Determination of the genome size and the number of markers for saturation of linkage maps of citrus

Roberto Pedroso de Oliveira*[1]; Carlos Ivan Aguilar-Vildoso[2] and Marcos Antônio Machado[2]
[1]Embrapa-Centro de Pesquisa Agropecuária de Clima Temperado (CPACT), Caixa Postal 403, CEP 96001-970, Pelotas, RS, Brazil. Fellowship of CNPq; [2]Centro de Citricultura 'Sylvio Moreira' (CCSM-IAC), Caixa Postal 04, CEP 13490-970, Cordeirópolis, SP, Brazil. (* Corresponding Author. E-mail: rpedroso@cpact.embrapa.br)

ABSTRACT

The objective of this study was to determine the size of the genome of citrus and correlate it with the number of markers necessary to saturate linkage maps. Linkage maps of 'Pêra' sweet orange (*Citrus sinensis* (L.) Osbeck) and 'Cravo' mandarin (*C. reticulata* Blanco) were constructed using 123 and 53 RAPD markers, respectively. The estimation of the genome size was conducted using regression models, the method of moments and Poisson probability. These approaches revealed a significant variation when estimating the genomes of 'Pêra' (612 to 1731 cM) and 'Cravo' (392 to 698 cM). The average number of markers necessary to saturate 99% of the genetic length determined for the genomes of 'Pêra' and 'Cravo' was 183 and 98, respectively. The linkage maps of these two varieties showed that the markers were distributed in clusters and this affected the accuracy of the estimates, especially of the 'Cravo' mandarin.

KEY WORDS: Method of Moments, Regression Method, Poisson Probability, RAPD

INTRODUCTION

The genus *Citrus* is predominately made up of diploid species with nine pairs of homologous chromosomes, which breed among themselves producing fertile hybrids (Barrett, 1985). Factors contributing to the wide variability of this genus are natural hybridization, spontaneous mutation, apomixis and selection through cultivation (Herrero et al., 1996).

Molecular markers such as AFLP, RAPD, RFLP and microsatellites have been extensively applied to construct genetic linkage maps of various citrus species, with the purpose of seeking genes and/or quantitative traits loci (QTLs) for resistance/tolerance to salts and cold (Moore et al., 2000), tristeza virus (Cristofani, 1997), citrus variegated chlorosis (Oliveira et al., 2002), latency, juvenility and vigor (Roose et al., 1992), plant height and fruit acidity (Gmitter Jr. et al., 1996).

The length of the genome (cM), the number of markers necessary to saturate the linkage maps and the ratio of the physical (kb) to the genetic lengths (cM) are important genetic traits that have broad application during the planning of mapping experiments and studies of the quality of the linkage maps (Chakravarti et al., 1991). A few approaches have been proposed to estimate these variables using partial linkage data obtained from molecular markers (Lange and Boehnke, 1982; Hulbert et al., 1988; Liou, 1990; Fjellstrom and Parfitt, 1994), which have revealed different results.

The studies conducted by Liou (1990) and Jarrell et al. (1992) have been the only ones that estimated the genetic length of *Citrus*, using integrated linkage maps and obtaining the values of 1500 and 1700 cM, respectively. These were used as references in later mapping studies (Durhan et al., 1992; Roose et al., 1992; Luro et al., 1994; Cristofani, 1997; Oliveira et al., 2002). However, since the size of the *Citrus* genome varies according to the specie of interest (Ollitrault et al., 1994), and the methodologies used have limitations (Chakravarti et al., 1991; Fjellstrom and Parfitt, 1994), significant errors occur when one attempts to generalize the known data.

The objective of this study was to estimate the genetic length, the number of markers necessary to saturate linkage maps and the ratio between physical (kb) and genetic lengths (cM) of *Citrus sinensis* (L.) Osbeck cv. 'Pêra' and *C. reticulata* Blanco cv. 'Cravo', by comparing different methodologies and correlating the results with the genetic of citrus.

MATERIAL AND METHODS

Partial linkage data related to the segregation of 123

RAPD markers of *Citrus sinensis* (L.) Osbeck cv. 'Pêra' and 53 of *C. reticulata* Blanco cv. 'Cravo' in a F_1 progeny composed by 94 hybrids were used in this study. The markers were used independently, regardless of their segregation type (Mendelian or skewed), and were assumed to be randomly distributed.

The following methods were utilized to estimate the length and the number of markers necessary to saturate the genetic maps.

Regression method (Liou, 1990)

Four samples with 10, 20, 30, 40 and 50 markers, randomly chosen from a total of 53 of 'Cravo' mandarin, and four other samples with 10, 30, 50, 70, 90 and 110 markers, from a total of 123 samples of 'Pêra' sweet orange, were separately submitted to simulation linkage analyses.

These studies utilized the MapMaker version 3.0 (Lander et al., 1987), with the pseudo-testcross strategy, Kosambi function, LOD ≥ 6.0, and highest recombination frequency (θ) of 0.25. The mean results obtained from these models, associated to those derived from all markers, were utilized to establish the functions *P(N)* and *D(N)*, where *P(N)* refers to the regression observed from the ratio between the percentage of linked markers with the respective numbers of markers used *(N)*, while *D(N)* pertains to the regression of the ratio between the mean distances of the adjacent loci in the maps and the respective numbers of markers utilized *(N)*.

Using *Microsoft Excel 97*, the following regression models were estimated: linear, cubic, quadratic, potency, exponential, hyperbolic and logarithmic for the functions *P(N)* and *D(N)* in relationship to the distribution of the data obtained. The chosen function was the one that showed the highest regression correlation coefficient (R^2) and had biological meaning.

The genetic length *(G)*, in centiMorgan (cM) unit, was determined by the equation $G = N' D(N)$, considering N' the number of markers at the saturation point. The number of markers necessary to saturate the map was estimated through the regression *P(N)* with y = 99%.

Method of moments (Hulbert et al., 1988)

The genetic length was determined by the equation $G = N (N-1) X / K$, where G is the estimated size of the genome (cM); N is the total number of linked markers, considering the markers with null rate of recombination as only one marker; X is the highest distance, in cM, between two markers, according to the criteria of the established maps, i. e., $LOD = 6.0$ and $\theta = 0.25$ (25.38 cM), and K is the number of loci pairs observed with $LOD = 6.0$. The values for K were obtained from the function *Lod Table* of *Mapmaker*, where $KPêra = 427$ and $KCravo = 82$.

The number of markers necessary to saturate the linkage map was estimated from these data using the equation of Lange and Boehnke (1982): $N = [\ [\log (1-p)\ ] / \log [1-(2c) / G]\ ]\ 1.25$, where N is the number of markers necessary for the saturation; p is the proportional value needed to cover the map; G is the estimated genetic length, and c is the highest distance between markers. The present study utilized $p = 0.99$ and $c = 15$ cM.

Method of the Poisson probability (Fjellstrom and Parfitt, 1994)

The genetic length was estimated by the equation $G = n\ d / \{1 - \sqrt{}\ [1 + (n \ln p) / (k - 1)\ ]\ \}$, where G is the estimated size of the genome (cm); n is the haploid number of the chromosomes of the specie (9); d is the highest distance between linked markers (25.38 cm); p is the ratio of the number of unlinked markers by the total number of markers; and k is the total number of used markers. These analyses did not consider the markers with null rate of recombination, and the linkage maps were constructed with the parameters described above.

The number of markers *(N)* necessary to saturate the linkage maps was obtained by the equation $N = - n\ ln (1- p') / [1 - (1 - d/L)^2\ ]$, where n is the haploid number of chromosomes (9); p' is the proportion value needed to cover the genome (99%); d is the highest desired distance between linked markers (15 cM); and L is the mean length of the linkage groups.

The ratio between physical (kb) and genetic lengths (cM) of the genome of each variety was estimated considering the physical average length of *Citrus* of 5.63×10^5 kb of DNA (Liou, 1990), and the estimated genetic length was obtained by the three determination methods.

RESULTS AND DISCUSSION

A significant variation was observed when estimating the genetic length of 'Pêra' sweet orange (612 to 1731

cM) and the 'Cravo' mandarin (392 to 698 cM) based upon the three approaches utilized (Table 1).

Variations at high proportions had already been reported for other species even when using the same method for the estimation, while varying the markers and/or mapping criteria (Liou, 1990; Jarrell et al., 1992; Fjellstrom and Parfitt, 1994; Liu, 1998). These facts have been observed in the literature because the methods used to estimate the genetic length are very susceptible to alterations of LOD, maximum frequency of recombination, function of mapping, number and type of markers, as well as size and type of populations studied (Liu, 1998). Due to the large number of variables involved, there are no conclusive studies on the magnitude of errors derived from the methods used to estimate the genetic length. However, the estimation studies done are important for planning genetic mapping experiments and to analyze the quality of the maps produced (Liu, 1998).

The mean genetic length estimated for 'Pêra' sweet orange (1113 cM) was 95% higher than that of 'Cravo' mandarin (571 cM) (Table 1). The large value documented for the 'Pêra' genome size was expected, however in a lower proportion considering the data collected by Ollitrault et al. (1994) who reported, through cytological analyses that consisted of a straight measurement of the amount of DNA, a ratio of only 1.027 cM between the genomes of *C. sinensis* and *C. reticulata*. Although these authors did not study the varieties 'Pêra' and 'Cravo', they documented that the variations of the genome sizes within the evaluated *Citrus* species were not higher than 3.6%.

Up to now, only two groups of researchers have conducted studies on estimating the genetic length of *Citrus* using partial linkage data. Liou (1990) used regression methods and Jarrell et al. (1992) utilized the method of moments by Hulbert et al. (1988) to analyze integrated linkage maps. These approaches revealed a genetic length of 1500 and 1700 cM, respectively. These values were higher than those found in this study, especially the one related to the genetic length of 'Cravo' mandarin. The mean for the 'Pêra' genome size estimated (1113 cM) could be considered close to the estimated by other authors, since Jarrell et al. (1992) believed that the average length of each chromosome, approximately 200 cM, was extremely large when comparing to most of the other species studied. Even though Liou (1990) and Jarrell et al. (1992) conducted their studies using a smaller number of markers (less than 40) and few progenies (less than 65), it is assumed that their results are precise because the markers used were randomly distributed in the respective linkage maps.

Based upon the data from the literature and the three methods applied to estimate the genetic length, a lower variation was expected for the results obtained (Table 1). According to Oliveira et al. (2002), one of the main characteristics of the linkage maps of 'Pêra' and 'Cravo' is the occurrence of clustered markers. Contrary to the premise of random distribution of the markers, this fact may have been the main reason for the magnitude of the variability found among the methods used in this study when compared to those obtained by Liou (1990) and Jarrell et al. (1992).

Although, theoretically, the distribution of the RAPD markers is random in the genomes, this has not been observed in linkage maps of *Citrus* (Luro et al., 1994; Cristofani, 1997) and other plants that were partially saturated (Vallejos et al., 1992; Grattapaglia and Sederoff, 1994). Therefore, the existing methods that estimate the genetic length should be adjusted to consider this observation, so that they will be more precise.

In regard to the usage of the methods of interest to estimate the genetic length, it was observed that the

Table 1. Determination, through different methods, of the genetic length (cM), number of markers necessary for saturation, and the ratio between physical and genetic lengths of the linkage maps of *C. sinensis* (L.) Osbeck cv. 'Pêra' and *C. reticulata* Blanco cv. 'Cravo'.

Method	Genetic length (cM)			Number of markers for saturation (99%)		Ratio physical/genetic lengths (kb/cM)	
	'Pêra'	'Cravo'	Difference	'Pêra'	'Cravo'	'Pêra'	'Cravo'
Regression	995	392	154%	158	55	566	1436
Moments	612	698	14%	115	131	920	807
Poisson	1731	624	177%	277	107	325	902
Mean	1113	571	95%	183	98	604	1048

methods of moments (Hulbert et al., 1988) provided the fastest determination in function of the "*k*" variable (number of pairs of loci at a given LOD and at the maximum distance between markers) be calculated by the known mapping program. Moreover, this method is based upon the maximum distances found between markers in the respective linkage maps, which improves the consistency of the estimates. Thus, making it the most utilized method in the literature (Jarrell et al., 1992; Vallejos et al., 1992; Grattapaglia and Sederoff, 1994).

The method of Poisson probability (Fjellstrom and Parfitt, 1994) was also an easy determination. This method, which presents high sensibility due to the logarithmic function, however, is subjected to many errors when low numbers of markers are evaluated. Moreover, this method is based upon the proportion of linked and unlinked markers besides being the only method tested that considers the number of chromosomes of the specie studied. Furthermore, it is very useful for species that have a known number of chromosomes.

On the other hand, the regression method (Liou, 1990) was very laborious, requiring the construction of many linkage maps followed by analyzes of various regression models. However, this method gave very precise estimates once the premises were met. In addition, the various regression models showed estimates very close to the genetic length.

The equations of the regression method that represented the ratio of the percentage of the linked markers and the mean distance between adjacent loci in function of the total number of markers used for the construction of linkage maps of 'Pêra' and 'Cravo' showed that the cubic, hyperbolic, logarithmic and quadratic regression models were the ones that best fit when considering the determination coefficient R^2 (Tables 2 and 3). The study conducted by Liou (1990) was based upon the premises that the percentage of markers that are mapped increase proportionally (up to 100%) to the number of markers used in the linkage analyzes, and that the mean distance between adjacent loci decreases as markers are added to the map. Therefore, the cubic, hyperbolic, logarithmic and quadratic regression models did not meet the premises. The first two did not fit due to the type of curve produced in the graphic, and the hyperbolic model had an asymptote value different from 100% for the function *P(N)*, especially for the data obtained from 'Cravo' mandarin. Therefore, as in the studies done by Liou (1990), the logarithnic regression model was chosen for the functions *P(N)* and *D(N)*, which were then used to estimate the genetic length for both varieties.

The linkage analyses for both varieties did not show the expected decrease in the mean distance between adjacent loci when more markers were added to the maps. This fact contradicted the premises of the estimation of the genetic length according to the method done by Liou (1990). This must be related to the occurrence of some process of selecting markers and to the genetics of *Citrus*. Even though primers were randomly chosen, only the most consistent

Table 2. Regression models of the percentage of linked markers (*P(N)*) and of the mean distance of the adjacent loci (*D(N)*) in function of the total number of markers used in the construction of linkage maps of *C. sinensis* (L.) Osbeck cv. 'Pêra'.

Regression Model	Function *P(N)*	R^2
Linear	$Y = 0.2123x + 72.0274$	0.7145
Cubic	$Y = 0.0001x^3 – 0.0190x^2 – 1.5186x + 51.6383$	0.9913
Quadratic	$Y = -0.0035x^2 + 0.6768x + 61.5450$	0.9053
Potency	$Y = 48.4939x^{0.1441}$	0.9219
Exponential	$Y = 71.7535e^{0.0026x}$	0.6821
Hyperbolic	$Y = 97.3805x / (5.0238 + x)$	0.9859
Logarithmic	$Y = 11.4182\ln(x) + 41.2068$	0.9351
Regression Model	Function *D(N)*	R^2
Linear	$Y = 0.0075x + 4.7248$	0.3948
Cubic	$y = 0.000002x^3 – 0.000702x^2 + 0.067765x + 3.608818$	0.9817
Quadratic	$y = -0.0003x^2 + 0.4509x + 3.8757$	0.9539
Potency	$y = 3.5406x^{0.0974}$	0.7257
Exponential	$y = 4.6898e^{0.0015x}$	0.4046
Hyperbolic	$y = 5.6962x / (3.4530 + x)$	0.9124
Logarithmic	$y = 0.4715\ln(x) + 3.3626$	0.7122

polymorphic bands in the agarose gels were selected for the linkage analyses.

As a hypothesis, these could be related to the sequence of multiple copies in the genome of the varieties studied and usually found in regions rich in heterochromatin, which are abundant and irregularly distributed in the chromosomes of *Citrus* (Guerra, 1993). According to Liou (1990), the non-methylated DNA regions of the *Citrus* genome are comprised of approximately 73% single copy sequences, 18.9% multiple copy sequences, and 8.1% repetitive sequences. If this hypothesis is correct, in this study there was a selection of markers from regions rich in heterochromatin, thus making it difficult to analyze the map and to estimate the genetic length. Therefore, it caused a higher tendency for the markers to be clustered, and a lower tendency for them to be located in other regions of the linkage groups. This probably caused errors in the estimation done especially for 'Cravo' mandarin, which showed a lower number of markers.

The average number of markers necessary to saturate 99% of the mean length estimated for the genomes of 'Pêra' sweet orange and 'Cravo' mandarin, at a maximum distance between markers of 15 cM, was of 183 and 98, respectively (Table 1). It is important to know this variable when planning experiments on genetic mapping, even with the occurrence of eventual errors that arise due to non-precise estimations.

When the physical size of the *Citrus* genome of 5.63 x 10^5 kb DNA (Liou, 1990) was used, the average of the ratio between the physical and genetic lengths for the 'Pêra' sweet orange was 604 kb/cM, and 1048 kb/cM for the 'Cravo' mandarin (Table 1). These values were much higher than those obtained by Liou (1990), i.e., 375 kb/cM, that estimated the genetic length of *Citrus* to be 1500 cM. The ratio of the physical to the genetic lengths is used only to compare species because this ratio can be highly variable depending on the genome region. According to Lee (1995), this ratio can vary up to 10,000 fold in regions with high recombination frequency (hot spots) and in those that correspond to the centromers and telomers.

CONCLUSIONS

The estimation of the genetic length of the genome of 'Pêra' sweet orange, through the regression methods, the method of moments, and the method of Poisson probability, was very different from that of 'Cravo' mandarin.

The estimation of the genetic length according to the method of moments is less laborious than that of regression and less susceptible to errors than that of Poisson probability. This is because the method of moments is based upon the maximum distances between markers of the linkage maps.

The genetic length of the genome of 'Pêra' sweet orange is larger than that of 'Cravo' mandarin.

The genetic linkage maps of 'Pêra' sweet orange and 'Cravo' mandarin show that the markers are distributed in a clustered manner, which could be related to the occurrence of some selection process

Table 3. Regression models of the percentage of linked markers (*P(N)*) and of the mean distance of the adjacent loci (*D(N)*) in function of the total number of markers used in the construction of linkage maps of *C. reticulata* Blanco cv. 'Cravo'.

Regression Model	Function *P(N)*	R^2
Linear	$y = 1.1156x + 42.0230$	0.9142
Cubic	$y = -0.0003x^3 - 0.0011x^2 - 2.1456x + 25.8190$	0.9899
Quadratic	$y = -0.0269x^2 + 2.8496x + 20.6590$	0.9891
Potency	$y = 17.6150x^{0.4380}$	0.9732
Exponential	$y = 45.6050e^{0.0156x}$	0.8760
Hyperbolic	$y = 127.7955x / (17.2941 + x)$	0.9816
Logarithmic	$y = 30.6880\ln(x) - 23.9080$	0.9773
Regression Model	Function *D(N)*	R^2
Linear	$y = 0.0215x + 5.7532$	0.6830
Cubic	$y = 0.00007x^3 - 0.00704x^2 + 0.22396x + 4.17763$	0.8161
Quadratic	$y = -0.0003x^2 + 0.0397x + 5.5285$	0.6997
Potency	$y = 4.7645x^{0.0944}$	0.7377
Exponential	$y = 5{,}7645e^{0{,}0034x}$	0.6774
Hyperbolic	$y = 7.0472x / (2.2262 + x)$	0.7687
Logarithmic	$y = 0.5924\ln(x) + 4.4785$	0.7340

and to the genetics of *Citrus*.

The methods to estimate the genetic length must be adapted to the genome in study because there are species that do not show markers that are randomly distributed.

The use of a high number of markers can overcome the errors obtained through the methods utilized to estimate the genetic length based upon partial linkage data.

RESUMO

Determinação do Tamanho do Genoma e do Número de Marcadores Para Saturação de Mapas de Ligação de Citros

Objetivou-se estimar o tamanho do genoma e o número de marcadores necessários para saturação de mapas de ligação de citros. Foram utilizados mapas de laranja 'Pêra' (*Citrus sinensis* (L.) Osbeck) e tangerina 'Cravo' (*C. reticulata* Blanco) construídos a partir de 123 e 53 marcadores RAPD, respectivamente. As estimativas de tamanho do genoma foram realizadas pelos métodos das regressões, momentos e probabilidade de Poisson. Obteve-se variação pronunciada nas estimativas de tamanho do genoma de laranja 'Pêra' (612 a 1731 cM) e tangerina 'Cravo' (392 a 698 cM). O número médio de marcadores necessários para saturar 99% do genoma estimado foi de 183 para laranja 'Pêra' e 98 para tangerina 'Cravo'. Os mapas de ligação das duas espécies apresentaram uma distribuição dos marcadores formando agrupamentos, o que comprometeu a precisão das estimativas, principalmente de tangerina 'Cravo'.

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