appendix C. Yield performance of selections at the Billings Regional Variety Trials showing particularly high yields of Nonpareil clonal source (3-8-2-70) (from Bruce Lampinen 2011/12 RVT Annual Report)

				Kernel pounds per			
2011 Variety	No. of nuts/tree	Average kernel wt (g)	Shelling percentage	unit PAR int.	Tree	Acre	Cumulative kernel yield (lbs/acre)
Nonpareil-Nico	18776.9 a	0.99 bcde	68.0 abc	86.7 a	41.0 a	4964.2 a	19522.7 a
Nonpareil-3-8-2-70	17744.2 abc	1.05 bc	70.7 a	87.9 a	41.0 a	4962.3 a	18878.1 ab
Nonpareil-Newell	17790.9 abc	1.00 bcd	70.1 ab	81.0 ab	39.2 a	4744.7 a	18746.5 ab
Nonpareil-Driver	17943.0 ab	0.98 bcde	66.0 abcd	84.3 a	38.7 ab	4682.6 ab	18593.4 abc
Nonpareil-5	15744.6 de	1.03 bc	70.4 ab	78.0 ab	35.9 abc	4341.9 abc	17886.9 bcd
Nonpareil-6	16630.0 bcde	1.04 bc	70.0 ab	81.6 ab	38.1 ab	4618.5 ab	17838.3 bcd
2-19e	18253.3 ab	0.91 bcde	64.8 abcd	73.6 ab	36.8 ab	4459.7 ab	17560.0 bcd
Nonpareil-7	17078.8 abcd	0.83 e	69.2 abc	76.1 ab	31.4 bcd	3804.0 bcd	17235.0 cd
Nonpareil-Jones	16992.6 abcd	0.96 bcde	70.0 ab	81.6 ab	36.0 abc	4359.4 abc	17050.7 d
Winters	15979.0 cde	0.83 e	58.7 ef	76.3 bc	29.3 cde	3553.5 cde	14757.0 e
Chips	11900.6 f	0.94 bcde	60.3 de	51.4 de	24.6 de	2984.7 de	13917.8 e
Sweetheart	14969.2 e	0.86 de	64.1 bcde	52.5 de	28.2 de	3411.8 de	13712.5 e
Kahl	12420.0 f	0.89 cde	53.5 f	59.1 cd	24.4 de	2953.2 de	13514.3 e
Marcona	9633.4 g	1.07 b	30.8 g	51.8 de	22.7 e	2746.0 e	12053.7 f
Kochi	8701.4 g	1.22 a	63.5 cde	43.4 e	23.3 e	2825.2 e	11246.5 f

Appendix D. Improved almond by peach molecular marker map developed by our program (Citation 22 and in review) using 864 markers (860 SNPs and 4 SSRs). Molecular markers in almond by peach test progeny which may be found to be highly correlated with BF expression can then be used both as a marker or predictor of BF as well as a starting point to identify the specific gene(s) controlling this trait.



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