Construction of genetic maps for *Citrus aurantium* and *C. latipes* based on AFLP, RAPD and RFLP markers

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Construction of genetic maps for *Citrus aurantium* and *C. latipes* based on AFLP, RAPD and RFLP markers.

Abstract — Introduction. Citrus have a word-wide economic importance. Their breeding is a long process complicated by a long juvenile period, heterozygosity and nucellar embryony. Molecular markers are an important tool for the identification of chromosomal regions carrying loci controlling important agronomic traits in citrus and for marker assisted rootstock breeding. Then, they were tested for the construction of linkage maps for *C. aurantium* and *C. latipes*. **Materials and methods**. Polymorphism of 50 F1 individual of a single controlled cross between *C. latipes* and *C. aurantium* were carried out from the study of AFLP markers (7 AFLP EcoRI/MseI primer combinations), RAPD markers (280 primers) and the use of 50 RFLP probes, isolated from a *Citrus Pst*I genomic library. Markers were scored according to the pseudo-testcross strategy and two linkage maps – one for *C. latipes* and one for *C. aurantium* – were constructed using a suitable software. **Results and discussion**. In *C. latipes*, 110 segregating markers (60 AFLPs, 46 RAPDs and 4 RFLPs) were identified. Among them, 92 formed 12 linkage groups (7 major and 5 minor ones) spanning 600cM, while 18 markers remained unlinked. In *C. aurantium*, 268 segregating markers (144 AFLP, 111 RAPD and 13 RFLP) were identified. Among them, 247 formed 20 linkage groups (10 major and 10 minor ones) spanning 1 000cM, while 21 markers resulted unlinked. The typing of a larger number of individuals seems necessary to increase the robustness of the map and permit quantitative trait (QTL) analysis. (© Elsevier, Paris)

***Citrus aurantium* / *Citrus latipes* / molecular biology / analytical methods / genetic markers / genetic maps**

Marquage par AFLP, RAPD et RFLP pour la construction de cartes génétiques de *Citrus aurantium* et *C. latipes*.

Résumé — Introduction. Les agrumes ont, à l'échelle mondiale, une grande importance économique. Leur sélection est longue du fait de la durée de leur période juvénile, de leur hétérozygotie et de l'embryonnie nucellaire. Les techniques de marquage moléculaire sont un outil important pour identifier les régions chromosomiques portant les loci des caractères agronomiques importants et pour effectuer une sélection, assistée par marqueurs, de porte-greffes d'agrumes. Ils ont donc été utilisés pour la construction de cartes de linkages pour *C. aurantium* et *C. latipes*. **Matériel et méthodes**. L'évaluation du polymorphisme de 50 individus F1, issus d'un croisement entre *C. latipes* et *C. aurantium*, a été faite à partir de l'étude par marqueurs AFLP (sept combinaisons d'amorces EcoRI/MseI), RADP (280 amorces) et RFLP (50 sondes isolées d'une bibliothèque génomique de *Pst*I d'agrumes). Les marqueurs ont été identifiés en utilisant une stratégie de pseudo-testcross et deux cartes de linkage – l'une pour *C. latipes*, l'autre pour *C. aurantium* – ont été construites en utilisant un logiciel adapté. **Résultats et discussion**. Pour *C. latipes*, 110 marqueurs de ségrégation (60 AFLP, 46 RAPD et 4 RFLP) ont été identifiés. Parmi eux, 92 ont formé 12 groupes de linkage (7 principaux et 5 mineurs) couvrant 600 cM, alors que 18 marqueurs sont restés non liés. Pour *C. aurantium*, 268 marqueurs de ségrégation (144 AFLP, 111 RAPD et 13 RFLP) ont été identifiés, dont 247 ont formé 20 groupes de linkage (10 majeurs et 10 mineurs) couvrant 1 000cM, tandis que 21 marqueurs restaient non liés. L'étude d'un plus grand nombre d'individus apparaît nécessaire pour augmenter la fiabilité de la carte obtenue et entreprendre l'analyse des loci des caractères quantitatifs (QTL). (© Elsevier, Paris)

***Citrus aurantium* / *Citrus latipes* / biologie moléculaire /technique analytique / marqueur génétique / carte génétique**

1. introduction

Citrus is a fruit tree crop of word-wide economic importance. Its breeding is a long process that requires large investments in land and time for the evaluation of progenies. In addition, it is complicated by a long juvenile period, heterozygosity and nucellar embryony. Citrus cultivars susceptible to root disease are propagated by grafting onto tolerant highly heterozygous and vigorous seedling rootstocks. In spite of their heterozygosity, seedling populations of many rootstocks are genetically uniform, because they largely derive from apomictic seed through nucellar embryony.

Sour orange (*Citrus aurantium* L.) is tolerant to *Phytophthora* highly vigorous and has grafting compatibility with most *Citrus* species and cultivars. This species is widely used as rootstock, however, the susceptibility to tristeza virus limits its use in countries where this virus is endemic. *C. aurantium* is polyembryonic and propagates through apomictic nucellar embryony. On the one hand this represents an advantage for its multiplication, on the other, the scarcity of sexual embryos imposes severe limits to the breeding of this species.

Some taxa of *Citrus* are entirely sexual in reproduction and their use as rootstock is problematic due to the segregation of heterozygous loci and traits occuring during their multiplication. However, their use in breeding programmes as females may be advantageous because it will eliminate apomictic seedlings. *C. latipes* Swing (Tar.) [1, 2] is a sexual monoembryonic species resistant to several diseases, including tristeza virus. The introgression of disease resistance into polyembryonic species and of polyembryony in disease resistant species may give rise to agronomically and economically improved *Citrus* rootstocks.

The advent of molecular markers, in particular of those based on polymerase chain reactions (PCR), now permits the construction of linkage maps, the investigation and mapping of agronomic traits [3–5] and the marker assisted selection in several crop tree species, including *Citrus*. To date, three linkage maps have been produced within *Citrus*, based on RFLP and RAPD markers. All were constructed from intergeneric hybrid crosses [6–9]. The richest map contains 109 RAPD markers and covers 70–80% of the *Citrus* genome [8]. The most recent [10] contains also microsatellite markers, together with RFLPs.

The purpose of this paper is to report preliminary data on *C. aurantium* and *C. latipes* linkage maps, produced adopting a two-way pseudo-testcross mapping strategy [11, 12]. The F_1 progeny analysed was obtained by the testcross mating between the two *Citrus* species. We report the linkage analysis of AFLP, RAPD and RFLP markers and the maps of the major linkage groups.

2. materials and methods

2.1. plant material

The tested material consisted of a single controlled cross made in Acireale (Sicily) during 1970. The monoembryonic *C. latipes* was used as female in a cross with the polyembryonic *C. aurantium*, used as male. Two hundred and fifty F_1 individuals were planted as seedlings at the experimental field at Fonti Ciane (Syracuse, Sicily). Fifty F_1 individuals were genotyped for the construction of linkage maps.

2.2. DNA extraction

Total DNA was extracted from 3.5 g of fresh leaves according to the procedure described by Doyle and Doyle [13].

2.3. AFLP assay

The procedure for the production and detection of EcoRI/MseI AFLP markers followed the protocol described in detail by Vos et al. [14]. Genomic DNA was

digested with EcoRI and MseI restriction endonucleases; synthetic EcoRI and MseI adapters were ligated to restriction fragments; the products of ligation were diluted and amplified with primers complementary to the adapters and carrying one selective nucleotide beyond the restriction site; the products of amplification were diluted again and amplified with a ^{33}P labelled EcoRI primer and an unlabelled MseI primer, both carrying three selective nucleotides. Finally, labelled fragments were resolved on sequencing gels and autoradiographed. Nine primers were designed following the indications of Vos et al. [14]: E31 (extension AAA), E38 (extension ACT), E40 (extension AGC), M33 (extension AAG), M34 (extension AAT), M37 (extension ACG), M38 (extension ACT), M39 (extension AGA) and M44 (extension ATC). The following primers pairs were used: E31/M33, E31/M38, E31/M39, E31/M44, E38/M33, E38/M37 and E40/M34.

2.4. RAPD assay

A total of 280 decamer of random sequence (kits A, B, C, D. F, H. I, J, K, L, N, P, W, Z; Operon Technologies Inc., Alameda, CA, USA) were used as primers to amplify genomic DNA from the two parents and a sample of four progenies. Twenty-five ng of genomic DNA were used in each amplification reaction according to Williams et al. [15]. The amplification products were separated on 1.3% agarose gel and detected by ethidium bromide staining.

2.5. RFLP assay

Approximately 2 µg of DNA was digested with 10 U of the appropriate 6-bp-recognition restriction enzymes (*Eco*RI, *Hind*III and *Xba*I). Restriction fragments were separated on 1% agarose gel and transferred to nylon membranes Hybond N^+ according to manufactorer's instructions (Amersham, Arlington, IL, USA). The 50 probes tested were isolated from a *Pst*I genomic library obtained from a *C. latipes* × *C. aurantium* hybrid, ^{32}P-labelled using a 'prime-a-gene' labelling system kit (Promega Co., Madison, WI, USA), and hybridised according to Feinberg and Vogelstein [16].

2.6. scoring of markers and statistical analysis

In the 50 F_1 individuals obtained, markers were scored following the pseudo-testcross mapping strategy suggested by Grattapaglia and Sederoff [11]. Only bands polymorphic between parents (present in *C. aurantium* and absent in *C. latipes*, or vice-versa) were considered. Polymorphic bands that were heterozygous in either parent segregated in the F_1 progenies according to an 1:1 expected ratio were used for map construction. Segregating markers were scored for their presence or absence in each F_1. Two datasets and two maps were constructed (one comprising all markers heterozygous in *C. aurantium* and the other comprising all markers heterozygous in *C. latipes*). The software MAPMAKER 3.0 [17] was used to construct linkage maps. Data were treated as backcross data. Since, in the pseudo test-cross strategy, marker configuration remains unknown, two point analysis was performed on a duplicated data-set, with all markers having either normal and inverted allele score. This enabled the identification of linked markers even when showing band presence in repulsion phase. The preliminary grouping was performed at LOD score 4.0 and 30 cM maximum distance. Markers from each linkage group were used to create a framework map using full multipoint analysis and a LOD score of 2.0. Markers not ordered at this LOD in multipoint analysis were mapped relative to the framework map using the Place command, but were not included in the map skeleton. The use of a threshold LOD score of 2.0 in multipoint analysis indicates that the relative order of markers in the framework map is at least 100 fold more likely than any alternative order.

3. results and discussion

The total number of AFLP, RAPD and RFLP markers used for linkage maps construction is listed in *table I*. In the AFLP assay, four *Eco*RI and five *Mse*I primers were used in 7 different combinations. In the 50 F_1 progenies, 144 and 60 AFLP markers were scored for *C. aurantium* and *C. latipes*, respectively. The efficiency of AFLP technology, in terms of the average number of polymorphic bands produced per assay, was 17.8 and 7.7 for *C. aurantium* and *C. latipes*, respectively. In *C. aurantium*, on average, 106 bands per primer pair were observed, with a range between 51 and 161. On average, 34.7 bands per primer pair were polymorphic and 17.8 segregated in the F1 progenies. In *C. latipes*, values observed for these parameters were lower: on average, 92.6 bands were observed per primer pair, with a range between 62 and 142, 25.3 bands were polymorphic and 7.7 segregated. The ratio between the number of segregating bands and the total number of polymorphic DNA bands can be considered as an estimate of the proportion of heterozygous loci. This estimate ranged from 51.3% (17.8/34.7) for *C. aurantium* to 30.5% (7.7/25.3) for *C. latipes*.

In the RAPD assay, 170 (61%) out of the 280 primers tested were discarded, due to lack of polymorphism or low amplification intensity. The remaining 110 primers produced 157 segregating RAPD markers that were mapped (111 in *C. aurantium* and 46 in *C. latipes*). The efficiency of the method, in terms of mean number of polymorphic bands produced per assay, was 0.4 and 0.16 for *C. aurantium* and *C. latipes*, respectively.

The screening of 50 genomic clones resulted in the identification of 6 probes (12%) that identified 17 segregating RFLP bands (13 in *C. aurantium* and 4 in *C. latipes*). Two *Pst*I clones hybridised to single copy sequences (1–2 bands per lane) and 4 *Pst*I clones probably identified multiple-copy sequences (3–6 bands per lane). The efficiency of RFLP markers, in terms of mean number of polymorphic bands produced per assay, was quite low: 0.26 and 0.08 for *C. aurantium* and *C. latipes*, respectively.

Our results suggest that AFLP is by far the most efficient technology for generating polymorphic markers in *Citrus*. This observation is in agreement with data reported in other plant species where different marker technologies have been compared for their capacity of identifying polymorphisms [18, 19]. The very low level of RFLP polymorphism found in the present investigation is in agreement with data reported in *C. grandis* and *Poncirus trifoliata* [6, 7]. No statistical differences in the number of bands amplified and in the number of polymorphic bands detected were identified between the two species. Conversely, a significantly ($P \geq 0.05$) larger number of segregating AFLP markers have been observed in *C. aurantium*, compared to *C. latipes*. This result is attributable to the higher level of heterozigosity (51.3% versus 30.5%) observed in *C. aurantium* compared to *C. latipes*. Estimates of heterozygosity at AFLP loci investigated indicated high values for both species, in agreement with RAPD data reported by Cai et al. [8] for *C. grandis* and *P. trifoliata*.

In *C. aurantium*, the two point analysis grouped 247 out of the 268 AFLP,

Table I.
AFLP, RAPD and RFLP markers used to construct linkage maps of *Citrus aurantium* and *C. latipes*.

Citrus species	Total markers	Markers unlinked	Markers used for linkage analysis	Markers in the core map
C. aurantium	268	21	247	130
C. latipes	110	18	92	55

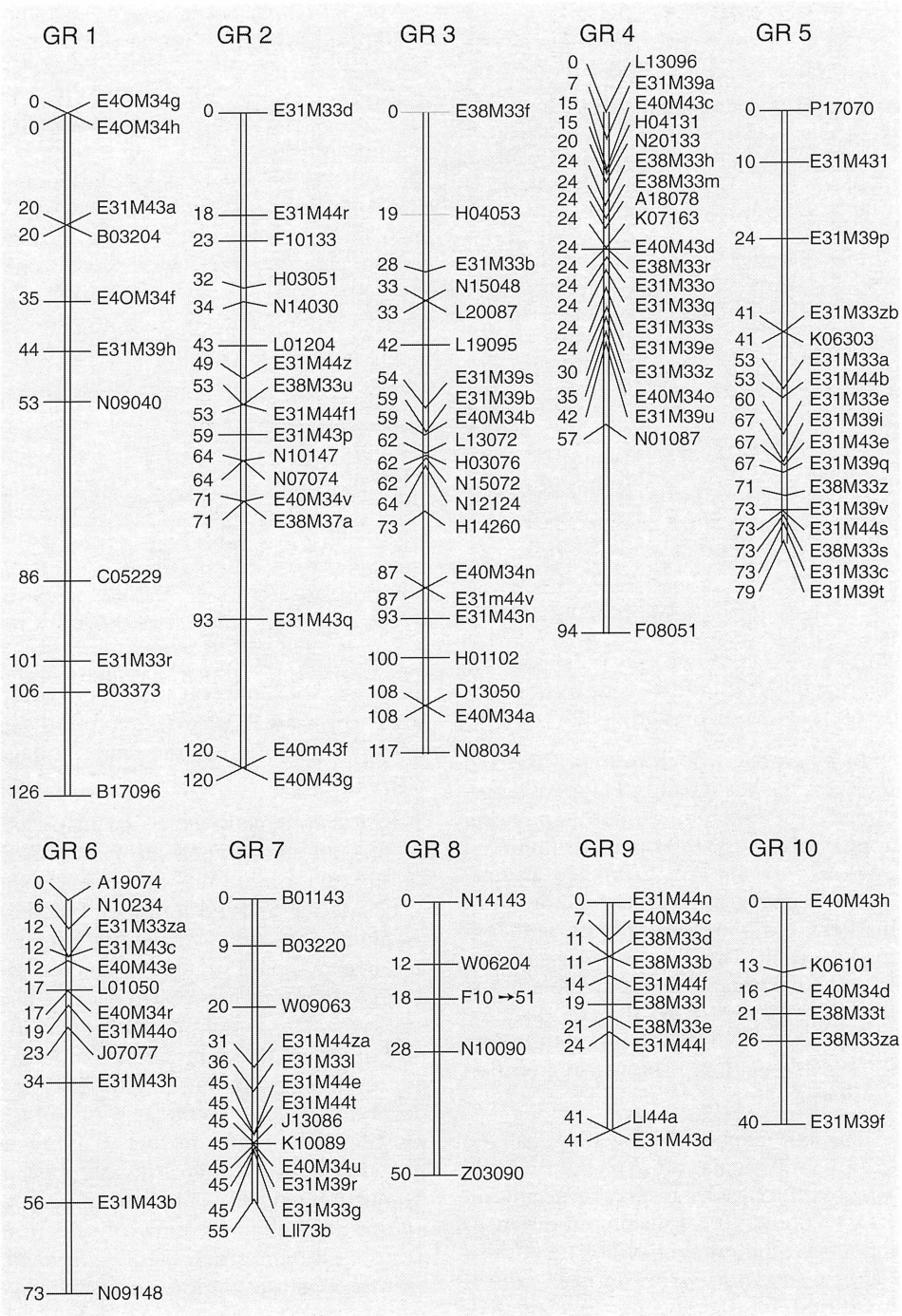

Figure 1.
The ten major framework linkage maps of *Citrus aurantium* based on AFLP, RAPD and RFLP markers. Map distances in centiMorgans are listed on the left and loci on the right of each linkage group (GR). AFLP loci are labeled with the primer combination from which they are produced, followed by a number indicating the relative position detected in the gel lane. RAPD markers are identified by the primer designation and the size in base pairs of the segregating allele scored. RFLP nomenclature includes, from left to right, the probe number and the size in base pairs. In both cases, a zero must be added to obtain the size in bp.

RAPD and RFLP markers in 20 linkage groups. Among them, 10 major groups were formed by 5–21 markers and 10 small linkage groups comprised 2–4 markers. Twenty-one markers remained unlinked. The whole map spanned approximately 1,000 cM. The 10 major linkage groups (*figure 1*) contain 132 markers and span approximatively 795 cM (range from 40 to 126 cM). Thirty-eight markers (14% of the

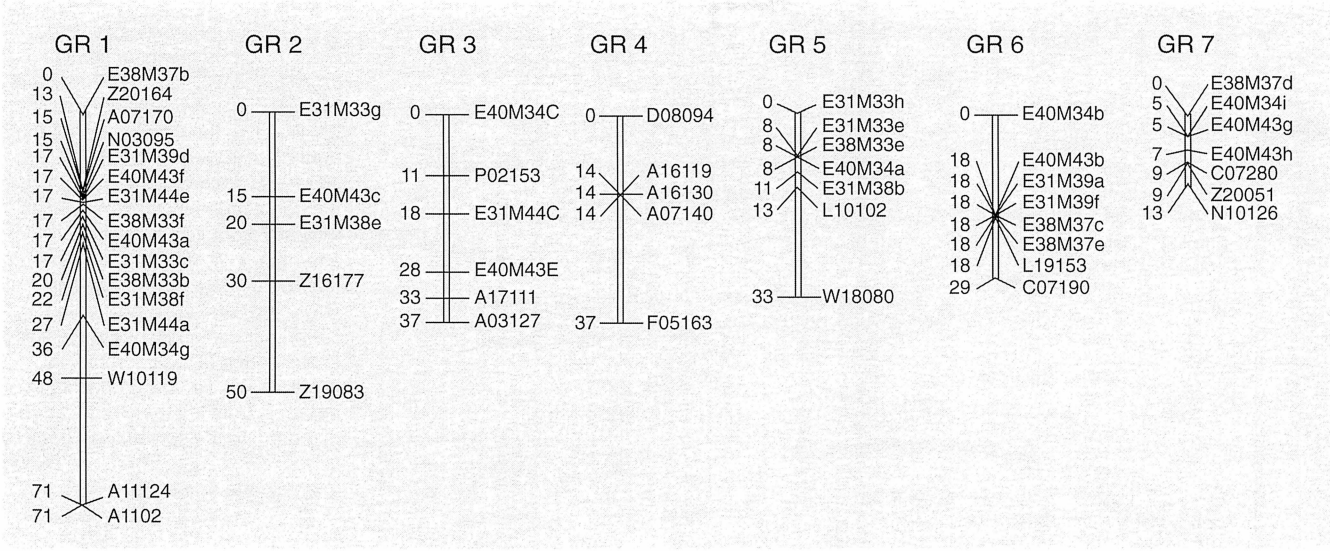

Figure 2.
The seven major framework linkage maps of *Citrus latipes* based on AFLP, RAPD and RFLP markers. Map distances in centiMorgans are listed on the left and loci on the right of each linkage group (GR). AFLP, RAPD and RFLP loci are labeled as reported in *figure 1*.

total) showed segregation distortion and 12 of these mapped to group 6.

In *C. latipes*, the grouping of the 110 scored AFLP, RAPD and RFLP bands produced 12 linkage groups, spanning approximately 600 cM, and 18 unlinked markers. Among the 12 linkage groups, 7 are major groups that include 5–17 markers and 5 are minor ones, with 2–4 markers each. The 7 major groups (*figure 2*) contain 55 markers and define approximately 270 cM of total map distance (range 13–71 cM). Three AFLP and three RFLP markers (5.4%) showed segregation distortion.

The low number of progenies analysed (50 F1 individuals) and the tight criteria adopted to consider linkage as significant (LOD 4.0 and 30 cM maximum distance) influenced the number of linkage groups (20 in *C. aurantium* and 14 in *C. latipes*) identified in the species investigated, higher than the *Citrus* basic chromosome number ($n = x = 9$). Marker distribution on the major linkage groups is sometimes uneven (*figures 1, 2*), with AFLP and RAPD markers that have a tendency to form clusters (i.e., group 4 in *C. aurantium* and group 1 in *C. latipes*). No comment may be done on RFLP behaviour, since only two RFLP markers found significant association to the major linkage groups.

In *Citrus*, genetic molecular maps will become an important tool to locate markers linked to interesting agronomic traits to be used in assisted rootstock breeding programs. We are now using the pseudotestcross mapping strategy to analyze the quantitative trait loci (QTL) for nucellar embryony in which major genes are probably involved in the quantitative expression of this trait [2, 20]. The production of nucellar seedlings is one of the most important traits in rootstock breeding althought it limits the number of zygotic individuals available from a cross and reduces the efficiency of selection. To identify genes involved in apomixis and to construct a more robust linkage map, a larger number of F1 individuals are being analyzed.

Further marker applications in *Citrus* breeding could be seen in the localisation of genes controlling resistance to pathogens, cold and salt tolerance, plant juvenility, and vigor.

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note

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Marcado mediante AFLP, RAPD y RFLP para la construcción de mapas genéticos de *Citrus aurantium* y *C. latipes*.

Resumen — Introducción. Los agrios tienen, a nivel mundial, una gran importancia económica. Su selección es larga debido a la duración de su periodo juvenil, de su heterozigotia y de la embrionia nucelar. Las técnicas de marcado molecular son una herramienta importante para identificar las regiones cromosómicas llevando los loci de los caracteres agronómicos importantes y para efectuar una selección, asistida por marcadores, de porta injertos de agrios. Por lo tanto, se utilizan para la construcción de mapas de enlaces para *C. aurantium* and *C. latipes*. **Material y métodos**. Se realizó la evaluación del polimorfismo de 50 individuos F1, oriundos de un cruzamiento entre *C. latipes* y *C. aurantium*, a partir del estudio por marcadores AFLP (siete combinaciones de cebos EcoRI/MseI), RADP (280) y RFLP (50 sondas aisladas de una biblioteca geonómica de *Pst* I de agrios). Se identificaron los marcadores al utilizar una estrategia de pseudo-testcross y dos mapas de enlace – uno para *C. latipes* el otro para *C. aurantium* – fueron construidos al utilizar un programa adaptado. **Resultados y discusión**. Para *C. latipes*, se identificaron 110 marcadores de segregación (60 AFLP, 46 RAPD y 4 RFLP). Entre ellos, 92 formaron 12 grupos de enlace (7 principales y 5 minores) cubriendo 600 cM, mientras que 18 marcadores se quedaron no enlazados. Para *C. aurantium*, se identificaron 268 marcadores de segregación (144 AFLP, 111 RAPD y 13 RFLP), de los cuales 247 formaron 20 grupos de enlace (10 mayores y 10 menores) cubriendo 1000cM, mientras que 21 marcadores permanecían no enlazados. Es considerado necesario estudiar un mayor número de individuos para aumentar la fiabilidad del mapa logrado y emprender el análisis de los loci de los caracteres cuantitativos (QTL).

***Citrus aurantium* / *Citrus latipes* / biologia molecular / tecnicas analíticas / marcadores genéticos / mapas genéticos**