

Investigating the Parentage of ‘Orri’ and ‘Fortune’ Mandarin Hybrids

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Abstract

Germplasm characterization is now possible and more reliable using improved molecular markers and genomic tools. Previously citrus cultivars were primarily described according to their morphological or horticultural traits, but trueness-to-type can now be confirmed using molecular markers, thereby limiting inadvertent cultivar misidentification. Following routine testing the reported parentage of two mandarin hybrids is now questioned. ‘Orri’ mandarin is derived from ‘Orah’ mandarin by induced mutation, and was reportedly bred by crossing ‘Temple’ tangor and ‘Dancy’ mandarin. ‘Orri’ has excellent organoleptic qualities, and other favorable traits. With ‘Dancy’ as the pollen parent, at least some degree of susceptibility of ‘Orri’ to *Alternaria* brown spot (ABS) might be expected, but it is more likely to have inherited a high degree of susceptibility; yet ‘Orri’ appears to be immune to ABS. SSR analysis provided evidence that excludes ‘Dancy’ as the pollen parent of ‘Orah’. Further testing revealed the likely pollen parent candidate as ‘Kinnow’ mandarin. ‘Fortune’ mandarin was a popular late-maturing mandarin cultivar until the high incidence of ABS rendered the cultivar uneconomical to produce in certain citrus production regions. This exceptionally late, high quality, attractive cultivar is reported to have originated from a ‘Clementine’ mandarin × ‘Dancy’ cross. However, two independent molecular marker studies revealed that the pollen parent of ‘Fortune’ is possibly ‘Orlando’ tangelo and not ‘Dancy’. In both cases, the pollen parent of ‘Orri’ and ‘Fortune’ mandarin hybrids appears not to be ‘Dancy’ as published. This information will assist geneticists and breeders to re-interpret heritability studies on ABS susceptibility of mandarins and their hybrids.

Keywords: *Citrus reticulata*, *Alternaria* brown spot, heritability, molecular markers, SSR

INTRODUCTION

With the advent of improved technology and the greater ability to detect genetic polymorphisms among citrus cultivars using molecular markers, the characterization and authentication of germplasm entering gene banks is now possible and more reliable. Previously, the characteristics of citrus cultivars were primarily described according to their morphological or horticultural traits. It is now possible, however, to confirm trueness-to-type of citrus cultivars using molecular markers, thereby limiting the inadvertent misidentification of cultivars. Molecular markers also provide a useful tool for identification of zygotic vs. nucellar seedlings and marker-assisted selection of potentially promising progeny. Compared to other molecular markers, SSR (simple sequence repeat) markers are now more widely used in genetic, systematic, and comparative genomic studies largely due to their co-dominance, multi-alleles among closely related genomes, and relatively inexpensive development and detection procedures (Chen et al., 2006). These polymorphic, co-dominant, and segregating alleles greatly facilitate authentication and tracking of genetically-related cultivars and hybrids (Chen et al., 2008).

'Orah' mandarin was reportedly bred by Pinhas Spiegel-Roy and Aliza Vardi at The Volcani Center, Israel, with 'Temple' tangor (♀) (*Citrus reticulata* × *C. sinensis*) and 'Dancy' mandarin (♂) (*C. reticulata*) reported as the parents (Jaffa, undated; Saunt, 2000; Greenberg et al., 2004; Tietel et al., 2009; Citrogold, 2012). Whereas, 'Orah' mandarin is produced commercially only in Israel and Thailand (Mr. Tal Amit, pers. commun., 2010) due to its excessive seediness, 'Orah' mandarin is also the progenitor of 'Orri' (= 'Or') mandarin, as 'Orri' mandarin was derived from 'Orah' mandarin by induced mutation using gamma irradiation, thereby producing a low-seeded variant with low pollen viability. 'Orri' mandarin is one of only a few commercially successful mandarin cultivars bred and released in the modern era of citriculture, and is highly prized in many European markets for its excellent organoleptic qualities (Tietel et al., 2009), and other favourable traits, viz. ease of peeling, having few to no seeds, appearance and time of maturity (typically after 'Clementine' and 'Nova' mandarins).

However, with 'Dancy' mandarin reported as the pollen parent, one might expect 'Orri' mandarin to exhibit at least a low degree of susceptibility to *Alternaria* brown spot (*Alternaria alternata* Fr. (Keissler) pv. *citri* Solel), but it is more likely to have inherited a high degree of susceptibility to *Alternaria* brown spot. However, 'Orri' mandarin appears to be immune to this fungus. Further investigation into the fruit and leaf morphology of 'Orri' mandarin compared with both parents as well as other mandarin cultivars led the authors to investigate the parentage of 'Orah' mandarin using molecular tools. Therefore, the parentage of 'Orri' mandarin was in question given that 'Orri' mandarin does not have the predicted sensitivity to *Alternaria* brown spot, especially for progeny of 'Dancy' mandarin, and that 'Orri' mandarin exhibits remarkably similar leaf and fruit morphological traits as 'Kinnow' mandarin ('King' mandarin [*C. nobilis*] × 'Willowleaf' mandarin [*C. deliciosa*]) – overall, 'Orah' and 'Orri' mandarins look like an early-maturing 'Kinnow' mandarin, except that 'Orri' mandarin peels easier and cleaner than 'Kinnow' mandarin. Therefore, could the parentage of 'Orah' actually be 'Temple' × 'Kinnow', rather than 'Temple' × 'Dancy'? It should be noted that Spiegel-Roy and Vardi developed a 'Temple' × 'Kinnow' hybrid, named 'Pazit', at more-or-less the same time as when 'Orah' mandarin was developed.

'Fortune' mandarin was a popular late-maturing mandarin cultivar, particularly in Spain, until the high incidence of *Alternaria* brown spot rendered the cultivar uneconomical to produce in certain citrus production regions. This exceptionally late, high quality, attractive cultivar is reported to have originated from a 'Clementine' mandarin (♀) × 'Dancy' mandarin (♂) cross made by J.R. Furr (1964) of the United States Department of Agriculture at the U.S. Date and Citrus Station, Indio, California, USA, and was released in 1964 (Furr, 1964; Hodgson, 1967). 'Fortune' mandarin has also been extensively used as the female parent in numerous citrus breeding programs as its seeds are monoembryonic and, notably, some of the progeny are not susceptible to *Alternaria* brown spot, e.g. 'Safor' ('Fortune' mandarin × 'Kara' mandarin [*C. unshiu* × *C. nobilis*]) and 'Garbí' ('Fortune' mandarin × 'Murcott' tangor) (Aleza et al., 2010; Cuenca et al., 2010).

Therefore, the reported pollen parent, coincidentally 'Dancy' mandarin, of these two mandarin hybrids, 'Orri' and 'Fortune', has been called into question. So, the purpose of this study was: a) to determine whether 'Dancy' mandarin is in fact the pollen parent of 'Orri' and 'Fortune' mandarins; and b) if not 'Dancy' mandarin, to determine what the probable pollen parents are.

MATERIALS AND METHODS

'Orri' Mandarin DNA Profiling Using SSR Molecular Markers: UF Study

The SSR primer search, design, and genotyping procedure are described in detail by Chen et al. (2006, 2008). The 24 SSR primers selected in this study (Table 1) were previously used in authentication among mandarins and other cultivars and hybrids (Chen et al., 2008). In brief, fluorescent PCR products were amplified in Bio-Rad (Hercules,

CA, USA) iCyclers in 15 µl volume consisting of 1x PCR buffer, 0.2 mM dNTP, 2 mM MgCl₂, 0.3 µM M13-tailed forward primer, 0.3 µM reverse primer, 0.05 µM dye-labeled M13, 1 U Taq DNA polymerase (Promega, Madison, WI, USA), and 20 ng genomic DNA templates. The products were loaded and analyzed in a 3130xl Genetic Analyzer to generate chromatographic files, and GeneMarker (SoftGenetics LLC., State College, PA) to analyze the files and to generate allele tables.

‘Fortune’ Mandarin DNA Profiling Using SSR Molecular Markers: UCR Study

The M13-tailed primer strategy (Oetting et al., 1995) was used to incorporate dye label into the PCR products for SSR markers. For most markers, duplex PCR reactions were used to amplify two markers simultaneously. Primer pairs compatible with duplex reactions were identified as those giving identical marker phenotypes in single and duplex reactions using templates of the parental cultivars of ‘Fortune’ mandarin and two progeny of those parental cultivars. For a few loci no duplex compatible combinations were found, so those were amplified as single locus reactions, and pooled before loading on the gel. Ten µl PCR reactions contained 1X Promega (Madison, WI, USA) thermophilic buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), and 0.1% Triton X-100), 0.5 units Promega Taq DNA polymerase, 0.2 mM dNTPs, 2 mM MgCl₂, 25 ng DNA, 0.025 µM IRD700 labelled M13 forward or 0.025 µM IRD800 labelled M13 reverse primers (or both if duplex), 0.025 µM of forward and reverse locus-specific primers. The F primer for one locus was 5’-tailed with M13 forward sequence, and the F primer for the second locus was 5’-tailed with M13 reverse sequence for any duplex combination. The PCR program was 5 min 94°C, 38 cycles (1 min 94°C, 1 min 55°C, 1.5 min 72°C), 15 min 72°C. PCR reactions were performed on PTC-100 (MJ Research Inc., Watertown, MA, USA) or Mastercycler Gradient (Eppendorf, AG, Hamburg, Germany) thermocyclers. PCR products were analysed on a LI-COR IR2 4200LR Global DNA sequencer dual dye system (Barkley et al., 2006).

Fifteen loci were screened for ‘Fortune’, ‘Fairchild’, ‘Dancy’, ‘Algerian Clementine’ and ‘Fina Clementine’ mandarins, and ‘Orlando’ tangelo. One of these loci apparently had null alleles and was excluded from further analysis. Multiple samples of ‘Dancy’ and ‘Fortune’ mandarins were obtained from the UCR Citrus Variety Collection and from Lindcove Research and Extension Center. The trees used as parents of ‘Fortune’ mandarin and the original ‘Fortune’ mandarin tree no longer exist, so they could not be analyzed. The seven samples of ‘Dancy’ mandarin and four samples of ‘Fortune’ mandarin were tested with the five loci that had produced patterns incompatible with ‘Clementine’ × ‘Dancy’ parentage of ‘Fortune’ mandarin. An additional 107 loci were screened in multiplex reactions using one sample each of ‘Fortune’, ‘Fairchild’ and ‘Algerian Clementine’ mandarins, and ‘Orlando’ tangelo. (A full list of locus names and primer sequences used at UCR is available from Roose or Federici upon request.)

‘Fortune’ Mandarin DNA Profiling Using SSR Molecular Markers: USDA Study

Citrus SSRs were identified from citrus ESTs using MISA software (Thiel et al., 2003; <http://pgrc.ipk-gatersleben.de/misa/>) as described by Chen et al. (2006). Target motifs were 2 or 3 bp that repeated at least five times. Sequencher software (Gene Codes Corp., USA) was used to align candidate EST sequences and Oligo software (Molecular Biology Insights, Inc., USA) to design primers. A total of 200 SSR primer pairs were identified; primers were synthesized by MWG Biotech with fluorescent label on the forward primer of each pair.

Each of the primer pairs was used in combination with ‘Succari’ sweet orange (*C. sinensis*) DNA to verify amplification products. Those primer pairs producing unambiguous amplicons were analyzed using the ABI 310 sequence analyzer and GeneMapper software.

Following extensive screening, three of the primer pairs (designated: 32241388, MVF46-G09, and MVF08-E02) were found to be polymorphic in ‘Clementine’ and ‘Dancy’ mandarins and ‘Orlando’ tangelo. These primer pairs were used in combination

with DNA extracted from ‘Fortune’, ‘Clementine’ and ‘Dancy’ mandarins, and ‘Orlando’ tangelo.

RESULTS AND DISCUSSION

‘Orri’ Mandarin DNA Profiling Using SSR Molecular Markers: UF Study

The alleles were successfully generated from 23 of the 24 primers in the four cultivars tested (Table 1).

Based on markers CX6F17 and CX6F30, ‘Dancy’ mandarin was unequivocally excluded as a paternal or maternal parent of ‘Orri’ mandarin because none of the alleles at the two loci in ‘Orri’ mandarin were found in ‘Dancy’ mandarin. However, neither ‘Temple’ tangor nor ‘Kinnow’ mandarin can be excluded as parents of ‘Orri’ mandarin on the basis of their allelic content; this is not the same as saying that they are the parents of ‘Orri’ mandarin, and there could conceivably be several other parents that might have been involved, but we have not pursued this to the point of certainty. In addition, it was also confirmed that ‘Orri’ mandarin is not a spontaneous mutation of ‘Kinnow’ mandarin because the alleles at most heterozygous loci in ‘Orri’ mandarin were not found to be identical to those in ‘Kinnow’ mandarin.

Table 1. The alleles of 24 primers in ‘Orri’, ‘Dancy’ and ‘Kinnow’ mandarins, and ‘Temple’ tangor. ‘Dancy’ mandarin was excluded as a parent of ‘Orri’ mandarin, according to CX6F17 and CX6F30, in which no allele was shared between the two cultivars.

Locus name	Orri mandarin	Dancy mandarin	Kinnow mandarin	Temple tangor
CX0010	220/230	220/230	220/230	220
CX0035	172/181	172	172/181	172/181
CX2018	173/181	173	173/181	173/181
CX2021	150	148/150	150	148/150
CX6F04	162/173	162	162	162/173
CX6F18	153	153	153	153/159
CX6F29	153/154	153/154	153/154	151/154
CX5F57	156/166	156/166	156/166	156/166
CX6F02	-	-	-	-
CX6F03	269	269	269	269/280
CX6F06	169	169/172	169	169
CX6F07	107	107	107	107
CX6F09	157	157/176	157	157/176
CX6F10	173/179	173/179	173/179	173/179
CX6F13	171/177	177	171/177	171/177
CX6F14	109/114	109/114	109/114	109/114
CX6F15	160	160	160	160
CX6F17	132/157	135/138	148/157	132/138
CX6F19	150/168	150	150	150/168
CX6F21	142/155	149/155	142/155	149/155
CX6F27	159	159	159	159
CX6F30	101	107	101	89/101
CX6F32	161	161/167	161/167	161
CX5F38	139	139	139	139

‘Fortune’ Mandarin DNA Profiling Using SSR Molecular Markers: UCR Study

All samples of ‘Dancy’ mandarin produced identical band patterns with all five loci analyzed, and all four samples of ‘Fortune’ mandarin also produced identical patterns. Of the 14 loci screened with ‘Fortune’, ‘Fairchild’, ‘Dancy’ and ‘Algerian Clementine’ mandarins, and ‘Orlando’ tangelo, nine produced band patterns in ‘Fortune’ mandarin that could have been derived from ‘Clementine’ × ‘Dancy’ or ‘Clementine’ × ‘Orlando’ (Table 2). Five loci (JK-TAA41, CF-TTC01, CF-AT01, CF-CAT04, and CTV2745) produced band patterns in ‘Fortune’ mandarin that could not have been derived from ‘Clementine’ × ‘Dancy’, but could have been derived from ‘Clementine’ × ‘Orlando’ (Fig. 1). ‘Clementine’ mandarin is apparently heterozygous for a null allele at locus NB-CAG01 that explains the apparent absence of a 122 allele in ‘Fortune’. The reverse primer for this locus has a one base insertion relative to the haploid ‘Clementine’ genome sequence and progeny phenotypes observed in the ‘Clementine’ × ‘Chandler’ pummelo (*C. maxima*) cross (Ollitrault et al., 2012) were consistent with the presence of a null allele in ‘Clementine’ mandarin (Y. Kacar, pers. commun.). All of the 107 loci screened with ‘Fortune’, ‘Fairchild’ and ‘Algerian Clementine’ mandarins, and ‘Orlando’ tangelo produced bands in ‘Fortune’ mandarin that could have been derived from ‘Clementine’ × ‘Orlando’, with the assumption that ‘Clementine’ or ‘Orlando’ was heterozygous for a null allele at two loci. This evidence leads to the conclusion that ‘Orlando’ tangelo was possibly the pollen parent of ‘Fortune’ mandarin, rather than ‘Dancy’ mandarin.

Table 2. SSR marker analysis at UCR showing allele sizes at 15 loci for which data was collected on ‘Fortune’ and ‘Fairchild’ mandarins and their possible parents.

Locus name	Dancy mandarin	Algerian clementine	Fortune mandarin	Fairchild mandarin	Orlando tangelo
CMS25	160/162	160/178	160/178	160/178	162/178
CMS30	149	149/153	149	149/169	149/169
CMS7	150/152	150/152	150/152	150/152	150
JK-CAC15	158	149/158	149/158	158/160	158/160
JK-CAC39	174	174	174	174	174
JK-cAGG9	118	118	118	118	118
JK-TAA15	192/204	192/195	195/204	192/195	192/204
NB-CAG01	122/124	122	124	122/124	124
NB-CT21	145	145	145	145	145
CF-AT01	269	269	260/269	269	260/269
CF-CAT04	264	264	255/264	264	255/264
CF-TTC01	192	162/192	192/198	162/192	192/198
JK-TAA41	140/150	145/150	136/145	136/145	136/150
CTV2745	303/315	303/315	300/303	303/315	300/303

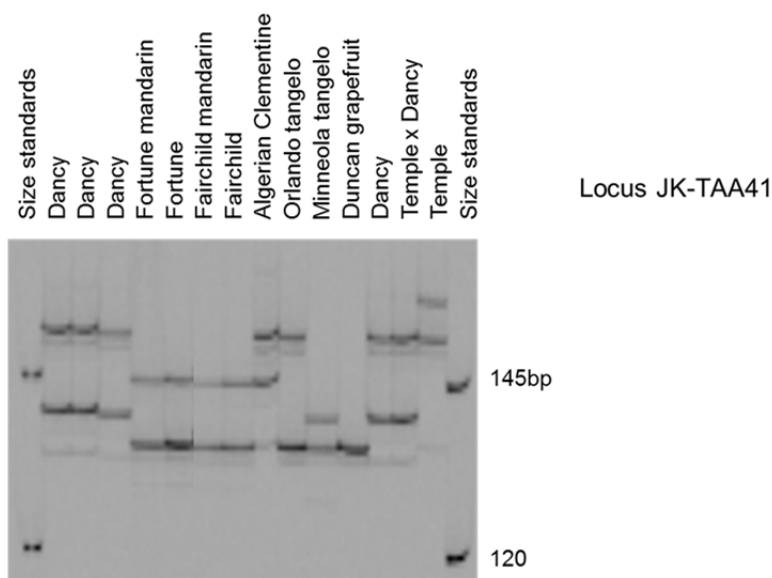


Fig. 1. Investigating the parentage of ‘Fortune’ and ‘Fairchild’ mandarins: parent samples from several different trees at UCR were compared with JK-TAA41 locus. Neither band in ‘Fortune’ mandarin is found in ‘Dancy’ mandarin, but the ‘Fortune’ mandarin bands are found in ‘Clementine’ mandarin and ‘Orlando’ tangelo.

‘Fortune’ Mandarin DNA Profiling Using SSR Molecular Markers: USDA Study

Three loci were screened with DNA extracted from ‘Fortune’, ‘Dancy’ and ‘Monreal Clementine’ mandarins and ‘Orlando’ tangelo, and in agreement with the UCR results but using independent sources of the parental varieties, none produced amplification products with ‘Fortune’ mandarin DNA that could have been derived from ‘Clementine’ × ‘Dancy’ (Table 3) further supporting the likelihood that ‘Fortune’ mandarin was derived from ‘Clementine’ × ‘Orlando’.

Table 3. SSR marker analysis at USHRL showing allele sizes at loci for which data was collected on ‘Fortune’ mandarin and possible parents.

Locus name	Dancy mandarin	Monreal clementine	Fortune mandarin	Orlando tangelo
32241388	373	373	362/373	362/373
MVF46-G09	213/216	213/216	195/216	195/213
MVF08-E02	247/250	247/250	247/257	250/257

CONCLUSIONS

SSR analysis using primers that previously revealed substantial polymorphisms among mandarin cultivars has provided evidence that excludes ‘Dancy’ mandarin as the pollen parent of ‘Orah’ mandarin, but suggests that ‘Kinnow’ mandarin is the likely pollen parent. During the same era that ‘Orah’ mandarin was developed, ‘Pazit’ mandarin was also bred by Spiegel-Roy and Vardi at The Volcani Center. Note that ‘Pazit’ mandarin is a hybrid of ‘Temple’ tangor × ‘Kinnow’ mandarin.

Whereas the published parentage of ‘Fortune’ mandarin is ‘Clementine’ × ‘Dancy’, two independent studies using molecular markers have revealed that this is not correct – the markers are consistent with ‘Clementine’ × ‘Orlando’ being the parentage of

‘Fortune’ mandarin. Therefore, the pollen parent of ‘Fortune’ mandarin is probably ‘Orlando’ tangelo and not ‘Dancy’ mandarin, which is the same parentage as ‘Fairchild’ mandarin, a hybrid made by Furr of the USDA at about the same time as ‘Fortune’ mandarin. This error may have been due to mislabelling or possibly natural pollination having taken place as bagging of pollinated flowers to prevent natural cross-pollination was not always used in that breeding program. A note from Dr. Bill Bitters to Dr. Mikeal Roose (concerning some rootstock hybrids) states: “Now – be careful – Furr didn’t bag etc. at pollination – he felt the first pollen to reach the stigma did the trick.” Well, apparently, not always!

In both cases, the pollen parent of ‘Orah’ (and therefore ‘Orri’) and ‘Fortune’ mandarins appears not to be ‘Dancy’ mandarin as previously published. Further paternity testing revealed the likely candidates as the pollen parent. The suggested pollen parent of ‘Orah’ mandarin, and hence ‘Orri’ mandarin, is ‘Kinnow’ mandarin, and the suggested pollen parent of ‘Fortune’ mandarin is ‘Orlando’ tangelo.

Given the interpretation regarding the parentage of ‘Orah’ and ‘Fortune’ mandarin hybrids, then of intermediate interest is that this knowledge will assist geneticists and breeders to re-interpret heritability studies on *Alternaria* brown spot susceptibility of mandarins and their hybrids (Kohmoto et al., 1991; Schutte, 1993; Dalkilic et al., 2005; Dr. David Ezra, personal communication, 2010); these studies could be re-interpreted to demonstrate that direct progeny of ‘Dancy’ mandarin will be sensitive to *Alternaria* brown spot, e.g. ‘Michal’ and ‘Nova’ mandarins and ‘Minneola’ and ‘Orlando’ tangelos, whereas second generation progeny will not necessarily be susceptible, e.g. ‘Orah’, ‘Safor’ and ‘Garbí’ mandarins. *Alternaria* brown spot susceptibility appears to be a dominant trait that is transferred from ‘Dancy’ mandarin to its progeny, and resistance is presumed to be controlled by a single dominant allele found in ‘Clementine’ mandarin (Kohmoto et al., 1991; Dalkilic et al., 2005). Furthermore, such conclusions may encourage the use of ‘Fortune’ and ‘Orah’ mandarins as breeding parents.

Identitatus patri semper incertus est.
The identity of the father is always uncertain!

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