Paper 23

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Assessment of genetic diversity of Oryza Sativa L and weedy rices by microsatellite markers

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Abstract

Rice varieties show an odd characteristic in producing spontaneous adventitious forms in spite of the absence of any endemic wild forms. This phenomenon is being observed in most irrigated rice fields where direct seedlings is being employed whether the geographic area as the botanical sub-species of cultivated varieties (indica or japonica) are. These spontaneous forms are strong adventices at the starting point of populations able to persist and scatter easily. Adventitious rices are nowadays main problem in irrigate rice-growing. Unhusked seeds are scarcely distinguished from varieties. They increase seeds certification work amount. Infestation by adventitious rices lessens either rice quality and production.

Based on culture rotation or chemical means, the way of control are uneasy and their effect are short lasting. It is yet impossible to guess about long term genic fluxes between adventitious forms and varieties and, thus, to tell genetic pollution risks in developing transgenic varieties.

Adventitious rices today's main locations are intense irrigated culture with direct seedlings. But it might soon extend to high level production spots such as south-east Asia where direct seedlings replaces planting out.

The constitution of plant material and the analysis of situations observed on the ground have been accomplished in rice fields of Camargue.

Thus, our objective is to characterise adventitious rices by the use of molecular markers. One first study has permitted to distinguish the different varieties cultivated in Camargue before to compare them to their adventitious forms. To improve discrimination between neighbouring varieties (cultivated varieties belong to the japonica sub-species), microsatellites markers have been used. Microsatellites offer a high allelic variability thanks to specific PCR primers. These markers have proved particularly powerful for varieties identification. 25 microsatellites have been tested on 78 rice varieties issued of a collection available at the laboratory. This study has allowed the characterisation of the microsatellites that distinguish the more as possible Camargue's varieties as well as their adventices forms. These two informations -specific markers of varieties and markers of adventitious rices- will permit the identification of origins of adventitious rices and the interpretation of situations observed on the ground. The application of these markers to adventitious rices detection in seeds share will permit to detect red rices.

Keywords

- Oryza Sativa, weedy rice, micro satellites, genetic diversity
- France, Europe

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Introduction

Rice varieties show an odd characteristic in producing weedy forms in spite of the absence of any endemic wild forms. This phenomenon is being observed in most irrigated rice fields where direct seeding is being employed whether the geographic area as the botanical sub-species of cultivated varieties (indica or japonica) are. These spontaneous forms are strong adventices at the starting point of populations able to persist and scatter easily. Some weedy rice strains are similar to cultivar,s but other strains are similar to wild rice in that they have a red pericarp (they are called « red rice), a high degree of seed shattering, a high degree of seed dormancy, a vigour at the start, tillering most important, precocity, awning more or less pronounced and anthocyan pigmentations (base of the stem, ligule, grain apiculus, stigma, awns, colour of the pericarp (Marie, 1986; Cho et al., 1995; Suh et al., 1997). A high degree of cold tolerance at the seeding stage as well as a high degree of shoot-emergence ability in deep water have been observed in some weedy strains. A wide range of tolerance to various adverse conditions is expected in weedy rice (Suh et al., 1997).

Experimental observations have been made on weedy rices in Camargue (Marie, 1986), USA (Smith, 1979, 1981), Korea (Suh et Ha, 1994). Studies on dormancy are of the uttermost importance for controlling these weedy forms (Cohn et Hugues, 1981).

Weedy rices are nowadays main problem in irrigated rice-growing like in Camargue. Unhusked seeds are scarcely distinguished from varieties. They increase seeds certification work amount. Infestation by weedy rices lessens either rice quality and production.

Based on culture rotation or chemical means, the ways of control are uneasy and their effects are short lasting. It is yet impossible to guess about long term gene flow between weedy forms and varieties and, thus, to know if there are genetic pollution risks in developing transgenic varieties.

Weedy rices today's main locations are intense irrigated culture with direct seeding. But it might soon extend to high level production spots such as south-east Asia where direct seeding replaces planting out.

The primary objective of these study was to collect, identify and characterise weedy rices by the use of molecular markers, to know better the origin of this phenomenon, to trace the varietal origin of weeds, and finally to test the dynamic of the weedy populations.

The cultivated varieties in Camargue belong to the japonica sub-species ; so, to improve discrimination between neighbouring varieties and their derived weedy forms, microsatellites have been used. This class of co-dominant DNA markers permit to detect higher levels of allelic variation than RFLP or RAPD markers do, is easily and economically assayed by the Polymerase Chain Reaction (PCR) and can be efficiently distributed throughout the world by publication of the PCR primers sequences. Preliminary studies suggest that SSRs (Simple Sequence Repeat) are abundant and widely distributed in the rice genome (Wu and Tanksley, 1993, Panaud et al., 1996, Akagi et al., 1996). Closely related bread-wheat cultivars have also been distinguished by microsatellite polymorphism (Plaschke et al., 1995). In rice, hypervariable microsatellites consisting in AT repeats were used to distinguish fifty-nine closely related japonica cultivars in Japan (Akagi et al., 1996). These results suggest that highly polymorphic microsatellites could be used to identify closely related rice cultivars, such as Mediterranean cultivars and maybe their weedy forms, using PCR.

Material and methods

Plants material

Fifty-nine cultivars, including seventeen indica, thirty japonica, six Aus and six Basmati varieties (respectively isozyme group 1, 6, 2 and 5, Glaszmann, 1987) and one Oryza glaberrima accession providing from a rice collection gathered by CIRAD and ORSTOM and the G0 seeds of the eleven main varieties cultivated in Camargue were studied for polymorphism analysis. For the assessment of genetic diversity of weedy rice, 23 weedy plants from a multiplication rice field of Thaïbonnet (a japonica cultivar) were collected in Camargue in 1997 (with the help of Semences de Provence). This field consists in three parcels a, b and c, in which alfalfa had been cultivated during a lot of years and 'Thaïbonnet' was cultivated since 1996 for seeds production. One thaïbonnet panicle ('Thaïbonnet a') from this field was collected to be compared to 'Thaïbonnet G0' in order to test the homogeneity of the cultivar (Tab. 1). Plants were grown under greenhouse conditions.

DNA extraction

Young leaves issued from germination of 5 to 10 seeds were ground in 1 millilitre of CTAB extraction buffer (1M Tris-HCl pH 7.5, 5M NaCl, 0.5 M EDTA pH 8, Matab and 1 per cent b-mercaptoethanol). The samples were mixed and incubated at 65°C for one hour. The samples were extracted once with chloroform : isoamyl alcohol (24 : 1) and DNA were precipitated with 2 volumes of ethanol for 30 min at -20°C. The DNA was centrifuged, air-dried and re-suspended in 100 µl 10 mM Tris-HCl, 1 mM EDTA (pH 8.0).

Microsatellite assays

Nine microsatellite markers (designated RM for « Rice MapPairs ») were used to analyse all individuals. These microsatellites were selected because of their position in genetic map and their high polymorphism (Panaud et al., 1995; Wu et Tanksley, 1992).

PCR amplifications were performed in a total volume of 25µl containing 25ng of genomic DNA, 0,5µmol of each primer, 200µM of each of dGTP, dCTP, dTTP and 2,5µM dATP, 0,7µCi [a-33P] dATP, 50mM KCl, 10mM TrisHCl pH 8,3, 2,5mM MgCl2 and 1 unit of Taq polymerase. Samples were processed through 35 temperature cycles consisting of 94°C for 1 min, 62°C for 2 min and 72°C for 2 min with a final extension step at 72°C for 5 min. PCR products were denatured by the addition of 25µl stop solution (95% formamide) and heating at 95°C for 5 min 2,5µl of each reaction was then analysed on a 6% polyacrylamide denaturing gel containing 8M urea and separated for 2h at 50W constant power. The sequencing reactions of the four nucleotides of M13 DNA were used as molecular-weight standards to determine the exact nucleotide length of the denatured PCR products (kit T7, pharmacia). The gel was fixed in 20% ethanol and 10% glacial acetic acid for 15 min, then dried in a vacuum dryer at 80°C and exposed to a Kodak Biomax film for one night to produce autoradiographs.

Data analysis

Allelic frequencies in O. Sativa, japonica group, indica group and Mediterranean varieties were calculated for all the microsatellite loci. The Polymorphism Information Content (PIC) value, described by Botstein et al. (1980), was used to refer to the value of a marker for detecting polymorphism within a population, depending on the number of detectable alleles and the distribution of their frequency. Occurrences of rare alleles have a lesser impact on the PIC than those occurring with high frequency. Here, we use a simplified formula according to Anderson et al. (1993). The calculation for the PIC of a marker i is :

$PIC_i = 1 - S\ p^2_{ij}$

where pij is the frequency of the jth pattern for marker i and the summation extends over n patterns.

Genetic similarity matrices were calculated and subjected to cluster analysis by Unweighted Pair-Group Method Analysis (UPGMA).

Results and discussion

Polymorphism of the microsatellite markers

The degrees of polymorphism of the nine microsatellites were examined in the fifty-nine cultivars. These primer set revealed a total of 97 alleles among the 59 cultivars. They showed 3 to 18 alleles with an average of 10.7 alleles per locus (Table 2). Among the 97 microsatellite alleles, 24 (26.6%) were detected in only one of the 60 cultivars ; 30 (30.9%) were specific to the 30 japonica cultivars, and 27 (27.8%) were specific to the 17 indica cultivars.

PIC values for SSRs are presented in table 2 and ranged from 0.45 to 0.896. The mean PIC for SSRs was 0.771.

The rice materials included in this study probably represent a major component of the gene pool of the cultivated rice. Our SSR survey clearly detected high levels of polymorphism among these rice lines. These results from the microsatellite analysis of 59 cultivars of O. sativa indicate that the number of alleles detectable in rice by SSRs is very high. This is consistent with results of previous next reports (Panaud et al., 1996 ; Wu et Tanksley, 1993 ; Zhang et al., 1995). It is very important to detect the maximum number of alleles to try to explain the genetic diversity of weedy rices.

Genetic similarity of the rice varieties

The estimated similarity between the cultivars was calculated (Data not shown). The 59 varieties were clearly separated into two groups, all the 29 japonica varieties were tightly clustered in one group and all the 17 indica varieties were in the other group. Mediterranean varieties were clustered in the japonica group as expected. However, the Mediterranean varieties were clearly separated in the japonica group except ‘Cigalon’ and ‘Ballila’ In the indica group, the two Brazilian varieties ‘Vermelho’ and ‘Preto’ were indistinguishable to (Fig. 1).

Fig. 2 represents a cluster dendrogram of Mediterranean varieties used in this study ; two clusters were observed : a group with ‘Thaïbonnet’ and ‘Miara’ and a second group with others varieties.

The classical pattern of genetic variation previously observed in O. Sativa with other markers (Isozymes, RFLPs) is easily recognised namely the major groups indica and japonica. The dendrogram presented in Fig. 2 clearly demonstrates the ability of microsatellites to detect a large amount of genetic variation in genetically closely related varieties of rice cultivated in Camargue, and to identify groups with different levels of genetic distance. The grouping of this cultivars based on SSR markers is consistent with isozyme taxonomy and pedigree information. Therefore, the relatively small number of SSR used in the present study was sufficient to distinguish the different cultivars used in this study. This demonstrates the usefulness of SSR in assigning rice germplasm into appropriate subspecies or variety groups. Thus, it should be possible to establish a set of highly polymorphic SSRs for cultivar identification and plant variety protection in rice as it has been proposed for soybean (Rongwen et al., 1995) and for wheat (Plaschke et al., 1995).

Analysis of weedy forms

Comparison between ‘Thaïbonnet a’ and ‘Thaïbonnet G0’

‘Thaïbonnet a’ alleles are similar to ‘Thaïbonnet G0’ alleles for six loci and differ for 3 loci : RM1, RM11, RM168 (Tab. 3). At RM1 locus, ‘Thaïbonnet a’ allele is ‘n-32’ and ‘Thaïbonnet G0’ allele is n-22 ; n-32 has not been found in Mediterranean varieties though it exists in other O. sativa varieties. At RM168 locus, ‘Thaïbonnet a’ allele, n-18, is very common in japonica group. At RM11 locus, ‘Thaïbonnet a’ has an allele (n-12) which has not been found in other varieties.
Two hypothesis can be invoked to explain the difference between ‘Thaïbonnet a’ allele is different from ‘Thaïbonnet G0’ allele : whether it is an allele which exist in other varieties or it is a new allele. In the last case, two assumptions can be raised. First, the sampling falls short therefore alleles which are found in japonica subspecies are under-evaluated. Whether, there are variation within cultivars. But, these observations were made on two plants only. Furthermore, panicle of ‘Thaïbonnet a ‘ collected in this field was more precocious than other plants. So, it was a plant no different on the base of morphological characters but different about maturity date. The within-cultivars variation must be confirmed on a higher range of plants.

Genetic diversity of weedy rices

We observed a high diversity for all loci, whatever plants, parcels at which they were owned, with « new » allelic forms or weakly represent in collection. These allelic types are interpreted as function of collection alleles.
RM19 show the typical expected pattern : weedy allele is the same as cultivar allele.
At RM1 locus, there were two alleles, n-14 and n-32. These 2 alleles are different from ‘Thaïbonnet G0’ (n-22), but ‘Thaïbonnet a’ allele is n-32 ; this allele is also found in some indica varieties. Even if it is frequent in weedy population, it is scarcely found in O. sativa ; n-14 is found in two Mediterranean varieties, ‘Koral’ and ‘Carillon’.
At RM168 locus, we found 5 alleles : ‘Thaïbonnet a’ allele (n-18), ‘Thaïbonnet G0’ allele (n-20), one allele often found in other Mediterranean varieties (n-22), one allele met only one time in O. sativa (n-24) and one « new » allele. It is the typical pattern of variation : n-18 and n-22 are found scarcely in japonica group but the most common allele is not indexed. In some cases, some weedy samples could come from other Mediterranean varieties. For example, at RM1 locus, the allele n-14 was also found in Koral. So weedy plants with n-14 could be originated from Koral. Alleles of other Mediterranean varieties were found in these weedy plants.
Many weedy forms showed simultaneously these rare allelic forms at all loci. A plant can present only « new alleles ».
Now, loci are all not link on the genetic map and no heterozygous individuals have been detected.

In a field where they was adapted conditions for assessment of origin of weeds, we have found a tremendous amount of diversity.

If some markers seemed stable and appropriate to follow diversity (weedy allele is the same as cultivar allele like RM19), other showed a diversity more difficult to explain. A lot of « new alleles » were observed. Population size we used could be insufficient because of the tremendous diversity of these markers. Nevertheless, because of number of the different alleles identify , it seems possible that there is a relationship between appearance of this « new » polymorphism and the weedy forms, all the more because frequency of these « new » allelic forms is high in weedy population.

Two hypothesis can explain origin of these weedy forms :

- 1) An external contribution (tools, tractors...) or hybridisation with other weeds.
  Because of this high diversity, it is difficult to assign an other variety origin by crossing or weed external contribution. Furthermore, no heterozygous were observed, rice is self-pollinated, outcrossing with other varieties are rare and weedy forms are more early.
- 2) High diversity could be originated from within-cultivar variation. For neutral markers, we can detect a little of residual polymorphism. But we can suppose, it will be too low to generate a so high number of alleles with a so high frequency. So, a study of within-cultivar variation must be carried with seeds coming from multiplication and production fields, to test their potential to product weedy forms.

The weedy rice population studied contained high levels of genetic variation greater than that found in their putative parental variety 'Thaïbonnet'. To make sure that these new alleles do not yet exist in other varieties, we should increase the size of the collection to detect the greatest diversity as possible in O. sativa. So, we are carrying on a study of variety pedigrees and within-cultivar variation with other loci.

Tab. 1 : List of the rice varieties used in this study

Cultivars	Origin	isozyme group	Cultivars	Origin	isozyme group
Canaroxa	*japonica*	6	Sinna Sithina Kali	*indica*	1
PTB 30	Aus	2	CI 5319	*japonica*	6
Da 28	Aus	2	TOG 568113	*glaberrima*	
Dourado Aghula	*japonica*	6	IR 64	*indica*	1
OS 4	*japonica*	6	TKM 6	*indica*	1
Tres Meses	*japonica*	6	IR 56 381-139-2-2	*indica*	1
Tchampa 32368	Basmati	5	Azucena	*japonica*	6
Tchampa 32362	Aus	2	T 571	*japonica*	6
Da 16	*japonica*	6	Tongil	*indica*	1
Rexoro	*japonica*	6	Patik	*indica*	1
Binulawan	*japonica*	6	Honduras	*japonica*	6
Carolina Gold	*japonica*	6	Shoemed	*japonica*	6
Pate Blanc MNI	*japonica*	6	Lemont	*japonica*	6
Basmati 370	Basmati	5	IR36	*indica*	1
Arc 13829	Basmati	5	Hill Rice Mishima	*japonica*	6
Dom Sofid	Basmati	5	T564	*japonica*	6
Pankhari 203	Basmati	5	Thaïbonnet	*japonica*	6
T 26	Basmati	5	Miara	*japonica*	6
N 22	Aus	2	Ariete	*japonica*	6
Dular	Aus	2	Cigalon	*japonica*	6
FR13A	Aus	2	Inca	*japonica*	6
Nam Sagui 19	*indica*	1	Koral	*japonica*	6
IR 5	*indica*	1	Balilla	*japonica*	6
ASD 1	*indica*	1	Carillon	*japonica*	6
PTB 19	*indica*	1	LV1	*japonica*	6
Guan-Yin-Tsan	*indica*	1	Crodogéribe	*japonica*	6
Carreon	*indica*	1	Loto	*japonica*	6
Leuang-Pratew	*indica*	1	Preto BR	*indica*	6
Peta	*indica*	1	Vermelho BR	*indica*	6
Irat 13	*japonica*	6			

Table 2 : Allelic variation and polymorphism information contents (PICs) of microsatellite markers in collection

Microsatellites	Chromosome No.	allele size (bp) in IR36	No. of alleles	PIC
RM1	1	113	14	0,9
RM5	1	113	8	0,764
RM11	7	140	10	0,856
RM18	7	157	9	0,78
RM19	12	226	12	0,807
RM21	11	157	18	0,896
RM148	3	129	3	0,45
RM167	11	128	12	0,726
RM168	3	116	11	0,76

Table 3 : Allelic frequencies of microsatellite markers in O sativa, indica, japonica, varieties cultivated in Camargue and weedy rices. In bold, the most frequent allele.

SSR	Alleles	Allelic frequencies					Allele in	
		O. sativa	*indica*	*japonica*	Mediterranean varieties	weedy plants	Thaibonnet G_0	Thaibonnet from parcel a
RM1	n+10	0,01	0,06					
	n+2	0,03	0,17					
	n	0,12	0,41					
	n-2	0,03		0,03				
	n-14	0,05		0,1	0,17	0,72		
	n-18	0,05		0,1	0,17			
	n-20	0,17		0,26				
	n-22	**0,18**	0,06	0,23	0,17		X	
	n-24	0,1		0,2	0,42			
	n-26	0,05						
	n-28	0,03	0,06					
	n-30	0,01	0,06					
	n-32	0,11	0,17		0,08	0,28		X
	n-34	0,03		0,06				
RM5	n+24	0,03		0,06	0,08			
	n+12	0,08		0,1		0,95		
	n+10	0,25		0,4	0,25		X	X
	n+8	**0,38**	0,53	0,43	0,7	0,05		
	n+6	0,03	0,12					
	n+4	0,06	0,17					
	n+2	0,1	0,06					
	n	0,05	0,12					
RM11	n	0,11	0,41					
	n-2	0,05	0,17					
	n-4	0,08	0,17	0,03				
	n-6	0,03	0,12					
	n-12				0,08			X
	n-14	0,15	0,06	0,26	0,17		X	
	n-16	0,01						
	n-18	0,03		0,03		0,63		
	n-20	**0,23**	0,06	0,43	0,75	0,27		
	n-22	0,16				0,1		
	n-24	0,11		0,23				

RM18	n-10	0,01						
	n-6	0,13	0,47					
	n-4	0,01						
	n	0,13	0,41	0,03				
	n+2	**0,38**	0,06	0,6	0,6			
	n+4	0,13	0,06	0,16				
	n+6	0,1		0,03		0,045		
	n+8	0,01		0,03	0,25	0,1	X	X
	n+10	0,06		0,1	0,17	0,045		
	n+12					0,31		
	n+16					0,4		
	n+18					0,1		
RM19	n+27	0,01	0,06					
	n+24	0,03	0,12					
	n+21	0,15	0,47	0,03				
	n+18	0,03	0,12					
	n+9	0,01		0,03				
	n+6	0,01						
	n+3	0,03	0,12					
	n	0,01	0,06					
	n-6	0,11		0,23				
	n-9	**0,28**		0,23	0,34			
	n-12	**0,26**	0,06	0,43	0,66	1	X	X
	n-21	0,01		0,03				
RM21	n+10	0,01	0,06					
	n+8	0,01	0,06					
	n+4	0,08	0,23	0,03				
	n+2	0,05		0,1				
	n	0,05	0,17					
	n-2	0,11		0,2	0,17		X	X
	n-4	0,05	0,06	0,06	0,08			
	n-6	0,03		0,06				
	n-8	0,03		0,06				
	n-10	0,01						
	n-14	0,01		0,03				
	n-18	0,01						
	n-20	0,16		0,3	0,66	0,05		
	n-22	0,03						
	n-24	**0,18**	0,17	0,06	0,08	0,2		
	n-26	0,01	0,06			0,23		
	n-28	0,01		0,03		0,52		
	n-30	0,08	0,17	0,03				
RM148	**n**	**0,7**	0,82	0,64	0,17	1		
	n+2	0,23		0,36	0,83		X	X
	n+6	0,06	0,18					
RM167	**n**	**0,43**	0,7	0,23	0,25		X	X
	n+4	0,1		0,03				
	n+21	0,26	0,12	0,43	0,5	0,1		
	n+22	0,01				0,86		
	n+23	0,1		0,16	0,25			
	n+24	0,01	0,06					
	n+25	0,03		0,03				
	n+26	0,01	0,06			0,04		
	n+27	0,03		0,06				
	n+31	0,01		0,03				
	n+32	0,01						
	n+38	0,01	0,06					

RM168	n+14	0,03	0,12					
	n+10	0,01						
	n	0,13	0,47					
	n-2	0,03						
	n-6	0,06						
	n-10	0,01		0,03				
	n-16	0,03						
	n-18	0,08		0,16	0,25	0,09		X
	n-20	**0,45**	0,35	0,63	0,33	0,09	X	
	n-22	0,11	0,06	0,16	0,42	0,28		
	n-24	0,01				0,09		
	n-26					0,45		

Figure 1 : Dendrogram showing a cluster analysis of 58 Oryza sativa cultivars, based on polymorphism of 9 microsatellite loci

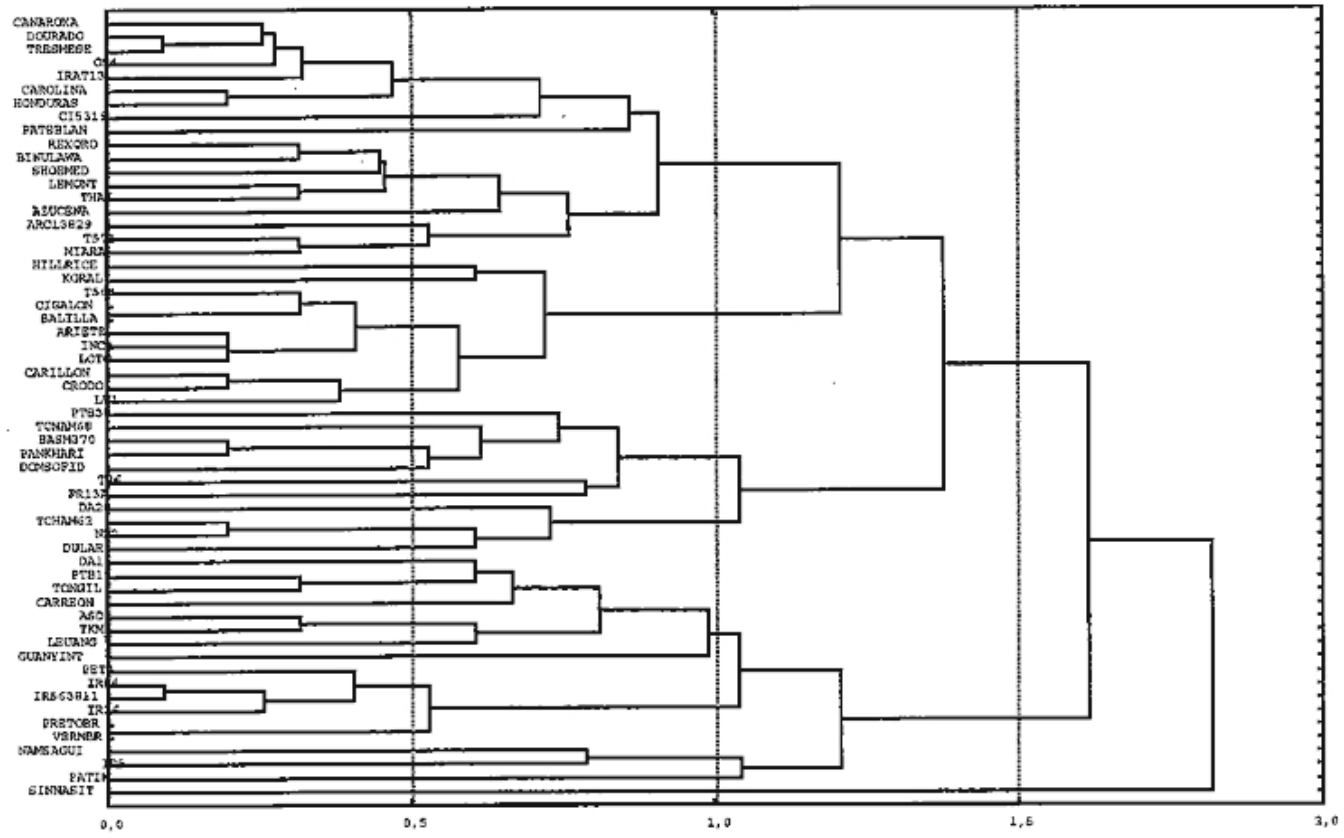
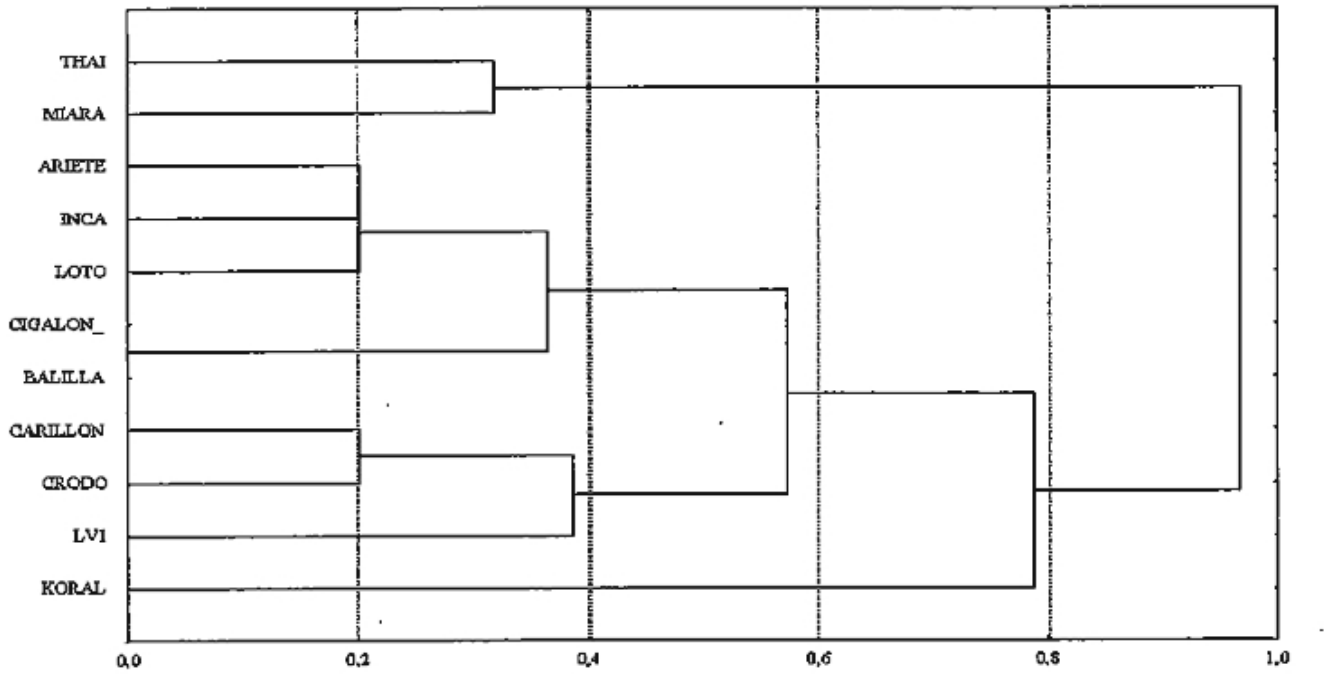


Figure 2 : Dendrogram showing a cluster analysis of 11 Mediterranean japonica cultivars, based on polymorphism of 9 microsatellite loci



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Figures and tables summary

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