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Genetic maps of RAPD, AFLP and ISSR markers in *Ananas bracteatus* and *A. comosus* using the pseudo-testcross strategy

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Abstract

Genetic maps of random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) and inter simple sequence repeats (ISSR) markers in pineapple ($2n = 2x = 50$) are reported for the first time. On the basis of a segregating population of 46 F1 individuals from a cross *Ananas comosus* × *A. bracteatus*, genetic maps of these two species were constructed using the two-way pseudo-testcross approach. The *A. bracteatus* map consists of 335 markers (60 RAPDs, 264 AFLPs and 11 ISSRs) assembled into 50 linkage groups, 26 of them with at least four markers. The *A. comosus* map consists of 157 markers (33 RAPDs, 115 AFLPs, eight ISSRs and the 'piping' trait locus) organized into 30 linkage groups, 18 of them with at least four markers. These maps cover, respectively, 57.2% of the *A. bracteatus* genome estimated as 3693 cM long, and 31.6% of the *A. comosus* genome calculated as 4146 cM. A rough estimate of 120 and 127 kbp/cM on average was found for the relationship between physical and genetic distance for *A. bracteatus* and *A. comosus*, respectively.

Key words: *Ananas* spec. — genetic mapping — molecular markers — pseudo-testcross

Pineapple, *Ananas comosus*, is an important tropical fruit crop cultivated in all tropical and subtropical countries and commercialized worldwide as fresh fruit, canned slices and chunks, or juice concentrates. *Ananas bracteatus* is cultivated as an ornamental, for fibre production and for fencing (Coppens d'Eeckenbrugge et al. 1997). Crosses between these two species, as well as between all the seven species within the *Ananas* genus, are fully fertile, which is certainly favoured by their similarity in floral structure and chromosome number ($2n = 2x = 50$) (Coppens d'Eeckenbrugge et al. 1997). The fact that all species included in the *Ananas* genus belong to the same primary gene pool, extends the importance of genetic studies, including the construction of linkage maps, over the whole genus.

The relatively high level of heterozygosity of *A. comosus* and *A. bracteatus* and the high degree of genetic polymorphism between both species permitted map construction to be carried out employing the 'two-way pseudo-testcross' or 'double pseudo-testcross' strategy, using an F1 progeny (Grattapaglia and Sederoff 1994, Hemmat et al. 1994). Genetic maps of diverse plant species have been constructed employing the same strategy, e.g. apple – *Malus* spp. (Hemmat et al. 1994), eucalypts – *Eucalyptus* spp. (Grattapaglia and Sederoff 1994), sugar beet – *Beta vulgaris* L. (Schumacher et al. 1997), alfalfa – *Medicago* spp. (Barcaccia et al. 1999), oil palm – *Elaeis guineensis* Jacq. (Moretzsohn et al. 2000), kiwifruit – *Actinidia* spp. (Testolin et al. 2001).

Random amplified polymorphic DNA (RAPD) (Williams et al. 1990), inter simple sequence repeats (ISSRs) (Zietkiewicz et al. 1994) and amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995) were used for map construction.

The gene *P*, whose dominant allele determines the 'piping' phenotype with suppressed leaf margin spines characteristic of *A. comosus*, was mapped in one of the linkage groups. Selection has been carried out for this trait since the early domestication of *A. comosus* because it drastically reduces the inconvenience resulting from leaf spines during the cultivation and manipulation of this crop. In contrast, in *A. bracteatus*, selection has not been thoroughly exercised against leaf spines, a valuable trait for fencing. Nevertheless, spineless mutations have been selected in ornamental forms of this species (Coppens d'Eeckenbrugge et al. 1997).

Materials and Methods

Plant materials: The mapping population consisted of 46 F1 hybrid plants obtained from a cross between *Ananas comosus* (L.) Merrill 'Rondon', clone BR 50 and *Ananas bracteatus* (Lindley) Schultes 'Branco do mato', clone BR 20. Crosses were carried out in the CIRAD-FLHOR, Martinique and leaves from progenitors and progeny plants were sent to the University of Algarve for molecular marker analysis and map construction.

DNA isolation: Leaves were cut into small pieces and ground in a mortar with liquid nitrogen. The resulting fine powder was resuspended in approximately two volumes of extraction buffer containing 200 mM Tris–HCl pH 8.0; 25 mM EDTA; 250 mM NaCl; 1% SDS and 2% PVP and kept at 65°C for 15 min, then purified by RNase A (40 μg/ml) at 37°C for 30 min, and the total genomic DNA purified by successive phenol:chloroform and chloroform extractions (Sambrook et al. 1989).

RAPD assay: RAPD analyses were carried out as previously described (Elisiário et al. 1999). Primers that revealed clearly defined polymorphic bands between parental genotypes were further tested with a random sample of six progeny plants. The absence of the polymorphic band in at least one of those progeny plants was assumed as an indication of possible heterozygosity at the marker locus and the analysis proceeded among all the individuals of the remaining progeny. Inversely, putative polymorphic markers that were present in all the six progeny individuals were assumed not to be segregating and were not used for further analysis.

AFLP assay: AFLP analyses were performed using standardized procedures (Vos et al. 1995) as earlier described (Monte-Corvo et al.

Table 1: AFLP *Mse*I/*Eco*RI primer combinations and respective code numbers

*Mse*I + 3/*Eco*RI + 3 AFLP primers	Code number
M-CTA/E-AAG	01
M-CAT/E-ACG	02
M-CTC/E-ACG	03
M-CTT/E-AGC	04
M-CAA/E-ACC	05
M-CAT/E-ACC	06
M-CTT/E-ACC	07
M-CTT/E-AGG	08
M-CAC/E-ACC	09
M-CTG/E-AAC	10
M-CAG/E-AGC	11
M-CAA/E-AAC	12
M-CAG/E-ACC	13
M-CTC/E-ACC	14
M-CTG/E-ACT	15
M-CTG/E-ACC	16
M-CTA/E-ACC	17
M-CAC/E-ACT	18
M-CAT/E-ACT	19
M-CAG/E-AAC	20
M-CTC/E-ACT	21
M-CTC/E-ACA	22
M-CTC/E-AAC	23
M-CAT/E-AAC	24
M-CTA/E-ACT	25
M-CTC/E-AAG	26
M-CTG/E-AAG	27
M-CAA/E-AAG	28
M-CAT/E-AAG	29
M-CTT/E-AAG	30
M-CAC/E-AGG	31
M-CAA/E-AGG	32
M-CAT/E-AGG	33
M-CTA/E-ACG	34
M-CTA/E-AAC	35
M-CAC/E-AAG	36
M-CTA/E-ACA	37
M-CAG/E-ACG	38
M-CAG/E-ACA	39
M-CAG/E-ACT	40
M-CTT/E-ACT	41
M-CTT/E-AAC	42
M-CTT/E-ACA	43
M-CTT/E-ACG	44
M-CAA/E-ACT	45
M-CTG/E-ACA	46
M-CAG/E-AGG	47
M-CTG/E-ACG	48

2000). The 48 AFLP primer combinations used for mapping are listed in Table 1. Taking into account the relatively high number of polymorphisms between the progenitor genotypes revealed by the RAPD technique and the expected high multiplex ratio of the AFLP technique, the AFLP analyses were carried out directly on all F1 plants.

ISSR assay: ISSR markers (Zietkiewicz et al. 1994) were produced using 18 bp primers with 3′ anchored arbitrary nucleotides (Table 2) directly on all F1 progeny. Each reaction mix (10 µl) consisted of 10 ng of total genomic DNA, 1 µM of primer, 0.3 mM of each dNTP and 1 U of Taq DNA polymerase (Amersham Bioscience, Uppsala, Sweden) in reaction buffer: 10 mM Tris–HCl pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$. To each reaction, 0.5 µCi of ^{33}P dCTP (Amersham Bioscience) was added for marker detection by autoradiography. ISSR products were resolved in non-denaturing 6% polyacrylamide gels (Long Ranger gel solution; BMA, Rockland, ME, USA). Autoradiographic images were obtained by exposing X-ray films to gels dried on 3 MM paper.

Table 2: ISSR primers and respective code numbers

Primers[1] (5′ to 3′)	Code number
(CA)8ARG[1]	01
(CA)8ARY[1]	02
(GA)8YT	03
(GA)8YC	04
(GA)8AYC	05
(AG)8GYT	06
(AG)8YC	07
(AC)8YA	08
(AC)8YG	09

[1] Y represents the pYrimidines and R the puRines.

Map construction: The goodness-of-fit between observed and expected segregation data was assessed using the chi-square (χ^2) test. Polymorphic markers and common heterozygous markers segregating, respectively, at a 1:1 and a 3:1 Mendelian ratio ($\chi^2 \leq \chi^2_{\alpha=0.05}$) or to some extent deviating from it ($\chi^2_{\alpha=0.05} < \chi^2 \leq \chi^2_{\alpha=0.01}$) were used for map construction. The JoinMap® 3.0 program (Van Ooijen and Voorrips 2001) set for the Kosambi's mapping function was used for map construction and for graphical representation of the linkage groups. Maps were constructed using a minimum LOD of 4.0 for linkage analysis and a log-likelihood support of 1.0 for linkage group ordering.

RAPD markers were designated by the letter(s) identifying the primer kit followed by primer code number (from 01 to 20) and estimated fragment size (bp), e.g. OPA03700 stands for the 700 bp marker generated by primer 03, kit A, from Operon Technologies Inc. (Alameda, CA, USA). The AFLP and ISSR markers were identified, respectively, by the capital letters AFLP or ISSR, the two-digit number ascribed to each AFLP primer combination (Table 1) or ISSR primer (Table 2) followed by the estimated fragment size, e.g. AFLP01200 stands for a 200-bp fragment generated by the primer combination 01 and ISSR06300 for an ISSR marker with 300 bp generated by primer 06. Markers that segregate with slight deviation from the expected ratio ($\chi^2_{\alpha=0.05} < \chi^2 \leq \chi^2_{\alpha=0.01}$) are identified with an asterisk. Heterozygous markers common to both parents are labelled by the indication '_cb'. The symbol # identifies common heterozygous markers linked at least to three non-common markers in both maps.

Results

Among the 300 RAPD primers initially screened against both parents, 70 primers (23.3%) amplified 134 polymorphic heterozygous markers (83 from *A. bracteatus* and 51 from *A. comosus*) and nine common heterozygous markers suitable for map analysis. The 48 AFLP primer combinations revealed 491 polymorphic AFLP markers (338 from *A. bracteatus* and 153 from *A. comosus*) and 46 common heterozygous markers. The nine ISSR primers identified 49 polymorphic markers (26 for *A. bracteatus* and 23 for *A. comosus*) and eight common heterozygous markers, whose segregation could be clearly monitored among the mapping population.

As 34 polymorphic DNA markers of *A. bracteatus* and 15 of *A. comosus* evidenced a pronounced segregation distortion ($\chi^2 > \chi^2_{\alpha=0.01}$), only 413 markers of *A. bracteatus* and 213 markers of *A. comosus* (including the 'piping' trait) and 63 markers common to both parents were actually used for map construction.

Despite the dominant mode of inheritance of the molecular markers used in this study, some markers could be identified as allelic because of codominant segregation (Fig. 1).

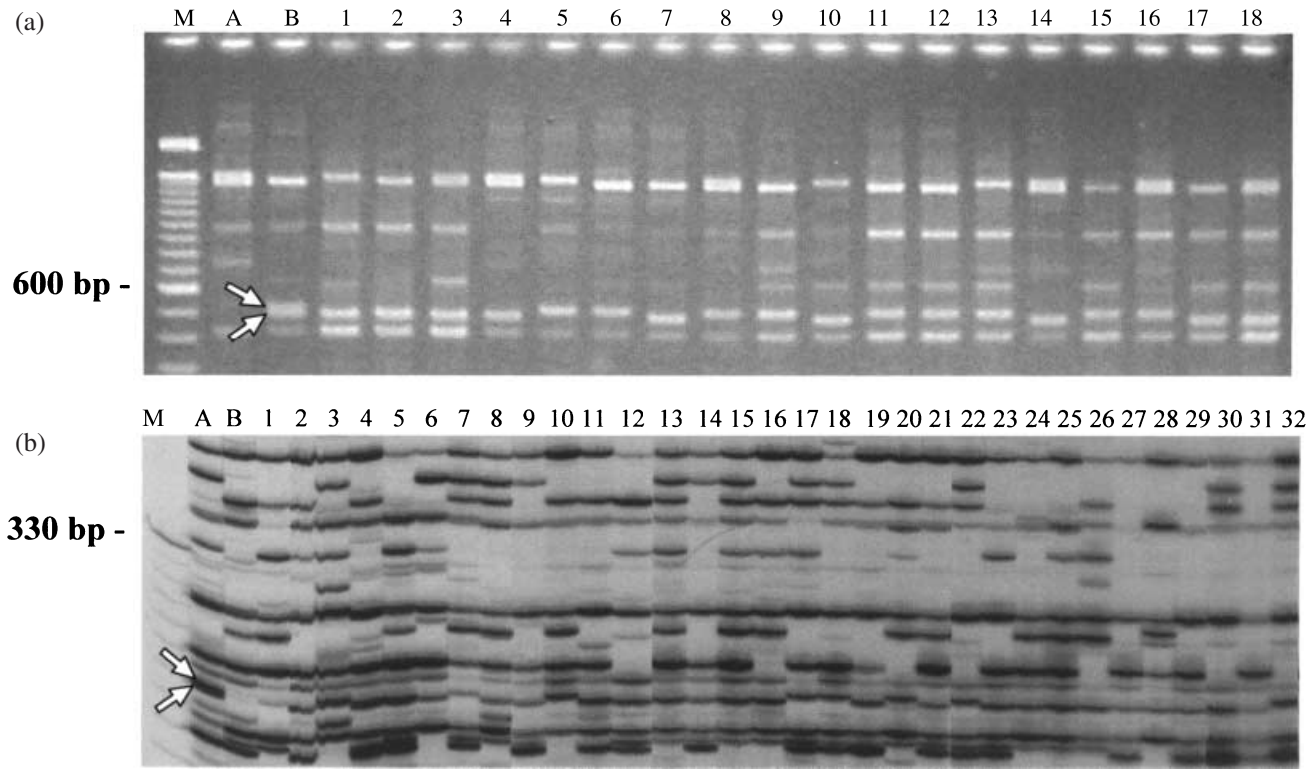

Fig. 1: (a) RAPD amplification patterns of 18 F1 plants (1–18) and of the parents, *Ananas bracteatus* (A) and *A. comosus* (B), obtained with primer OPB15. Arrows indicate two markers of *A. comosus* whose alternate segregation suggests an allelic relationship. (b) AFLP patterns of 32 F1 plants (1–32) and parents, *A. bracteatus* (A) and *A. comosus* (B), amplified with the M-CTA/E-AAG primer combination. Arrows indicate two markers, specific to *A. bracteatus*, apparently alleles of the same locus

Ananas bracteatus map

Among the 413 polymorphic markers of *A. bracteatus* used for map construction, 37 showed a partially deviated segregation ($\chi^2_{\alpha=0.05} < \chi^2 \leq \chi^2_{\alpha=0.01}$). Three of the 63 common markers showed the same level of distortion. Linkage relationships were found for 314 polymorphic DNA markers and 21 common markers (60 RAPDs, 264 AFLPs and 11 ISSRs), which were assigned to 50 groups. Twenty-six linkage groups assemble at least four markers each, six groups gather three markers each, and 18 groups consist of pairs of markers (Fig. 2a). Ninety-nine polymorphic markers (24%) and 42 common markers (67%) remained unlinked.

Ananas comosus map

The 213 polymorphic mapping markers obtained for *A. comosus* are about half of those identified for *A. bracteatus*. Eighteen of these markers showed a slightly distorted segregation ($\chi^2_{\alpha=0.05} < \chi^2 \leq \chi^2_{\alpha=0.01}$). The 'piping' phenotype segregated in agreement with a ratio of 1:1, confirming the monogenic inheritance of this trait (Cabral et al. 1997). One hundred and fifty-seven markers (33 RAPD, 115 AFLP, eight ISSR and the morphological 'piping' character) were organized into a map with 30 linkage groups, 18 of which assemble at least four markers, four groups congregate three markers each and eight groups consist of simple pairs of markers (Fig. 2b). Seventy-eight polymorphic markers (38.5%) and 41 (65%) common markers remained unlinked.

Combination of maps

AFLP16370_cb# was the single common heterozygous marker linked to at least three polymorphic markers in both maps, a constraint suggested by Debener and Mattiesch (1999) for using a marker in map combination. This single marker permitted the establishment of homology between linkage groups Ab6 and Ac13. Nevertheless, due to lack of additional common markers, the relative orientation between these two groups was not determined.

Map length and genome coverage

In order to estimate the total span of each map the framework of each linkage group, as suggested by Marques et al. (1998), was enlarged at each end point with the average distance between adjacent markers, 5.48 cM for *A. bracteatus* and 7.01 cM for *A. comosus*.

Estimated in this way, the 50 linkage groups of *A. bracteatus* span over 2111 cM, covering 57.2% of the 3693 cM estimated as the genome length (G) of this species using the method 3 of Chakravarti et al. (1991). Using the same methodology, the *A. comosus* map was found to span over 1311 cM, representing 31.6% of the species genome, which was computed as being 4146 cM long.

Bearing in mind that, based on flow cytometry data, Arumuganathan and Earle (1991) have estimated the sizes of the haploid genome of *A. bracteatus* and *A. comosus*, respectively, to be 444 and 526 Mbp, on average, a rough relationship between physical and genetic distance of about 120 kbp/cM

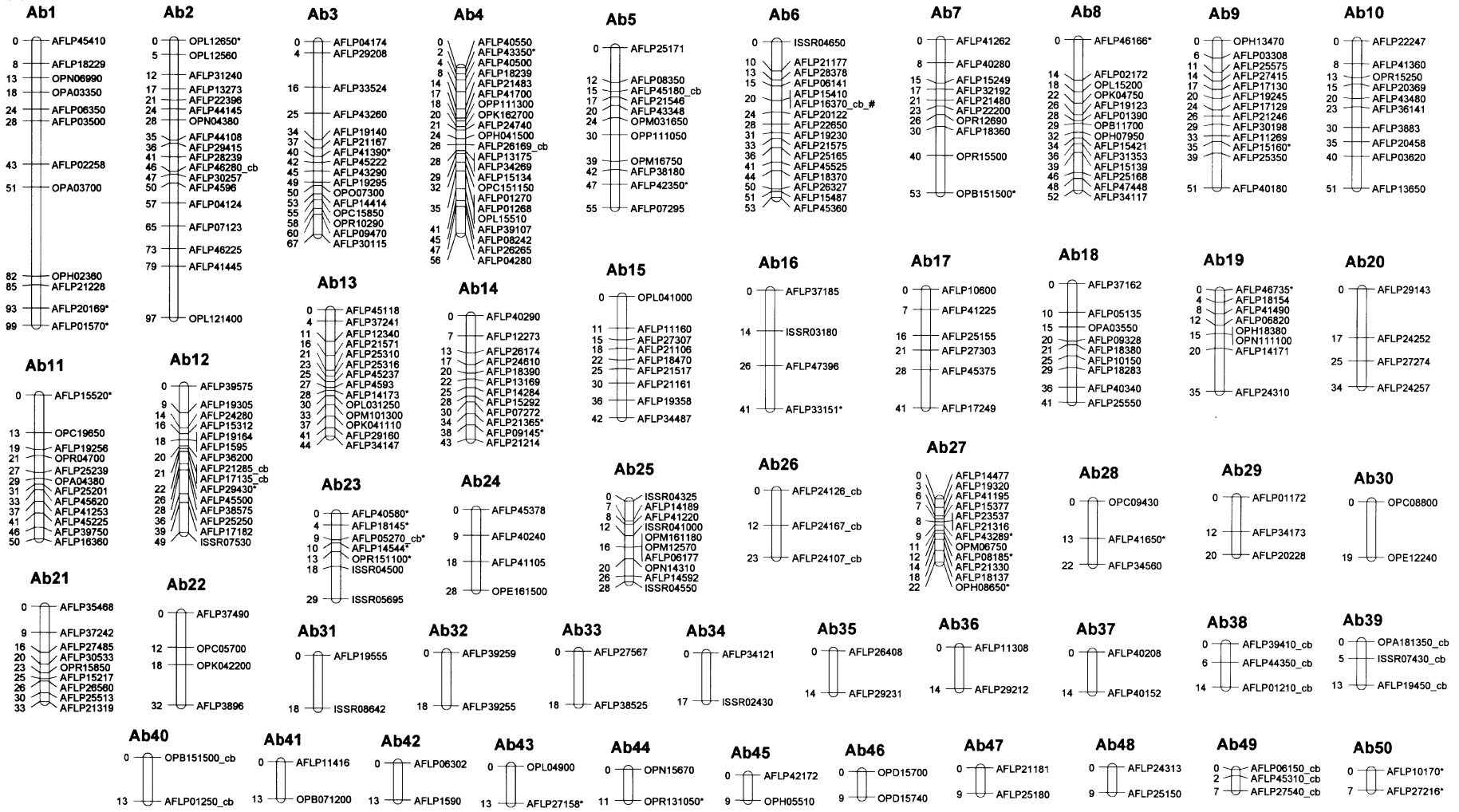

Fig. 2: Linkage maps of *Ananas bracteatus* (a) and *Ananas comosus* (b). Linkage groups were assembled with an LOD score of 4 and ordered with a minimum log-likelihood support of 1.0. Numbers on the left indicate absolute positions in cM

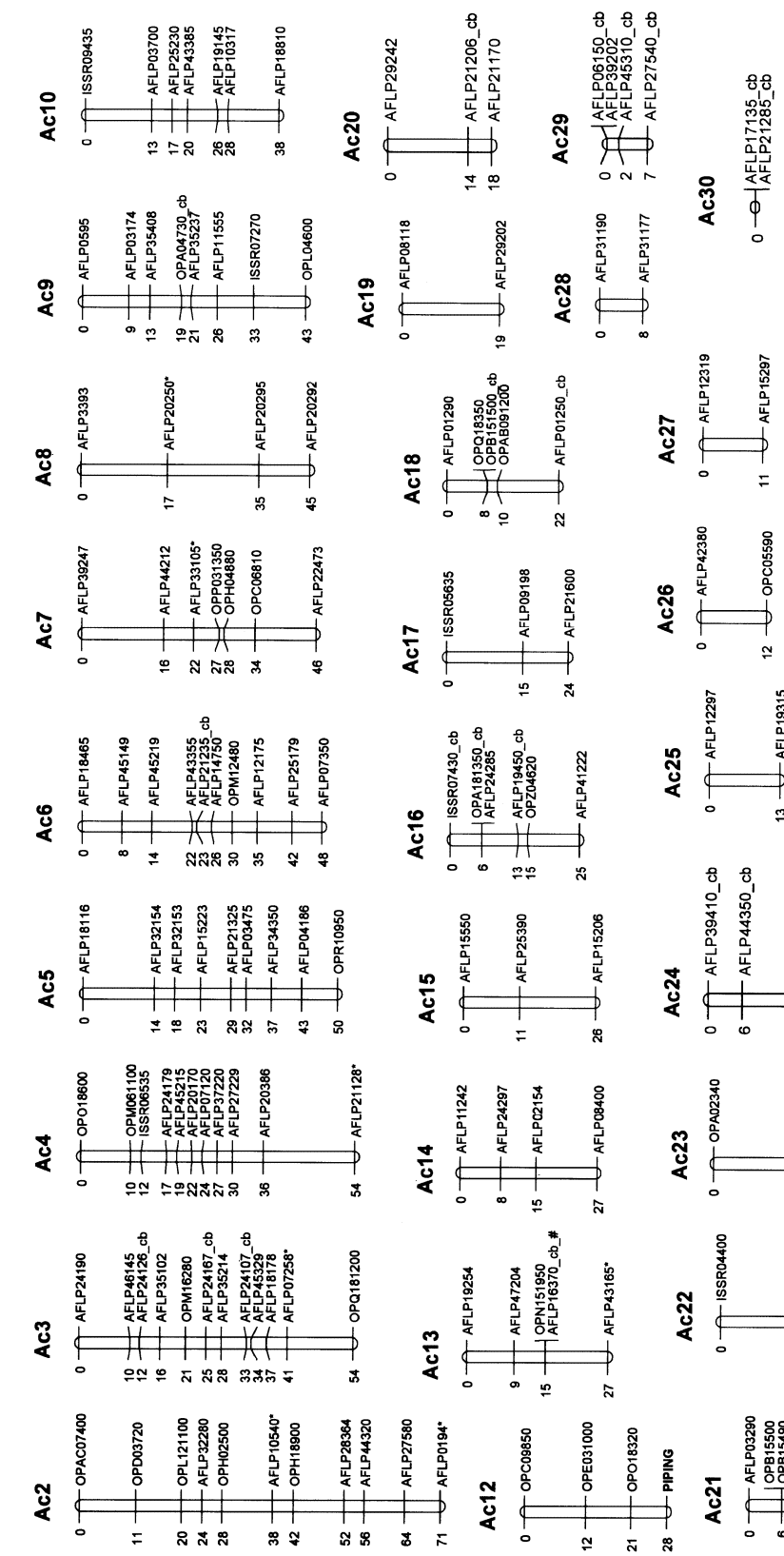

Fig. 2: Continued

was estimated for *A. bracteatus* and of 127 kbp/cM for *A. comosus*.

Discussion

In the present study, map construction was twice as efficient in *A. bracteatus* than in *A. comosus*, apparently in consequence of the higher average heterozygosity of the first species: 93.8% of heterozygous marker loci compared with 62.9% in *A. comosus*, computed on the basis of 150 polymorphic makers. Furthermore, the fact that *A. bracteatus* possesses a larger number of specific DNA markers, which are not present in the other parent – *A. comosus*, has also certainly contributed to the differences found in map construction efficiency. Higher efficiency in the map construction of the higher heterozygous progenitor was also reported by Lespinasse et al. (2000) in rubber tree and by Hurtado et al. (2002) in apricot. In both cases the more heterozygous parent was of hybrid origin. For both the pineapple species used in this study, no evidence has been established for their origin. Nevertheless, *A. bracteatus* displays an array of specific rare isozyme alleles that clearly distinguish it from the other pineapple species (Aradhya et al. 1994).

The proportion of skewed markers found, about 12–15% for both *Ananas* species, ranks among the values commonly estimated for other plant species (Jenczewski et al. 1997). Nevertheless, as a variable degree of expression of incompatibility relationships and inbreeding depression has been observed in different *Ananas* species (Coppens d'Eeckenbrugge et al. 1997, Cabral et al. 2000), a relative amount of segregation distortion among molecular markers was expected due to unfavourable intra-locus or inter-loci allelic interactions. Distortions in the segregation of some morphological traits were registered by Cabral et al. (1997).

Compared with other mapping studies, one unusual result obtained is the relatively high proportion of unlinked markers: 30 and 43%, respectively, for *A. bracteatus* and *A. comosus*, a phenomenon probably due to the small size of the mapping population, one of the most frequently mentioned causes for a large number of markers remaining unlinked (Liu 1998). Furthermore, the high number of chromosomes involved in the cross used to generate the mapping population, $2n = 50$ chromosomes for each parent, and the large dimension of the both genomes, over 3500 cM, would play a decisive role leading to the fragmentation of the map, increasing the number of small linkage groups and unlinked markers. High percentages of linkage groups with two and three molecular markers were also obtained by Testolin et al. (2001) in *Actinidia* spp., which also possess a large chromosome number ($2n = 58$) and an estimated genome length > 3000 cM. Although a significant reduction in the number of unlinked markers can be achieved for both *Ananas* species if an LOD score of 3 is used for linkage analysis, the incorporation of more markers into the maps is accompanied by the formation of unusually large linkage groups which it is not possible to order.

The maps presented in this paper are the first reported genetic maps of *Ananas* sp. Currently, these maps are being complemented with additional molecular markers. The inclusion of polymorphic sequence characterized amplified regions (SCARs), strategically distributed along the genome, will allow the easier location, and further isolation, of genes controlling morphological and agronomical traits relevant for plant breeding using different *Ananas* species.

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