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UNIVERSITY OF CALIFORNIA
RIVERSIDE

Cross-Compatibility, Graft-Compatibility, and Phylogenetic Relationships in the
Aurantioideae: New Data From the Balsamocitrinae

A Thesis submitted in partial satisfaction of the requirements for the degree of

Master of Science

in

Plant Biology

by

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December 2016

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ACKNOWLEDGEMENTS

I am indebted to many people who have been an integral part of my research and supportive throughout my graduate studies:

A huge thank you to Dr. Norman Ellstrand as my major professor and graduate advisor, and to my supervisor, Dr. Tracy Kahn, who helped influence my decision to go back to graduate school while allowing me to continue my full-time employment with the UC Riverside Citrus Variety Collection. Norm and Tracy, my UCR parents, provided such amazing enthusiasm, guidance and friendship while I was working, going to school and caring for my growing family. Their support was critical and I could not have done this without them.

My committee members, Dr. Timothy Close and Dr. Robert Krueger for their valuable advice, feedback and suggestions.

Robert Krueger for mentoring me over the past twelve years. He was the first person I met at UCR and his willingness to help expand my knowledge base on *Citrus* varieties has been a generous gift. He is also an amazing friend.

Tim Williams for teaching me everything I know about breeding *Citrus* and without whom I'd have never discovered my love for the art. I will always appreciate his attention, encouragement and mentoring.

Dr. Shana Welles, who taught me the various laboratory techniques essential to completing my research, even though she was busy writing her own dissertation.

Toots Bier who recruited me, introduced me to *Citrus*, and showed me how to forge a path for myself in *Citrus* research while guiding me until her own retirement.

Vicki Newman and Brittany Moreland of the USDA Citrus Germplasm Repository for not only their technical assistance during my experiments, but who provided endless comfort, laughter and friendship.

David Karp, my friend and fellow *Citrus* admirer, who has spent the last ten years photographing the entire Citrus Variety Collection with me.

My laboratory assistants, Le Nguyen, Tom Shea, and Karene Trunnelle who helped a great deal during the peak of my laboratory research to make the rest of our *Citrus* research come together as planned. Thank you also for all the laughs.

Jammy Yang in Graduate Student Services and anyone else who pushed the astonishing amount of forms, documents and other paperwork for me in order to make this degree attainable.

I could not have done this without the love and encouragement of my family including my in-laws, who have been supportive throughout my career and provided countless hours of daycare. I appreciate it. So much of my success I owe to the love of my parents, who allowed me to follow my ambitions throughout my childhood and have also instilled in me the value of hard work. Lastly, Dexter and Charli, who I love with all my heart, may you always know that anything you want out of life is possible.

This work is dedicated to my husband, Kirby Wooldridge. You were instrumental in the completion of this degree by not only working countless overtime shifts and caring for our young son and daughter, but also providing your unconditional encouragement, love and support. I am thankful for every day that I have you in my life.

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INTRODUCTION

Loss of genetic diversity is a threat to some of our most vital food crops. Although *Citrus* is one of the most important tree crops in terms of global production (FAO 2014), it is vulnerable to environmental stresses, land development, exotic pests, and recently appearing pathogens such as Huanglongbing (HLB) (Bové 2006). Consequently, wild relatives with a close genetic relationship to *Citrus* have been deemed a priority for worldwide conservation as key resources for the improvement of *Citrus* crops because many are at risk of genetic erosion and even extinction (Vincent *et al* 2013). In 2015, a global inventory of all collections of *Citrus* and wild *Citrus* relative germplasm was piloted by the Global Citrus Network (GCN). Strategies to safeguard at-risk *Citrus* germplasm have been implemented by Global Crop Diversity Trust which began cataloging wild relatives of 22 major food crops in 2010 and recently made accessible a searchable database online that contains the global inventory of wild *Citrus* relatives (Roose *et al* 2015). Now it is possible to locate a resource of a rare distant *Citrus* relative species on the other side of the globe within minutes.

To understand the relationship between *Citrus* and its wild relatives, we must appreciate some of the complications regarding *Citrus* taxonomy. Currently, the phylogenetic relationship between *Citrus* and its related taxa is problematical due to competing taxonomic systems based on opposing species concepts (Krueger 2010). The most broadly accepted classification systems are based on traditional plant descriptors such as geographic distribution, anatomy, and morphology, which were developed by

Swingle (1943), then revised as Swingle and Reece (1967), and expanded into an alternative system by Tanaka (1932,1977). *Citrus* exists in the Aurantioideae, one of the seven subfamilies in the family Rutaceae (Engler 1989, 1931). Traditionally, Aurantioideae was divided into tribes, with Tanaka (1932) recognizing as many as eight distinct tribes. The system most commonly used, and for which preference will be made to in this thesis, is the one proposed by Swingle (1943) which recognized only two tribes, Clauseneae and Citreae (Table 1). The following synopsis of the Aurantioideae is based upon Swingle (1943).

The Clauseneae tribe consists of taxa comprising the Very Remote and Remote Citroid Fruit Trees, and in itself is comprised of three subtribes: the Micromelinae, containing the genus *Micromelum*; the Clauseninae, containing three genera (*Glycosmis*, *Clausena*, and *Murraya*); and the Merrillinae, containing one genus *Merrillia*.

The Citreae tribe contains familiar genera such as *Citrus*, *Poncirus*, *Fortunella*, and *Microcitrus*. This tribe is comprised of three subtribes: the Triphasiinae, containing the eight Minor Citroid genera: *Wenzelia*, *Monanthocitrus*, *Oxanthera*, *Merope*, *Triphasia*, *Pamburus*, *Luvunga*, and *Paramignya*; the Citrinae, containing the thirteen genera: *Severinia*, *Pleiospermium*, *Burkillanthus*, *Limnocitrus*, *Hesperethusa*, *Citropsis*, *Atalantia*, *Fortunella*, *Eremocitrus*, *Poncirus*, *Clymenia*, *Microcitrus*, and *Citrus*; and the Balsamocitrinae, containing the seven Hard-Shelled Citroid genera: *Swinglea*, *Aegle*, *Afraegle*, *Aeglopsis*, *Balsamocitrus*, *Feronia*, and *Feroniella*.

Table 1. Tribes, subtribes and genera of the Aurantioideae subfamily as summarized by Swingle (1943).

Tribe I. Clauseneae: Very Remote and Remote Citroid Fruit Trees
Subtribe 1. Micromelinae: Very Remote Citroid Fruit Trees
I. <i>Micromelum</i>
Subtribe 2. Clauseninae: Remote Citroid Fruit Trees
II. <i>Glycosmis</i>
III. <i>Clausena</i>
IV. <i>Murraya</i>
Subtribe 3. Merrillinae: Large-Fruited Remote Citroid Fruit Trees
V. <i>Merrillia</i>
Tribe II. Citreae: Citrus and Citroid Fruit
Subtribe 1. Triphasiinae: Minor Citroid Fruit Trees
VI. <i>Wenzelia</i>
VII. <i>Monanthocitrus</i>
VIII. <i>Oxanthera</i>
IX. <i>Merope</i>
X. <i>Triphasia</i>
XI. <i>Pamburus</i>
XII. <i>Luvunga</i>
XIII. <i>Paramignya</i>
Subtribe 2. Citrinae: Citrus Fruit Trees
Group A. Primitive Citrus Fruit Trees
XIV. <i>Severinia</i>
XV. <i>Pleiospermium</i>
XVI. <i>Burkillanthus</i>
XVII. <i>Limnocitrus</i>
XVIII. <i>Hesperethusa</i>
Group B. Near-Citrus Fruit Trees
XIX. <i>Citropsis</i>
XX. <i>Atalantia</i>
Group C. True Citrus Fruit Trees
XXI. <i>Fortunella</i>
XXII. <i>Eremocitrus</i>
XXIII. <i>Poncirus</i>
XXIV. <i>Clymenia</i>
XXV. <i>Microcitrus</i>
XXVI. <i>Citrus</i>
Subtribe 3. Balsamocitrinae: Hard-Shelled Citroid Fruit Trees
XXVII. <i>Swinglea</i>
XXVIII. <i>Aegle</i>
XXIX. <i>Afraegle</i>
XXX. <i>Aeglopsis</i>
XXXI. <i>Balsamocitrus</i>
XXXII. <i>Feronia</i>
XXXIII. <i>Feroniella</i>

However, more recent phylogenetic studies based on molecular analysis or chemotaxonomy have aligned some of the relationships between taxa in the Aurantioideae in a different way. Furthermore, some species and genera have been subject to various taxonomic revisions (Table 2). While new perspectives have produced some uncertainty of the Tribe-Subtribe division, they have reinforced the validity of the Citrus Fruit Trees in the Citrinae subtribe as a distinct interrelated group (Krueger 2010).

Additionally, Rutaceous plants may contain the largest number of genera and species that exhibit a reproductive system known as nucellar polyembryony, a type of apomixis (Lim 1996). Apomixis is a term generally used to describe reproduction without fertilization. The seeds produced in this system will contain embryos identical to the maternal parent (Grant 1981). The current use of the term “agamospermous” is also widely accepted and considered synonymous with apomixis (Bicknell 2004). Some

Table 2. Examples of taxonomic revisions in the Aurantioideae

Species recognized by Swingle (1943)	Taxonomic synonym	Reference
<i>Murraya koenigii</i>	<i>Bergera koenigii</i>	Kong <i>et al</i> (1986) Li <i>et al</i> (1988) Guerra <i>et al</i> (2000) Samuel <i>et al</i> (2001) Ranade <i>et al</i> (2006)
<i>Hesperethusa crenulata</i>	<i>Naringi crenulata</i>	Panigrahi (1975) Weirsem & Leon (1999)
<i>Feronia limonia</i>	<i>Feronia acidissima</i>	Airy-Shaw (1939)
<i>Feronia limonia</i>	<i>Limonia elephantum</i>	Panigrahi (1975) Weirsem & Leon (1999)
<i>Feronia limonia</i>	<i>Limonia acidissima</i>	Stone & Nicolson (1978)

apomictic species are facultative, in which case apomixis does not always take place and sexual reproduction also occurs in the species such that some plants create both asexual and sexual seed, or it may be obligate, where apomixis is the main or only method of reproduction. Apomictic plants may be one of two main types: gametophytic or adventitious (also known as sporophytic). In a gametophytic system, there is a gametophytic phase involved in the development of the embryo. Gametophytic apomixis can be further divided into two categories: apospory and diplospory.

However, in adventitious agamospermy, the embryo does not arise from the cells of the gametophyte. Rather, the embryo develops directly from vegetative cells that surround the ovule (either the nucellus or the integument), and within one of two cell layers away from the embryo sac (Lim 1996). Since the cells do not enter a gametophytic phase, the embryo is also considered a somatic embryo, and because the tissue used to create the embryo is called the nucellus, this type of apomixis is often referred to as nucellar embryony. Nucellar embryony is the predominant form of agamospermy in *Citrus* and some other species of tropical tree crops like *Mangifera* (Webber 1931). Zygotic embryos occur in the system, and they frequently develop beside the nucellar embryo, producing two or more seedlings from the same seed, a condition referred to as polyembryony (Aleza 2010). Sometimes the zygotic embryo may not complete development. In this case, all seedlings are of nucellar origin and thus are same genotype as the female parent (Koltunow 1993). The occurrence rate of nucellar seedlings in *Citrus* can be highly variable and is often dependent on environmental influences and genotype (Khan and Roose 1988).

One of the oldest known records of nucellar polyembryony in *Citrus* is from Antonie van Leeuwenhoek, who discovered its existence in sweet orange in 1719 (Cook 1907). With the exception of citron, pummelo, clementine, and some mandarin hybrids, most *Citrus* genotypes are agamospermous and frequently produce excessive nucellar embryos in the embryo sac (Frost 1926). Studies on nucellar embryony in *Citrus* have received much attention due to their ability to provide virus-free, genetically uniform offspring---an important feature for citrus rootstock cultivar development (Lim 1996). However, at the same time it can often interfere with conventional hybridization methods utilized in the improvement of commercial *Citrus* scion cultivars.

Despite extensive work done in the reproductive biology and phylogenetic relationships of *Citrus*, not much is known regarding the reproductive biologies of the majority of wild *Citrus* relatives (Table 3). Additionally, limited work has been accomplished in the area of fertility relationships between *Citrus* and other members of

Table 3. Reported reproductive biologies of some lesser known wild *Citrus* relatives.

Taxa	Tribe-Subtribe	Reproduction type	Reference
<i>Clausena</i> spp.	Clauseneae-Clauseninae	Nucellar embryony	Bitters (1986)
<i>Bergera</i> (<i>Murraya</i>) <i>koenigii</i> ¹	Clauseneae-Clauseninae	Nucellar polyembryony	Chakravarthy (1935) Chakravarthy (1936)
<i>Murraya paniculata</i> (<i>exotica</i>) ²	Clauseneae-Clauseninae	Nucellar polyembryony	Chakravarthy (1935) Chakravarthy (1936)
<i>Triphasia trifolia</i> (<i>aurantifolia</i>) ³	Citreae-Triphasiinae	Nucellar polyembryony	Mauritzon (1935) Lim (1996)
<i>Citropsis</i> spp.	Citreae-Citrinae	Nucellar embryony	Bitters (1986)
<i>Aegle marmelos</i>	Citreae-Balsamocitrinae	Nucellar embryony	Chakravarthy (1935) Johri and Ahuja (1956)

1. After the publication of Chakravarthy (1935), *Murraya koenigii* was moved to a new genus *Bergera koenigii*. 2. *Murraya paniculata* and *Murraya exotica* may be considered synonymous (Krueger 2010). 3. After the publication of Mauritzon (1935), *Triphasia aurantifolia* was moved to a new species, *Triphasia trifolia*.

the Aurantioideae. It has been well established that genes in wild relatives of crops often possess traits that could provide benefit to the related crop, such as natural resistance to diseases and insect pests, special adaptations to local environments, or even improvement of yield and quality (Hajjar 2007; Tanksley 1997). There are many successful examples of crop improvement accomplished by human-mediated introgression of novel wild relative genes into standard cultivars of domesticated plants (Hajjar 2007; Tanksley 1997). In spite of this precedence, the cross compatibility of the numerous Aurantioideae taxa remains largely unknown. This type of data is of immediate practical value to not only germplasm banks and collections, but also *Citrus* improvement programs. Additionally, the correlation of cross and graft compatibility could be evolutionarily significant as evidence of the phylogenetic relationships in the subfamily.

This work, then, has two goals. First, it investigates what is currently known regarding cross compatibilities in the Aurantioideae so that deficiencies in knowledge in this area may be addressed. Second, it reviews known intergeneric graft congenialities and attempts to better define the subfamilies interrelationships when comparing these findings with more contemporary molecular based phylogenetic associations.

CHAPTER I: CROSS COMPATIBILITIES IN THE AURANTIOIDEAE WITH EMPHASIS ON THE BALSAMOCITRINAE SUBTRIBE

Distant citrus relatives often possess genetically-based traits that can be useful for the improvement of standard Citrus cultivars, such as natural resistance to pathogens. Thus, cross compatibility data between Citrus and its relatives has potential value to Citrus improvement programs as it can be used to predict the introgressability of these genes. This chapter reviews the current knowledge of cross compatibilities among Citrus and related genera and identifies gaps in the data. To address these deficiencies, a selection of diverse germplasm from the Aurantioideae subfamily was utilized to perform cross pollinations. These findings are an important supplement in what is known regarding cross compatibilities within the Aurantioideae

The modern *Citrus* industry faces many challenges as it contends with destructive diseases like Huanglongbing (HLB), which has already caused the loss of more than half of Florida's citrus tree acreage (Rusnak 2015). It is plausible that tolerance or resistance to HLB and other *Citrus* diseases may only be accomplished through conventional breeding (Roose, M., pers. comm., ca. 2014). Thus, it is imperative that the possibility of combining the unique qualities of *Citrus* wild relatives with *Citrus* be investigated. An impressive screening of over one hundred Aurantioideae accessions and nearly 900 seedlings and their response to HLB exposure has found resistant (low levels of the pathogen were detected in the host but the pathogen was unable to establish), tolerant (the pathogen was detected in the host but the host was able to recover despite exhibiting symptoms), and even a few immune (the pathogen was undetectable in the host despite repeat exposure) accessions in wild *Citrus* relatives (Ramadugu et al 2016).

Therefore, cross compatibility data between *Citrus* and its wild relatives must be collected so that potential intergeneric hybrids can be identified.

Historically, breeders have only turned to intergeneric hybrids as a last-ditch effort for many reasons besides the lack of available germplasm. They can be physically challenging to cross due to small floral morphology. If seeds are produced they may not be viable. Any seedlings obtained from the union may be not survive in the field, may have sterility issues, not flower at all, or bring many other undesirable morphological traits to the table with them (Barrett 1985). Unfortunately, also as the phylogenetic distance between taxa increases, so do many pre- and post-zygotic barriers (Ladizinsky 1992).

Cross compatibility between a few genera in the Aurantioideae subfamily is well-established. For example, many commercial citrus rootstocks were created by combining *Poncirus trifoliata* (L.) Raf. with a sweet orange, mandarin or sometimes grapefruit. The ability of *Citrus* and *Poncirus* to hybridize was not recognized until the beginning of the 20th century when the first organized breeding program to intentionally produce intergeneric hybrids was formed in response to a period of catastrophic cold temperatures that occurred in Florida in 1894 (Barrett 1977). Working for the United States Department of Agriculture (USDA), W.T. Swingle and H.J. Webber sought to develop scion varieties with adaptations to cold tolerance. The progeny from these crosses were indeed resilient in colder climates, but were also sour, bitter, and nearly inedible, one of the more undesirable traits acquired from the *Poncirus* parent (Barrett

1977). Willard P. Bitters, the UC Riverside Citrus Variety Collection (CVC) curator from 1947 to 1982, performed extensive field testing on these types of hybrids during his career, making significant contributions to what we now know regarding rootstocks that are tolerant or resistant to viral and fungal pathogens, soil-borne parasites, or unfavorable soil conditions (Bitters 1986). Other early intergeneric hybrids were made between *Citrus* and *Fortunella*, *Citrus* and *Microcitrus*, and *Fortunella* and *Poncirus* (Barrett 1977). Today the compatibility of these genera is established and presently used in *Citrus* breeding programs. In reviewing what is known about cross compatibilities among *Citrus* and its relatives subsequent to this time, we find uneven answers.

Barrett (1977) was part of a later generation of researchers who attempted to create wide intergeneric hybrids for citrus cultivar improvement. He faced many challenges in procuring the germplasm he needed in order to conduct breeding experiments of this type, as movement of the material had become regulated and germplasm resources were increasingly scarce. His attempts to hybridize *Citrus* × *Glycosmis* and *Citrus* × *Severinia* were failures. He was able to produce F₁ hybrids using different species in crosses of *Citrus* × *Eremocitrus*, and different species in crosses of *Microcitrus* × *Citrus*. F₁ hybrids between genera other than *Citrus* included *Eremocitrus* × *Fortunella*, *Eremocitrus* × *Microcitrus*, and *Microcitrus* × *Fortunella*. Crosses of *Microcitrus* × *Poncirus*, *Eremocitrus* × *Poncirus*, and *Citrus* × *Citropsis*, produced seeds that failed to germinate and some seedlings that died soon after germination. *Fortunella* × *Poncirus* progeny were reported to be fairly weak.

Table 4. Crosses attempted between Aurantioideae taxa as reported by Iwamasa *et al* (1988).¹

Female parent	Male parent	Results
<i>Glycosmis</i>	<i>Murraya</i>	No seed
<i>Glycosmis</i>	<i>Poncirus trifoliata</i>	No seed
<i>Glycosmis</i>	<i>Microcitrus</i>	No seed
<i>Glycosmis</i>	<i>Citrus</i>	No seed
<i>Murraya</i>	<i>Poncirus trifoliata</i>	No seed
<i>Murraya</i>	<i>Microcitrus</i>	No seed
<i>Murraya</i>	<i>Citrus</i>	No seed
<i>Murraya</i>	<i>Fortunella</i>	No seed
<i>Murraya paniculata</i>	<i>Murraya koenigii</i> (Bergera koenigii) ²	No seed
<i>Triphasia</i>	<i>Poncirus trifoliata</i>	No seed
<i>Triphasia</i>	<i>Microcitrus</i>	No seed
<i>Triphasia</i>	<i>Citrus</i>	No seed
<i>Atalantia</i>	<i>Severinia</i>	No seed
<i>Atalantia</i>	<i>Fortunella</i>	No seed
<i>Atalantia</i>	<i>Microcitrus</i>	No seed
<i>Atalantia</i>	<i>Poncirus trifoliata</i>	No seed
<i>Atalantia</i>	<i>Citrus</i>	No seed
<i>Fortunella</i>	<i>Severinia</i>	No seed
<i>Fortunella</i>	<i>Citropsis</i>	No seed
<i>Citrus</i>	<i>Severinia</i>	No seed
<i>Citrus</i>	<i>Hesperethusa crenulata</i> (<i>Naringi crenulata</i>) ³	No seed
<i>Citrus</i>	<i>Citropsis schweinfurthii</i>	Several seeds, no seedlings
<i>Citrus</i>	<i>Clausena</i>	No seed
<i>Citrus</i>	<i>Murraya</i>	No seed
<i>Citrus grandis</i>	<i>Microcitrus australasica</i>	Seedlings obtained
<i>Citrus grandis</i>	<i>Microcitrus virgata</i>	Seedlings obtained
<i>Citrus grandis</i>	<i>Microcitrus inodora</i>	Seedlings obtained
<i>Citrus grandis</i>	<i>Poncirus trifoliata</i>	Seedlings obtained
<i>Citrus iyo</i>	<i>Microcitrus australasica</i>	Seedlings obtained
<i>Citrus iyo</i>	<i>Microcitrus virgata</i>	Seedlings obtained
<i>Citrus iyo</i>	<i>Microcitrus inodora</i>	Seedlings obtained
<i>Citrus macroptera</i>	<i>Microcitrus australasica</i>	Seedlings obtained
<i>Citrus macroptera</i>	<i>Poncirus trifoliata</i>	Seedlings obtained
<i>Severinia buxifolia</i>	<i>Fortunella</i>	No seed
<i>Severinia buxifolia</i>	<i>Citrus</i>	No seed
<i>Hesperethusa crenulata</i> (<i>Naringi crenulata</i>) ³	<i>Citrus</i>	No seed
<i>Citropsis</i>	<i>Atalantia</i>	No seed
<i>Citropsis</i>	<i>Poncirus</i>	No seed
<i>Citropsis gabunensis</i>	<i>Fortunella margarita</i>	Several seeds, no seedlings
<i>Citropsis schweinfurthii</i>	<i>Citropsis gabunensis</i>	No seed
<i>Eremocitrus</i>	<i>Poncirus trifoliata</i>	No seed
<i>Fortunella margarita</i>	<i>Fortunella hindsii</i>	Seedlings obtained
<i>Fortunella margarita</i>	<i>Poncirus trifoliata</i>	Seedlings obtained
<i>Fortunella margarita</i>	<i>Microcitrus australasica</i>	Seedlings obtained

<i>Fortunella margarita</i>	<i>Citrus aurantium</i>	Seedlings obtained
<i>Fortunella margarita</i>	<i>Citropsis gabunensis</i>	Several seeds, no seedlings
<i>Microcitrus australasica</i>	<i>Eremocitrus</i>	No seed
<i>Microcitrus australis</i>	<i>Eremocitrus</i>	No seed
<i>Microcitrus inodora</i>	<i>Eremocitrus</i>	Seedlings obtained
<i>Microcitrus virgata</i>	<i>Eremocitrus</i>	No seed
<i>Microcitrus warburgiana</i>	<i>Eremocitrus</i>	No seed
<i>Microcitrus australasica</i>	<i>Poncirus trifoliata</i>	Seedlings obtained
<i>Microcitrus australis</i>	<i>Poncirus trifoliata</i>	No seed
<i>Microcitrus inodora</i>	<i>Poncirus trifoliata</i>	Seedlings obtained
<i>Microcitrus virgata</i>	<i>Poncirus trifoliata</i>	Seedlings obtained
<i>Microcitrus warburgiana</i>	<i>Poncirus trifoliata</i>	No seed
<i>Microcitrus australasica</i>	<i>Fortunella margarita</i>	Seedlings obtained
<i>Microcitrus australis</i>	<i>Fortunella margarita</i>	No seed
<i>Microcitrus inodora</i>	<i>Fortunella margarita</i>	No seed
<i>Microcitrus virgata</i>	<i>Fortunella margarita</i>	No seed
<i>Microcitrus warburgiana</i>	<i>Fortunella margarita</i>	Seedlings obtained
<i>Microcitrus australasica</i>	<i>Citrus</i>	No seed
<i>Microcitrus australis</i>	<i>Citrus latipes</i>	Seedlings obtained
<i>Microcitrus inodora</i>	<i>Citrus</i>	No seed
<i>Microcitrus virgata</i>	<i>Citrus ichangensis</i>	Seedlings obtained
<i>Microcitrus warburgiana</i>	<i>Citrus</i>	No seed
<i>Microcitrus australasica</i>	<i>Microcitrus</i>	No seed
<i>Microcitrus australis</i>	<i>Microcitrus</i>	No seed
<i>Microcitrus inodora</i>	<i>Microcitrus australis</i>	Seedlings obtained
<i>Microcitrus virgata</i>	<i>Microcitrus australis</i>	Seedlings obtained
<i>Microcitrus warburgiana</i>	<i>Microcitrus</i>	No seed
<i>Microcitrus inodora</i>	<i>Microcitrus australasica</i>	Seedlings obtained
<i>Microcitrus inodora</i>	<i>Microcitrus virgata</i>	Seedlings obtained
<i>Microcitrus virgata</i>	<i>Microcitrus inodora</i>	Seedlings obtained

1. Many species names were not provided by original authors. 2. After the publication of the investigation by Iwamasa *et al*, *Murraya koenigii* was moved to a new genus *Bergera koenigii*. 3. After the publication of the investigation by Iwamasa *et al*, *Hesperethusa crenulata* was moved to a new genus *Naringi crenulata*

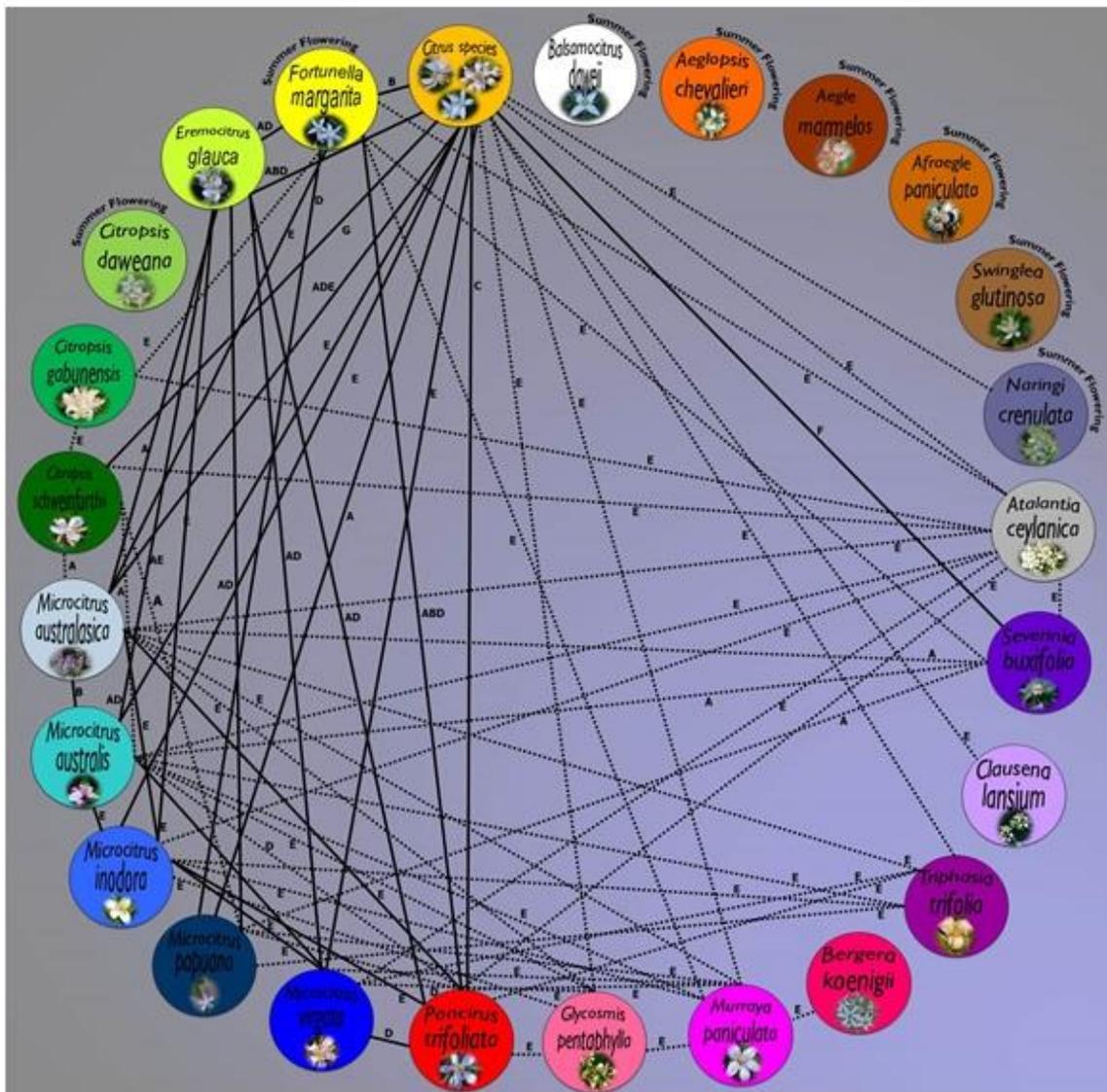
In 1988 wider crosses were attempted between more remote genera in the Aurantioideae (Iwamasa *et al* 1988) as seen in Table 4. The only hybrids obtained from this study were again from various species of *Microcitrus* × *Poncirus*, *Microcitrus* × *Fortunella*, *Microcitrus* × *Eremocitrus*, *Microcitrus* × *Citrus*, *Citrus* × *Microcitrus*, and *Citrus* × *Poncirus*. Barrett (1977) was unable to obtain seedlings from *Microcitrus* × *Poncirus* crosses, but Iwamasa *et al* (1988) were successful in their study. Incompletely

developed seeds were produced from *Citropsis* × *Citrus* and *Citropsis* × *Fortunella* crosses. These results, along with the earlier study from Barrett (1977), suggested that intergeneric hybrids from these species might be possible with larger numbers of crosses.

The first sexually obtained hybrids between *Citrus* and *Severinia* and *Severinia* and *Citrus* were reported by Medina-Filho *et al* (1998), a particularly wide cross between genera in the Citrinae subtribe. 297 pollinations of *Citrus sunki* (Hayata) hort. ex Tanaka × *Severinia buxifolia* (Poir.) Ten. were performed along with 219 pollinations of the reciprocal cross, yielding 3 hybrid plants of *Citrus sunki* × *Severinia buxifolia*, and 2 hybrid plants of *Severinia buxifolia* × *Citrus sunki*, with conspicuous intermediate morphology between the parent types.

Yahata *et al* (2006) and Yasuda *et al* (2010) tried to complete earlier work started by Barrett (1997) and Iwamasa (1988) by finally producing intergeneric sexual hybrids of *Citrus* × *Citropsis* and *Citropsis* × *Citrus*. Yahata (2006) was able to obtain a set of developed and underdeveloped seeds from reciprocal crosses of *Citrus* and *Citropsis*. Embryo rescue was performed with the developed seeds, which were grown in culture, but did poorly after being transplanted to soil until they were micrografted onto *Poncirus trifoliata* seedlings. Additionally, Smith *et al* (2013) hybridized *Citrus wakonai* P.I.Forst. & M.W.Sm. × *Citropsis gabunensis* (Engl.) Swingle & M. Kellerm. and successfully produced the first fruiting intergeneric hybrids with these genera within a few years.

Figure 1. Results of all known intergeneric crossing studies within the Rutaceae subfamily Aurantioideae are shown.



A: Barrett 1977
B: Swingle & Reece 1967
C: Bitters 1969
D: Barrett 1985
E: Iwamasa et al. 1988
F: Medina et al. 1998
G: Yahata et al. 2006

— = SUCCESSFUL CROSS
 - - - = FAILED CROSS
 NO LINE = NO CROSS ATTEMPTED

A more recent report of an even wider cross has been attempted with distant germplasm in the subfamily Toddalioideae and *Citrus*. Munter (2015) pollinated *in situ* *Zanthoxylum americanum* Mill. with various *Citrus* cultivars obtained from the USDA National Clonal Germplasm Repository for Citrus and Dates (USDA-NCGRCD) in Riverside, CA. *Citrus* pollen germinated and pollen tubes grew on *Zanthoxylum americanum* stigmas and styles. However, the seedlings obtained from these crosses showed only *Zanthoxylum americanum* morphology, suggesting the seeds were produced by apomixis (Munter, 2015). Like *Citrus*, nucellar embryony is the dominant reproduction system in this dioecious plant species.

Figure 1 displays information that is presently known regarding cross compatibility among *Citrus* and *Citrus* relative taxa. Much of the data collected was extracted as collateral information from the above-mentioned breeding studies. However, these prior studies have left us with many gaps in the data. Although several gaps were identified, compatibility data within the Hard-Shelled Citroid subtribe, Balsamocitrinae, was largely lacking. To address this deficiency, a selection of diverse germplasm from the Aurantioideae subfamily was cross pollinated onto *Aegle marmelos* (L.) Correa (Indian bael fruit), the most economically important species in the Balsamocitrinae.

Methods

The Citrus Variety Collection (CVC) is a resource of more than 1,000 varieties of *Citrus* and *Citrus* relative taxa, consisting of 28 of the 33 different genera existing in the

Aurantioideae subfamily. Many of the *Citrus* relative accessions held by the CVC don't visually appear to have any relationship to *Citrus* (See Appendix A). *Aegle marmelos* was chosen as the female parent due to its abundant flowering, tree size, location and number of accessions available for use in the CVC. Male parents for this study were selected based upon pollen availability and taxonomic distance from *Aegle marmelos*. The cross combinations and number of pollinations can be seen in Table 5. Comprehensive morphological descriptor information on *Citrus* relative taxa is available in CVC accession databases, including flowering and fruiting phenology. It is important to note that within the Balsamocitrinae subtribe, flowering mainly occurs in the summer months, specifically July and August in Riverside, CA. This is also true for species of *Fortunella*. However, the main flowering season for *Citrus* is in early spring, largely March through April in Riverside. Only fresh pollen was utilized, so in the cases of differing flowering seasons sometimes flowers from off-blooms were used. Pollinations were conducted in the field, and took place during years 2012, 2013 and 2014.

Pollen collection

Flowers from trees used as a male parent were selected prior to anthesis. For most types of citrus, the anthers will not dehisce prior to the flower opening, but care still must be taken to prevent possible cross-contamination from outside pollen sources (Williams, T., pers. comm., ca. 2007). Flowers were collected into small paper bags and placed into a cooler until transported to the laboratory. Stigmas or entire stylar stalks were removed to prevent pollen from sticking to the stigma using forceps. Anthers

were then removed from the filaments using forceps and placed into a plastic petri dish. Petri dishes were placed under an incandescent light source at room temperature overnight to encourage dehiscence. When the majority of the anthers had opened and pollen was visible, pollen was then collected directly into a labeled centrifuge tube. Tubes of pollen were then stored in a refrigerator until use for up to one week to retain good viability.

Pollinations

Pollinations took place over a two-week period from late July through early August in 2012, 2013 and 2014, with fruit maturing roughly 52-56 weeks later. Pollinations were performed using methods routinely used by the UC Riverside Citrus Breeding program (Williams, T., personal communications, 2007-current). *Citrus* pollen germinates well at temperatures above 65 degrees Fahrenheit (Williams, T., personal communication, ca. 2007), and germination is maintained at the average high of 95 degrees Fahrenheit in Riverside in July and August. Closed flowers that are receptive to pollinations are those with petal tips just starting to open, indicating they would open spontaneously within a few hours. Open flowers were never used as they could have already been pollinated by other pollen sources. Once a cluster or branch with flowers was chosen, any underdeveloped or completely open flowers were removed to prevent geitonogamy within a bagged cluster. Using forceps dipped in alcohol and dried, petals were spread open, and anthers were removed. Care was taken to avoid removing any

Table 5. Parental combinations with *Aegle marmelos* female parent, number of pollinations, number of fruit set, and fruit set percentage.

Male Parent and CRC #	Year 2012		
	# of pollinations	# of fruit set	% fruit set
<i>Aegle marmelos</i> (CRC 3140)	30	0	0%
<i>Aeglopsis chevalieri</i> (CRC 2878)	27	6	22.2%
<i>Balsamocitrus dawei</i> (CRC 3514)	20	4	20%
<i>Citrus madurensis</i> (CRC 2592)	40	3	7.5%
<i>Poncirus trifoliata</i> (CRC 3795)	24	3	12.5%
Male Parent and CRC #	Year 2013		
	# of pollinations	# of fruit set	% fruit set
<i>Aegle marmelos</i> (CRC 3140)	22	0	0%
<i>Afraegle paniculata</i> (CRC 4033)	23	2	8.7%
<i>Atalantia ceylanica</i> (CRC 3287)	23	0	0%
<i>Balsamocitrus dawei</i> (CRC 3514)	20	2	10%
<i>Citropsis gilletiana</i> (CRC 3296)	23	0	0%
<i>Citropsis schweinfurthii</i> (CRC 3126)	21	0	0%
<i>Citrus ichangensis</i> (CRC 2327)	25	5	20%
<i>Citrus medica</i> (CRC 3891)	21	2	9.5%
<i>Citrus x floridana</i> (CRC 3069)	23	4	17.4%
<i>Clausena excavata</i> (CRC 3166)	22	0	0%
<i>Fortunella crassifolia</i> (CRC 3818)	23	1	4.3%
<i>Fortunella polyandra</i> (CRC 3901)	21	1	4.8%
<i>Microcitrus australasica</i> (CRC 1484)	22	1	4.5%
<i>Microcitrus australis</i> (CRC 3669)	21	3	14.3%
<i>Severinia buxifolia</i> (CRC 1491)	25	0	0%
<i>Wenzelia dolichophylla</i> (CRC 3966)	22	0	0%
Male Parent and CRC #	Year 2014		
	# of pollinations	# of fruit set	% fruit set
<i>Aeglopsis chevalieri</i> (CRC 2878)	22	3	13.6%
<i>Atalantia ceylanica</i> (CRC 3287)	20	0	0%
<i>Citrus madurensis</i> (CRC 2592)	21	3	14.3%
<i>Citropsis gilletiana</i> (CRC 3296)	20	0	0%
<i>Citropsis schweinfurthii</i> (CRC 3126)	21	2	9.5%
<i>Citrus ichangensis</i> (CRC 2327)	20	2	10%
<i>Citrus medica</i> (CRC 3891)	23	1	4.3%
<i>Citrus x floridana</i> (CRC 3069)	24	3	12.5%
<i>Clausena excavata</i> (CRC 3166)	22	0	0%
<i>Fortunella polyandra</i> (CRC 3901)	20	6	30%
<i>Microcitrus australasica</i> (CRC 1484)	24	0	0%
<i>Severinia buxifolia</i> (CRC 1491)	21	0	0%

stigmas or styles. Using a small paintbrush dipped in alcohol and dried, the brush was dipped into centrifuge tube to collect pollen grains onto the brush. Pollen was immediately brushed onto the stigma of the flowers. Once all the flowers on the cluster or branch had been pollinated it was immediately covered with large organza type bag and tied above the cluster. All branches with bags were labeled with male parent identity, date and number of flowers pollinated. Pollinations were also recorded in a record book. *Aegle marmelos* requires approximately 12 to 13 months to produce a mature fruit, so pollinations were periodically examined for fruit set. After 4 to 6 months the organza bags were removed and a larger mesh bag was put into place to allow better air circulation, room for the growing fruit, and to catch any fruits that may fall from the branch.

Fruit harvest, seed extraction and germination

Fruits were determined to be physiologically mature and ready for harvest at 12 to 13 months beyond pollinations. These dates were based upon prior seed collection records from the National Clonal Germplasm Repository for Citrus and Date (USDA-NCGRCD) in Riverside, Calif. This period is also about the same time the tree is flowering for next year's crop. The branch containing fruits inside of mesh bags were cut at a point above the bag, and tags were removed and placed inside of the mesh bag and retied. All mesh bags were collected into a larger bag and taken to the laboratory. Each fruit was given an identification number on a waterproof label. *Aegle marmelos* is a hard-shelled citroid so it is near impossible to cut open the fruit with a knife as one

would with standard *Citrus*, so each individual fruit was placed inside of another mesh bag and carefully smashed open with a rubber mallet. A strainer was used to collect seed from the pulp and after rinsing the mass with warm water.

Following the protocol used by the USDA-NCGRCD for extracting and cleaning Aurantioideae seeds (USDA ARS NCGRCD, 2010), seeds were then immersed in a 50% solution of Clariseb RL 2X pectinase (Specialty Enzymes & Biotechnologies, Chino, Calif.) and left to sit overnight. Using cheesecloth and strainer, seeds were strained from the pectinase treatment and rinsed with warm water. Seeds were then immersed in a 5% bleach solution for 10 minutes and then rinsed thoroughly with warm water. Seeds were placed in a cloth bag and put into a hot water bath for 10 minutes at 125 degrees Fahrenheit and then rinsed in room temperature water. Seeds were then submerged into a 1% solution of 8-hydroxyquinoline sulfate for 3 minutes. Treated seeds were then spread evenly onto drying racks and left to dry for 24-48 hours. Lastly, seeds were stored in a refrigerated cold room until ready to be weighed and planted.

Seeds were sorted by cross and by individual fruit from each cross. Each set was counted and a total weight and 10-seed weight was recorded for each (Table 6). Seeds were then planted in a pasteurized UC *Citrus* soil mix in Ray Leach “Cone-tainers”™ (UV stabilized plastic tubes 1.5 in. x 8.25 in.) (Stuewe and Sons, Tangent, Oregon). The Cone-tainers™ were organized as 98 tubes per rack, labeled with identification information, and placed in a greenhouse certified for Asian Citrus Psyllid (ACP)

exclusion. Any germinated seedlings were observed periodically for morphological features indicating hybrid status such as leaf shape, color or leaflet type.

Results

Results in Table 6 show the parental combinations, number of fruit generated, and fruit set percentage. Self-pollinations with *Aegle marmelos* as a male parent in 2012 and 2013 yielded no fruit set, indicative of self-incompatibility. *Atalantia ceylanica* (Arn.) Oliv. and *Severinia buxifolia* of the Citrinae subtribe as a male parent in 2013 and 2014 yielded no fruit set. *Wenzelia dolichophylla* (Lauterb. & K. Schum.) Tanaka of the Triphasiinae subtribe yielded no fruit set in 2013. A species from the Clauseneae tribe, *Clausena excavata* Burm. f., used as male parent in 2013 and 2014 yielded no fruit set. *Citropsis gilletiana* Swingle & M. Kellerm. from the Citrinae subtribe was used unsuccessfully as a male parent in 2013 and 2014, however a different species, *Citropsis schweinfurthii* (Engl.) Swingle & M. Kellerm, although yielding no fruit set in 2013, had a 9.5% fruit set from 2014 pollinations.

Other pollinations utilizing male parents from Citrinae subtribe gave mixed results: *Fortunella crassifolia* Swingle produced a 4.3% fruit set from pollinations in 2013, *Fortunella polyandra* (Ridl.) Tanaka produced 4.8% fruit set in 2013 and 30% fruit set from pollinations performed in 2014, *Citrus x floridana* (*Citrofortunella* spp.) produced a 17.4% fruit set in 2013 and a 12.5% fruit set in 2014, *Poncirus trifoliata* produced a 12.5% fruit set from 2012 pollinations, *Citrus madurensis* Lour. produced a fruit set of 7.5% in 2012 and 14.3% in 2014, *Citrus ichangensis* Swingle produced a 20% fruit set in 2013 and a 10%

fruit set in 2014, and *Citrus medica* L. produced 9.5% fruit set in 2013 and a 4.3% fruit set in 2014. Pollinations with *Microcitrus australis* (A. Cunn. ex Mudie) Swingle pollen generated 14.3% fruit set in 2013 and *Microcitrus australasica* (F. Muell.) Swingle pollen generated no fruit set in 2014, but 4.5% fruit set the prior year. When male parents from the Balsamocitrinae subtribe were used, fruit set rates were higher. *Aeglopsis chevalieri* Swingle pollen produced a fruit set of 22.2% in 2012 and 13.6% in 2014, *Balsamocitrus daweyi* Stapf pollen produced a fruit set of 20% in 2012 and 10% in 2013, and *Afraegle paniculata* (Schumach.) Engl. pollen produced a fruit set of 8.7% in 2013.

Results in Table 6 show the amount of seed per fruit, weight of seeds and germination percentage. Male parents that caused fruit set in *Aegle marmelos* were *Aeglopsis chevalieri*, *Afraegle paniculata*, *Balsamocitrus daweyi*, *Citropsis schweinfurthii*, *Citrus ichangensis*, *Citrus madurensis*, *Citrus medica*, *Citrus x floridana*, *Fortunella crassifolia*, *Fortunella polyandra*, *Microcitrus australis* and *Microcitrus australasica*. However, there were notable differences in the size and weight of the seeds that were produced from each combination. A comparison of seed size and shape from all seeds obtained from the intergeneric crosses is shown in Appendix B.

The average 10-seed weight was 0.76 grams for seeds produced by male parent *Aeglopsis chevalieri*, 0.77 grams for male parent *Afraegle paniculata*, and 1.04 grams for male parent *Balsamocitrus daweyi*. However, we see that as the phylogenetic distance between *Aegle marmelos* and the male parent taxa increases, the 10-seed weight of the seeds produced by the union decreases.

Table 6. Fruit development after intergeneric crosses performed with seed parent *Aegle marmelos*.

Pollen Parent	CRC #	Year	ID #	Total seeds	Weight (g)	10 seed weight (g)	# of Seedlings	Germ. %
<i>Aeglopsis chevalieri</i> ♂	2878	2013	8.1	131	10.683	1.098	122	93%
<i>Aeglopsis chevalieri</i> ♂	2878	2013	8.2	152	10.780	0.895	137	90%
<i>Aeglopsis chevalieri</i> ♂	2878	2013	8.3	82	7.341	0.996	76	93%
<i>Aeglopsis chevalieri</i> ♂	2878	2013	8.4	76	6.856	0.886	73	96%
<i>Aeglopsis chevalieri</i> ♂	2878	2013	8.5	126	8.603	0.76	109	87%
<i>Aeglopsis chevalieri</i> ♂	2878	2013	8.6	150	9.699	0.726	129	86%
<i>Aeglopsis chevalieri</i> ♂	2878	2015	7.1	20	0.674	0.404	7	35%
<i>Aeglopsis chevalieri</i> ♂	2878	2015	7.2	60	2.256	0.597	26	43%
<i>Aeglopsis chevalieri</i> ♂	2878	2015	7.3	50	1.808	0.458	18	36%
<i>Afraegle paniculata</i> ♂	4033	2014	9.1	51	2.938	0.723	27	53%
<i>Afraegle paniculata</i> ♂	4033	2014	9.2	138	9.882	0.818	118	86%
<i>Balsamocitrus dawei</i> ♂	3514	2013	9.1	67	6.752	1.114	54	81%
<i>Balsamocitrus dawei</i> ♂	3514	2013	9.2	120	11.316	1.038	106	88%
<i>Balsamocitrus dawei</i> ♂	3514	2013	9.3	102	9.053	0.97	84	82%
<i>Balsamocitrus dawei</i> ♂	3514	2013	9.4	61	5.823	1.122	55	90%
<i>Balsamocitrus dawei</i> ♂	3514	2014	8.1	44	3.404	0.976	40	91%
<i>Balsamocitrus dawei</i> ♂	3514	2014	8.2	25	2.401	1.019	23	92%
<i>Citropsis schweinfurthii</i> ♂	3126	2015	5.1	22	0.282	0.135	0	0%
<i>Citropsis schweinfurthii</i> ♂	3126	2015	5.2	33	0.436	0.12	0	0%
<i>Citrus ichangensis</i> ♂	2327	2014	3.1	64	0.506	0.074	0	0%
<i>Citrus ichangensis</i> ♂	2327	2014	3.2	51	0.433	0.087	0	0%
<i>Citrus ichangensis</i> ♂	2327	2014	3.3	43	0.312	0.079	0	0%
<i>Citrus ichangensis</i> ♂	2327	2014	3.4	65	0.533	0.092	0	0%
<i>Citrus ichangensis</i> ♂	2327	2014	3.5	65	0.563	0.094	0	0%
<i>Citrus ichangensis</i> ♂	2327	2015	2.1	67	0.591	0.088	0	0%
<i>Citrus madurensis</i> ♂	2592	2013	5.1	22	0.155	0.060	0	0%
<i>Citrus madurensis</i> ♂	2592	2013	5.2	66	0.428	0.068	0	0%
<i>Citrus madurensis</i> ♂	2592	2013	5.3	71	0.545	0.083	0	0%
<i>Citrus madurensis</i> ♂	2592	2015	4.1	76	0.575	0.068	0	0%
<i>Citrus madurensis</i> ♂	2592	2015	4.2	41	0.215	0.058	0	0%
<i>Citrus madurensis</i> ♂	2592	2015	4.3	62	0.54	0.070	0	0%
<i>Citrus medica</i> ♂	3891	2014	4.1	55	0.580	0.113	0	0%
<i>Citrus medica</i> ♂	3891	2014	4.2	24	0.243	0.103	0	0%
<i>Citrus medica</i> ♂	3891	2015	3.1	20	0.144	0.085	0	0%

<i>Citrus x floridana</i> ♂	3069	2014	6.1	35	0.321	0.099	0	0%
<i>Citrus x floridana</i> ♂	3069	2014	6.2	35	0.296	0.098	0	0%
<i>Citrus x floridana</i> ♂	3069	2014	6.3	34	0.292	0.094	0	0%
<i>Citrus x floridana</i> ♂	3069	2014	6.4	18	0.113	0.058	0	0%
<i>Citrus x floridana</i> ♂	3069	2015	6.1	23	0.197	0.087	0	0%
<i>Citrus x floridana</i> ♂	3069	2015	6.2	29	0.159	0.046	0	0%
<i>Citrus x floridana</i> ♂	3069	2015	6.3	17	0.151	0.084	0	0%
<i>Fortunella crassifolia</i> ♂	3818	2014	2.1	53	0.304	0.066	0	0%
<i>Fortunella polyandra</i> ♂	3901	2014	1.1	114	1.098	0.093	0	0%
<i>Fortunella polyandra</i> ♂	3901	2015	1.1	81	0.420	0.058	0	0%
<i>Fortunella polyandra</i> ♂	3901	2015	1.2	27	0.152	0.056	0	0%
<i>Fortunella polyandra</i> ♂	3901	2015	1.3	49	0.278	0.052	0	0%
<i>Fortunella polyandra</i> ♂	3901	2015	1.4	72	0.377	0.065	0	0%
<i>Fortunella polyandra</i> ♂	3901	2015	1.5	62	0.300	0.058	0	0%
<i>Microcitrus australasica</i> ♂	1484	2014	7.1	30	0.241	0.084	0	0%
<i>Microcitrus australis</i> ♂	3669	2014	5.1	49	0.371	0.085	0	0%
<i>Microcitrus australis</i> ♂	3669	2014	5.2	50	0.417	0.094	0	0%
<i>Microcitrus australis</i> ♂	3669	2014	5.3	47	0.389	0.084	0	0%
<i>Poncirus trifoliata</i> ♂	3795	2013	7.1	23	0.234	0.102	0	0%
<i>Poncirus trifoliata</i> ♂	3795	2013	7.2	48	0.480	0.106	0	0%
<i>Poncirus trifoliata</i> ♂	3795	2013	7.3	39	0.402	0.113	0	0%

The average 10-seed weight was 0.13 grams for seeds produced by male parent *Citropsis schweinfurthii*, 0.09 grams for seeds produced by male parent *Citrus ichangensis*, 0.07 grams for seeds produced by male parent *Citrus madurensis*, 0.10 grams for seeds produced by male parent *Citrus medica*, 0.08 grams for seeds produced by male parent *Citrus x floridana*, 0.07 grams for seeds produced by male parent *Fortunella crassifolia*, 0.06 grams for seeds produced by male parent *Fortunella polyandra*, 0.08 grams for seeds produced by male parent *Microcitrus australasica*, 0.09 grams for seeds produced by male parent *Microcitrus australasica*, and 0.11 grams for seeds produced by male parent

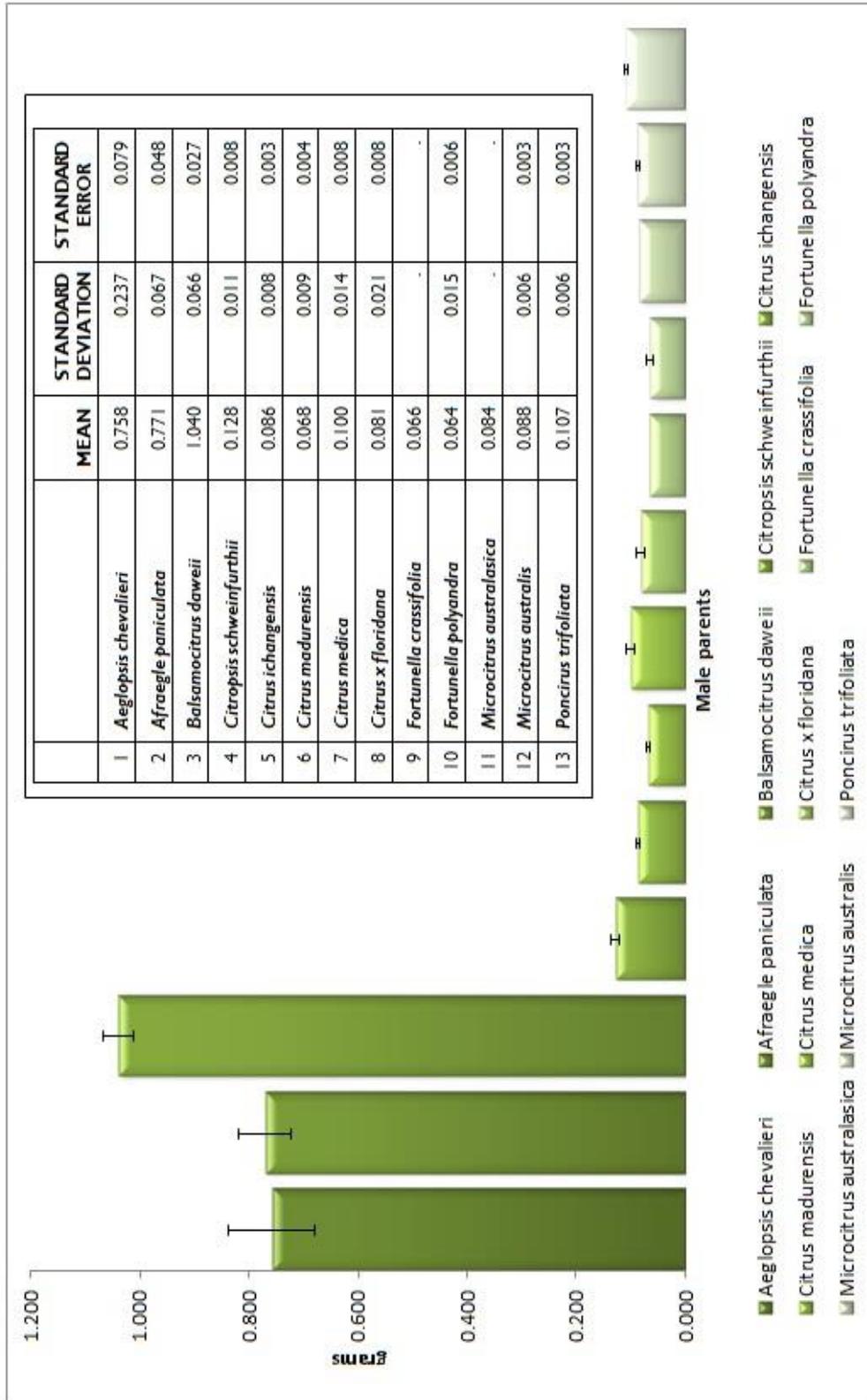
Poncirus trifoliata. The average 10-seed weight from all seeds produced across each combination exhibited clear differences in seed morphology (Fig. 2).

The only seeds that germinated were those from the intergeneric hybrid combinations within the Balsamocitrinae subtribe. For crosses with male parent *Aeglopsis chevalieri* we see an average of 91% germination rate for seed obtained in year 2013 and 38% germination rate for seed obtained in year 2015. The lower rate for year 2015 was likely due to the timing of the harvest which can affect seed quality. If *Aegle marmelos* fruits are harvested too early the fruits still contain high percentage of immature seeds. Crosses with male parent *Afraegle paniculata* had an average of a 69% germination rate for seeds obtained in year 2014. Lastly, crosses with male parent *Balsamocitrus daweyi* produced an average germination rate of 85% for seed obtained in 2013 and 91% for seed obtained in 2015.

Discussion

This crossing study has provided a preliminary picture of the fertility relationship that exists between the Balsamocitrinae subtribe and other members of the Aurantioideae subfamily. Additionally, it increases our current knowledge of cross compatibility among *Citrus* and *Citrus* relative taxa (Fig 3). It is clear that seed development in this study took two distinct developmental pathways: seed abortion and viable seed development. It is also clear that *Aegle marmelos* is obligately self-incompatible since geitonogamy caused flower abortion and thus no fruit set. A study by Bhardwaj (2013) further supports this as they also found a complete lack of fruit set

Figure 2. Mean and standard error bars per 10-seed lot weight for individual fruits resulting from each cross combination

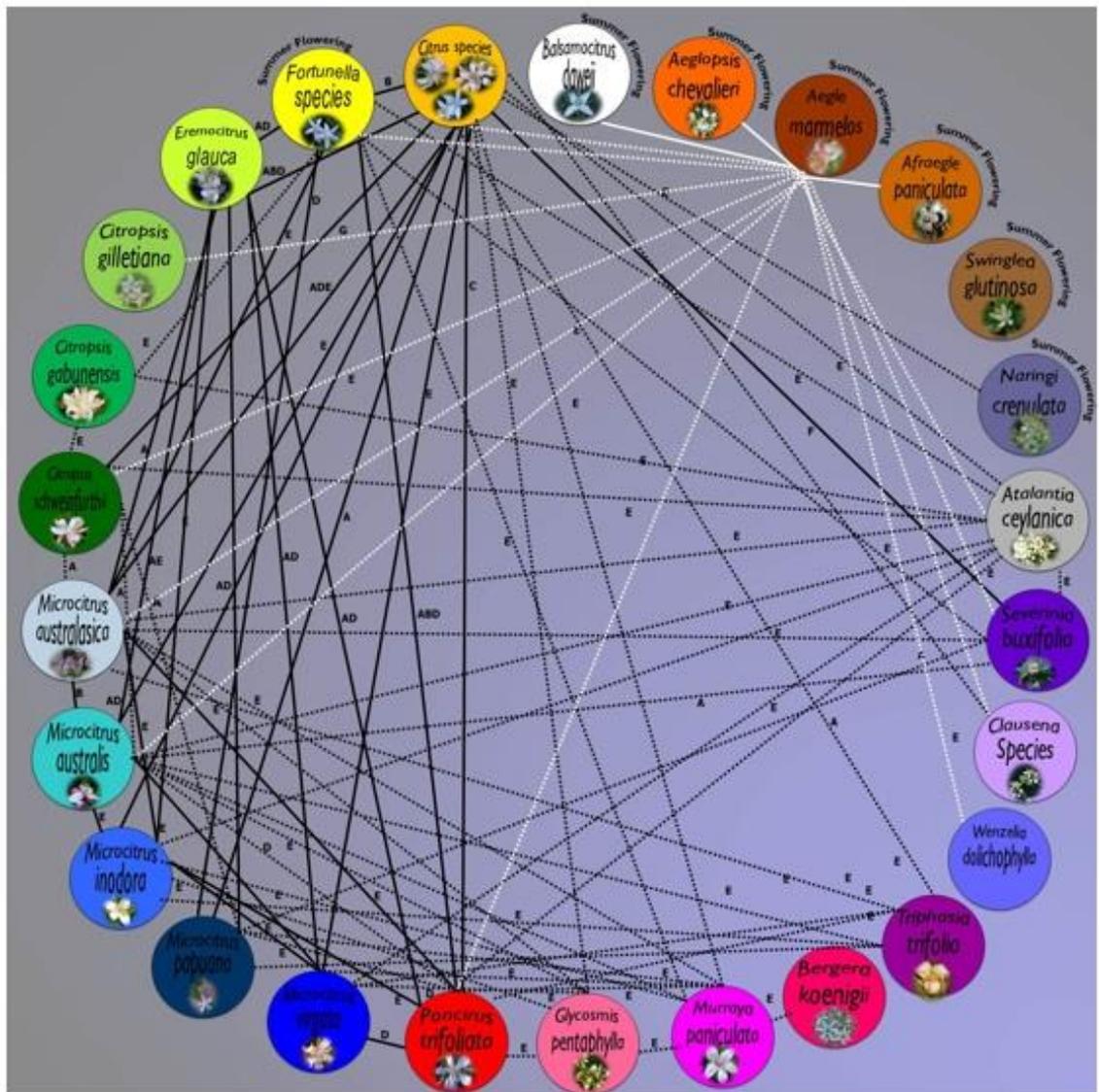


when *Aegle marmelos* trees in their study were also self-pollinated. Therefore, there is some minimum requirement that the pollen parent be from other closely related taxa. Due to a lack of additional accessions of *Aegle marmelos*, I was unable to test whether pollen from a different selection of *Aegle marmelos* would be sufficient to induce viable seed.

The unsuccessful rate for fruit set when male parents were utilized from the Clauseneae tribe and the subtribe Triphasiinae demonstrates the challenge of producing intergeneric hybrids across wide phylogenetic distances within the Aurantioideae subfamily. Conversely, the ability of some of the male parents in the Citrinae subtribe to cause fruit set in the Balsamocitrus subtribe reveals some limited compatibility. Although pollinations from male parents in the Citrinae subtribe caused fruit set in *Aegle marmelos*, not one of the nearly 1800 seeds produced from these unions were viable. These seeds were empty and less robust. Seeds recently observed in the CVC collected from open-pollinated *Swinglea glutinosa* and *Balsamocitrus daweei* fruits, also from the Balsamocitrinae subtribe, were found to be in a similar condition. The cause for this outcome is conceivably due to the absence of endosperm.

For proper endosperm development, a 2:1 endosperm balance number (EBN) of maternal-paternal genome ratio is essential, but there may be a lack of sufficient compatibility of the polar nuclei and the sperm cells for endosperm development to proceed. Johri and Ahuja (1956) examined the reproductive biology of *Aegle marmelos* and observed nearly all gametophytes out of approximately 200 ovules were in a

Figure 3. Updated results of all known intergeneric crossing studies within the Rutaceae subfamily Aurantioideae are shown.



A: Barrett 1977
B: Swingle & Reece 1967
C: Bitters 1969
D: Barrett 1985
E: Iwamasa et al. 1988
F: Medina et al. 1998
G: Yahata et al. 2006

——— = SUCCESSFUL CROSS NEW
 ——— = SUCCESSFUL CROSS
 - - - - = FAILED CROSS NEW
 - - - - = FAILED CROSS
 NO LINE = NO CROSS ATTEMPTED

degenerated condition even before the entry of the pollen tube. In their study the endosperm was observed to be in a free nuclear state during which several nucellar embryos initially developed, but only one of them completed development.

The response of *Aegle marmelos* to pollination by a male parent from the Citrinae subtribe was possibly to initiate nucellar embryo development. The embryos may progress to a degree in response to stimulation from the growth of the pollen tube. However, in this circumstance the endosperm fails to advance and we witness the starvation of the embryo, and thus seed abortion. According to crop production studies for *Aegle marmelos*, cultural practices have relied heavily on vegetative propagation of these trees due similar encounters of low fruit and seed set, and poor seed germination rates (Zaman 1988).

It is considered extremely rare for a plant species to be obligately apomictic, relying on variation in the progeny to be the result of genetic mutation only (Richards 1997). Many apomictic species retain moderate to low levels of sexual seed production and can therefore be considered facultative apomicts (Grant, 1981). According to Esen and Soost (1977), normal endosperm development within the course of reproduction through facultative apomixis in the Aurantioideae subfamily is significant for normal embryo development. In this study the intergeneric crosses that produced viable seed were between *Aegle marmelos* and other members in the Balsamocitrus subtribe, further supporting a case for preference to outbreeding. Although these crosses are intergeneric the parents exist in the same subtribe and therefore compatibility within

these genera was more expected than in crosses with more distantly related parents.

However, are any viable zygotic seeds generated from these unions?

Aegle marmelos is a slow growing tree that can take up to twenty years to fruit from seed (Fairchild 1944) and we have limited differing descriptors of which we can use to identify hybrid seedlings visually. Many seedlings appear identical, fitting morphological descriptions of the maternal parent, *Aegle marmelos*. *Aegle marmelos*, *Afraegle paniculata*, and *Balsamocitrus dawsonii* are generally identified as trifoliolate, with *Aeglopsis chevalieri* being the only unifoliolate type species of this group (See Appendix C). However, according to Swingle (1943) different *Aegle marmelos* seedling trees can have dimorphic twigs and leaves, display significant variability in leaf type, and substantial variation of other taxonomically important characters. Some seedlings in this study did indeed exhibit variability in leaf size, shape, type, apex and margin characters. Although the seedlings demonstrated symptoms of a facultative apomictic breeding system, due the capricious nature of *Aegle marmelos* we cannot know for sure based on visual observation alone whether the seedlings are truly hybrids or not. These uncertainties suggest the need for further research.

CHAPTER 2: CROSSING, GRAFTING AND PHYLOGENETIC RELATIONSHIPS IN THE AURANTIOIDEAE

Graft compatibility can be a useful tool in investigations of cross compatibility as it is potentially suggestive of the degree of comparative cross compatibility between two species of distant taxa. This chapter reviews historical documentation of congeniality between graft partners within the Aurantioideae subfamily as well as recounts current observations of grafted accessions maintained in the CVC and USDA-NCGRCD germplasm collections. These findings, along with current knowledge of cross compatibility in the Aurantioideae, enhance our understanding of the subfamily's interrelationships as supported by more contemporary phylogenetic associations.

In the early part of the twentieth century it was common to see massive seedling *Citrus* trees growing on commercial farms and in the backyards of homeowners. They were dependably productive and long lived until an epidemic of *Phytophthora* root rot fungi caused worldwide destruction in once healthy expansive sweet orange orchards (Fawcett 1936). Grafting, a centuries-old horticultural technique, was already an accepted practice in *Citrus* nursery production after the commercialization of the seedless 'Washington' navel orange in the late 1800s, but became essential when certain *Citrus* genotypes, such as sour orange (*Citrus aurantium* L.), were found to be tolerant of *Phytophthora* (Castle 2010). Many *Citrus* trees were grafted onto sour orange rootstock for decades until a different disease outbreak of quick decline strains of *Citrus tristeza* virus (CTV), to which the common sweet orange/sour orange rootstock combination is

susceptible, encouraged the switch to citrange (*Poncirus trifoliata* (L.) Raf. × *Citrus sinensis* (L.) Osbeck) type rootstocks, as they were found to be tolerant of CTV.

As previously discussed in Chapter 1, many commercial rootstocks were created by combining a near citrus relative known as *Poncirus trifoliata* with another *Citrus* species. Seedlings derived from the combination of *Poncirus trifoliata* and *Citrus sinensis* are known as citranges, *Poncirus trifoliata* and *Citrus reticulata* Blanco are known as citrandarins, and *Poncirus trifoliata* and *Citrus paradisi* Macfad. are known as citrumelos. There are many other combinations with different names but these are the most common, and have historically been the main taxa associated with graft compatibility studies between Aurantioideae and *Citrus*.

Bitters *et al* (1964) saw many advantages to studying wild *Citrus* relatives as potential alternatives to the commercial rootstocks in use by the mid-twentieth century. Wild *Citrus* relatives are known for having some distinct advantages over species of *Citrus* such as tolerance to elevated salinity levels, drought resistance, nematode resistance, and *Phytophthora* resistance (Bitters 1986). More recently, there have been reports of huanglongbing (HLB) resistance found in *Citrus* relatives (Ramadugu *et al* 2016). Certainly, these taxa often have special traits and adaptations that can be useful for *Citrus* rootstock improvement. Therefore, graft compatibility between *Citrus* and other members of the Aurantioideae have been investigated many different times in *Citrus* research. This is largely due to the fact that rootstocks for the *Citrus* industry are

continuously evolving and the ones in current use today may not be ideal for problems that may arise in *Citrus* production in the future (Bitters 1986).

This chapter will report the present knowledge of graft compatibilities by reviewing historical documentation of congeniality between graft partners of related Aurantioideae taxa, as well as describe current observations of grafted accessions maintained in the CVC and USDA-NCGRCD germplasm collections. These data along with our current understanding of cross compatibility within the Aurantioideae subtribe can be used to better define the subfamilies interrelationships when augmented with more modern molecular-based phylogenetic associations.

Graft compatibility and incompatibility in Aurantioideae taxa

Grafting has been described as the merger of at least two living plant tissues with the intention to grow them together as a single plant (Andrews *et al* 1993). Bitters (1986) described the union as an artificially induced symbiosis, with each part dependent on the other for survival. According to Moore and Walker (1981) the adhesion of the scion and rootstock during the grafting procedure is a direct result of the healing process, which commences in response to the wounding of the two trees. The process itself is a completely passive event (Moore 1984). Undoubtedly, a complex relationship exists between the roots and the canopy of grafted *Citrus* trees.

Factors that may lead to unsuccessful grafts can include using rootstock-scion partners at the incorrect physiological stage, pest infestations or disease complications in one or both of the graft partners (Andrews *et al* 1993). For example, as part of

biological indexing practices, budwood is grafted onto indicator plants that are known to be sensitive to specific pathogens and will express particular symptoms that “indicate” the presence of the disease in the budwood (Roistacher 1991). These trees ultimately perish from this disease transmission. CTV contributes to some unsuccessful grafts as the toxin it produces often kills phloem cells at the bud union of the grafted tree (Schneider 1984). However, most frequently the misalignment of tissues leads to failures, which is often due to the inexperience of the grafter. Certain types of grafts are tricky and demand a level of artistry best left to someone skilled.

However, an unsuccessful graft is not always the same as an incompatible graft, just as a successful graft is not always the same a compatible graft. Indeed, Bitters (1986) asserted “it is possible to graft any *Citrus* with *Citrus*, and apparently many genera with many other genera, but the degree of success may vary greatly.” He claimed the ability to keep *Citrus sinensis* buds alive for at least one year when he grafted a few onto *Casimiroa edulis* La Llave & Lex., which is particularly surprising as this plant species resides in a completely different subfamily, Toddalioideae (Bitters 1986). Likewise, in a study by Yoshida (1996) all species tested within the Aurantioideae subtribe were certainly *graftable* to *Citrus*, but the success rate of the scion growing into a healthy tree was uneven.

Incompatibilities between *Citrus* rootstocks and scions can preclude many otherwise desirable combinations. Research has suggested it may be due to a lack of cell-to-cell recognition, differing wound responses, growth regulators, or even

incompatibility toxins generated from secondary metabolites (Andrews *et al* 1993). Nito *et al* (2005) saw in their evaluation of callus graft interfaces of different taxa in the Aurantioideae that within combinations of more distantly related species with *Citrus*, such as *Citropsis schweinfurthii* (Engl.) Swingle & M. Kellerm, *Atalantia monophylla* (L.) DC., *Triphasia trifolia* (Burm. f.) P. Wilson, and *Bergera koenigii* L., the border of the graft interface was visible, possibly indicating an element of premature incompatibility. Since grafts between the same genera in the study (i.e. *Citrus* with *Citrus*) revealed no obvious border, this may be an indication of an early recognition response.

In the Aurantioideae, most often the incompatibility is demonstrated in a breakdown in the continuity of the vascular system at the graft union, which can happen in the grafted tree even at an advanced age. Symptoms of incompatibilities in grafted *Citrus* trees of all types are sometimes expressed by the amount overgrowth at the bud union due to gaps in the vascular systems. Sometimes the overgrowth is displayed only on the side of the scion, while others only on the side of the rootstock. This observation is not always a reliable indication of incompatibility as overgrowths like these may occur due to a difference in growth rates between distantly related species (Andrew *et al* 1993). Although uncommon, mature grafted trees in the CVC have even completely severed at the union, indicating very few connections in the vascular system between the rootstock and the scion were ever constructed.

Addressing the complications of graft compatibilities was significant in early *Citrus* research. As a result, HJ Webber (1926) developed a method to rate the congeniality of

rootstocks and scions where “C” represented a normal, smooth bud union and all other conditions of the rootstock-scion interactions were graded with a numbered series of “minus” and “plus” ratings (i.e. -3, -2, -1, C, +1, +2, +3). If the scion overgrew the rootstock, it received a minus rating. If the rootstock overgrew the scion it received a plus rating. In addition, as the overgrowth grew larger, so did the number rating. Bitters (1986) later modified Webber’s ratings as he decided that unions he observed to be healthy and compatible would be labeled as incompatible under Webber’s system. Certainly, within intergeneric grafts in the Aurantioideae, we see many cases where a smooth union does not result under a particular rootstock-scion combination (Siebert *et al* 2015). However, despite this the tree continues to grow well for decades with good health and vigor. Therefore, graft unions between different genera in the Aurantioideae (See Appendix D) can be more informative of congenialities when they also show supplementary irregularities such as asymmetry, odd shapes or difference in bark textures (Bitters 1986).

Methods

Documentations of graft compatibilities between Aurantioideae taxa were obtained not only from historical records, but also from observations of grafted Aurantioideae trees currently maintained in the CVC and USDA-NCGRCD. As a normal part of CVC and USDA-NCGRCD operations, information on the compatibility of *Citrus* relatives as scions on *Citrus* rootstocks is important for the maintenance of germplasm accessions where they are often retained as grafted trees for the lifetime of

the plant (Siebert *et al* 2015). The grafts have been made by several generations of researchers within UC Riverside and USDA-NCGRCD, and have not been a part of any known replicated trials. Many observations were made of trees grown in greenhouse pots in cases when the accession has been observed to be too fragile to be grown under field conditions in Riverside, CA., Irvine, CA., or Thermal, CA. Most of the grafts made for CVC accessions were T-buds but occasionally cleft grafts were made. The affinities of *Poncirus*, *Citrus*, and *Fortunella* with each other are well known commercially, as well as in decades of research, and will not be reported here in detail (Siebert *et al* 2015). In many cases, *Citrus* relative accessions in the CVC are grown on their own root system, and therefore graft compatibility information could not be obtained. Particular combinations have been observed to be entirely incompatible, whereas others have shown either short or long-term incompatibility.

Results

Information on grafting relationships within Aurantioideae taxa recovered from the literature search and observations of grafted Aurantioideae germplasm accessions retained by the CVC and USDA-NCGRCD are summarized in Table 7. In the Clauseneae tribe, intergeneric grafts of *Glycosmis* with *Citrus* have short-term incompatibilities. *Clausena* with *Citrus* or *Poncirus* hybrids exhibit long-term incompatibilities. *Murraya* and *Bergera* are considered not graft compatible with *Citrus*. All genera in the Triphasiinae subtribe, except for *Oxanthera*, have shown incompatibilities with *Citrus*. *Wenzelia* and *Triphasia* have been particularly difficult to

Table 7. Observed and reported graft compatibilities between *Citrus* and related Aurantioidae taxa

Taxa	Tribe	Subtribe	Graft compatibility	Reference
<i>Glycosmis</i>	Clauseneae	Clauseniinae	Short term incompatibilities with <i>Citrus</i> and other species of <i>Glycosmis</i>	Bitters (1964); Yoshida (1996)
<i>Clausena</i>	Clauseneae	Clauseniinae	Long term incompatibilities with <i>Citrus</i> and <i>Poncirus</i> hybrids	Bitters (1964); Swingle and Reece (1967); Yoshida (1996)
<i>Murraya</i> (Bergera)	Clauseneae	Clauseniinae	Incompatible with <i>Citrus</i>	Bitters (1964); Swingle and Reece (1967); Yoshida (1996)
<i>Wenzelia</i>	Clauseneae	Clauseniinae	Incompatible with <i>Citrus</i>	Bitters (1964); Yoshida (1996)
<i>Oxanthera</i>	Citreae	Triphasinae	Possibly compatible with <i>Citrus</i> and <i>Poncirus</i> hybrids. Limited information.	Siebert et al (2015)
<i>Triphasia</i>	Citreae	Triphasinae	Compatible with <i>Citrus</i>	Siebert et al (2015)
<i>Severinia</i>	Citreae	Triphasinae	Short term incompatibilities with <i>Citrus</i>	Bitters (1964); Yoshida (1996); Siebert et al (2015)
<i>Pleiospermium</i>	Citreae	Citrinae	Compatible with <i>Citrus</i> and <i>Poncirus</i> hybrids	Bitters (1964); Yoshida (1996); Siebert et al (2015)
<i>Pleiospermium</i>	Citreae	Citrinae	Compatible with <i>Citrus</i> and <i>Poncirus</i> hybrids	Bitters (1964); Yoshida (1996); Siebert et al (2015)
<i>Limocitrus</i>	Citreae	Citrinae	Compatible with <i>Citrus</i>	Bitters (1964); Yoshida (1996); Siebert et al (2015)
<i>Hesperethusa</i> (Naringi)	Citreae	Citrinae	Compatible with <i>Citrus</i> , <i>Poncirus</i> , <i>Poncirus</i> hybrids, and <i>Swinglea</i>	Bitters (1964); Swingle and Reece (1967); Yoshida (1996); Siebert et al (2015)
<i>Citropsis</i>	Citreae	Citrinae	Compatible with <i>Citrus</i> , <i>Poncirus</i> , <i>Poncirus</i> hybrids, <i>Feronia</i> (<i>Limonia</i>), <i>Swinglea</i> , and <i>Clymenia</i>	Bitters (1969, 1977); Swingle and Reece (1967); Yoshida (1996); Siebert (2015)
<i>Atalantia</i>	Citreae	Citrinae	Compatible with <i>Citrus</i> and <i>Poncirus</i> hybrids	Bitters (1964); Yoshida (1996); Siebert et al (2015)
<i>Eremocitrus</i>	Citreae	Citrinae	Compatible with <i>Citrus</i> and <i>Poncirus</i> hybrids	Bitters (1964); Yoshida (1996); Siebert et al (2015)
<i>Clymenia</i>	Citreae	Citrinae	Compatible with <i>Citrus</i> , <i>Poncirus</i> hybrids, and <i>Citropsis</i>	Siebert et al (2015)
<i>Microcitrus</i>	Citreae	Citrinae	Compatible with <i>Citrus</i> , <i>Poncirus</i> , <i>Poncirus</i> hybrids	Siebert et al (2015)
<i>Swinglea</i>	Citreae	Balsamocitrinae	Compatible with <i>Citrus</i> , <i>Poncirus</i> , <i>Poncirus</i> hybrids, <i>Citropsis</i> , and <i>Hesperethusa</i> (Naringi)	Bitters (1964, 1969); Swingle and Reece (1967); Yoshida (1996); Siebert (2015)
<i>Aegle</i>	Citreae	Balsamocitrinae	Compatible with <i>Citrus</i> , <i>Poncirus</i> hybrids, <i>Afraegle</i> , <i>Aeglopsis</i> , and <i>Balsamocitrus</i>	Siebert et al (2015); Swingle and Reece (1967)
<i>Afraegle</i>	Citreae	Balsamocitrinae	Incompatible with <i>Citrus</i> . Compatible with <i>Aegle</i> , <i>Aeglopsis</i> , and <i>Balsamocitrus</i> .	Bitters (1964); Swingle and Reece (1967)
<i>Aeglopsis</i>	Citreae	Balsamocitrinae	Compatible with <i>Citrus</i> , <i>Aegle</i> , <i>Balsamocitrus</i> , and <i>Afraegle</i>	Bitters (1964, 1969); Swingle and Reece (1967); Yoshida (1996)
<i>Balsamocitrus</i>	Citreae	Balsamocitrinae	Compatible with <i>Aegle</i> , <i>Afraegle</i> , and <i>Aeglopsis</i>	Swingle and Reece (1967)
<i>Feronia</i> (<i>Limonia</i>)	Citreae	Balsamocitrinae	Compatible with <i>Citrus</i> , <i>Poncirus</i> hybrids and <i>Citropsis</i>	Bitters (1964, 1969); Swingle and Reece (1967); Yoshida (1996); Siebert (2015)
<i>Feroniella</i>	Citreae	Balsamocitrinae	Compatible with <i>Citrus</i> and <i>Poncirus</i> hybrids	Yoshida (1996); Siebert (2015)

graft with *Citrus* and are generally considered to be not very incompatible. *Wenzelia* may possibly be somewhat compatible with *Citrus* and/or *Poncirus* due to its brief survival on Troyer citrange rootstock until its death during a greenhouse malfunction.

Citrus and *Poncirus* tend to be most graft-compatible to other genera residing within the Citrinae subtribe. In this subtribe, *Citrus* can grow successfully on a wide variety of genera either directly, or with the use of certain interstocks (Siebert *et al* 2015). All genera in the Balsamocitrinae subtribe with the exception of *Afraegle* have shown to be congenial to intergeneric grafts with *Citrus* and *Poncirus*, as well as other members in the Balsamocitrinae tribe.

Discussion

A compilation of the graft and cross compatibilities between all genera mentioned in this study is shown in Table 8. Remarkable correspondence was found between cross and graft compatibilities within Aurantioideae taxa. In nearly all cases where information was found in both compatibility categories, we see that if the two genera are cross-compatible, they are also graft-compatible and vice versa, with a few disagreements. In their study, Rao and Ram (1971) suggested the degree of cross compatibility between two different taxa could be predicted by the comparative graft compatibility of the two species. One could assume these responses should be analogous since the two taxa would have to possess some functional and structural likenesses (Rao and Ram 1971). If this is accurate, then the performance of intergeneric hybrids and intergenic grafts of *Citrus* could possibly serve as a guide for future

combinations between distant taxa. Graft compatibility may be potentially suggestive of the crossability of the parents. In contrast, where intergeneric sexual hybrids have not occurred, we may also see graft-incompatibilities and/or graft union disorders. Additionally, these data may be used to better define the group's phylogenetic relationships if we surmise cross and/or graft compatibility indicates a close phylogenetic relationship and conversely, a cross and/or graft incompatibility suggests a more distant one.

Barrett (1977) and Iwamasa *et al* (1988) reported the success, or lack thereof, in creating intergeneric hybrids comprised of the genera *Citrus*, *Microcitrus*, *Eremocitrus*, *Fortunella*, and *Poncirus*. These genera are also well known to be graft-compatible with each other. However, Bayer *et al* (2009) argues in a recent molecular study, based on nine cpDNA sequences, that the decision by Swingle (1943) to segregate these genera was often based on a single morphological character, and under their more conservative taxonomic treatment, these hybrids would only represent congeneric hybrids (Smith *et al* 2013). The interpretation of their relationship, as detailed by the molecular study of Bayer *et al* (2009), leaves us with little doubt as to why these taxa would also have an intimate graft- and cross-compatible relationship. Barrett (1977, 1985) seemed to have some trouble producing hybrids between certain species of *Microcitrus* with *Poncirus*, which has led to their labeling as cross-incompatible in Table 8. However, due to other species of *Microcitrus* being cross-compatible with *Poncirus*, and new opinions on the phylogenetic relationship of these species, I believe that there was some other element

(environmental, cultural conditions etc.) involved in the lack of germination of the hybrid seeds from Barrett's crosses.

The molecular analysis by Bayer *et al* (2009) also reorganized the genera *Oxanthera*, *Feroniella*, *Clymenia*, *Microcitrus*, *Eremocitrus*, *Fortunella*, and *Poncirus* into the genus *Citrus* in the True Citrus Fruit Trees clade. *Feroniella* is the first species with pinnate leaves to be included in the genus *Citrus* (Mabberley 2010). The graft compatibility of *Feroniella* with *Citrus* and *Poncirus* is supportive of the assignment of *Feroniella* to *Citrus* by Bayer *et al* (2009). Cross compatibility of *Feroniella* with other taxa has not been tested. In addition, *Feronia* (*Limonia*) and *Feroniella* have leaves of similar morphology to those of *Citropsis* and *Hesperethusa* (*Naringi*), and the graft compatibility of these taxa with *Citrus* and *Poncirus*, further supports the belief they are more closely related than originally believed.

As the taxonomic treatment by Swingle (1943) stands, one would assume that since members in the Citreae tribe are phylogenetically closer to *Citrus* than members in the Clauseneae tribe, they would be more graft-compatible; however, this is simply not true. All genera in the Triphasiinae subtribe, except for *Oxanthera*, have shown short- and long-term incompatibilities with *Citrus*. However, Bayer *et al* (2009) returns *Oxanthera* to the Citrinae clade. This rearrangement is well supported by observations of its degree of high graft compatibility with *Citrus*. *Oxanthera* was unable to be tested as a male parent with *Aegle marmelos* (L.) Correa in the cross-compatibility study as it only flowers from Dec.-Mar. in Riverside, Calif., while *Aegle marmelos* only flowers from Jul.-

Aug. in Riverside, CA. In addition, further evaluation of *Oxanthera* as a female parent is impossible as the only accession retained by the CVC and USDA-NCGRCD is seedless.

Bayer *et al* (2009) proposed *Feronia* (*Limonia*) remain under the Citreae tribe, but it was not included within the Balsamocitrinae. Otherwise, it was determined the genera *Aegle*, *Aeglopsis*, *Afraegle*, and *Balsamocitrus* corresponded exactly as described by Swingle (1943). Since these taxa were observed to be graft-compatible with each other, and *Aegle* is cross compatible with *Aeglopsis*, *Afraegle*, and *Balsamocitrus*, these data are in complete agreement of remaining in the Balsamocitrinae as proposed by Bayer *et al* (2009). *Aegle marmelos* is graft-compatible with *Citrus* and *Poncirus*, however through the investigations of this study, was found to be cross-incompatible in terms of producing inviable seeds due to its breeding system. Thus, the phylogenetic relationship between these taxa is near enough for graft compatibility, yet too distant for cross compatibility.

Both Bayer *et al* (2009), and Samuel *et al* (2001) using plastid sequence and phytochemical analysis, concluded *Glycosmis* and *Clausena* were strongly monophyletic, but that *Murraya* be removed from the Clauseneae tribe, reorganized together under the Merrilliinae subtribe and then reassigned to the Citreae Tribe. This reordering is in contrast with the results of our study. Bayer *et al* (2009) and Samuel *et al* (2001) suggest a closer phylogenetic relationship to *Citrus* by this reassignment, but our findings report firm graft- and/or cross-incompatibilities for *Murraya* with *Citrus*, *Microcitrus*, *Fortunella*, and *Poncirus*. However, the lack of cross compatibility with *Berbera* supports the separation *Murraya* into a different subtribe as proposed by Bayer *et al* (2009).

Bayer *et al* (2009) concluded *Citropsis* and *Naringi* to have the same close phylogenetic relationship as described by Swingle (1943). *Hesperethusa (Naringi) crenulata* (Roxb.) Nicolson is graft-compatible with *Citrus*, but not cross-compatible according to the investigation by Iwamasa *et al* (1988). In the CVC, *Hesperethusa (Naringi) crenulata* is a summer flowering accession and viable pollen has not yet been obtained. Crosses could be performed using *Hesperethusa (Naringi) crenulata* as a female parent to further test compatibilities, perhaps initially with different species *Citropsis* as its close relationship with this genus is supported by both Bayer *et al* (2009) and Swingle (1943).

Atalantia and *Severinia* are very graft-compatible with *Citrus* and *Poncirus* but found not to be cross-compatible with *Citrus* by Iwamasa *et al* (1988). *Atalantia* and *Severinia* were also found to be cross-incompatible with *Fortunella*, *Microcitrus*, *Poncirus*, and each other, even though these taxa reside in Citrinae. It is possible that an insufficient number of attempts have been made with these combinations given the amount of work the hybridization of *Severinia buxifolia* (Poir.) Ten. with *Citrus* required in the investigation by Medina-Filho *et al* (1998). *Severinia buxifolia*, which has small floral morphology similar to *Atalantia*, necessitated over 500 tedious pollinations with *Citrus* before hybrids were obtained. Perhaps with more effort sexual hybrids of *Atalantia* and *Citrus*, and perhaps others, are possible.

Despite some uncertainties, our findings generally agree with many accepted viewpoints of the relationships that exist between taxa within the Aurantioideae subfamily. Furthermore, in comparing molecular phylogenetic studies, cross-, and graft-compatibilities, we see primarily reinforcement of long-held perspectives of the

interrelationships. To my knowledge, this type of “three-way relationship” study has not been done before for any economic plant. As molecular phylogenetic studies of the Aurantioideae subfamily evolve, there will be an ongoing need to further clarify and bolster proposed phylogenetic associations with graft and cross compatibility studies.

However, much work still lies ahead in determining all compatibility relationships between the Aurantioideae taxa. Clearly, numerous gaps of information in intergeneric graft and cross compatibilities remain. As most of the cross-compatibility data was obtained as collateral information from breeding studies with the intent to improve *Citrus* or rootstocks for *Citrus*, most data on cross compatibilities within the Aurantioideae frequently had one *Citrus* or *Poncirus* parent. Similarly, most graft compatibility data within the Aurantioideae was obtained from rootstock investigations with the intent to improve rootstocks for *Citrus*, and thus usually had a *Citrus* graft partner. Therefore, almost no graft and cross compatibility information exists between genera in the Clauseneae tribe, Triphasiinae subtribe, and Balsamocitrinae subtribe. We also cannot be sure that failures are not the result of lack of skill or poor sample size. Furthermore, breeding systems of some Aurantioideae taxa are still undetermined. Unfortunately, this germplasm is not very accessible to researchers for these types of investigations, rendering a broad-scale evaluation of all Aurantioideae taxa, a potential barrier to completing this valuable work.

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APPENDICES

Appendix A. Examples of Citrus Relative Germplasm Accessions held by the UC Riverside Citrus Variety Collection

Atalantia citroides



Murraya (Bergera) koenigii



Hesperethusa crenulata



Citropsis gabunensis



Clausena excavata



Eremocitrus glauca



Glycosmis pentaphylla



Limonia acidissima



Swinglea glutinosa



Severinia buxifolia



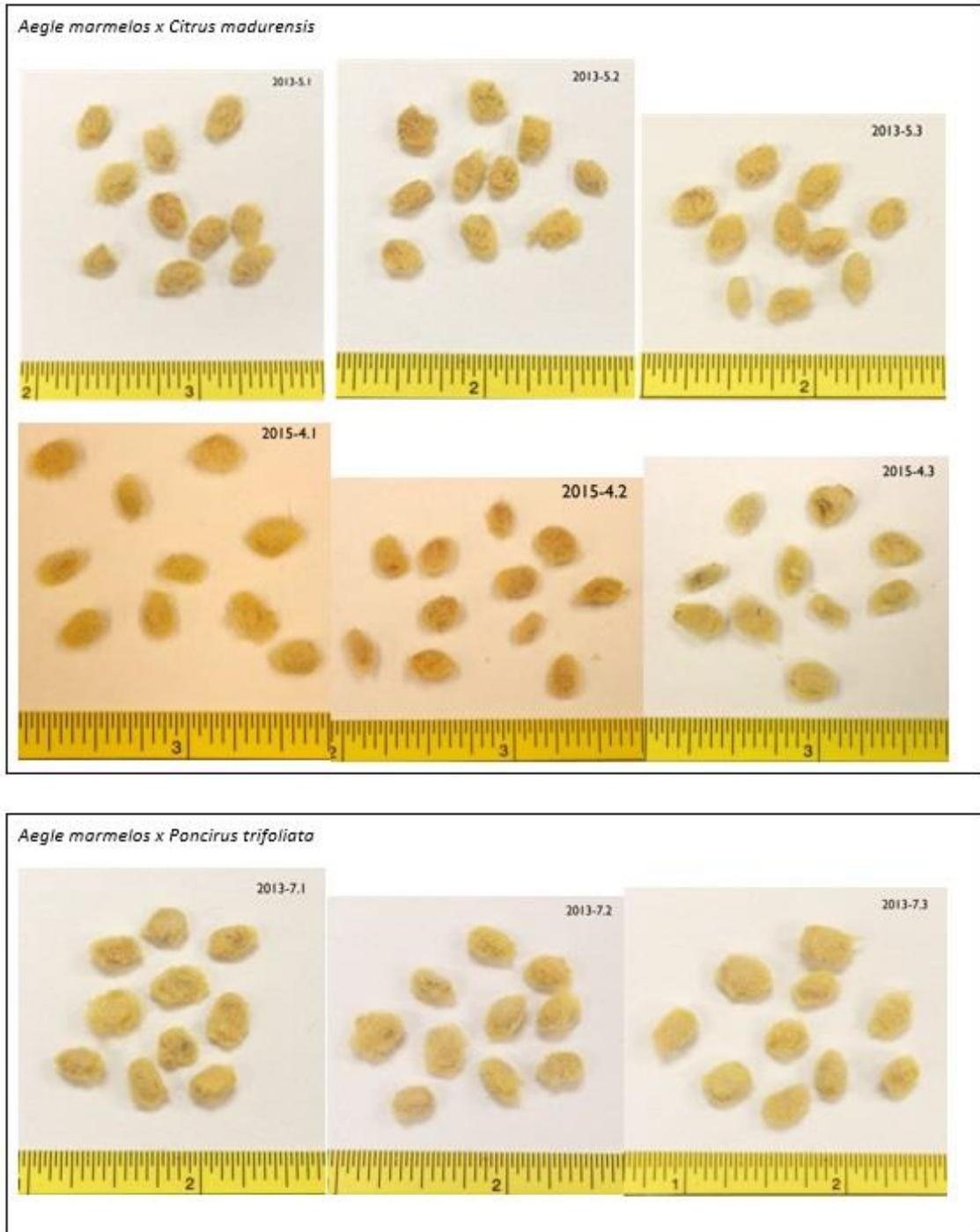
Microcitrus australasica



Microcitrus inodora



Appendix B: A comparison of seed size and shape of all seeds obtained from intergeneric crosses



Aegle marmelos x *Aeglopsis chevalieri*



Aegle marmelos x *Balsamocitrus dawei*



Aegle marmelos x *Fortunella crassifolia*



Aegle marmelos x *Afraegle paniculata*



Aegle marmelos x *Fortunella polyandra*



Aegle marmelos x *Citrus medica*



Aegle marmelos x (*Citrus* x *floridana*)



Aegle marmelos x *Microcitrus australis*



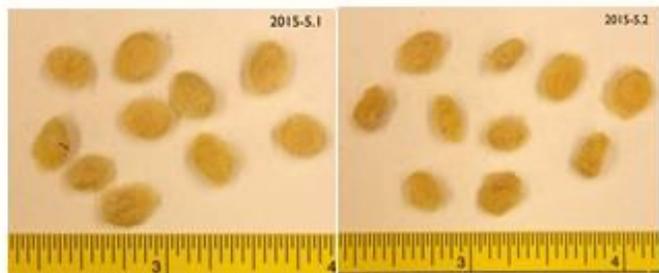
Aegle marmelos x *Citrus ichangensis*



Aegle marmelos x *Microcitrus australasica*

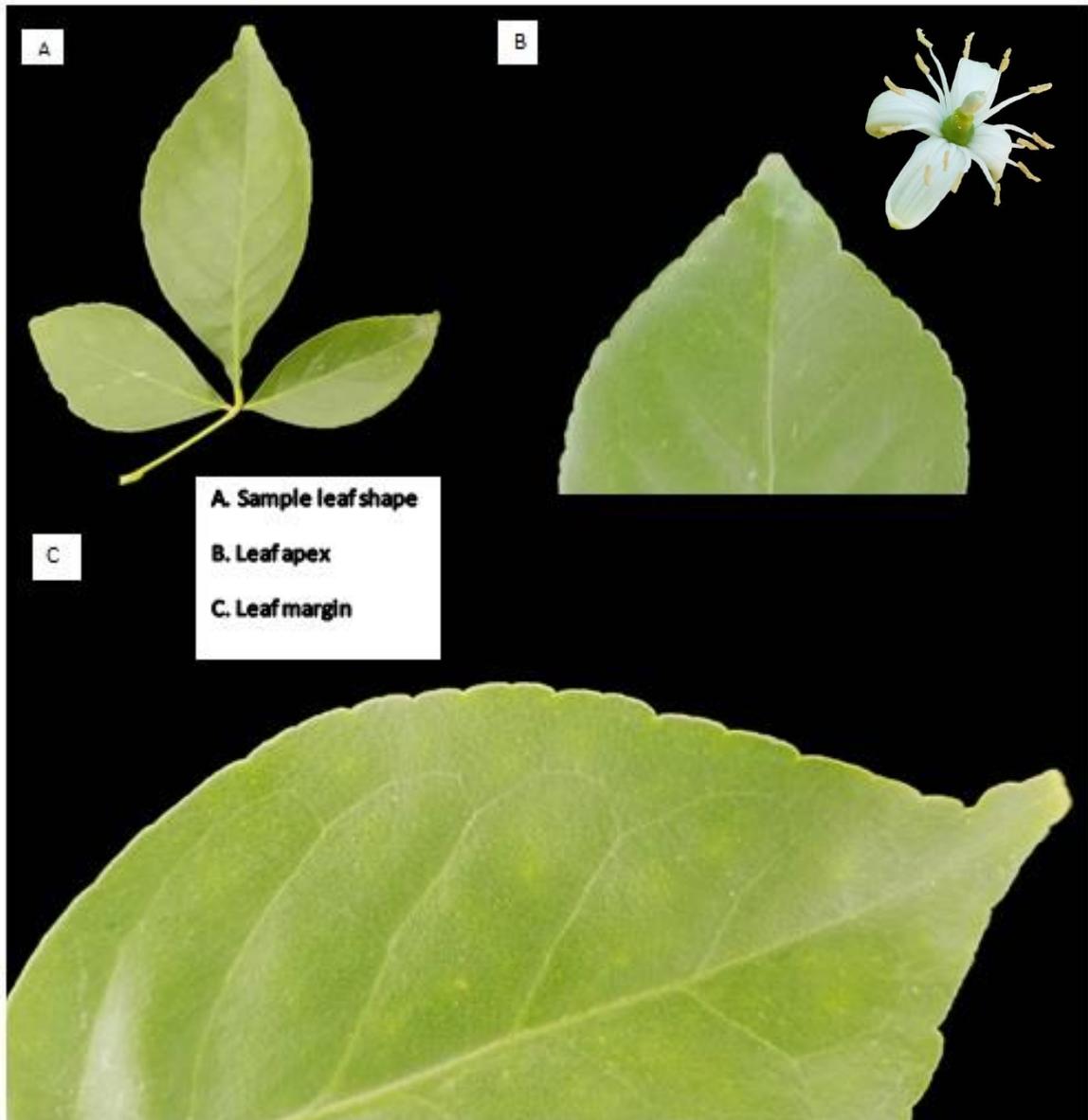


Aegle marmelos x *Citropsis schweinfurthii*



Appendix C: Parental taxa that produced viable seed from the Balsamocitrinae subtribe

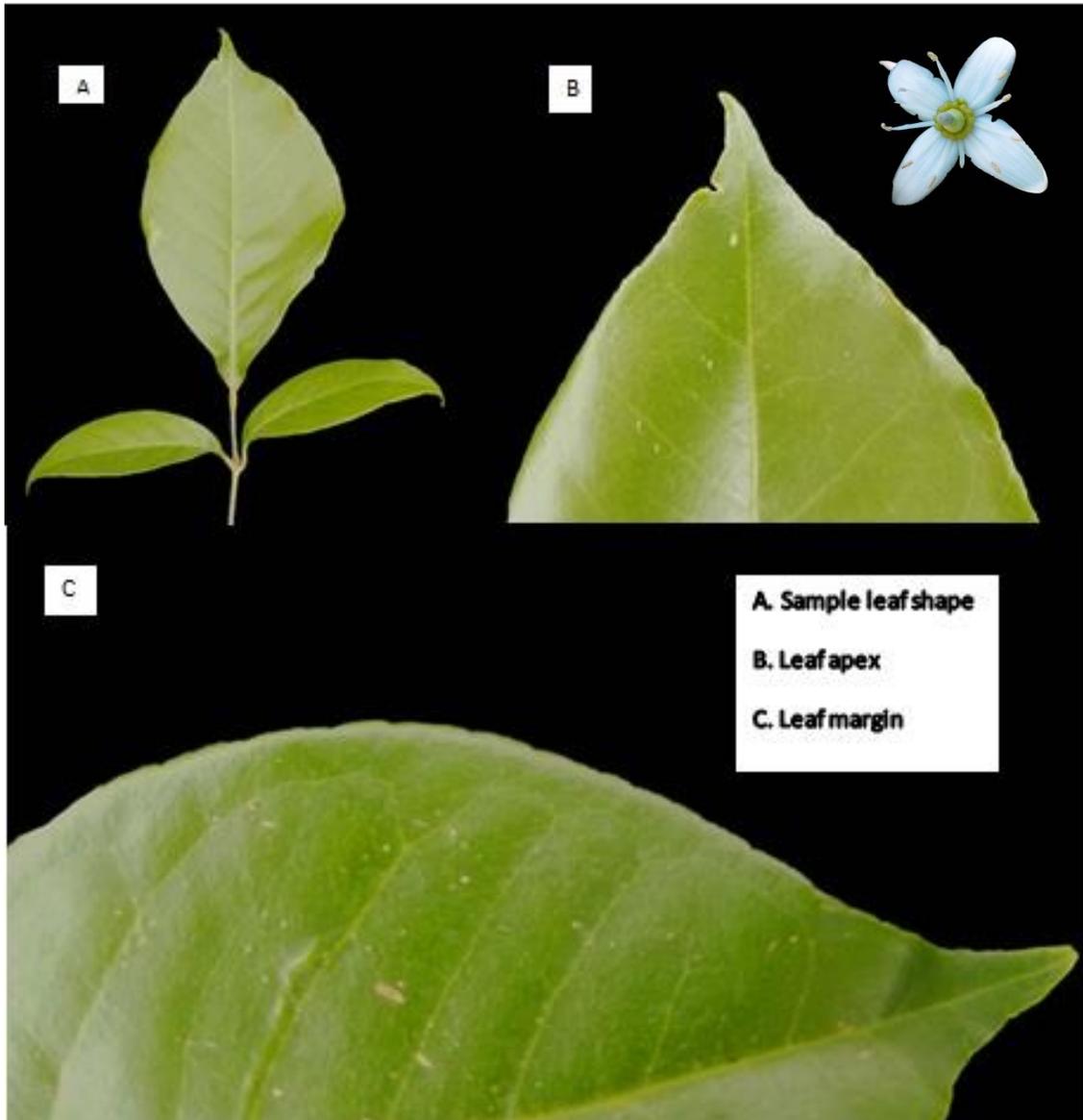
Aegle marmelos (CRC 3140) **FEMALE PARENT**



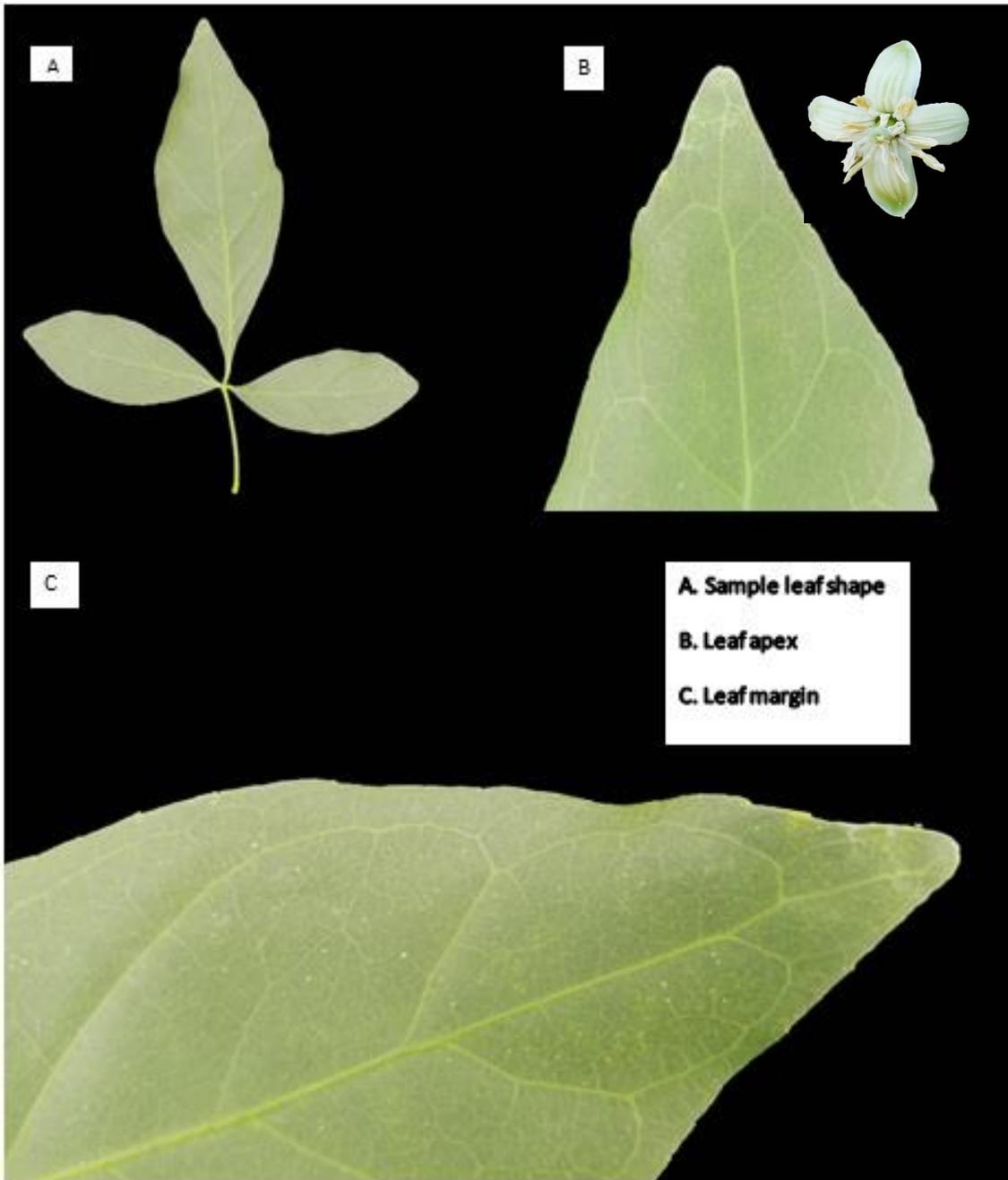
Aeglopsis chevalieri (CRC 2878) **MALE PARENT**



Balsamocitrus daweyi (CRC 3514) MALE PARENT



Afraegle paniculata (CRC 4033) **MALE PARENT**



Appendix D: Examples of normal and abnormal budunions occurring in intergeneric *Citrus* and *Citrus* relative grafts

