Hort Innovation

Final Report

Building a genetic foundation for Australia's citrus future

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Delivery partner:

Queensland Department of Agriculture and Fisheries

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CT15017

Project:

Building a genetic foundation for Australia's citrus future (CT15017)

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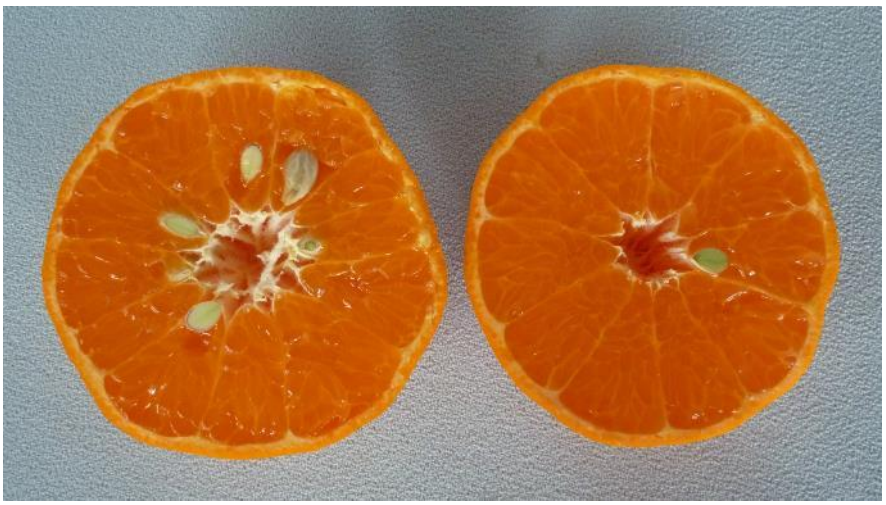
Content

Summary

The main highlight of this phase of the Australian Citrus Breeding program (CT15017 – Building a genetic foundation for Australia's citrus future) was the release of two new mandarin varieties and their commercial adoption. Known as 'Premier Murcott' and 'CB Murcott' these new varieties were selected from large field populations of hybrids and then re-selected to reduce their seed number. The development of these two new varieties represent a concerted effort to combine attractive fruit appearance with good eating quality.



'Premier Murcott', released in 2019 and now in commercial production. Bottom image shows '01C011' (RHS) fruit prior to mutation breeding with 'Premier Murcott' (LHS) the result.

'CB Murcott' released in 2021.
A later maturing variety with good colour, flavour, ease of peeling and low seeded.

At project conclusion over 40,000 unique hybrids are growing in the field representing genetic combinations aimed at addressing all the traits required for commercially successful new citrus varieties.

A large focus through this program has been on achieving seedlessness and the multiple strategies tested to achieve this goal. Work with a single gene for seedlessness has demonstrated its stability and effectiveness under Australian conditions and there are already more than 7,000 hybrids in the field from families carrying this gene. It has become the main seedlessness strategy and most crosses made in the final year of the project will segregate for this gene. Conversely, there has been a staged retreat from triploid breeding, recognizing that the Australian industry cannot justify the level of resourcing needed to generate large enough populations of triploids with high fruit quality; along with disappointing results in terms of fruit quality, productivity, thorns and seediness. A new opportunity for seedlessness was serendipitously discovered during the project, occurring in one small family where approximately 50% of the progeny were seedless. This cross has now been repeated in far higher numbers along with other combinations using both parents, with the objective of understanding the mechanism involved. The tried-and-proven strategy of mutation breeding has been applied to a high-quality orange-like hybrid to reduce seed numbers with good survival of treated buds at a high dose rate. These multiple strategies for seedlessness reflect the growing importance of this trait and the enormous challenge it has been for citrus breeders. New and effective strategies have been quickly adopted in the program with large progenies already well established under field conditions. The new releases 'Premier Murcott' and 'CB Murcott' are low-seeded and it is expected that future genetics developed from these varieties and their families will have even less seeds.

Orange breeding has become an important component of the Australian breeding effort, made possible by an understanding of the original parents of the Sweet orange and the availability of a high quality pummelo to re-construct this citrus type. This high quality pummelo is now the single largest seed parent represented in field plantings. This work complements the mandarin breeding activities because of the opportunity to also recover Citrus black spot resistance from this parent. We believe there are considerable opportunities to improve the sweetness of oranges using a strategy of backcrossing to recover the required peel colour.

Natural genetic disease resistance has been a cornerstone of the Australian breeding work and the commercial relevance of this continues to increase. Market pressures are bringing a real urgency to the need for non-chemical growing methods and these pressures threaten to out-pace the breeding effort. All hybrids field-planted during the life of this project are genetically resistant to Alternaria brown spot and Citrus scab thanks to efficient screening techniques developed at Bundaberg. The program also successfully challenged the long-held view that all citrus are susceptible to Citrus black spot by demonstrating the occurrence of natural resistance in pummelo and the ability to transfer this resistance to the program's pipeline of genetics via conventional hybridisation. Converting this new discovery into an efficient screening technique remains a challenge. Significant progress has also been made in capturing Citrus tristeza virus resistance in scion breeding material and the use of molecular markers to incorporate additional disease resistance regions from *Poncirus*.

Australian citrus growers and industry are encouraged to contact the DAF Citrus breeding team or Hort Innovation if they would like more information.

Keywords

citrus, orange, mandarin, breeding, triploidy, disease resistance, seedlessness, irradiation, alternaria brown spot, citrus scab, citrus black spot, citrus tristeza virus, huanglongbing

Introduction

A comprehensive breeding program with access to diverse genetics is critical to sustaining the long-term viability of one of Australia's largest horticultural industries. This program efficiently employs all breeding technologies and germplasm in an integrated program focused on market outcomes and economic benefits. Excellent grower and industry involvement, linkages with domestic and international collaborators, and a strong breeding team continue to drive genetic progress while maximising returns on investment.

Methodology

The program is predominantly a conventional breeding effort, predicated on purposeful parent selection and the efficient screening of large hybrid populations. It is built on multiple generations of hybridization with high selection intensity designed to 'fix' important traits within the breeding populations. Improvements are sought for many traits and almost all of these traits segregate widely even when both parents share a similar phenotype. Such high levels of heterozygosity make it challenging to simultaneously improve multiple traits, hence the need for large field populations and the desire to fix as many traits as possible within breeding populations. Even more challenging is the phenotyping of fruit quality traits, which can vary enormously within the same genotype. "What you see is not what you get" and promising selections must be phenotyped on multiple occasions across multiple seasons before any decision can be made of their merit. Consumers have high expectations for citrus and the Australian industry is based on supplying the upper end of the high-quality export market. There is no place for a breeding program that delivers mediocre new varieties.

Perhaps the biggest methodological challenge for citrus breeding over the past 100 years has been seedlessness. It has been a curse for breeders and prevented them from tackling so many other opportunities that exist within the genus. Many different strategies have been pursued (e.g. triploid, irradiation, pollen sterility, self-incompatibility) most of which are cumbersome and inefficient and/or result in genotypes which are low-seeded rather than truly seedless. All of these strategies have been used by the breeding team with triploid breeding being a major focus at the start of this phase of the program. More recently a new methodology became available, enabling breeders to incorporate a single gene for seedlessness using conventional hybridization. This is now our main seedlessness methodology and large segregating field populations are already established at Bundaberg. F1 populations demonstrated the effectiveness of the gene but fruit quality traits were poor so backcross populations (using high quality parents from the program) have been constructed. This is an exciting methodology development for citrus breeding, not only because it addresses a major concern of consumers (seedlessness), but also because it enables breeders to shift their focus onto so many of the other traits that require improvement.

Amongst these other traits is disease resistance, and citrus is afflicted with more devastating diseases than any other tree crop. Methodologies for efficiently screening for Alternaria brown spot and Citrus scab were established by the project team (Smith et al. 2016a) and are now standard practice in the Australian breeding program. All hybrids field planted during CT15017 have natural genetic resistance to these two diseases. A methodology for virus resistance breeding and screening was also devised (Smith et al. 2016b) and this is applied to all populations with a *Poncirus* background.

Molecular methods have also been incorporated at appropriate places within the breeding program. A SNP marker for Alternarian brown spot resistance has been run on all the major parents and this information was used in the last two years of the project to confirm parent choices in the crossing program and sowing density in the nursery. Markers linked to disease resistance regions on the *Poncirus trifoliata* genome have been check on parents chosen for the next generation of crossing. They are of marginal value but can now be generated at moderately low cost and so are likely to be used more widely to aid with parent selection decisions.

The methodology for industry engagement and consultation also warrants mention because it is efficient and effective. Major field plantings are located on commercial properties which regularly exposes the breeding team to industry concerns while also providing an opportunity for other growers, consultants and packers to see the varieties under real conditions. Secrecy is a dangerous thing in tree crop breeding because it hides germplasm from the people who have the most useful input to contribute. Close industry engagement also provides the driver for commercialization because commercial people push the need for release of germplasm they believe will benefit their business. This has been the case for both 'Premier Murcott' and 'CB Murcott'.

Outputs

Background:

Successful citrus breeding is the result of many years of forward thinking and field plantings, well beyond the timeframe of modern project funding cycles. It is therefore important to document progenies maintained and developed during the life of the program, so that they are available for future breeding activity.

An Intellectual Property (IP) Register has been supplied to Hort Innovation at the time of completing this Final Report. This document will protect the long-standing investment of all parties including the Queensland Government, Australian citrus levy payers, and Hort Innovation.

Summary:

The Australian citrus breeding program has a relatively fast turn-around of progeny blocks (compared with other international breeding sites) and no field plantings are held for longer than 10 years. Furthermore, because of the high-density plantings it is often the case that poor-quality families are identified well within 10 years and are immediately removed using an industrial mulching contractor. This means that field progeny blocks have already been subject to significant tree removal within 5 years of planting. This is on top of the major nursery culling (for Alternarian brown spot and Citrus scab) that occurs prior to field planting.

At the end of the project a stock take exercise of field plantings was carried out and the parentage and number of all families was documented to form an Intellectual Property (IP) Register. While the IP Register is maintained as confidential document, general observations were made and are presented in this final report. The exercise showed that there were over 40,000 hybrids in the field at Bundaberg at the completion of the project, resulting from the use of more than 130 different seed parents and almost 200 different pollen parents. The majority of these parents (>80%) were derived from previous breeding work at Bundaberg representing efforts to fix important commercial traits within breeding populations.

The largest single pollen parent is '17Q015' with 4,634 progeny spread across 32 seed parents. '17Q015' carries the single-gene for seedlessness and has the highest fruit quality of all available parents with this gene. Having large populations already established in the field reflects a concerted effort by the breeding team to quickly establish backcross populations with the best F1 hybrid carrying the seedlessness trait.

Other important pollen parent are the satsumas (Clausellina, Okitus, Miho) with 6,286 hybrids collectively. The Australian breeding program is one of the few in the world that has been able to develop large populations using satsuma pollen parents (this citrus type is pollen-sterile in most growing regions) and it was a major focus of the work at the start of CT15017. Hopes of obtaining seedless progeny were quickly dashed, and the fruit quality of these satsuma hybrids was terrible (puffy, poor colour, watery taste). Hence a major cull of satsuma families was performed mid-way through the project and those that remain (6,286) represent the best combinations with other Bundaberg parents. A few promising satsuma hybrids were selected in the 2020 season and used as parents within the program to incorporate satsuma genetics, yielding some benefits to the program from this research activity. However, crosses with satsuma will not be repeated moving forward.

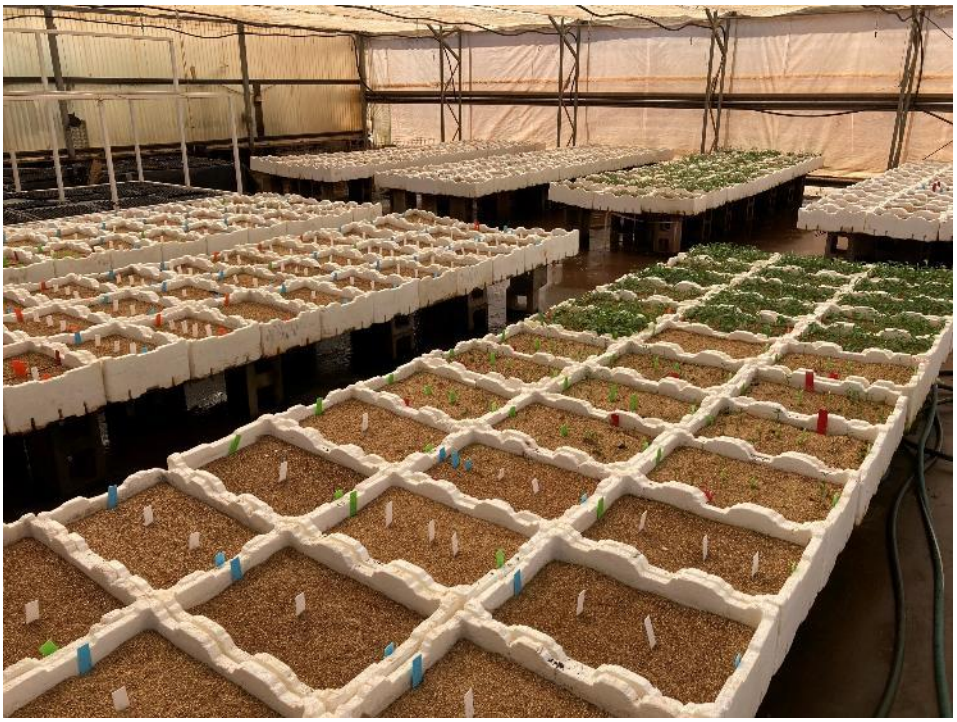
'20Q016' a satsuma hybrid, unusual in having good colour, firm flesh, non-puffy skin and high Brix.

Another important pollen parent has been '08C008', a Daisy x IrM1 hybrid, with 2,520 progeny currently in the field. This parent has produced a high frequency of seedless progeny in some crosses and we are eager to confirm this result on larger populations and with more diverse combinations.

Major seed parents are the high-quality mandarin selections '07C007' (4,374 progeny), '05C016' (2,568 progeny), '07C005' (2,274 progeny) and '01C011' (1,942 progeny). Orange breeding work is represented by K15 (4,092 progeny) and its F1 hybrids such as '16Q028', '16Q029', and '16Q030' (collectively 1,562 progeny). The fact that our best quality hybrids also now constitute the bulk of the parentage in progeny blocks reflects the:

- Fast turn-around of progeny blocks
- Early culling of families that are performing poorly
- Annual crossing programs that incorporate the latest seasons phenotyping and selection information
- New crosses and new field plantings in every season.

The stock take and cataloguing of the program's genetic pipeline also demonstrated the transition to seedlessness within the program. Multiple strategies for seedlessness were investigated in the program, and as it became obvious which approaches were the most effective and efficient then the crossing program evolved to reflect this. Thus, while triploid breeding was a major drain on project resources at the start of the project, the emergences of a more efficient system based on a single gene meant that no triploid pollinations were conducted in the final season (Aug-Sep 2020). Instead, the final season of pollinations was dominated by five key F1 hybrids carrying the single gene for seedlessness.



Hybrid seed sown in the nursery at Bundaberg Research Station, at the end of the project, May 2021. The result of pollinations performed during the Aug-Sep 2020 flowering season where hybrids carrying the single gene for seedlessness were the dominant parents.

Seed contamination in commercial Afourer orchards

Background:

Afourer mandarin is an important commercial variety in many Australian citrus growing areas. When grown in isolation (away from other citrus varieties that could provide pollen) it produces seedless fruit that is in high demand from consumers. However, it is sometimes the case that supposedly 'isolated' blocks of Afourer produce fruit with seed (seed contamination) and this can cause major marketing issues. The breeding program sought to help solve this problem using molecular markers and a library of marker data that had been developed. The objective was to identify which varieties were the source of pollen that was causing seed contamination. The study was conducted using two large commercial orchards, one in southern Australia and the other in the subtropics.

Summary:

Seed contamination in Afourer orchards in both regions was likely the result of cross pollination with other commercial scion varieties. Rootstock varieties, home garden types, a wide range of mandarin varieties, native citrus and Afourer itself can be confidently dismissed as the culprit. It was not possible to distinguish which selection(s) of Murcott were involved in the seed contamination and all types (e.g. Murcott, IrM1, Phoenix) must be considered as possibilities. Similarly it was not possible to distinguish between Sweet orange selections. Marker results from the orchard in northern Australia were distinctly different from a matching set of Afourer seedlings from a southern Australian orchard analysed in tandem, demonstrating the usefulness of the process in identifying sources of pollen contamination in different environments.

Afourer is polyembryonic but produces a surprisingly high frequency of zygotic seedlings making it possible to readily identify a population of hybrids from which the likely sources of pollen contamination can be determined. The original zygotic seedlings showed consistent marker patterns on two separate sampling and analysis dates, demonstrating the robustness of the technology. Additional zygotics were identified in a second round of analysis.

A simple method now exists to identify the likely cause(s) of seediness in Afourer orchards.

Methods:

Plant material: The collaborating growers were asked to visit those sections of their orchard where seed contamination had been a problem and to then cut Afourer fruit in these areas and extract any seed they could find. The resulting seed lots were sent to Bundaberg Research Station in May 2019. Upon arrival, seeds were surface sterilised and then sown at wide spacing in pasteurised potting mix and kept in a heated growth chamber to ensure rapid germination. About 60 plants were obtain from each of 5 seed lots. Leaves from 44 of these young seedlings were sampled, using a 6mm diameter pathology punch, on the 3rd July 2019, and records made of their individual morphology and whether they were from multiple-seedling seeds or single-seedling seeds. Samples were desiccated at low temperature over silica gel before dispatching to the laboratory.

These original seed lots were re-examined in July 2020 and 7 additional plants were identified as morphologically different and sampled for molecular analysis. A single seed from low-seeded Afourer was also sent to Bundaberg Research Station on the 21st May 2020, and 2 plants were successfully germinated from it; both of which were submitted for molecular analysis.

Molecular markers: An initial panel of 24 molecular markers was developed to address a wide range of project interests in the national citrus breeding program (CT15017). The majority of these markers were designed to distinguish cultivated citrus varieties and were spread across all 9 linkage groups on the citrus genome. Confidential information for these markers was supplied by international colleagues as well as design work by colleagues in DAF. The author was also supplied with data on an extensive citrus germplasm collection that could be used to cross-check genotyping data from the Bundaberg arboretum. The seedlings from the initial 44 plants were included in a much larger number of samples submitted by the national citrus breeding program on the 12th July 2019, in a pilot project designed to test the usefulness and cost-effectiveness of new techniques for molecular markers.

For the second round of molecular testing, a different panel of 24 markers was developed based on the initial results, with 14 in common with the first set. A further 10 new experimental markers were included, mostly aimed at

identifying traits of importance in the breeding program.

Data analysis: For the initial analysis, results were received from the laboratory on the 30th September 2019. These were then colour coded to enable visual assessment and edited to analyse for the presence of zygotic seedlings and their likely parentage. Data from standard varieties in the Bundaberg germplasm collection were compared with the results from an overseas collection in order to validate the project.

Data obtained in the second round of testing was integrated into the results in December 2020.

Results and interpretation:

Northern orchard: An initial set of 44 seedlings were individually analysed using the molecular markers, with 14 seedlings derived from Bay 4-8 and the remaining 13 seedlings from Bay 18-20 (Table 1). Four seedlings were found to be zygotic (hybrids) and the rest were nucellar (identical to Afourer). All 4 zygotic seedlings came from Bay 18-20, although this may simply be a consequence of the small number of seedlings analysed (27). Prior to this exercise we had no idea what the frequency of zygotic seedlings was likely to be in Afourer. In choosing the 44 seedlings for analysis, we attempted to include any that looked slightly different morphologically, and we also sampled seedlings that were the only plant to emerge from a seed as well as seedlings where multiple plants had emerged from a single seed. The 6 seedlings chosen because they "looked" slightly different, all proved to be nucellar. Two of the 4 zygotics had come from single plant seeds (out of 11) and the other 2 from multiple-plant seeds (out of 16). Thus, trying to identify zygotics based on appearance or the presence of multiple plants per seed seems futile.

From the second set of 7 seedlings an additional 2 zygotic hybrids were found, representing a much higher rate of detection (2/7) than the initial set (4/27); probably indicating that true morphological difference become more obvious in older seedlings. None-the-less, a detection rate of 2/7 is still very low considering that all 7 seedlings were thought to look slightly different from Afourer.

The detection of a zygotic in the 2 seedlings that emerged from the seedless Afourer seed provided an opportunity to examine pollen flow into an orchard of this seedless variety.

We can be confident of having accurately identified true hybrid seedlings because they each differ by at least 4 marker positions. Similarly, we can be confident that the remaining seedlings are just Afourer because they are identical with mature Afourer at all markers. Only 13 of the 28 markers proved useful in distinguishing zygotic from nucellar seedlings, although the remaining markers were needed to identify likely pollen parents.

Having successfully found 12 zygotic seedlings, the task now shifted to identifying their pollen-parent. This is a process of elimination and requires knowledge of the same 28 markers for all varieties suspected of being involved. There are 4 groups of suspects:

Afourer: Even though this variety is considered self-incompatibly, we should not discount the possibility that it may have an extremely low level of self-pollination under the right conditions and thus occasionally produce seed from its own pollen.

Rootstock varieties: It is possible that rootstock suckers have unknowingly developed within Afourer orchards and are the source of pollen contamination.

Scion varieties: Mistakes during nursery propagation may have resulted in varieties other than Afourer being planted within solid blocks. Different varieties being grown in nearby blocks may also be the source of pollen contamination as bees (and other pollinating insects/birds) transfer pollen into the Afourer blocks. There is much debate about what constitutes a "nearby" block for pollen transfer so all varieties grown in the region need to be considered.

Backyard and native citrus: It is possible that non-commercial varieties could be the source of pollen contamination, such as a Meyer lemon tree growing in a nearby backyard or pollen from a wild citrus species growing in native vegetation.

We examined all of these possibilities and were able to completely dismiss most of them, based on the presence of molecular differences for each of the 12 zygotic seedlings. Evidence for dismissing each of the possible pollen sources was based on differences at multiple genome locations and the number of differences is shown in the tables below. The vast majority of possible pollen contamination sources can be confidently dismissed based on the marker data. In terms of the four possible sources of pollen contamination:

Afourer: There is no evidence for self-pollination and Afourer is not the pollen parent of the seven hybrids.

Rootstock varieties: Most of the rootstock varieties could not be the source of pollen, although Benton is a possibility for four of the hybrids. None of these four plants show any morphology associated with Poncirus or Poncirus hybrids (Benton is a Poncirus hybrid). The only other rootstock possibility is if Sweet orange has been used as a rootstock.

Scion varieties: Nearly all of the possible commercial scion varieties can be easily dismissed based on data at multiple marker locations. The additional commercial varieties Shiranui and Orri were tested in the second round and were not pollen parents. Lemon, lime and pomelo are clearly not responsible for the pollen contamination and neither are the two tangelo varieties. Very few of the varieties listed in the table are present in region but were included just to illustrate how easily they can be excluded as possible parents. The most likely possibilities are Murcott, Sweet orange and Ellendale.

Backyard and native citrus: These are not the source of contamination, since Meyer lemon, Kumquat and native Desert lime have distinct differences at multiple marker locations.

Southern orchard: In the southern orchard, seed contaminations sources were more complex. No single variety could be held responsible for all five hybrids, although the evidence for dismissing Clementine is based on only one marker difference in just one hybrid. Similarly, the possibility of Sweet orange being the only source of pollen contamination is compromised only by the presence of a single marker difference in three of the hybrids, and this is the same marker in all three cases. There is a low likelihood of Nova or Daisy being the pollen source. It is a possibility for only three of the hybrids and each of these hybrids has other candidate pollen parents, which curiously are just Sweet orange and Clementine. Available evidence suggests that Sweet orange and/or Clementine are causing seeds in these Afourer orchards.

Results from the five zygotic seedlings included in the second batch of molecular markers served to reinforce the initial findings that Sweet orange and/or Clementine were the most likely source of pollen contamination. This second analysis was able to dismiss Orri and Shiranui (Sumo, Decapon, Hallobong) as possible pollen sources (since leaf material was not available for the first round of testing). The second analysis also demonstrated consistency of results, not only with these five zygotic seedlings but with hundreds of other samples that had been included from the Australian national breeding program. An additional marker specifically designed to help distinguish between Sweet orange/Clementine/Murcott parentage, was included in the second panel of markers but failed to function properly (see above). The orchards studied in both northern and southern Australia would benefit from having a marker that clearly distinguishes these three potential pollen sources.

Conclusions regarding the source of pollen contamination in the southern and northern orchards differed. In the northern Afourer orchard, four varieties were possible sources of contamination. Only Murcott could be responsible for all seven hybrids. Although we cannot completely dismiss the possibility of a nursery mistake resulting in a Sweet orange scion or rootstock tree being present, we know that no Sweet orange selection (e.g. navel, Valencia, CaraCara) has ever been grown in this region. A new marker capable of distinguishing between Murcott and Sweet orange parentage was tested in the second submission of samples but failed to function properly. This marker has now been modified and is being tested in the third submission of samples. If we can get this new marker to function properly then it will be useful in removing any doubt about Murcott vs Sweet orange parentage. The existing molecular and horticultural information suggests that Murcott is causing seeds in these northern Afourer orchards, while Sweet orange and Clementine are the main culprit in the south. Whether this pollen contamination is coming from rouge trees within the Afourer blocks, or transfer from "nearby" blocks of citrus is not clear, however it is clear that scion varieties (rather than rootstock suckers, backyard trees, or Afourer itself) are causing seediness in solid blocks.

<u>Northern orchard</u>: Visualisation of allele calls for 36 seedlings assessed against 24-28 molecular markers. At least two markers were chosen on each linkage group. Seedlings in red are zygotic while those in black are identical to Afourer and therefore considered nucellar.

	1a	1b	2a	2b	2c	2d	3a	3b	3c	4a	4b	5a	5b	6a	6b	6c	6d	7a	7b	8a	8b	9a	9b	9c	1c	3d	6e	8c
Afourer	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G
seedling 1	G:A	G:A	G:G	G:A	T:T	A:A	G:G	C:C	T:G	T:T	T:C	C:A	T:T	G:A	G:A	C:C	C:C	C:C	T:C	C:A	T:C	T:C	T:C	?	T:C	T:C	A:A	G:G
seedling 6	G:A	G:A	G:G	G:A	T:T	A:A	G:G	C:C	T:G	T:T	T:C	A:A	T:T	G:A	A:A	C:C	?	C:C	T:C	C:A	T:C	C:C	T:C	T:T	T:T	T:C	A:A	G:G
seedling 10	G:G	G:A	G:G	G:G	T:T	A:A	G:G	C:C	T:G	T:T	T:T	C:A	T:T	A:A	G:A	C:C	?	C:C	T:C	C:A	T:C	T:C	T:C	T:T	T:C	T:T	A:A	G:G
seedling 13	G:A	G:A	G:G	?	T:T	A:A	G:G	C:C	T:G	?	T:T	C:A	T:T	G:A	A:A	C:C	?	C:C	T:T	C:A	T:C	T:C	T:C	T:T	T:C	T:C	A:A	G:G
Afourer Bay4-8 2		G:A	G:G		T:T		G:G	C:C	T:G		T:T	C:A		G:A	G:A	C:C			T:C			T:C	T:C	T:T	T:C	T:C	A:A	G:G
Afourer Bay4-8 7		?	G:G		T:T		G:G	C:C	T:G		?	C:A		G:A	G:A	C:C			T:T			T:C	T:C	T:T	T:C	T:C	A:A	G:G
seedling 1		G:A	G:G		T:T		G:G	C:C	T:G		T:T	C:A		A:A	G:G	C:C			T:T			C:C	C:C	T:T	T:C	T:T	A:A	G:G
seedling 11	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 12	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 14	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 15	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 16	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 17	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 18	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	?	T:C	C:C	T:C	T:T				
seedling 19	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 2	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 20	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 21	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 22	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 23	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 24	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 25	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 26	G:A	G:G	G:G	?	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 27	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 3	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 4	G:A	G:G	G:G	?	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 5	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 7	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 8	G:A	G:G	G:G	?	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 9	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
Afourer Bay4-8 1	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G
Afourer Bay4-8 3	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G
Afourer Bay4-8 4	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G
Afourer Bay4-8 5	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G
Afourer Bay4-8 6	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G
seedling 2	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G

<u>Southern orchard</u>: Visualisation of allele calls for 17 seedlings assessed against 24 molecular markers, plus an additional four markers tested on the five identified zygotic seedlings. At least two markers were chosen on each linkage group. Seedlings in red are zygotic while those in black are identical to Afourer and therefore considered nucellar.

	1a	1b	2a	2b	2c	2d	3a	3b	3c	4a	4b	5a	5b	6a	6b	6c	6d	7a	7b	8a	8b	9a	9b	9c	1c	3d	6e	8c
Afourer	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G
seedling 6	A:A	G:G	G:G	A:A	T:T	A:A	G:A	T:C	T:T	T:T	T:T	C:C	T:T	A:A	G:A	C:C	?	C:C	T:C	C:A	T:C	T:C	T:C	T:T	T:T	T:T	A:A	G:G
seedling 8	A:A	G:G	G:G	G:A	T:T	A:A	G:A	C:C	T:G	T:T	T:C	A:A	T:T	A:A	Uncallable	C:C	?	C:C	T:C	C:C	T:C	T:C	T:C	T:T	T:C	T:T	A:A	G:G
seedling 12	G:A	G:A	G:G	A:A	T:T	A:A	G:G	C:C	T:T	T:T	T:C	A:A	T:T	G:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:T	T:T	A:A	?
seedling 14	G:A	G:G	G:G	?	T:T	A:A	G:A	T:C	T:T	T:T	T:C	C:C	T:T	A:A	G:G	C:C	C:C	T:C	T:C	A:A	T:C	T:C	T:C	T:T	T:T	T:T	A:A	G:G
seedling 17	G:A	G:G	G:G	A:A	T:T	A:A	G:G	T:C	T:T	T:T	T:C	C:A	T:C	G:A	G:A	C:C	C:C	T:C	T:T	C:C	T:C	T:C	T:T	T:T	T:T	T:C	A:A	G:G
seedling 1	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 10	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 11	G:A	G:G	G:G	G:A	T:T	?	G:G	?	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 13	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 15	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 16	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 2	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 3	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 4	G:A	G:G	G:G	G:A	T:T	A:A	G:G	?	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 5	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 7	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
seedling 9	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T				
Afourer	G:A	G:G	G:G	G:A	T:T	A:A	G:G	C:C	T:T	T:T	T:T	C:A	T:T	A:A	G:A	C:C	C:C	C:C	T:T	C:A	T:C	C:C	T:C	T:T	T:C	T:T	A:A	G:G

<u>Northern orchard</u>: Number of impossible allele combinations for seven Afourer hybrids predicted against 28 possible parents. Values in red indicate a possible pollen parent.

		1	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4
Zygotic seedling	Marker differences	Afourer	Benton	Cleopatra	Rough lemon	Swingle	Trifoliata	Troyer/Carrizo	Eureka/Lisbon	Tahiti lime	WI lime	Minneola	Orlando	Pomelo	Clementine	Daisy	Ellendale	Fremont	Imperial	Murcott/Phoenix	Nova	Orri	Pixie	Satsuma	Shiranui	Sweet orange	Desert lime	Kumquat	Meyer
1	7	7	2	4	4	1	6	3	5	5	8	3	3	8	1	4	1	5	2	0	3	4	3	2	2	0	8	8	3
6	9	6	1	3	3	2	3	4	6	5	7	3	5	8	1	4	1	6	2	0	5	4	4	1	4	0	6	8	5
10	6	4	2	5	3	1	4	1	4	2	7	2	2	7	2	3	0	4	3	0	2	3	3	4	2	0	7	7	4
13	6	5	0	3	3	2	7	4	6	6	8	3	4	9	1	5	1	4	2	0	4	3	2	2	5	0	9	8	5
Bay4-8 2	6	6	0	4	3	2	3	2	4	4	5	2	3	5	1	4	1	5	2	0	3	2	2	2	3	0	4	4	2
Bay4-8 7	4	4	0	2	3	2	4	3	5	5	6	2	3	6	1	3	1	3	2	0	2	2	1	2	3	0	5	5	3
Seed. 1	4	2	0	2	1	1	2	2	3	2	5	3	4	4	1	2	0	3	2	0	2	3	4	3	4	1	3	2	4

<u>Southern orchard</u>: Number of impossible allele combinations for five Afourer hybrids predicted against 28 possible parents. Values in red indicate a possible pollen parent.

		1	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3		3	3	3	3	4	4	4
	Marker difference	Afourer	Benton	Cleopatra	Rough lemon	Swingle	Trifoliata	Troyer/Carrizo	Eureka/Lisbon	Tahiti lime	WI lime	Minneola	Orlando	Pomelo	Clementine	Daisy	Ellendale	Fremont	Imperial	Murcott	Nova	Orri	Pixie	Satsuma	Shiranui	Sweet orange	Desert lime	Kumquat	Meyer
6 R11	8	4	4	5	3	2	5	4	5	4	8	3	4	8	0	0	2	1	4	3	0	3	3	4	2	1	8	9	5
8 R101	10	5	3	5	6	2	6	4	5	4	9	3	3	10	1	0	1	2	3	1	3	5	3	4	2	0	8	9	5
12 R41	6	3	3	2	3	2	5	4	5	4	8	5	5	9	0	2	0	2	0	1	3	3	4	2	3	1	9	7	4
14 R41	9	6	7	7	7	2	7	6	6	5	11	4	3	8	0	1	3	2	5	3	0	2	5	3	1	0	9	10	5
17 R41	9	6	3	4	6	2	9	5	7	7	10	3	3	11	0	3	2	4	3	4	2	2	4	2	3	1	12	11	4

Future work:

The current project has shown that Afourer produces a sufficient frequency of zygotic seedlings to enable the prediction of pollen contamination sources in different production environments (northern and southern Australia). Analysis of 44 seedlings with 28 markers has succeeded in pointing to just a few possible sources of pollen contamination in both production regions.

The techniques now exist to efficiently determine which varieties are causing seed contamination in Australian Afourer orchards. This is critical to the successful development of management strategies to overcome this significant commercial constraint. If growers know what pollen caused their Afourer orchards to become seedy then they can better target their efforts to eliminate the problem.

Genetics of acidity development in ancestral taxa

Background

The breeding program was approached by one of their international collaborators regarding the origins of acidity in the genus Citrus. These collaborators have in the past supplied the Australian breeding program with very useful molecular markers and so the breeding team undertook assessments of germplasm held at Bundaberg to generate the requested information. Bundaberg Research Station now houses one of the world's most diverse collections of Citrus germplasm and it is increasingly useful in building collaborations that benefit the Australian citrus industry.

Introduction

Many citrus relatives lack the plump juice-filled sacks that characterise commercial citrus species, which may partly explain a general lack of information about acidity and sugar contents for this germplasm. To address this problem we sampled mature fruit from a range of citrus relatives held at Bundaberg Research Station, extracted juice from the flesh, and then determined acidity and Brix. Recovering a juice sample was challenging for most of the species and required the use of a pressurised plant press followed by centrifugation to remove debris. The resulting samples revealed interesting characteristics of these species, including Brix values as high as 26° and acids less than 0.1% citric-acid equivalent. To calibrate these values, a range of domesticated citrus species known to have high or low acidity were sampled at the same time (even though some of them were a few months from full maturity). This showed that the low acidity (less than 0.5%) seen in the 5 species (*Atalantia ceylanica, Citropsis gilletiana, Triphasia trifolia, Glycosmis trifoliata*, and *Clausena brevistyla*) was similar to that seen in the local acidless orange mutation 'Bakers Sweet'.

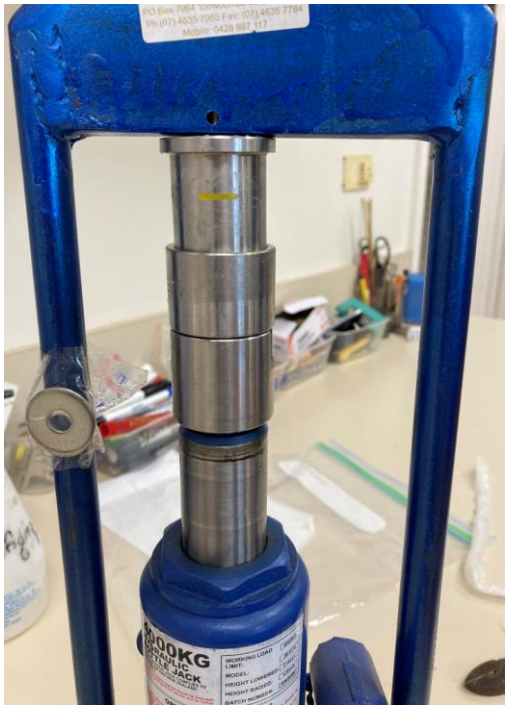
Methods

Fully mature fruit were harvested from the collection of citrus relatives held at Bundaberg Research Station on the 1st of February 2021. Fruit of commercial varieties were sampled from the same orchard at this time (although some were immature), along with a sample of overmature Navel oranges from a local supermarket (imported from the USA).

Fruit of citrus relatives used in the determination of juice acidity and Brix,

with Tahitian lime included for comparison.

Juice was extracted from 3 replicates of fruit for each species, with replicates mostly consisting of 2 fruit each. The fruit were peeled and then cut to remove the seed, with the remaining flesh then inserted into the top canister of a plant press.



Plant press used to extract juice from all of the samples.

Most of the species resulted in press samples that were more like a paste than a liquid, and this needed to be centrifuged to separate out the juice. A speed of 14,000 rpm for 1 minute was used to avoid any possibility of sugar movement in the juice. *Clausena smyrelliana* (syn *Murraya crenulata*), *Clausena brevistyla*, and *Micromelum* minute proved particularly challenging to juice, whilst *Citropsis gilletiana* had a large amount of wax which rose to the surface during centrifugation.

Some of the challenges of obtaining juice samples from these citrus relatives. Top left, paste of Clausena brevistyla which was centrifuged to obtain a clear juice supernatant. Top right, paste of Micromelum minutum which needed to be mixed with water prior to centrifugation and the acid and Brix results corrected according to the dilution. Middle left, paste of Clausena smyrelliana which needed to be worked in with water prior to centrifugation but resulted in a clear sample (Bottom left). Middle right, centrifuged juice of Citropsis gilletiana showing the wax deposit on the surface.

Results

Data for the juice samples generate from 8 citrus relatives, 7 species/hybrids within the True Citrus Fruit, and one intergeneric hybrid are shown below:

Grouping	Genus	species	Reps	*% Acid*	*Brix*	Comment
Clauseneae: Micromelinae	Micromelum	minutum	1	1.535001	5.30508356	fruit overmature and dry, treat data with caution
Clauseneae: Clauseninae	Clausena	brevistyla	3	0.329629	25.5627864	
Clauseneae: Clauseninae	Clausena	lansium	3	4.333691	17.6653349	
Clauseneae: Clauseninae	Clausena	smyrelliana	3	1.015839	18.1982222	
Clauseneae: Clauseninae	Glycosmis	trifoliata	3	0.253684	14.221762	
Citreae: Triphasia group	Triphasia	trifolia	3	0.081695	22.8640295	tastes like sugary water, plus oil
Citreae: Near-Citrus Fruit	Atalantia	ceylanica	4	0.279751	18.8073192	
Citreae: Near-Citrus Fruit	Citropsis	gilletiana	3	0.442871	21.9199276	wax on juice surface
Citreae: True Citrus Fruit	Tahiti lime		3	5.503645	9.56024057	
Citreae: True Citrus Fruit	Navellina orange		3	1.392496	7.11012558	immature fruit
Citreae: True Citrus Fruit	USA Washington		3	0.624082	10.2948378	overmature
Citreae: True Citrus Fruit	Bakers Sweet orange		3	0.096744	7.86228301	immature fruit
Citreae: True Citrus Fruit	Fortunella	japonica	3	1.403	11.692713	
Citreae: True Citrus Fruit	17Q020 (F.hindsii x C.glauca)		3	6.687144	11.535014	
Citreae: True Citrus Fruit	F210 [(C.wakonai x C.glauca)xF.japonica)]		1	9.54216	16.8269296	
Citreae: True x Near	12Q031 (Citrus wakonai x Citropsis gabunensis)		2	8.792396	17.4334171	Brix 12.8 acid 8.7 in JASHS

Discussion

Five of the citrus relatives have very low acid content (<0.5%) with *Triphasia trifolia* being the lowest (0.08%). The low acidity of the 5 citrus relatives matched the sort of level found in the acidless sweet orange selection 'Bakers Sweet', which even though immature at the time of testing was still only 0.1% acid. Even the overmature USA fruit purchased in the supermarket had 6-times this acidity. *Clausena lansium* had surprisingly high acidity, given that it is a desert-type fruit, no doubt balanced by its high sugar content (Brix 17.7). Other high values of acidity were only found in the True Citrus Fruit and hybrids with them.

Sugar levels were remarkably high for many of the citrus relatives, with *Clausena brevistyla* being almost 26° Brix. With the exception of *Micromelum minutum* (which was not a good sample) the lowest Brix was only 14.2, which was well above nearly all of the samples from the True Citrus Fruit.

There was no relationship between acid and Brix, and indeed many of the relatives that had very low acidity often also had very high Brix. Perhaps the best example of this was *Triphasia trifolia*, with an acidity of 0.08 and a Brix of almost 23°. These results are entirely consistent with the feel and taste of this juice because it was very sticky to handle and tasted like sugary water.

Although low acidity is quite common in these citrus relatives it may not be consistent with genera. For example, within our 3 *Clausena* species the acidity ranged from very low (0.3) to high (4.3) with the third sample being intermediate (1.0).

Additional species held in the collection but not fruiting at the time will be available for analysis at a future date if the data is required. These include *Naringi crenulata* (*Hesperathus crenulata*), *Citropsis gabunensis*, and additional *Clausena* species.

Efficient screening for bioactive compounds

Background:

Citrus are well known for their diverse range of bioactive compounds. This is reflected in their importance as food flavourings, medicines, perfumes, and domestic products. However, not all of these compounds are desirable in fresh fruit and can lead to off-flavours or even interfere with medications. Part of the Australian breeding program involves the introduction of wild genetics (e.g. for natural disease resistance) but this can bring with it many undesirable traits. The project has tried to develop highly efficient screening techniques so that hybrids carrying undesirable levels of certain bioactive compounds can be eliminated at an early stage in the breeding process. We have had considerable success in this regard, having eliminated more than 50% of some segregating populations prior to field planting. This report records general progress in this important area because it represents a significant part of laboratory work conducted in this phase of the breeding program.

Discussion:

Screening technique for bioactive compounds continue to evolve, allowing ever more precise determinations of the chemical constituents of different plant parts. However, these newer techniques are best suited to analysis of relatively small numbers of samples. For breeding programs, it is critical and more efficient to process large numbers of samples at very low cost, and to be able to do this as early as possible. Work conducted early in this phase of the breeding program showed that seedlings could be screened for off-flavours at an early stage and eliminated prior to field planting. This work involved the development of efficient sample collection, compound extraction and sample analysis techniques. Surviving hybrids from this first round of screening have now started to fruit and the best of them have been used as parents. This second generation of hybrids resulting from the initial screening work are now growing in the nursery at Bundaberg and will be ready for screening in early 2022. This should demonstrate the usefulness of these new techniques in eliminating unwanted compounds in a backcrossing program.

International collaboration

Background:

The Australian citrus breeding effort is small by international standards, and indeed by comparison with most tree crop breeding programs, so collaboration is critical to leveraging genetic progress. An effective international network of breeders has been established and strengthened during this phase of the breeding program with tangible benefits to the Australian citrus industry.

Discussion:

International collaborations have provided access to germplasm, segregation populations and technology well beyond the capacity or resources of the program. These collaborations have now resulted in populations of hybrids growing at Bundaberg that were generated by colleagues in Italy, Korea, California and Florida. The populations capture the genetics of parents that are not present in Australia and jump-start the breeding process by avoiding the long delays (including high cost and risk) associated with introducing parents via quarantine. By getting colleagues to conduct the required pollinations in their country and then post the resulting seed to Bundaberg (via quarantine seed treatment on arrival) we can shorten the breeding process by almost 10 years. In return, the breeding team at Bundaberg have supplied hybrid seed (from non-IP parents) to colleagues in Florida, Italy, Korea and Spain.

Aside from this direct exchange of hybrid germplasm, the program also benefited from technologies share by international colleagues. For example, the panel of SNP markers used for Intellectual Property protection of the new varieties (as well as other privately owned varieties) and the determination of seed contamination in Afourer orchards is the result of information shared by colleagues in the USA. Likewise the disease-link SNPs associated with *Poncirus* were supplied by another group in the USA. Opportunities for canker resistance breeding arose from information and observations made by colleagues in Uruguay, and determining a path forwards in *C.glauca* breeding has been made possible via advice and sequencing information from France, Spain and Brazil. These are some examples of how international colleagues are making frequent and impactful contributions to the Australian breeding program.

Collaboration with international colleagues has managed to unravel most of the conflicting information that exists on different germplasm reactions to Alternaria brown spot. Through a workshop with other citrus breeders and strategic molecular testing of germplasm holdings at different sites, it has been possible to demonstrate that discrepancies in the literature are not the result of different strains of the pathogen, but instead mistakes in germplasm labelling and processing. This is important because it shows that everyone around the world is working on the same pathogen, rather than different biotypes with differing host ranges. A publication was prepared to document these corrections to previous literature (Arlotta et al. 2020).

agronomy

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Article

Disease Resistant Citrus Breeding Using Newly Developed High Resolution Melting and CAPS Protocols for Alternaria Brown Spot Marker Assisted Selection

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International collaboration provided an opportunity to correct some of the discrepancies in host-pathogen relationships that exist in the literature, via joint publication of this recent manuscript.

Citrus hybrids growing at Bundaberg, from hybrid seed supplied by international colleagues.

Top left, Korean hybrids generated using high quality mandarin varieties, top right: Ellendale x C.latipes hybrids produced in Sicily, middle left: Mandarin x Mikaku kishu hybrids produced by the Uni of Florida, middle right: Sundragon hybrids (HLB resistance) generated by USDA Florida, bottom left: Oxanthera x C.latipes hybrids from the Uni of California.

Improving the productivity of '15C001'

Background:

A major limitation of mutation breeding as a technique for seedlessness is its dependence on having diploid progenitors that are highly parthenocarpic in all production environments. In the absence of parthencarpy, truly seedless mutations may be created but never actually set fruit and so cannot be found (and would be of no commercial value because of low or zero fruit production). We are encountering this problem with '15C001' which is one of our best mutations of '00C018'; it is practically seedless but the hundreds of trees we have now established under commercial growing conditions produce very limited fruit. This selection has attracted intense commercial interest because of its outstanding fruit quality but increased grower confidence with regards to its productivity is required to commercialise this selection.

Methods:

Conscious of this impediment to commercialisation the breeding team invested significant effort in field trials designed to boost fruit production, and to investigate the cause of the low productivity. One trial harvested during the 2020 season involved an assessment of gibberelic acid (GA), both by itself and in combination with auxin and calcium. This experiment was conducted in two growing environments and contained a total of 32 replicates. It showed a clear benefit of GA application with the addition of auxin and calcium having no statistically significant additional benefit (although there would seem to be no harm and some horticultural benefit in its inclusion):

	Mean March + July 2020		
Spray	Pred Mean	se	BT Mean
Control	2.98 a	0.241	18.7
GA	3.72 b	0.241	40.3
All	4.07 b	0.241	57.6
p-value	<0.001		
sed	0.341		
95% lsd	0.678		

Despite the very promising response to GA, the yield on treated trees was still below commercial expectations and additional growth regulant strategies were tested by the breeding team on the 2020 fruit set. The new experiments set up in October 2020, were designed to prevent excessive fruitlet drop, since the project team have now demonstrated that the low yields of this variety are caused by excessive fruitlet drop rather than poor flowering or initial set; the variety flowers profusely and indeed one of the collaborating growers applied GA pre-flowering to reduce flower production and possible improve subsequent fruit set. These new experiments will be assessed in June 2021 (outside of the project period for CT15017) but early indications are that the anti-ethylene compound applied in October 2020 has not been as beneficial as the GA strategy that was tested the previous season. During the 2021 season the old GA experiment will be re-assessed to confirm whether there is a carry-over effect on the second year of cropping.

The promising response from the GA experiments was sufficient for the collaborating growers to treat their blocks at a commercial scale in 2020, and it is hoped that these applications, at a much higher volume than can be achieved in research trials, may produce an even better response. An assessment of this trial in May 2021 showed that '15C001' has the capacity to carry a commercial crop of fruit but that it is not consistent. There may be an opportunity to manage this variety so that it is sufficiently productive for commercial orchards but the exact techniques required have yet to be determined.

Trees of '15C001' carrying an acceptable quantity of fruit in May 2021. Some other trees of this variety have cropped poorly and ways to solve this were investigated within CT15017.

The picture below demonstrates that this low-yielding variety has very high initial fruit set:

Excellent initial fruit set on '15C001'. This selection is low-yielding, and the project team have demonstrated that this problem is caused by fruitlet drop rather than poor flowering. Growth regulator experiments are being conducted within the project to hopefully overcome this problem. Treatment occurred at the phenological stage shown in this photo (20th October 2020: GA applied 9th October 2019).

Particularly worthy of note in relation to the fruit-set problem of '15C001', is the apparent impact of rootstocks. The GA experiment described above (96 trees) contained a mixture of rootstocks and there was a strong indication that Benton performed the best:

Rootstock	Mean March + July 2020		
	Pred Mean	se	BT Mean
Benton	4.85 b	0.434	126.7
Troyer	3.04 a	0.137	20.0
US812	3.43 a	0.217	29.8
p-value	0.013		
Average sed	0.411		
Average 95% lsd	0.824		

However, these rootstocks could not be randomised across the trial sites and the number of replicates with Benton was low. This requires the need for caution when interpreting this result. As a result of strong forward thinking by the project team, the commercial sites were established using a range of rootstocks even though their main purpose was to compare scion selections/mutations. This strategy included properly designed rootstock evaluations with a couple of the new scions. Fortuitously, one such trial within the commercial planting at Gayndah was with '15C001' and although this trial does not contain Benton rootstock the data none-the-less reinforce the potential role of rootstocks in boosting productivity. Trees are still very young but the data below shows a dramatic impact on productivity.

Rootstock	Fruit count
CH414	0
D223	56
E397	71
F154	12
GLA36	
ICA5	8
Troyer	15
Grand Total	**27**

Six of the rootstocks are from the Bundaberg breeding program, with Troyer included as the industry control. No data is available for GLA36 because all trees have died (phytophthora).

This rootstock effect will be examined again during June 2021. As of May 2021, the growers hosting these trial sites are prepared to persist with '15C001' because the trials conducted by the breeding team, as well as their subsequent whole-row application of GA, give some hope that yields can be improved.

Significant work is being conducted by the project team to boost the productivity of '15C001', because unless yields can be improved then this very high-quality mandarin will have to be scrapped. Commercial release will not proceed until grower confidence with regards to its productivity is improved.

Application of molecular markers

Background:

The Australian citrus breeding work is a conventional breeding program based on purposeful parent selection and efficient generation and screen of large field populations of hybrids. Molecular markers are being used to assist with parent selection and to protect intellectual property.

Methods:

There are currently three trait related markers that have been used in this program. These are linked to the traits of Alternaria brown spot resistance, Citrus tristeza virus (CTV) resistance, and apomixis. All three traits have been managed via conventional screening techniques but the availability of molecular markers has served to strengthen this conventional phenotyping. In the case of Alternaria brown spot, the Australian program has a well-established methodology (Smith et al. 2016) that enables the efficient screening of progeny for both Alternaria brown spot and Citrus scab (for which a molecular marker is not yet available). This conventional screening technique is far more efficient and cost effective for culling large populations. Nonetheless, the Alternaria brown spot marker has been run on all of our important parents or potential parents to assist in devising the annual pollination plan. It supplements existing information on field disease phenotypes and progeny test results, providing confidence that the right parents are being chosen. This SNP marker has been run on over 600 accession in the breeding program.

The CTV marker was originally validated in an agarose methodology (Webb et al. 2017) but is now being used in SNP format. Virus resistance is far more difficult to determine than ABS which makes the molecular marker attractive, but unfortunately the accuracy of the marker is limited. Hence the Australian breeding program is reluctant to discard this marker (because it is somewhat useful) but also recognises that more development work is needed. CTV resistance may be thought of as a 'rootstock problem' rather than a 'scion problem', but the Australian breeding program is committed to developing scion varieties that have resistance to this virus. Problems such as 'Grapefruit stem pitting', which limit the useful life of Australian grapefruit orchards, and 'Orange stem pitting' are examples where single-gene virus resistance could play a role. The CTV molecular marker is being used in conjunction with field aphid exposure and serological techniques to capture virus resistance from *Poncirus trifoliata* in a mandarin/orange background. It is hoped that these hybrid populations may help in the development of more accurate markers for virus resistance.

The apomixis marker is mostly used in rootstock breeding but has found some application in this program. It proved useful in avoiding certain seed parents that morphologically appeared to be monoembryonic (and hence assumed to be zygotic) but were in fact nucellar. One such example was '07Q010' for which we already had some progeny in field blocks and did not realise it was apomictic until they started to fruit.

The greatest use of molecular markers within the program has been to protect intellectual property. We have been able to validate multiple SNP markers on all linkage groups and apply these across a large portion of the germplasm generated and used in the scion breeding work. These same markers have been used on privately owned varieties to confirm that differences can be detected. This represents a huge step forward in the protection of intellectual property developed through citrus breeding. Large environmental effects on phenotype have always made it difficult to prove whether two fruit are of the same genotype, resulting in significant piracy. The breeding team have publicised the existence of their data showing how easily it now is to prove theft, which should prove a significant deterrent.

Molecular information was generated prior to the lodging of PBR and US patent applications and shows clear differences. However, legal advice (confirmed by international breeding colleagues) was that this data should not be included with the lodgements because of the possibilities of legal manipulation. Instead, the lodgements indicate the existence of conclusive molecular differentiation, which can be called upon in future if needed.

Molecular markers were also applied to verify the true-to-type status held in Australia for certain citrus varieties with those existing in overseas collections. This proved a very worthwhile exercise particularly in relation to understanding discrepancies in Alternaria brown spot disease reaction.

Mutation breeding

Background:

Mutation breeding has proven to be the most reliable and rapid methodology for reducing seed numbers in citrus. It provides a way of reducing seed numbers without making obvious/frequent changes to other traits. It was the methodology successfully used in this program to produce the low-seededness of 'Premier Murcott' and 'CB Murcott', and has been widely used in other Australian and international breeding efforts.

Although the program has quickly transitioned to a single gene for seedlessness in new hybridisation work, there still exists many selections within the program that may be commercially useful if they had less seeds. The decision on which selections are of sufficient merit to warrant irradiation is quite difficult because it represents a significant cost and period of time before results are obtained. Furthermore, unless the selection has strong parthenocarpic capacity (can set fruit without seeds) then it is difficult to find low-seeded mutations that are productive.

One selection that always attracts the attention of the breeding team because of its excellent eating quality and high colour is '06C015', somewhat cruelly referred to as 'The Scab Orange' because of its high susceptibility to Citrus scab. Because of this Scab sensitivity (as well as its susceptibility to Alternaria brown spot) it has never been considered suitable for release in the subtropics. It has however been used extensively as a parent to transmit high eating quality.

The need for better tasting orange-like fruit in southern Australia prompted an effort to improve the commercial potential of this hybrid by reducing its seediness, and under southern growing conditions it will not suffer attack from Alternaria and scab.

Methods:

Budwood was collected from two source trees of '06C015' at Bundaberg Research Station on the 5th February 2021, de-leafed and immediately wrapped in damp newspaper. Approximately 500 sticks were collected and packaged into sealed containers designed for the mutation source. The following morning these three containers were subject to 65Gy from a Linear Accelerator located at GenesisCare Bundaberg. Assess to this facility was made possible by 2PH Farms who were irradiating citrus budwood at the same time and kindly allowed the breeding team to include these three containers.

For the following four days these treated budsticks were budded onto rootstocks and arranged in the nursery at Bundaberg Research Station. Each rootstock was triple-budded to maximise the chance of each rootstock having a surviving bud. Each of the three buds was cut from a different budstick. A total of 1,734 buds were inserted onto 578 rootstocks.

Buds were unwrapped on the 1st March 2021 (23 days after irradiation) with very promising results of 450 rootstocks having at least one green bud. However the health of many of these buds changed quickly once they were unwrapped, and after about five days many had shrivelled. By the 11th March 2021 (34 days after irradiation) the surviving buds were starting to push.

Irradiated bud of '06C015' starting to push, 34 days after being irradiated with 65Gy in a linear accelerator. Note how three buds were placed on each rootstock to maximise the number of mutation events available for screening.

By the end of the project period (May 2021) there were 229 trees with at least one green bud, making it possible that the eventual field population to screen for seedlessness will be around 200-300 trees. This should be adequate to find low-seeded selection that may make this high-quality orange-like hybrid suitable for commercial production in dry regions of Australia.

Trees of '06C015' having survived a 65Gy mutation dose and shooting away in the nursery at Bundaberg Research Station, May 2021.

Outcomes

- Two new mandarins are now available to Australian citrus growers, with promise of supplying consumers with fruit that is visually attractive, easy to peel, low seeded and tasty.
- Techniques have been developed and validated to identify the cause of seed contamination in Afourer orchards. Results from commercial orchards in southern and northern Australia suggest that the unwanted pollen is coming from scion varieties such as Sweet orange, Clementine and Murcott. It has been shown that Afourer produces a sufficiently high frequency of zygotic seedlings to make it possible to identify the pollen varieties causing problems. This technology can now be applied commercially.
- Experiments with gibberellic acid have shown promise in overcoming the low productivity of '15C001'. This high-quality seedless selection warrants persistence despite its productivity issues and commercial growers have taken up the challenge of overcoming the fruit set issue. The project management committee will reserve a decision on commercial release until these problems are resolved.
- Using the extensive collection of citrus relatives held at Bundaberg it has been possible to identify a number of very low-acid citrus ancestors as well as some with extremely high Brix (26°). This data will aid international colleagues in better understanding the domestication of citrus and which genes are involved in determining important commercial traits such as acidity and sugars.
- Molecular markers have been validated and deployed to clearly differentiate 'Premier Murcott' and 'CB Murcott' from all other varieties, thus strengthening their intellectual property protection via PBR and US Patent. Molecular data has also been generated for over 600 accessions developed or used within the Australian breeding program helping to protect against theft prior to future commercialization. This technology has also been applied to privately owned citrus varieties and to validate the correct naming of varieties in Australian germplasm collections.
- More effective and efficient techniques have been adopted to capture the seedlessness trait. We have demonstrated that a single gene for seedlessness works under local conditions and that it can be transmitted between generations. Backcross populations are now field established, aimed at improving fruit quality to an export standard.
- All hybrids generated during the project period were screened and culled for Alternaria brown spot and Citrus scab, thus ensuring that future selections will have these traits already incorporated. Other useful disease resistances and techniques for screening bioactive compounds have been developed and applied to breeding populations.
- More than 40,000 hybrids are field established and at various stages of evaluation and selection.

Monitoring and evaluation

The program was managed effectively by the DAF Citrus breeding team with substantial input and support of growers and industry. The project management committee and the Variety Leadership Group are mechanisms utilised by the program to monitor and seek input with regards to its on-going performance. The successful establishment of over 40,000 hybrids, and the release of two new high-quality mandarin varieties is evidence that the project has performed as per expectations.

An independent review of investments in Citrus breeding and variety evaluation was commissioned by Hort Innovation in 2021. This review was guided by a comprehensive Terms of Reference (TOR) aligned to the Citrus Levy Fund's Strategic Investment Plan with Key Evaluation Questions (KEQ) identified. The review was carried out by GHD. The review identified that the program was delivering as per intended and is aligned with the Citrus Levy Fund's Strategic Investment Plan. Please contact Hort Innovation for more information regarding this review.

Recommendations

- 'Premier Murcott' and 'CB Murcott' are recommended for commercial grower evaluation in all Australian growing regions.
- Seedlessness and high fruit quality (both external and internal) should remain key breeding objectives.
- Increased efforts to 'fix' disease resistance traits should be made within the breeding program by maintaining activities around Alternaria brown spot and Citrus scab while expanding into Huanglongbing and Citrus black spot. This will require additional project activity outside of the national breeding program to ensure that levy payers receive equitable benefit and are not subsidizing issues that are not relevant to them.
- Advanced selections should be quickly topworked at the Dareton site so that growers can assess performance under southern conditions.
- Fruit set research should continue with '15C001' to maximise the chance of this selection becoming commercially useful.
- A small group of international breeders should be brought to Australia to review the technical aspects of the local breeding program and to present on activities in their own countries.

Refereed scientific publications

Journal article

Smith M.W., Newman T.K., Gultzow D.L., Parfitt S.C., Barkley P.B. 2016. Citrus tristeza virus replication and movement in seedlings of 71 rootstock genotypes. *Citrus Research and Technology* **37:**156-164.

Smith M.W., Gultzow D.L., Newman T.K., Parfitt S.C., Miles A.K. 2016. A co-inoculation technique to rapidly screen citrus hybrids for resistance to both Alternaria Brown Spot caused by Alternaria alternata and Citrus Scab caused by Elsinoe fawcettii. *Citrus Research and Technology* **37**:82-87.

Tran N.T., Miles A.K., Smith M.W., Dietzgen R.G., Drenth A. 2018. Pathogenicity of Phyllosticta citricarpa Ascospores on Citrus spp. *Plant Disease* **102**:1386-1393.

Miles A.K., Smith M.W., Tran N.T., Shuey T.A., Dewdney M.M., Drenth A. 2019. Identification of Resistance to Citrus Black Spot Using a Novel In-field Inoculation Assay. *HortScience* **54**:1673-1681.

Arlotta C., Ciacciulli A., Strano M.C., Cafaro V., Salonia F., Caruso P., Licciardello C., Russo G., Smith M.W., Cuenca J., Aleza P., Caruso M. 2020. Disease Resistant Citrus Breeding Using Newly Developed High Resolution Melting and CAPS Protocols for Alternaria Brown Spot Marker Assisted Selection. *Agronomy* **10**: 2-21.

Whole book

Hardy, S., Barkley, P., Treeby, M., Smith, M., Sanderson, G. 2017. Australian mandarin production manual, State of New South Wales. ISBN 978 1 76058 056 8

Chapter in a book or Paper in conference proceedings

Webb M., Smith M.W. 2017. Assessment of molecular markers for improving citrus selections, Agri-Science Queensland Innovation Opportunity, Queensland Department of Agriculture & Fisheries, Brisbane, Australia. pp. 14.

Licciardello C., Russo M.P., Scalabrin S., Scaglione D., Salzano M., Cattonaro F., Smith M., Caruso M., Morgante M., Caruso P. 2019. Murraya paniculata genome and transcriptome as a source for the exploitation of resistance and susceptibility genes against Xanthomonas citri pv citri., LX111 Siga Annual Congress "Science and innovation for sustainable agriculture intensification: The contribution of plant genetics and breeding", Napoli, Italy.

Caruso M., Smith M.W., Froelicher Y., Russo G., Gmitter Jr F.G. 2020. Traditional breeding, In: M. Talon, et al. (Eds.), The Genus Citrus, Elsevier, United Kingdom. pp. 129-148. ISBN 978 0 12 812163 4

Intellectual property, commercialisation and confidentiality

An Intellectual Property Register, consisting of a listing of all field grown hybrid progenies currently held at Bundaberg Research Station, was submitted to Hort Innovation at the same time as this Final Report.

Background Intellectual Property, including all equity arrangements, were resolved in the CT15017 research agreement.

Acknowledgements

Debra Gultzow and Toni Newman provided outstanding technical support throughout CT15017 and without their care and commitment the program would not have been successful. Toni retired in early 2021 bringing to an end a remarkable 20-year period of stability for the 3-person breeding team. Michael Reid provided technical support in the later stages of the project as did Carola Parfitt in the first year. Bruce Boucher and more recently Justin Davies have efficiently managed the research station and worked hard to ensure the breeding work had the necessary facilities and equipment. Breeding director David Innes and commercialisation manager Gary Hopewell have been enthusiastic supports of the project and provided a great deal of help with complex funding and intellectual property issues. Rod Edmonds provided welcome help in developing the proposal and also recently retired, to be replaced by Anton Zbonak. Casual staff who have assisted at times during the period include Jesse Thomas and Tim Caflisch.

Andrew Miles, Patricia Barkley and Helen Hofman have been dependable sources of advice, guidance, and scientific information throughout the project. Matt Webb played a key role in the application of molecular techniques within the project and Paul Campbell was invaluable in establishing methodologies for testing bioactive compounds. Nah Tran and Andre Drenth provided an excellent opportunity for collaboration at the interface between pathology and plant breeding.

Many international colleagues have contributed in different ways to the project including Fred Gmitter, Pablo Aleza, Marco Caruso, Kwan Song, Robert Krueger, Toni Siebert, David Karp, Mike Rouse, Ed Stover and Randy Drigger.

Growers have made significant contributions to this project in addition to their compulsory levy's. Nick Ulcoq, Mark Trott and Paul Berthelsen, and their associates, provided land and all management inputs for the large commercial testing blocks which ultimately led to the release of 'Premier Murcott' and 'CB Murcott'. They have allowed access at all times and provided suggestions and feedback which have helped to guide the breeding work. Craig and (the late) Bindi Pressler provided funding for SNP marker analysis and made it possible to incorporate this technology into the national breeding program. They also provided free access to a linear accelerator for mutation breeding work within CT15017 and Afourer seed for the pollen contamination work. Andrew Harty supplied Afourer seed from solid blocks and suggestions regarding pollen contamination.