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Molecular analysis of introgressive breeding in coffee (*Coffea arabica* L.)

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Abstract Nineteen arabica coffee introgression lines (BC₁F₄) and two accessions derived from a spontaneous interspecific cross (i.e. Timor Hybrid) between *Coffea arabica* (2n=4x=44) and *C. canephora* (2n=2x=22) were analysed for the introgression of *C. canephora* genetic material. The Timor Hybrid-derived genotypes were evaluated by AFLP, using 42 different primer combinations, and compared to 23 accessions of *C. arabica* and 8 accessions of *C. canephora*. A total of 1062 polymorphic fragments were scored among the 52 accessions analysed. One hundred and seventy-eight markers consisting of 109 additional bands (i.e. introgressed markers) and 69 missing bands distinguished the group composed of the Timor Hybrid-derived genotypes from the accessions of *C. arabica*. AFLP therefore seemed to be an extremely efficient technique for DNA marker generation in coffee as well as for the detection of introgression in *C. arabica*. The genetic diversity observed in the Timor Hybrid-derived genotypes appeared to be approximately double that in *C. arabica*. Although representing only a small proportion of the genetic diversity available in *C. canephora*, the Timor Hybrid obviously constitutes a considerable source of genetic diversity for arabica breeding. Analysis of genetic relationships among the Timor Hybrid-derived genotypes suggested that introgression was not restricted to chromosome substitution

but also involved chromosome recombinations. Furthermore, the Timor Hybrid-derived genotypes varied considerably in the number of AFLP markers attributable to introgression. In this way, the introgressed markers identified in the analysed arabica coffee introgressed genotypes were estimated to represent from 9% to 29% of the *C. canephora* genome. Nevertheless, the amount of alien genetic material in the introgression arabica lines remains substantial and should justify the development of adapted breeding strategies.

Key words *Coffea arabica* · Introgression · Genetic diversity · Alien gene transfer · AFLP

Introduction

Cultivated coffee *Coffea arabica* L. (2n=4x=44) is an allotetraploid plant native to Africa containing two diploid genomes that originated from two different diploid wild ancestors (2n=2x=22), *C. canephora* and *C. eugenioides* (Lashermes et al. 1999a). *C. arabica* is characterised by a very low genetic diversity, which is attributable to its allotetraploid origin, reproductive biology and evolution. In contrast, considerable variability has been reported in diploid coffee species (Berthaud and Charrier 1988). The transfer of desired characters, in particular disease resistances, from diploid, related species such as *C. canephora* into cultivars of *C. arabica* has therefore become a priority in coffee breeding (Carvalho 1988).

The Timor Hybrid is an atypical tree which was identified in a *C. arabica* field (planted in 1927) on the island of Timor (Bettencourt 1973). Based on information relating to coffee germplasm introduced into Timor at the beginning of the century, the limited fertility of the original plant, characteristics of disease resistances and preliminary molecular investigations, it is believed that the Timor Hybrid originated from a spontaneous interspecific cross between *C. arabica* and *C. canephora* (Bettencourt 1973; Goncalves and Rodrigues 1976; Lashermes et al. 1993; Orozco-Castillo et al. 1994). Progenies of the

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Table 1 Accessions of *C. arabica*, *C. canephora* and Timor Hybrid-derived Arabica genotypes analysed by AFLP

Accession	Species	Description ^a	Origin	Source ^b /Code
1. Pluma Hidalgo	<i>C. arabica</i>	Cultivar – typica type	Mexico	T/T3629
2. Blue Mountain	<i>C. arabica</i>	Cultivar – typica type	Jamaica	T/T977
3. Typica	<i>C. arabica</i>	Cultivar – typica type	Brazil	T/T996
4. Bourbon	<i>C. arabica</i>	Cultivar – bourbon type	Brazil	T/T995
5. Villasarchi	<i>C. arabica</i>	Cultivar – bourbon type	Brazil	T/T17603
6. Caturra 7	<i>C. arabica</i>	Cultivar – bourbon type	Brazil	C
7. Catuaí 10	<i>C. arabica</i>	Cultivar – bourbon type × typica type	Brazil	C/T21399
8. PDRY 22	<i>C. arabica</i>	Cultivar	Yemen	T/T21242
9. PDRY 14	<i>C. arabica</i>	Cultivar	Yemen	T/T21239
10. E-18	<i>C. arabica</i>	Cultivar	Ethiopia	T/T4474
11. E-238	<i>C. arabica</i>	Cultivar	Ethiopia	T/T4759
12. E-536	<i>C. arabica</i>	Cultivar	Ethiopia	T/T4905
13. E-12	<i>C. arabica</i>	Cultivar	Ethiopia	T/T4950
14. Geisha	<i>C. arabica</i>	Cultivar	Ethiopia	T/T2722
15. Rume Sudan	<i>C. arabica</i>	Subspontaneous	Sudan	T/T2724
16. Amphilo	<i>C. arabica</i>	Cultivar	Ethiopia	T/T2754
17. Tafari Kella S-	<i>C. arabica</i>	Cultivar	Ethiopia	T/T2748
18. Dilla Alghe	<i>C. arabica</i>	Cultivar	Ethiopia	T/T2742
19. ET-5	<i>C. arabica</i>	Subspontaneous	Ethiopia	T/T16693
20. ET-6	<i>C. arabica</i>	Subspontaneous	Ethiopia	T/T17177
21. ET-32B	<i>C. arabica</i>	Subspontaneous	Ethiopia	T/T17205
22. ET-52	<i>C. arabica</i>	Subspontaneous	Ethiopia	T/T16733
23. ET-59	<i>C. arabica</i>	Subspontaneous	Ethiopia	T/T16739
24. IF 200	<i>C. canephora</i>	Cultivar	Ivory Coast	ORSTOM
25. IF 049	<i>C. canephora</i>	Cultivar	R D C (ex Zaire)	ORSTOM
26. IF 133	<i>C. canephora</i>	Cultivar	Gabon	ORSTOM
27. 02 0737	<i>C. canephora</i>	Spontaneous	Congo	ORSTOM
28. 02 0069	<i>C. canephora</i>	Spontaneous	Ivory Coast	ORSTOM
29. 02 9054	<i>C. canephora</i>	Spontaneous	Central African R.	ORSTOM
30. 02 0722	<i>C. canephora</i>	Spontaneous	Congo	ORSTOM
31. 02 0502	<i>C. canephora</i>	Spontaneous	Central African R.	ORSTOM
32. Timor Hybrid	Hybrid inter sp.	Spontaneous (progeny 832-1)	Timor	ORSTOM
33. Timor Hybrid	Hybrid inter sp.	Spontaneous (progeny 1343/349)	Timor	T/T4389
34. Cantimor 1	<i>C. arabica</i>	Introgressed cultivar "Costa Rica 95" (832-1)	Costa Rica	C/T8667
35. Catimor 2	<i>C. arabica</i>	Introgressed genotype (832.1)	Brazil	C/T8666
36. Catimor 3	<i>C. arabica</i>	Introgressed genotype (832.1)	Brazil	C/T18121
37. Catimor 4	<i>C. arabica</i>	Introgressed genotype (832.1)	Brazil	C/T18130
38. Catimor 5	<i>C. arabica</i>	Introgressed genotype (832.1)	Portugal	C/T5175
39. Catimor 6	<i>C. arabica</i>	Introgressed genotype (832.1)	Mexico	C/T12835
40. Catimor 7	<i>C. arabica</i>	Introgressed genotype	Costa Rica	C
41. Catimor 8	<i>C. arabica</i>	Introgressed cultivar "Colombia" (1343)	Colombia	C/17930
42. Catimor 9	<i>C. arabica</i>	Introgressed cultivar "Colombia" (1343)	Colombia	C/T17931A4
43. Catimor 10	<i>C. arabica</i>	Introgressed cultivar "Colombia" (1343)	Colombia	C/T17931A1
44. Catimor 11	<i>C. arabica</i>	Introgressed cultivar "Colombia" (1343)	Colombia	C/T17933
45. Sarchimor 1	<i>C. arabica</i>	Introgressed cultivar "IAPAR 59" (832.2)	Brazil	C
46. Sarchimor 2	<i>C. arabica</i>	Introgressed genotype (832.2)	Brazil	C/T18138
47. Sarchimor 3	<i>C. arabica</i>	Introgressed genotype (832.2)	Brazil	C/T18140
48. Sarchimor 4	<i>C. arabica</i>	Introgressed genotype (832.2)	Brazil	C/T18141
49. Sarchimor 5	<i>C. arabica</i>	Introgressed genotype (832.2)	Portugal	C/T5296
50. Sarchimor 6	<i>C. arabica</i>	Introgressed genotype (832.2)	Brazil	C/T16784
51. Sarchimor 7	<i>C. arabica</i>	Introgressed genotype (832.2)	Brazil	C/T16785
52. Sarchimor 8	<i>C. arabica</i>	Introgressed genotype (832.2)	Brazil	C/T16786

^a When known, the Timor-derived progenitor used in the introgression programme is indicated in parenthesis

^b T and C indicate CATIE and CICAFAE collections, respectively

Timor Hybrid have been distributed worldwide and, when observed, show $2n=44$ chromosomes (Rijo 1974). In recent decades, they have been used intensively (Charrier and Eskes 1998) in coffee breeding programmes as the main source of resistance to pests and diseases including coffee leaf rust (*Hemileia vastatrix*), Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae*, and root-knot nematode (*Meloidogyne exigua*). Exploitation of Timor Hybrid populations has so far re-

lied on conventional procedures in which a hybrid is produced with an outstanding arabica genotype, and the progeny is selfed and selected over three to four generations. Based on this strategy, improved cultivars have already been released in several important coffee-producing countries such as Brazil, Kenya and Colombia. However, considerable difficulties arise, such as genetic disturbances in such characters as agronomic, fertility and coffee quality traits (Moreno 1989; Bertrand et al. 1997).

Table 2 The 42 primer combinations used for AFLP analysis. E+3 and M+3: 3' end-selective nucleotides of the primers complementary to the *Eco*- and *Mse*-adapter, respectively

	E+3	M+3		E+3	M+3
1.	AAC	CAA	22.	ACG	CAT
2.	AAC	CAC	23.	ACG	CTA
3.	AAC	CAG	24.	ACG	CTC
4.	AAC	CTA	25.	ACG	CTG
5.	AAC	CTC	26.	ACG	CTT
6.	AAC	CTG	27.	ACT	CAA
7.	AAC	CTT	28.	ACT	CAC
8.	AAG	CAA	29.	ACT	CAT
9.	AAG	CTA	30.	ACT	CTA
10.	AAG	CTG	31.	ACT	CTG
11.	AAG	CTT	32.	ACT	CTT
12.	ACA	CAA	33.	AGC	CAA
13.	ACA	CAC	34.	AGC	CAC
14.	ACA	CAG	35.	AGC	CAG
15.	ACA	CAT	36.	AGC	CTC
16.	ACA	CTC	37.	AGC	CTG
17.	ACA	CTG	38.	AGG	CAC
18.	ACA	CTT	39.	AGG	CTA
19.	ACC	CAA	40.	AGG	CTC
20.	ACC	CAG	41.	AGG	CTG
21.	ACG	CAA	42.	AGG	CTT

The successful use of molecular markers to study alien gene introgression has been reported in a large number of crop species (Jena et al. 1992; Garcia et al. 1995; Wang et al. 1995). The study described here was therefore designed as a DNA analysis of arabica coffee introgressed genotypes. The recently developed amplified fragment length polymorphism (AFLP) approach (Vos et al. 1995), which enables simultaneous analysis of a large number of marker loci throughout the genome, appears to be remarkably powerful (Powell et al. 1996) and was therefore investigated. Introgressed genotypes derived from the Timor Hybrid have been evaluated using AFLP and compared with representative accession sets of *C. arabica* and *C. canephora*. In so doing, we sought to estimate the amount of introgression present in such material in order to gain insights into the mechanism of introgression in *C. arabica* and to improve the exploitation of genetic resources in arabica breeding.

Materials and methods

Plant material

The 52 accessions surveyed, with their origins, are indicated in Table 1. The Timor Hybrid-derived genotypes consisted of 2 accessions representing two different progenies of the Timor Hybrid (progenies 832-1 and 1343), and 19 introgression arabica lines (at least the F₄ generation) derived from different hybrids between accessions of various Timor Hybrid progenies (832-1, 832-2, and 1343) and commercial arabica cultivars. The introgression arabica lines belong to either the Catimor or Sarchimor series, depending on the arabica cultivar involved in the initial cross ('Caturra' or 'Villasarchi', respectively). Cultivated and wild accessions of *C. arabica* and *C. canephora* were also analysed. The samples of both coffee species were selected to represent the different genetic groups identified in previous genetic diversity analyses (Anthony et al. in preparation; Dussert et al. 1999).

AFLP protocol

Genomic DNA was isolated from lyophilised leaves through a nuclei isolation step as described by Agwanda et al. (1997). The AFLP procedure was performed essentially as described by Vos et al. (1995) with a minor adaptation for coffee DNA. A 500-ng sample of genomic DNA was digested with two restriction enzymes, *Eco*RI and *Mse*I. Restriction fragments were ligated with double-strand adapters using T4 DNA ligase (Boehringer). Digested-ligated DNA fragments were used as templates for a first amplification using primers which are complementary to the adapters with one additional selective 3' nucleotide. The reaction mix was diluted 1/30, and 10 µl was used for the final amplification using two sets of primers with three selective nucleotides (Table 2). All of the primers within set E (*Eco*RI end) include the sequence 5'-GACTGCGTACCAATTC, and M primers (*Mse*I end) have the sequence 5'-GATGAGTCCTGAGTAA in common. The code following E or M refers to additional selective nucleotides at the 3'-end of the primer. One of these primers (E) was end-labelled with γ -[33P]-ATP using T4 polynucleotide kinase. The polymerase chain reaction (PCR) amplifications were carried out in an MJ Research Thermal Controller programmed as described in Cervera et al. (1998). Amplification products were separated on 6% denaturing polyacrylamide gels. The gels were dried and exposed to Kodak Bio Max X-ray film.

Data evaluation

The AFLP amplification products were designated according to the restriction enzymes, the primer combinations used and in order of decreasing fragment size. Only AFLP bands showing a clear polymorphism were scored as present (1) or absent (0). Genetic distances (GD) between genotypes were estimated as follows: $GD_{xy} = (N_x + N_y) / (N_x + N_y + N_{xy})$, where N_x is the number of bands in line x and not in line y, N_y is the number of bands in line y and not in line x and N_{xy} is the number of bands in lines x and y. Cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA) was performed with the TREECON (version 1.1) package (Van der Peer and De Wachter 1994). The bootstrap method (Felsenstein 1985) was employed to evaluate the reliability of tree topologies.

Results

Levels of polymorphism

Forty-two AFLP primer combinations (Table 2) which generated clear patterns with coffee accessions in a preliminary study were used. The AFLP patterns are illustrated in Fig. 1 and were repeatedly found in different experiments. The size of the AFLP fragments in these experiments ranged from approximately 40 to 400 bp. The number of clearly amplified bands per sample and per gel varied between 20 and 50, depending on both the primer combination and the accession considered.

A total of 1062 polymorphic fragments among the 52 accessions analysed were scored (Fig. 2). The number of polymorphic bands was much higher within the canephora accessions (i.e. 945) than within the accessions of *C. arabica* (i.e. 109). Among the Timor Hybrid-derived genotypes, including the two Timor Hybrid offspring and the 19 introgression arabica lines, 207 polymorphic bands were observed. Of these 207 marker bands, 29 were also observed to be polymorphic within the arabica accessions, 69 were invariably present in the arabica ac-

Fig. 1 AFLP analysis of coffee accessions generated using primer combination E-AAG/M-CTG. Arrowheads indicate representative additional polymorphic bands associated with the introgression of Timor Hybrid-derived genotypes (i.e. band absent in *C. arabica* and present in at least 1 accession of *C. canephora*)

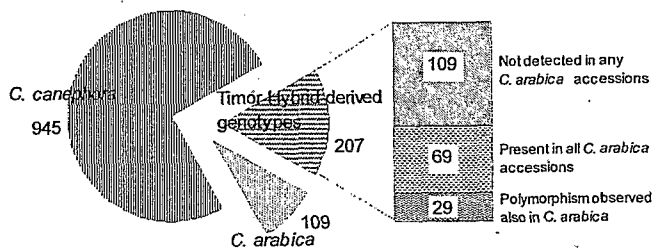
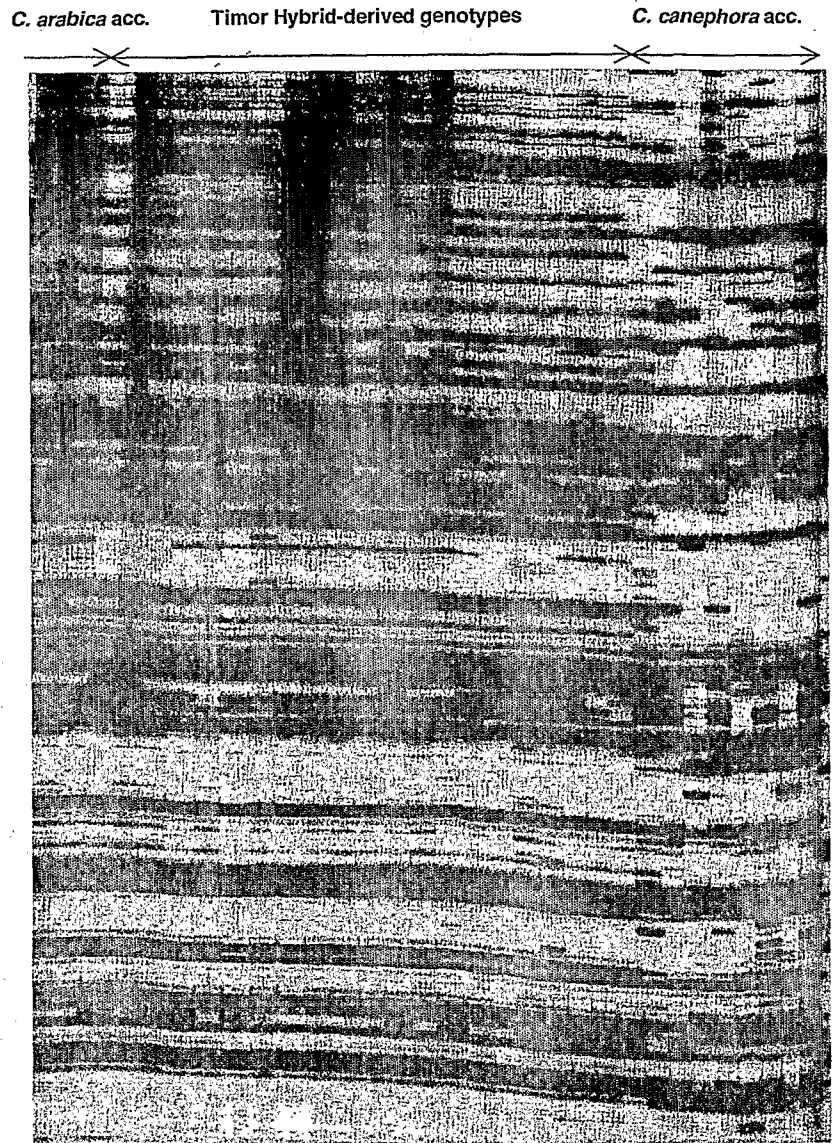


Fig. 2 Pie charts depicting the numbers of polymorphic AFLP bands observed among individuals within each group composed of accessions of *C. arabica*, *C. canephora* and introgressed Timor Hybrid-derived genotypes, respectively. For the introgressed material, the polymorphic markers either attributable to the Arabica parent or associated with the introgression of *C. canephora* chromosome segments were distinguished

cessions, while 109 were not detected in any of the accessions of *C. arabica* analysed in this study. Of the 109 additional marker bands detected in Timor Hybrid-derived genotypes, 101 were also observed in at least 1 of the canephora accessions analysed (Fig. 1). Those markers were therefore considered further as introgressed markers. Similarly, all 69 markers due to missing bands in Timor Hybrid-derived genotypes were found to be absent in at least 1 of the canephora accessions analysed. However, these markers could possibly represent introgressed fragments or, more likely, arise from the introgression process. They were therefore considered further as "related to introgression" but not as introgressed markers *per se*.

Comparison of Timor Hybrid-derived genotypes

The Timor Hybrid-derived genotypes were compared for the number of AFLP markers attributable to introgress-

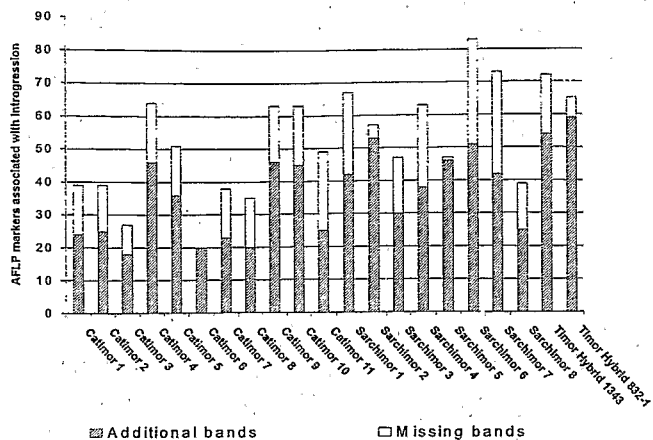


Fig. 3 Numbers of AFLP polymorphic bands attributable to introgression detected in Timor Hybrid-derived genotypes

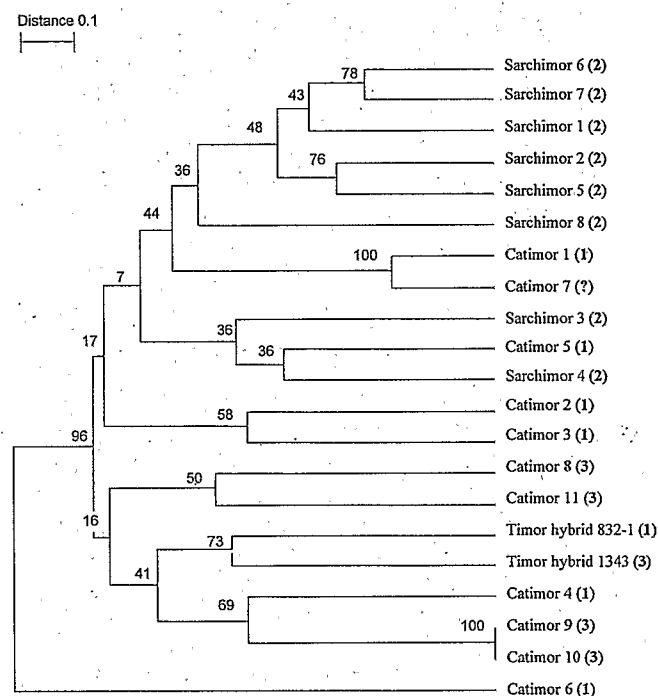


Fig. 4 Dendrogram of the introgressed Timor Hybrid-derived genotypes generated by group average clustering (UPGMA) using genetic distance based on introgression-AFLP marker bands. Numbers below branches are bootstrap values (%). The Timor Hybrid progenitor used in the introgression programme is indicated in parenthesis as 1, 2 and 3 for 832-1, 832-2 and 1343, respectively

sion (Fig. 3). The number of introgressed markers (i.e. additional bands) varied from 18 to 53 among the 19 introgression arabica lines, while the two Timor Hybrid offspring showed 54 and 59 markers, respectively. No single Timor Hybrid-derived genotype contained all of the introgressed markers. The mean number of introgressed markers in the introgression arabica lines (i.e. 34.5) appeared to be significantly lower than in the two Timor Hybrid offspring (*F* generated by one-way ANOVA significant at $P < 0.018$). In contrast, the average number of

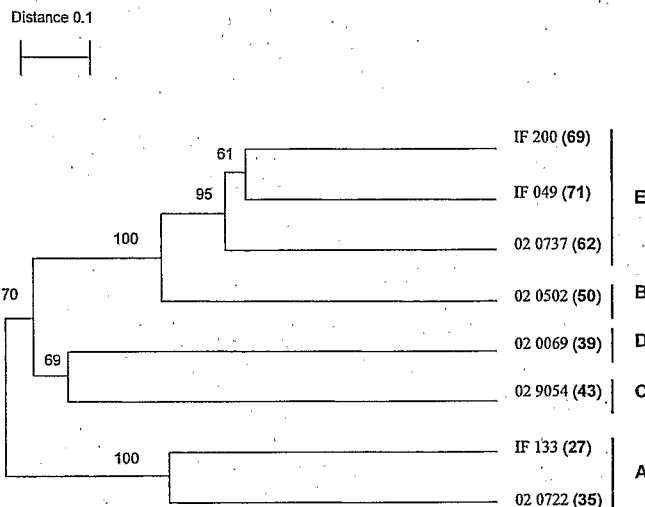


Fig. 5 Dendrogram of the *C. canephora* accessions generated by group average clustering (UPGMA) using AFLP-based genetic distance. The percentage of introgressed markers present in the Timor Hybrid-derived genotypes observed for each accession is indicated in parenthesis. Numbers below branches are bootstrap values (%). The letters following each clade correspond to the RFLP-based group as defined in Dussert et al. (1999)

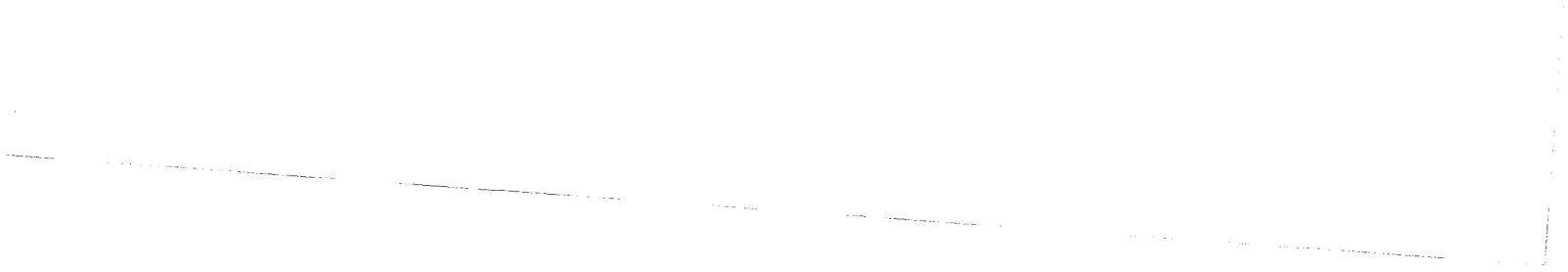
markers due to band absence associated with introgression (i.e. missing bands) in the introgression arabica lines (i.e. 16.2) did not differ significantly from those observed in the two Timor Hybrid offspring (i.e. 18 and 6, respectively). However, the number of missing bands among the introgression arabica lines varied considerably, ranging from 0 to 32. Moreover, the missing bands/additional bands ratio appeared to be significantly heterogeneous among the introgression arabica lines (χ^2 -test significant at $P < 0.001$).

The AFLP markers were used to estimate relationships among the Timor Hybrid-derived genotypes. A dendrogram constructed by cluster analysis using genetic distance based on the introgressed markers (i.e. 109 markers) is presented in Fig. 4. The introgressed genotypes derived from offspring Timor Hybrid progenitors belonging to related progenies showed a tendency to cluster. However, most associations appeared rather weak, as shown by the bootstrap values. Almost similar grouping-associations were obtained (data not shown) when using all polymorphic AFLP markers (i.e. 207 markers) or only the missing bands attributable to introgression (i.e. 69 markers).

Estimates of the amount of introgression

Groups among the accessions of *C. canephora* included in this study were clearly defined by cluster analysis using AFLP-based distances (Fig. 5). The observed grouping-association conformed to the groups previously defined by restriction fragment length polymorphism (RFLP) (Dussert et al. 1999). Furthermore, the different accessions of *C. canephora* were compared for the pres-

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