Assessment of coconut tree genetic divergence by compound sample RAPD marker analysis

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ABSTRACT

The coconut tree (*Cocos nucifera* L.) is a tropical species widely cultivated throughout the world, which is found in all intertropical regions. The species shows wide phenotypic variability, which, however, is little understood at the genetic level. This study of the variability among the various coconut tree populations is important to increase the efficiency of the development of superior cultivars adapted to different ecological conditions. It also helps the selection of divergent progenies that can maximize heterosis in hybridizations. Genetic divergence among 19 coconut tree populations available in the BAG - Coco at EMBRAPA/CPATC was estimated by RAPD. Leaf samples from 21 plants of each cultivar were squashed together (compound samples) in liquid nitrogen and the DNA extracted using the modified Doyle and Doyle (1990) protocol. Samples of these DNA were amplified with 24 primers of the OPERON Technologies series. One hundred and twenty-seven polymorphic and 61 monomorphic loci were obtained. Six different clusters, possibly heterotic groups, were formed by the Tocher optimized clustering analysis which used the matrix of the complement of the Jaccard index. Group 1 included the dwarf group cultivars. Giant accessions, abbreviated to GBR (Brazilian Giant), formed group 2, except GBRPF, which together with West African Giant (GOA) formed group 4. The most distant accession was the Tonga Giant cultivar (GTG) that did not group with the others and presents potential for hybridization with the six cultivars in the dwarf group cultivars and with the five in the GBR group. Group 3 consisted of GRL, GPY and GRT and Group 5 of GML and GVT. The dendrogram obtained by the nearest neighbor method was in line with the clustering obtained by the Tocher optimization method. The markers used permitted identification of each one of the populations showing that they were genetically different (absence of duplicity). The use of compound samples was effective to investigate the interpopulational genetic diversity. However, to understand the intrapopulation genetic variability, individual sampling should be used.

KEY WORDS: BAG, Jaccard similarity index, nearest neighbor clustering method, polymorphism, interpopulational genetic variability, Tocher clustering method.

INTRODUCTION

The coconut tree (*Cocos nucifera* L.) is a widely cultivated tropical species found in all intertropical regions. Two varieties, giant (var. typical) and dwarf (var. nana) are unique to the Cocus genus (2n = 2x = 32). There are three dwarf varieties distinguished by color: green, yellow and red dwarf. The giant variety also presents variations in its traits and can be divided into populations identified by the name of the country where the variety has been cultivated, but there is no universally accepted differentiating nomenclature (Purseglove, 1975). The two varieties can be naturally or artificially crossed and produce fertile hybrid descendants that present intermediate characteristics (Lyanage, 1950).

Genetic variability between the coconut tree varieties and between the different cultivars or populations (in the case of the giant variety) within a variety are primordial conditions for conducting efficient breeding programs, but little data are available on them. Morphological and physiological traits, such as seed germination time, floral biology, physical and chemical composition of the fruit and seed have been assessed without much success mainly because of environmental effects (Bourdeix et al., 1993; Ribei-

ro, 1993; Akpan, 1994; Lebrun et al., 1995). Zizumbo-Villareal and Pinero (1998) assessed 41 coconut tree populations using 17 traits linked to fruit morphology. The cluster and principal component analyses indicated the formation of four groups, in which there was high correlation between morphological and geographic distances. This corroborates the historical evidence of recent coconut tree introduction from different regions of the world in Mexico.

Ribeiro (1993) assessed fruit traits by multivariate statistical analysis, in five giant coconut tree populations in Brazil and identified one population (Gigante do Brasil, from Santa Rita) as more divergent than the others. However, only four of the nineteen traits assessed were significantly differentiated. In another study, the same author (Ribeiro et al., 1999) assessed five giant populations using canonic variables and Mahalanobis distances which suggested similarities between the Pacatuba and Merepe populations.

Santos et al. (1999) aiming at presenting electrophoresis profiles of total proteins of three types of cultivated coconut tree (Anão, Gigante and Híbrido) and at identifying specific bands, obtained preliminary results which demonstrated distinct profiles among the types assessed. Four proteins were present among the Hybrid and Giant types with molecular weight between 30 and 70 kDa, constituting possible molecular markers with differential genetic expression in the coconut tree. Cardena et al. (1998) analyzed the electrophoresis patterns of total proteins, peroxides and endopeptidases in the leaf. The polymorphism detected the expression of two alleles of a dimeric peroxide, two alleles of a monomeric endopeptidase and one pair of null alleles active in total proteins, which sufficed to screen the used genotypes.

Various molecular biology techniques are presently available to detect genetic variability at the DNA sequence level, that is, they are used to detect genetic polymorphism (Ferreira and Grattapaglia, 1996). The RAPD and RFLP techniques were applied to the coconut tree (Lebrun et al., 1995) and a geographic distribution pattern similar to that obtained with leaf polyphenols and morphological traits was found.

Wadt (1997) assessed genetic divergence between three giant coconut tree ecotypes (Rennell, West African and Brasil - Praia do Forte) by RAPD in compound samples. The Rennell ecotype was more genetically divergent from the others while the West African and Brazil - Praia do Forte were close to each other, but also different. Perera et al. (1998) assessed the genetic relationship among giant (Giant), dwarf (Nana) and intermediate (Aurantiaca) indigenous accessions from Sri Lanka by AFLP profiles obtained with eight primer pairs (EcoRI and MseI). The Aurantiaca group presented duplicates and was closer to Dwarf than to Giant, providing important implications for germplasm collection maintenance.

Lebrun et al. (1998) assessed the genetic diversity within giant and dwarf coconut tree ecotypes using nine cDNA rice clones and two wheat DNA clones (mitocondrial and genomic) as probes for hybridization in Southern blotting (RFLP). The results allowed identification of two main groups, one consisting of ecotypes from the Far East and South Pacific and the other consisting of ecotypes from India, Sri Lanka and West Africa. The technique also allowed the legitimization of two hybridizations and the determination of the inheritance (maternal) of the mitochondrial genome.

The study of the variability among the various coconut tree populations is important to help the development of superior cultivars adapted to different ecological conditions and selection of divergent parents to maximize heterosis in hybridizations. In this study we aimed at estimating by RAPD analysis the genetic divergence among 19 coconut tree populations available in the BAG-Coco at EMBRAPA/CPATC.

MATERIAL AND METHODS

Genetic material

Nineteen coconut accessions (*Cocos nucifera* L.) from the Germplasm Bank at Embrapa Trópicos Costeiros (Table 1) located in Aracaju, SE, Brazil, were used in this study. Young leaf blades were taken from twenty one plants per accession, squashed in liquid nitrogen and placed in an ultrafreezer (-86ºC) in closed 15mL `Falcon´ tubes. The study was carried out at the Laboratory of Plant Genetic Breeding at the Center of Agricultural Sciences and Technologies at the "Darcy Ribeiro" Northern Fluminense State University (LMGV/CCTA/UENF) in Campos dos Goytacazes, RJ.

DNA extraction

Approximately 200mg of squashed material from the leaf blades was transferred to 1.5mL eppendorf tubes and immersed in liquid nitrogen. DNA was extracted according to the protocol by Doyle and Doyle (1990) with modifications; 800 µl of pre-heated extraction

Table 1. Cocus nucifera L. accessions from the Germplasm Bank at Embrapa Trópicos Costeiros assessed in LMGV/CCTA/UENF.

Nb.	Abbreviation	Name	Type	Fruit Color
01	AAM	Malaysian Yellow	Dwarf	Yellow
02	AAG	Brazilian Yellow	Dwarf	Yellow
03	AVM	Malaysian Red	Dwarf	Red
04	AVG	Brazilian Red	Dwarf	Red
05	AVC	Cameroon red	Dwarf	Red
06	AVEJ	Brazilian Green	Dwarf	Green
07	GBRPC	Pacatuba	Giant	Green
08	GBRSR	Santa Rita	Giant	Green
09	GBRBF	Baia Formosa	Giant	Green
10	GBRSJM	São Jose do Mipibu	Giant	Green
11	GBRME	Merepe	Giant	Green
12	GML	Malaysian	Giant	Green
13	GBRPF	Praia do Forte	Giant	Green
14	GTG	Tonga	Giant	Green
15	GVT	Vanuatu	Giant	Green
16	GRL	Rennell	Giant	Green
17	GPY	Polynesia	Giant	Green
18	GRT	Rotuma	Giant	Green
19	GOA	West African	Giant	Green

buffer containing 1% CTAB, 1.4 M NaCl were added to the tube containing 1% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1% PVP and 0.1% 2-mercaptoetanol. It was incubated at 65ºC for 30 to 40 minutes and gently shaken every 10 minutes. It was then centrifuged at 13400g for five minutes. The supernatant (600 µl) was transferred to new tubes and an equal volume of chloroform: isoamilic alcohol (24:1) was added and continually inverted until an emulsion formed. This step was repeated, and after new centrifuging, the supernatant was transferred to new tubes and chilled isopropanol was added, with gentle inversion and placed overnight in the refrigerator. It was centrifuged at 13400g for 10 minutes, and a pellet obtained (precipitate) that was washed twice in 300 ml 70% ethanol and once in 300 ml 95% ethanol, dried under natural conditions, re-suspended in 200 µL TE solution (10mM Tris HCl, 1 mM EDTA, pH 8.0) and incubated with RNAse at a final concentration of 40ug/mL at 37ºC for 30 minutes. After adding 20 µL NaCl 5M and 140 µL chilled isopropanol the mixture was incubated for one night at 4ºC. It was then centrifuged at 13400g for 10 minutes, dried and finally the pellet was re-suspended in 200 µL of TE solution. The DNA concentrations in the samples were estimated using fluorometric methods (DyNA Quant 200 Fluorometer, Hoefer Scientific, San Francisco, USA) and then standardized at 10 ng. µL-1 concentration.

Polymerase chain reaction

The amplification reactions were carried out according to Williams et al. (1990) modified in a final volume of 25 ml containing the reagents at the following concentrations 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.4 mM MgCl2; 100 µM dATP, dCTP, dGTP and dTTP; 0.3 µM primer; 20 ng genomic DNA and a unit of Taq DNA polymerase (Pharmacia Biotech, EUA). A thermal cycler was used (Perkin Elmer GeneAmp PCR System 9600) programmed for 95ºC for 1 minute followed by 45 one minute cycles at 94ºC, 1 minute at 36ºC and 2 minutes at 72ºC and a final step for extension of 7 minutes at 72ºC, using the fastest temperature transition mode available (1ºC/1seg.). The amplification products (bands) were analyzed by electrophoresis and visualized after staining by ethydium bromide.

Band reading and data analysis

The RAPD profiles of each accession were obtained by the presence (1) or absence (0) of high intensity bands and the complement of the Jacard index (1 - IAB) (Alfenas et al., 1991) was calculated as IAB = a/(a+b+c) where: a = number of bands present in both accessions, simultaneously; b = number of bands present only in accession A; c = number of bands present only in accession B.

Statistical analysis

Hierarchical clustering methods (closest neighbor) were used, as well as the optimization method proposed by Tocher, quoted by Rao (1952) based on the matrix obtained from the arithmetical complement of the Jacard similarity index. The statistical analyses were performed by the programs GENES, 0.1.0 version and STATISTICA version 95 (Statsoft Inc, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

Randomly Amplified DNA Polymorphism (RAPD)

The primers used in the polymerase chain reaction are shown in Table 2. Twenty-four primers were analyzed and a total of 188 amplification products (bands) were obtained, resulting in a mean of 7.83 bands per primer. This value is similar to those reported by Virk et al. (1995) and Harvey and Botha (1996) in genetic divergence assessments using the RAPD technique. Out of the 188 bands, 127 resulted in polymorphic and 61 in monomorphic loci (5.29 polymorphic loci per primer and 2.54 monomorphic loci per primer). The OPK-19 primer presented the highest number of total (15) and polymorphic (11) bands.

Data analysis

Estimates of the genetic distances (arithmetic complement of the Jacard index) (Table 3) presented a wide variation, considering the extreme values (0.030) detected for the dwarf ACG (Brazilian Red) and ACV (Cameroon Red) accessions and for the dwarf AAM (Malaysian Yellow) and the giant GOA (West African) accession (0.368). These values confirm the existence of genetic variability among the various coconut tree populations available. Table 3 also shows that, in general, lower distances were observed between accessions and higher values between giant accessions and among giants and dwarf accessions.

Table 2. Operon Technologies Series primers with the respective number of polymorphic and monomorphic bands.

Operon Technologies Series Primers	Number of polymorphic loci	Number of monomorphic loci	Total band number per primer
OPA-09	10	0	10
OPA-11	5	6	11
OPA-15	7	3	10
OPA-19	3	5	8
OPAB-01	6	4	10
OPAB-05	3	5	8
OPH-03	8	0	8
OPH-04	6	1	7
OPH-07	2	3	5
OPI-7	4	2	6
OPI-11	2	1	3
OPJ-19	6	0	6
OPK-04	6	0	6
OPK-19	11	4	15
OPL-16	2	5	7
OPL-18	4	0	4
OPN-02	6	0	6
OPN-07	6	0	6
OPN-15	6	4	10
OPN-18	2	5	7
OPN-19	4	3	7
OPO-11	6	4	10
OPO-13	8	4	12
OPO-19	4	2	6
Total	127	61	188

This suggests that fruit color may be the main discriminating factor in the assessed dwarf accessions, while in the giant accessions (green colored fruit) the variability is present in other traits not considered in this study.

The cluster analysis by the Tocher optimization method (based on the matrix of the complement of the Jacard index) indicated the presence of six distinct groups, possibly forming heterotic groups (Table 4).

Cluster 1 contained all the dwarf group accessions. In the dendrogram of Figure 1, good agreement can be observed between the discriminatory capability of the fruit color variable and RAPD analysis. The AAM (Malaysian Yellow) and AAG (Brazilian Yellow) were placed on a single dendrogram branch. Similarly, AVM (Malaysian Red), AVG (Brazilian Red) and ACG (Cameroon Red) were also on a single branch. ACG and AVC, however, showed greater similarity, which can be mainly linked to their origin. The Brazilian Green Dwarf accession (AVEJ) was also linked to the dwarf accession branch, but presented greater dissimilarity compared to the others (Figure 1).

Giant accessions, abbreviated to GBR (Brazilian Giant) except GBRPF, formed the next group (Group 2) (Table 4). According to the dendrogram (Figure 1) all the GBR accessions and the GOA accession (West African) were placed on a different and quite divergent branch from the dwarf. Greater heterosis may result from crosses involving accessions from these two different groups. The GBRPC (Pacatuba) and GBRME (Merepe) accessions (or populations) were fairly similar, which is in line with the results obtained by Ribeiro et al. (1999) who described these populations as less divergent.

Group 3 (Table 4) consisted of the Rennell (GRL), Polynesia (GPY) and Rotuma (GRT) accessions. The GRL and GPY were slightly closer, as shown in Figure 1. Group 4, formed by the Giant Brazil Praia do Forte (GBRPF) and West African (GOA) accessions (Table 4) can also be seen in the dendrogram (Figure 1), indicating that the Giant Brazilian Praia do Forte accession may be a variation of the Giant West African accession that occurred after being introduced in Brazil.

There was also good agreement among the results of Tocher and nearest neighbor clustering methods in group 5, which placed together the Giant Malaysian (GML) and Giant Vanuatu (GVT) accessions. The Giant Tonga (GTG) did not group with the others, and formed a single accession group (Group 6)(Table 4). It (GTG) was located between groups 3 (GRL, GPY and GRT) and 5 (GML and GVT), according to its branch position in the dendrogram presented in Figure 1.

Table 3. Estimates of genetic distances (arithmetic complement of the Jaccard index) involving nineteen coconut tree (*Cocos nucifera* L.) accessions assessed in LMGV/CCTA/UENF, Campos dos Goytacazes, RJ.

2*	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
0.046	0.080	0.113	0.076	0.138	0.337	0.327	0.327	0.319	0.292	0.209	0.336	0.207	0.187	0.153	0.213	0.187	0.368	1
0	0.097	0.108	0.077	0.101	0.333	0.316	0.324	0.312	0.284	0.219	0.311	0.191	0.175	0.167	0.217	0.180	0.347	2
	0	0.065	0.031	0.106	0.353	0.333	0.333	0.325	0.292	0.192	0.359	0.221	0.169	0.146	0.227	0.181	0.371	3
		0	0.030	0.096	0.339	0.309	0.318	0.307	0.278	0.173	0.321	0.213	0.125	0.175	0.211	0.194	0.338	4
			0	0.088	0.323	0.301	0.306	0.303	0.292	0.190	0.313	0.213	0.153	0.164	0.230	0.191	0.336	5
				0	0.347	0.322	0.323	0.319	0.292	0.189	0.343	0.229	0.155	0.168	0.242	0.199	0.367	6
					0	0.097	0.113	0.117	0.102	0.259	0.178	0.279	0.302	0.290	0.259	0.310	0.178	7
						0	0.036	0.046	0.048	0.260	0.209	0.258	0.287	0.278	0.279	0.309	0.169	8
							0	0.043	0.074	0.273	0.197	0.253	0.285	0.295	0.281	0.316	0.169	9
								0	0.042	0.236	0.197	0.248	0.262	0.271	0.268	0.300	0.189	10
									0	0.204	0.163	0.195	0.250	0.229	0.244	0.243	0.190	11
										0	0.254	0.144	0.137	0.161	0.153	0.187	0.301	12
											0	0.209	0.292	0.260	0.257	0.273	0.132	13
												0	0.201	0.154	0.146	0.176	0.265	14
													0	0.135	0.164	0.164	0.342	15
														0	0.084	0.115	0.321	16
															0	0.161	0.333	17
																0	0.367	18
																	0	19

* Table 1 shows the accession identification.

Table 4. Cluster analysis by the Tocher Optimization method obtained based on the arithmetical complement of the Jaccard similarity index of nineteen coconut tree (Cocus nucifera L.) accessions assessed by LMGV/CCTA/ UENF, in Campos dos Goytacazes, RJ

Groups	Accessions
1	AVG [1/,2/], AVC, AVM, AAM, AAG and AVEJ
2	GBRSR, GBRBF, GBRSJM, GBRME and GBRPC
3	GRL, GPY and GRT
4	GBRPF and GOA
5	GML and GVT
6	GTG

[1/] The assortment of the accession in sequence indicates their entry in the group; [2/] These accessions are described in Table 1.

The clustering obtained by Wadt (1997) which assessed the genetic divergence among three ecotypes of giant coconut trees (Rennell, East African and Brazil - Praia do Forte) was in line with that presented in this study. The Rennell ecotype was more genetically divergent from the others, while the West African and Brazil - Praia do Forte populations were closer but different.

The use of compound samples was effective for investigating the interpopulational genetic diversity. However, individual sampling will be required for the intrapopulational genetic variability analyses.

CONCLUSIONS

There is genetic variability among the various coconut tree populations available in the species Germplasm Bank at Embrapa Trópicos Costeiros.

The RAPD analysis technique with compound samples discriminated between different types of coconut trees (dwarf and giant) and between different

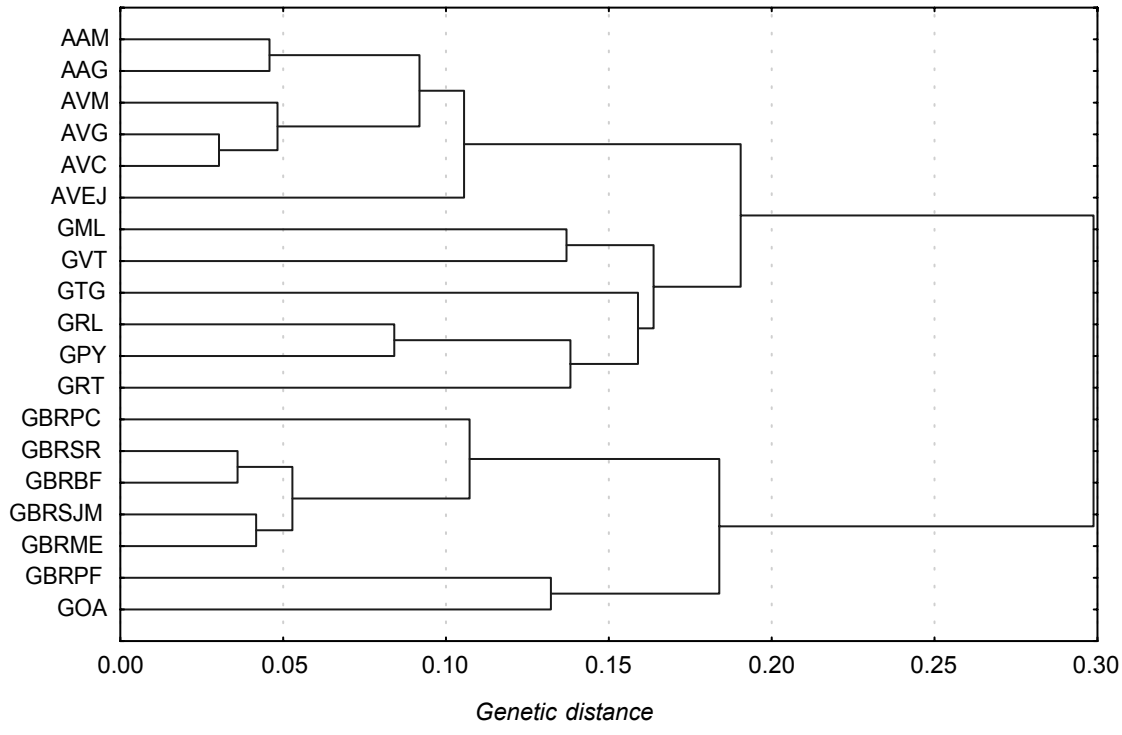

Figure 1. Clustering obtained by the hierarchical closest neighbor method based on the arithmetical complement of the Jacard similarity index of nineteen coconut tree (*Cocus nucifera L.*) accessions assessed by LMGV/CCTA/ UENF, in Campos dos Goytacazes, RJ.

accessions within each one of the assessed types.

The genetic divergence among dwarf accessions was smaller than that observed among giant accessions and between giant and dwarf accessions. This indicated that fruit color may be the main discriminating factor between the dwarf accessions, while variability between giant accessions is present in other traits not considered in this study.

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RESUMO

Divergência genética em coqueiro por marcadores RAPD em amostras compostas

O coco (*Cocos nucifera* L.) é uma espécie tropical amplamente cultivada no mundo, encontrada em todas as regiões intertropicais. Esta espécie possui uma grande variação fenotípica. Contudo, esta variabilidade é pouco conhecida ao nível genético. O estudo da variabilidade entre as diversas populações de coqueiro é importante para facilitar o desenvolvimento de cultivares superiores adaptados às diferentes condições ecológicas, além da seleção de progenitores divergentes que possibilitem maximizar a heterose em hibridações. Objetivou-se estimar a divergência genética entre 19 populações de coqueiro disponíveis no BAG-Coco da EMBRAPA/CPATC por meio de marcadores RAPD. Amostras de folíolos provenientes de 21 plantas por cultivar foram maceradas conjuntamente (amostras compostas) em nitrogênio líquido e, em seguida, procedeu-se à extração de DNA pelo protocolo de Doyle and Doyle, 1990, modificado. Amostras destes DNA foram submetidas à amplificação com 24 iniciadores da série OPERON Technologies. Foram obtidos 127 locos polimórficos e 61 monomórficos. Por meio da matriz do complemento do índice de Jacard, a análise de agrupamento pelo método de otimização de Tocher indicou a formação de 6 grupos distintos, possivelmente constituindo-se em grupos heteróticos. O grupo 1 reuniu os cultivares do grupo anão. Acessos gigantes, abreviados por GBR (Gigante do Brasil), formaram o grupo seguinte (2), exceto para GBRPF, que juntamente com Gigante Oeste Africano (GOA), formou outro grupo (4). Mais distante de todos, o cultivar Gigante de Tonga (GTG) não se agrupou com os demais, apresentando potencial para hibridação com os cultivares do grupo anão (em número de 6) e com os do grupo GBR (5 cultivares). O grupo 3 foi constituído de GRL, GPY e GRT e o grupo 5 de GML e GVT. O dendrograma obtido pelo método do vizinho mais próximo foi altamente concordante com o agrupamento obtido pelo método de otimização de Tocher. Os marcadores utilizados permitiram identificar cada uma das populações indicando serem geneticamente distintas (ausência de duplicidade). O uso de amostras compostas foi efetivo para concluir sobre a diversidade genética interpopulacional. Contudo, para o conhecimento da variabilidade genética intrapopulacional, será necessário o uso de amostras individuais.

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