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Short Communication

Skewed RAPD markers in linkage maps of *Citrus*

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Abstract

The objective of this work was to analyze the effects of RAPD markers with skewed segregation on genetic linkage maps. Segregation data for 123 *Citrus sinensis* (L.) Osbeck cv. Pêra markers and 53 *C. reticulata* Blanco cv. Cravo markers in F_1 progeny composed of 94 hybrids were used. Genetic linkage maps of the two varieties were constructed with non-skewed markers ($p < 0.05$ and $p < 0.01$) using the program MAPMAKER 3.0 and a pseudo-testcross strategy. The maps were compared to those constructed with all markers. Alterations in the genetic distances were observed based on the location of the skewed markers within the linkage groups. Generally, the skewed markers were located at the end of the linkage groups, sometimes forming entire linkage groups, without causing significant distance modifications. However, skewed markers located between non-skewed markers caused significant distance modifications and, in some cases, altered the order of the markers. Most of the skewed markers can be included in linkage maps, but in each case the degree of distance modification caused by each marker needs to be assessed.

Key words: genetic distance, Mendelian segregation, molecular markers, segregation distortion.

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Introduction

Genetic linkage maps have been obtained for several plant species and are the basis for advanced genetic studies that provide a better understanding of the inheritance, identification and isolation of genes (Roose *et al.,* 2000). Cloning and gene insertion through genetic transformation have been widely used in species with well-defined genetic maps (Gmitter Jr. *et al.,* 1996).

Different types of molecular markers have been used to obtain genetic linkage maps. Molecular markers are used because they are highly polymorphic and are not influenced by the environment. Among these, random amplified polymorphic DNA (RAPD) markers are the most common because the technique is easy, inexpensive, uses a low amount of genomic DNA, and produces markers that are highly polymorphic and that represent the whole genome (Ferreira and Grattapaglia, 1998). RAPD markers are detected by the random amplification of genomic DNA fragments of different sizes through the polymerase chain reaction (PCR) (Williams *et al.,* 1990).

The genetic features found in *Citrus* favor the construction of genetic maps. *Citrus* has a small genome (1C = 0.62 pg) (Guerra, 1984), is diploid with a small number of chromosomes (n = 9) (Soost and Cameron, 1975) and is highly polymorphic. In addition, interspecific and intergeneric hybrids can be produced (Barrett, 1985).

Several studies have described the mapping of *Citrus* in order to identify genes and/or quantitative trait loci (QTL) for traits such as resistance/susceptibility to mineral salts and cold (Cai *et al.,* 1994; Moore *et al.,* 2000), tristeza virus (Mestre *et al.,* 1997; Cristofani *et al.,* 1999) and citrus variegated chlorosis (CVC) (Oliveira *et al.,* 2004), dormancy, juvenile characters and vigor (Roose *et al.,* 1992), plant height and fruit acidity (Gmitter Jr. *et al.,* 1996).

The construction of linkage maps of *Citrus* based on molecular markers has been achieved using markers with an expected Mendelian segregation and with those that a skewed segregation (Cai *et al.,* 1994; Luro *et al.,* 1994; Kijas *et al.,* 1997; Cristofani *et al.,* 1999). For maps of other genera, some authors have used markers with a skewed segregation (Crouzillat *et al.,* 1996), while others have excluded such markers (Grattapaglia and Sederoff, 1994). Skewed loci can reveal important genetic information but can also alter the distances and the linear order of the other markers in the linkage groups (Jarrell *et al.,* 1992).

The objective of this work was to analyze and quantify the effects of RAPD markers with a skewed segrega-

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tion on genetic maps and on the location of these markers in linkage groups using *Citrus* species as a model.

Material and Methods

The species used were *Citrus reticulata* Blanco cv. Cravo, C. *sinensis* (L.) Osbeck cv. 'Pêra' and 94 F_1 progeny obtained by crossing these varieties. The hybrids were obtained by controlled crossing, with 'Cravo' mandarin as the female parent. Data from the segregation of 123 RAPD markers from 'Pêra' sweet orange and 53 from 'Cravo' mandarin, all obtained by Oliveira *et al.* (2004), were used for genetic analyses. These markers were heterozygous for one of the parents (Aa) and recessive homozygous (aa) for the other, based on the presence of the RAPD bands. The null hypothesis of a 1:1 Mendelian segregation was tested for each marker by χ^2 analyses ($p < 0.05$ and $p < 0.01$).

Linkage maps for each variety were constructed using the markers that did not have a skewed segregation at $p < 0.05$ and at $p < 0.01$. The pseudo-testcross strategy, the Kosambi function (Kosambi, 1944) and the parameters LOD (Likelihood of odds) ≥ 6.0 and a maximum frequency of recombination (θ) of 0.40 were applied to the linkage analyses among the markers using the software MAPMAKER v. 3.0 (Lander *et al.,* 1987). The compiled data were duplicated and recoded to allow the detection of linkage of the RAPD marker when in repulsion phase (Grattapaglia and Sederoff, 1994). The linkage groups were determined by grouping two points that were afterwards arranged by multipoint analyses. The groups were also constructed with markers from both linkage phases.

Once the maps of each variety were constructed, the number, size and composition of the linkage groups, the length of the maps, the linear order and the distances among the markers were compared to the results obtained by Oliveira *et al.* (2004). These authors used all markers to construct maps of *Citrus*, regardless of the absence or presence of skewed segregation.

The alterations in the map distances of adjacent markers caused by the removal of markers with a skewed segregation were analyzed. The position of the markers with a skewed segregation in the linkage groups (at the end of the groups or between markers with the expected segregation) was correlated with the conservation of the distances and the order of non-skewed segregation markers. The correlation among the distances of the markers with an expected segregation in the absence and presence of skewed markers at intermediate positions was also evaluated.

Results and Discussion

Significant deviations in the rates of segregation of the markers in relation to the proportion of expected Mendelian segregation (1:1) were observed at several levels in genetic linkage maps of *Citrus* obtained from interspecific and intergenera hybridizations, with extreme values ranging from 22.2% to 61% for $p < 0.05$. In the present study, the 'Pêra' variety showed 61% ($p < 0.05$) and 48% ($p < 0.01$) of the markers with a skewed segregation, while 'Cravo' mandarin had 26.4% ($p < 0.05$) and 15.1% ($p < 0.01$). The genetic nature of *Citrus* favors deviations in marker segregation in the progenies, frequently with selection during embryo formation (pre and/or post-zygotic stages) and during the development of the plants. Thus, *Citrus* is an appropriate model for studying the effects of these markers in genetic linkage maps of perennial species.

A comparison of the maps constructed with markers having the expected Mendelian segregation ratios (1:1) at $p < 0.05$ and $p < 0.01$ is given in Table 1. Although the

Table 1 - The effect of using RAPD markers with different levels of skewed segregation on the construction of linkage maps for *C. sinensis* Osb. 'Pêra' and *C. reticulata* Blanco 'Cravo'.

Variable	'Pêra' sweet orange			'Cravo' mandarin		
	All markers	$p < 0.01$[b]	$p < 0.05$[b]	All markers	$p < 0.01$[b]	$p < 0.05$[b]
Total number of markers	123	64	47	53	46	39
Linkage groups	12	8	7	12	12	10
Linked markers	117	59	43	51	43	38
Unlinked markers	6	5	4	2	3	1
Linked markers/group	9.8	7.4	6.1	4.3	3.6	3.8
Total length of the map[a]	612.1	300.6	210.1	353.3	233.9	199
Mean distance among markers[a]	5.2	5.1	4.9	6.9	5.4	5.2
Smallest distance among markers[a]	0	0	0	0	0	0
Largest distance among markers[a]	25.4	25.4	24.0	25.4	25.4	25.4
Smallest linkage group[a]	5.3	4.3	2.1	7.5	5.5	5.5
Largest linkage group[a]	101.1	87.8	87.8	60.7	36.6	36.6

[a]Quantified variables are in centiMorgan (cM) units.
[b]Probability of the expected Mendelian segregation ratio (1:1) of the markers used to construct the map.

'Pêra' variety had a higher number of markers when compared to those of 'Cravo' mandarin, primarily because of a high heterozygous index (Oliveira *et al.,* 2002), the number of markers in both varieties in the linkage map was very similar after removing the markers that had a skewed segregation.

Practically, no alteration in the number of linkage groups of 'Cravo' mandarin was observed when the markers with a skewed segregation were excluded. On the other hand, in 'Pêra' sweet orange, 12 groups were obtained when the map was constructed with all of the markers, whereas only seven and eight groups were obtained for maps constructed with markers that had the expected Mendelian segregation ratio (1:1) at $p < 0.05$ and $p < 0.01$, respectively. This occurred because the markers with a skewed segregation in 'Cravo' mandarin were present in a smaller percentage, and normally at the extremities of the linkage groups, while in 'Pêra' sweet orange, there were several linkage groups composed exclusively of markers with a skewed segregation (five groups at $p < 0.05$ and four at $p < 0.01$). In this case, removal of the markers with a skewed segregation resulted in the exclusion of the entire corresponding linkage groups.

The maps of both varieties showed no division of linkage groups when the markers with a skewed segregation were removed. Cristofani *et al.* (1999) showed that the number of linkage groups was inversely proportional to the number of markers because of the division of some groups caused by the absence of markers at intermediate positions. This trait was probably not seen here because of the short distance among the map markers and also because of the concentration of markers with a skewed segregation found in certain linkage groups or only at their extremities.

The segregation of the markers in the progeny studied showed low recombination frequencies and are therefore located very close to one another in the linkage groups. The average distance between adjacent markers varied from 4.9 to 5.2 cM for 'Pêra' sweet orange and from 5.2 to 6.9 cM for 'Cravo' mandarin. The values for the highest distance between adjacent markers for both varieties were conserved, thus preventing the formation of gaps, but causing a decrease in the average distance among markers after the exclusion of those with a skewed segregation (Table 1). Hence, the use of a cross with a high number of progenies (94) allowed the identification of distances among very close markers. The presence of a high amount of heterochromatin in the chromosomes of *Citrus* partially explains the tendency of markers to concentrate at certain positions in the linkage groups (Guerra, 1993).

The number of unlinked markers in the maps of both varieties decreased when the markers with a skewed segregation were removed (Table 1). These results were initially though to contradict the theories related to mapping since, according to Liou (1990), as the number of markers decreases in a map the number of unlinked markers increases. However, the reduction in the number of markers seen here was not random, but was caused by the exclusion of markers with a skewed segregation and that theoretically had a lower probability of linkage.

When the markers with a skewed segregation were removed, there was a proportional decrease in the absolute number of linked markers, in the number of linked markers per linkage group, in the size of the linkage groups, and in the total length of the maps of both varieties. Consequently, there was a lower coverage of the genome (Table 1).

Theoretically, the distances and the order of mapped markers should not change in the linkage groups when new markers are included or excluded. Small variations in the distances can be explained by the occurrence of double crossing overs, which are not very frequent between adjacent markers, whereas significant variations should only occur when the maps are constructed with a small number of markers (Liu , 1998).

The majority of skewed markers, 98.4% in 'Pêra' sweet orange and 96.2% in 'Cravo' mandarin, mapped at the extremities of the linkage groups and, in some cases, made up the entire linkage group. Similar behavior has been documented by others (Cai *et al.,* 1994; Luro *et al.,* 1994; Cristofani *et al.,* 1999). In 'Pêra' sweet orange, the markers with a skewed segregation were located at the extremities of four groups and formed five other complete groups, to give a total of 12 groups. In the case of 'Cravo' mandarin, four groups were found at the extremities and two others were completely formed, to give a total of 12 ($p < 0.05$). In these cases, removal of the markers with a skewed segregation did not alter the distances of the expected segregation markers (Figure 1).

A small number of markers with a skewed segregation occurred among the markers with an expected Mendelian segregation in the linkage groups. Two such markers

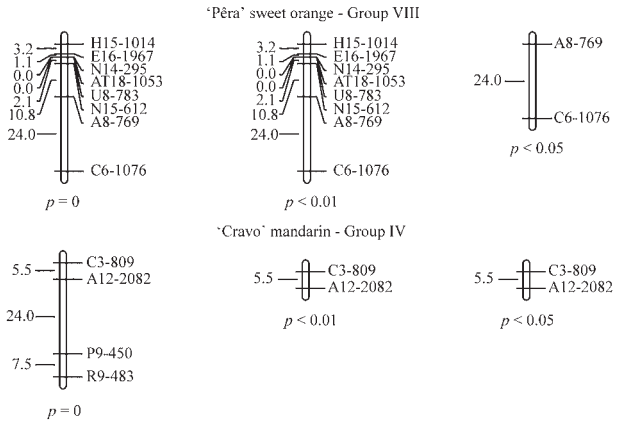

Figure 1 - Partial linkage map of *Citrus* showing the effect of excluding markers with a skewed segregation that are positioned at the extremities of the linkage groups of *C. sinensis* Osb. 'Pêra' and *C. reticulata* Blanco 'Cravo'. The linkage groups were constructed using MAPMAKER with a pseudo-testcross strategy, a Kosambi function, LOD ≥ 6.0 and $\theta \leq 0.40$. $p = 0$, $p < 0.05$ and $p < 0.01$ refer to the probability of the expected Mendelian segregation ratio (1:1) of the markers used to construct the map.

were found among the 123 markers in the maps of 'Pêra' sweet orange (AB14-1511 and B10-1986) and two among the 53 markers in the maps of 'Cravo' mandarin (G13-327 and E19-1282). In three cases (AB14-1511, B10-1986 and G13-327), the removal of markers with a skewed segregation from the linkage groups led to alterations in the distances of the adjacent markers that showed the expected Mendelian segregation (Figures 2 and 3). For the marker B10-1986, there were also alterations in the order of the remaining markers in the linkage groups (Figure 3). Such alterations have been documented by Jarrell *et al.* (1992) in maps of other *Citrus* species. The removal of the markers G13-327 and E19-1282 did not alter the order because only two markers were left in the respective linkage groups.

In some linkage groups, the exclusion of markers with a skewed segregation located at the extremities altered the distances by only 0.1 cM. These changes were probably not significant since they occurred only in the marker that occupied the extremity of the group. An exception was seen with the removal of the AV5-1555 marker of 'Cravo' mandarin, which also caused alterations in the order of the markers (Figure 3). In this case, the marker was considered to be an artifact that resulted from the limitations of the RAPD technique (Grattapaglia and Sederoff, 1994).

The alterations in the distances, expressed in map units, were significantly higher when the markers with a skewed segregation located among those with the expected segregation were removed. The interference varied from 11.8% to 104.8% in the maps studied, with a tendency to increase the distances in the presence of the skewed segregation markers. A linear correlation ($r^2 = 0.91$) was observed among the distances of the markers with the expected segregation in the absence and presence of markers with a skewed segregation. This correlation demonstrated that the presence of markers with a skewed segregation located among the markers with an expected segregation increased the distance among the markers by 1.485 fold for each cM. The existence of this correlation and, consequently, of a factor or equation for adjustment needs to be confirmed by adding new markers to the maps.

Every time a marker with a skewed segregation is added to linkage maps, there is a possibility that the distances may be altered, and this could jeopardize the identification of QTLs and the associated improvement programs (Cai *et al.,* 1994; Luro *et al.,* 1994). Therefore, the markers to be used in constructing the maps should be selected based on the degree of alteration caused by each marker. The markers with skewness that occur rarely among the markers with the expected segregation should be excluded or, if possible, have their distances corrected to prevent artifacts. On the other hand, the markers with a skewed segregation that are clustered and located at the extremity of groups or that completely occupy the linkage groups can be considered to be derived from chromosomal regions with structural differences (Cai *et al.,* 1994), from genetic re-

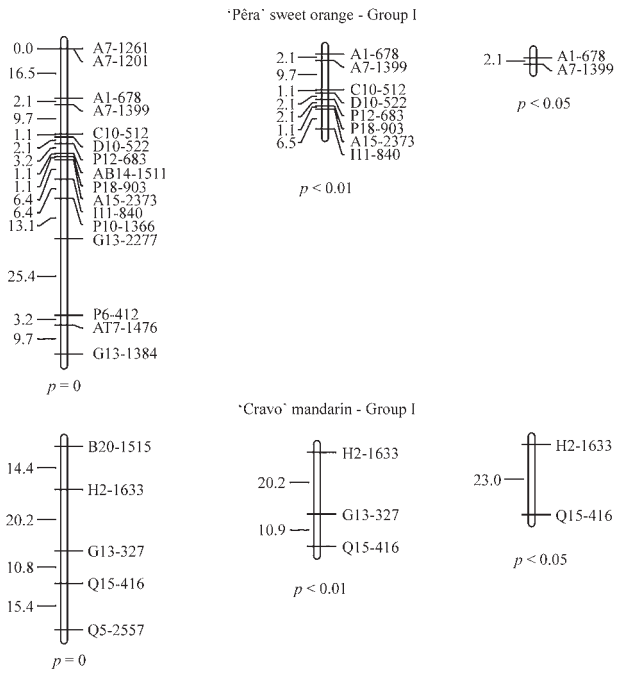

Figure 2 - Partial linkage map of *Citrus* showing the effect of excluding markers with a skewed segregation that are located within the linkage groups of *C. sinensis* Osb. 'Pêra' and *C. reticulata* Blanco 'Cravo'. The linkage groups were constructed using MAPMAKER with a pseudo-testcross strategy, a Kosambi function, LOD ≥ 6.0 and θ ≤ 0.40. p = 0, p < 0.05 and p < 0.01 refer to the probability of the expected Mendelian segregation ratio (1:1) of the markers used to construct the map.

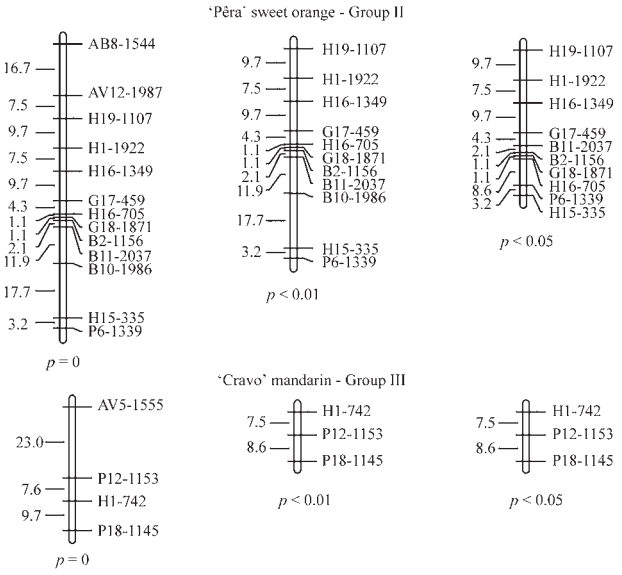

Figure 3 - Partial linkage map of *Citrus* showing the alterations in the order of the markers in the linkage groups of *C. sinensis* Osb. 'Pêra' and *C. reticulata* Blanco 'Cravo' following the exclusion of markers with a skewed segregation. The linkage groups were constructed using MAPMAKER with a pseudo-testcross strategy, a Kosambi function, LOD ≥ 6.0 and θ≤ 0.40. p = 0, p < 0.05 and p < 0.01 refer to the probability of the expected Mendelian segregation ratio (1:1) of the markers used to construct the map.

combination inhibited in heterochromatin-rich areas, from close association to the centromeres or telomeres, and/or from the expression of genes that affect the viability under selection (Grattapaglia and Sederoff, 1994). In all cases, the inclusion of such markers in the maps is essential for genetic studies and mapping.

Conclusion

The comparison of linkage maps with and without markers that have a skewed segregation is essential for understanding the genetics of a species and for selecting suitable markers for mapping studies. As shown here, most markers with a skewed segregation were located at the extremities of the linkage groups or formed entire linkage groups. The location of markers with a skewed segregation in a given linkage group generally affected the extent to which the genetic distances were altered. However, the inclusion of markers with a skewed segregation positioned at extremities of the linkage groups usually did not markedly affect the genetic distances of markers with an expected segregation. In contrast, the inclusion of markers with a skewed segregation located within linkage groups usually significantly altered the genetic distances of markers with an expected segregation. Although most markers with a skewed segregation can be included in linkage maps, it is still necessary to assess the interference of each marker in the genetic distances of markers with an expected segregation. Since these conclusions are based on *Citrus*, they may not be wholly applicable to other species.

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