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Full Length Research Paper

Assessment of the genetic diversity conservation in three tall coconut (*Cocos nucifera* L.) accessions regenerated by controlled pollination, using microsatellite markers

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Controlled pollination process is used during the regeneration of ageing accessions in the International Coconut Genebank for Africa and Indian Ocean (ICG-AIO). The effectiveness of this process has not yet been evaluated. This study aimed to evaluate the effectiveness of controlled pollination method by investigating the level of molecular resemblance between the regenerated (G1) and parental (G0) populations of three tall coconut accessions, Mozambique Tall (MZT), Gazelle Peninsula Tall (GPT) and Tahitian tall (THT) using 15 microsatellites (SSR) markers. The results indicate a relative reduction of gene diversity during regeneration. It decreased from 0.690 to 0.587, but, low values of Jaccard's dissimilarity index were found between regenerated and parental populations of tall coconut, varying from 0.072 to 0.133. Low values of genetic diversity between G0 and G1 tall coconut accessions (D_{ST}), ranging from 0.005 to 0.007, were recorded. Consequently, using controlled pollination technical as regeneration method of the genebank is effective for satisfying maintenance of the genetic integrity of the original coconut accessions.

Key words: Tall coconut palm, controlled pollination, regenerated accessions, genetic diversity, microsatellites markers.

INTRODUCTION

The coconut (*Cocos nucifera* L.) is a perennial oil crop from *Arecaceae* family (Teulat et al., 2000). This plant is cultivated in the wet tropics where it is an important source source of incomes for about 10 million families (IPGRI, 2004). It has a spectacular morphological diversity which is observed in the colour, size and shape of the fruits. The research programs in many countries are based on the exploitation of the ecotypes diversity (Bourdeix et al., 1992). In Côte d'Ivoire, there are 53 ecotypes whose 37 talls and 16 dwarves were introduced between 1960 and 1980 (Bourdeix et al., 2010) from Africa, Southern Pacific,

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Table 1. Populations of tall coconut palm including three parental (G0) and three regenerated (G1) populations investigated in the present study of the genetic diversity by microsatellite markers. Population code, origin and sample size are also shown.

Population	Generation	Code[a]	Origin	Sample size
Mozambique Tall	Parental	MZT G0	Mozambique	13
	Regenerated	MZT G1	Côte d'Ivoire	57
Gazelle Peninsula Tall	Parental	GPT G0	Papouasie	16
	Regenerated	GPT G1	Côte d'Ivoire	56
Tahitian Tall	Parental	THT G0	Polynésie	19
	Regenerated	THT G1	Côte d'Ivoire	51

[a]G0, parental population of coconut palm; G1, first cycle of regenerated population of the coconut palm.

Far East, Indian Ocean and Latin America. Nowadays, those accessions which are conserved at field are old. The inflorescences of the tall coconut genotypes which height exceeds 12 m become inaccessible (Bourdeix et al., 2010). Such situation restrains reproduction of the accessions and production of improved hybrid seeds for needs of research and development. Consequently, the regeneration of ageing accessions is regularly undertaken to continue the seed production. This regeneration is realized using controlled pollination method (De Nuce et al., 1980) which involves the risks of loss of genetic diversity into regenerated accessions. Indeed, sampling of parental palms from the initial accession can induce the fixing or elimination of some alleles, especially for the tall coconuts that are primary outcrossing (Vencovsky and Crossa, 2003; Konan et al., 2007). Moreover, the controlled pollination allows only 1.1 to 20.5% of seeds per inflorescence which reduces the regenerated accessions size (Yoboue, 2009). Consequently, the risks of gene losses from a generation to another seem to be feared.

In the International Coconut Genebank for Africa and Indian Ocean (ICG-AIO) hosted by Côte d'Ivoire, several authors had already studied the genetic diversity of the first introductions at the morphological (N'cho et al., 1993), biochemical (Jay et al., 1998) and molecular (Lebrun et al., 1998; Konan et al., 2007) levels, but, no information is yet available about the genetic diversity of the regenerated accessions and an eventual gap of genetic diversity which could result in the current method of controlled pollination used in the coconut genebank renewal. Microsatellites markers are known to be highly reliable for the studies of genetic diversity and identification of cultivars, for their extreme polymorphism and mendelian heredity (Pintaud et al., 2006). The results presented in this study relate to evaluation of the effectiveness of controlled pollination method by comparing molecular diversity level between parental and regenerated accessions from three tall coconut populations.

MATERIALS AND METHODS

Plant materials

212 genotypes were sampled from ICG-AIO at the Marc Delorme research station in Côte d'Ivoire (Table 1). Those genotypes were sampled from six populations of coconut palm including three parental (G0) and three regenerated (G1) accessions. Genotypes were chosen based on genealogical and homogeneity criteria. Genealogical criterion was based on parental information available among members of each population of coconut palm. Indeed, the regenerated populations Mozambique tall (MZT G1), Gazelle Peninsula tall (GPT G1) and Tahitian tall (THT G1) coconut palms were obtained by inter-crossing 13 MZT G0, 16 GPT G0 and 19 THT G0 genotypes, respectively according to controlled pollination method (De Nucé et al., 1980; Konan et al., 2008). The crossing design was MZT G0 x MZT G0, GPT G0 x GPT G0 and THT G0 x THT G0. According to the homogeneity criterion described by Ribeiro et al. (2010), populations exclusively composed of genotypes from the tall coconut generation were selected.

Methods

DNA extraction

DNA was extracted from 150 mg of fresh coconut palm leaflet using the MATLAB protocol as described by Risterucci et al. (2000). DNA extract was quantified by the comparison of its colour with a control sample under ultraviolet light after revelation with ethidium bromide.

PCR amplification

PCR amplification was carried out using 15 microsatellites (SSRs) markers synthesized by the Centre International de Recherche Agronomique pour le Developpement (CIRAD) (Table 2). PCR program consisted an initial denaturation at 94°C for 5 min, 35 cycles of amplification each consisting of 30 s denaturation at 94°C, 1 min annealing at 51°C and 1 min elongation at 72°C. PCR amplification was performed in 10 µl reactions containing 20 ng of template DNA, 1x amplification buffer, 0.25 µM of primer, 2.5 mM of $MgCl_2$, 0.2 mM of each dNTPs and 1 U of Taq polymerase. Reactions were overlayed with one drop of mineral oil and MJ-Research Peltier Thermocycler (PTC-100 Model) was used for amplification. 5 µl of PCR

Table 2. Number of alleles per locus, percentage of polymorphic allele and gene diversity (He) estimated for six populations of tall coconut palm including three parental (G0) and three regenerated (G1) populations, using 15 microsatellite markers.

Locus	Number of allele	Percentage of polymorphic allele (%)	Gene diversity (He)
CnCirA3	7	45.53	0.735
CnCirA9	8	44.60	0.724
CnCirB6	7	59.62	0.601
CnCirB12	14	28.32	0.850
CnCirC5	14	34.92	0.835
CnCirC7	7	32.37	0.777
CnCirC12	4	31.08	0.735
CnCirE2	13	21.29	0.869
CnCirE10	7	54.95	0.649
CnCirE12	4	70.63	0.434
CnCirF2	7	42.93	0.748
CnCirG11	7	49.49	0.690
CnCirH7	7	31.06	0.764
CnCirH11	5	36.84	0.731
CnZ40	12	16.14	0.870
Mean	8.2	39.98	0.734
Standard deviation	3.382	14.65	0.113

products were separated in a 6% polyacrylamide gel in 1x Tris-borate-EDTA (TBE) by electrophoresis using a sequencer (Ddh-400-33 Model) at 55 W for 2 h. Then, the products were revealed using a silver staining method as described by Creste et al. (2001).

Statistical analysis

The matrix of analysis was recorded according to the allele size. The diversity index such as percentage of polymorphic allele, the number of alleles, the allelic richness and expected heterozygosity also known as gene diversity (He =1-$\sum Pi^2$) according to Nei (1973) were given using GENETIX software version 4.05 (Belkhir et al., 2001). The significant differences of the mean allelic richness and the mean gene diversity between G0 and G1 accession generations were examined according to Mann–Whitney U test using software STATISTICA version 7.1 (StatSoft France, 2005). The genetic differentiation between regenerated and parental accessions was assessed using genetic diversity between generations of accessions ($D_{ST} = H_T - H_S$) and degree of genetic differentiation ($G_{ST} = 1-Hs / H_T$). A multivariate analysis termed factorial analysis of correspondence (FAC) was performed using the graphic menu FAC 2D from GENETIX software version 4.05 (Belkhir et al., 2001). It was carried out to structure the parental and regenerated individuals for each population of tall coconut palm and to check their genetic proximities. In addition, a dissimilarity matrix was created between regenerated and parental accessions with the obtained binary matrix using Jaccard's index. The dissimilarity matrix was then used for hierarchical cluster analysis. The hierarchical cluster analysis was examined in order to estimate genetic relationship between regenerated and parental populations of coconut palm. For the last analysis, data were scored in the disjunctive form (0 to 1) and analyzed using XLSTAT software version 7.5.3 (Xlstat, 2005).

RESULTS

Polymorphism of microsatellite (SSR) markers

15 SSR primers produced a total of 123 alleles (Table 2). The loci CnCirB12 and CnCirC5 gave higher allele number (14) whereas the locus CnCirE12 provided the lowest number of alleles (4). An average of 8.2 alleles was observed per locus. The mean gene diversity was 0.734, ranging from 0.434 to 0.870. All studied loci were polymorphic at 95 % significant level.

Diversity of parental G0 and regenerated G1 accessions

Variations within genetic diversity level between regenerated and parental accessions were revealed (Table 3). The number of alleles detected in the regenerated accessions (66 to 94) was relatively higher than the parental accessions one (65 to 88). However, the allelic richness variation was not significant ($p > 0.05$) between generations (Table 3). Also, the reduction of gene diversity from G0 to G1 generations, estimated between 2.5 and 9.2%, was not significant ($p > 0.05$). Four private alleles, with frequencies varying from 0.033 to 0.071, were not transmitted from parental accessions to regenerated ones (Table 4). Eight, three and five private alleles were identified in the regenerated accessions MZT G1, GPT G1 and THT G1, respectively (Table 4). These allele frequencies were lower than 0.05.

Molecular divergence between parental and regenerated accessions

The mean genetic diversity between each regenerated and parental accession (D_{ST}) was low. D_{ST} values recorded were 0.007, 0.005 and 0.006 for MZT, GPT and THT,

Table 3. Number of allele per population, allelic richness and gene diversity for six populations of tall coconut palm including three parental (G0) and three regenerated (G1) populations.

Index	MZT		GPT		THT	
	G0	G1	G0	G1	G0	G1
Number of alleles	75	82	65	66	88	92
Allelic richness	5	5.466	4.333	4.4	5.866	6.133
Standard deviation	0.543	0.608	0.333	0.349	0.646	0.613
P	0.583 ns		0.898 ns		0.690 ns	
Gene diversity (He)	0.676	0.619	0.647	0.587	0.690	0.672
Standard deviation	0.133	0.171	0.131	0.161	0.226	0.203
P	0.372 ns		0.213 ns		0.383 ns	
(G0-G1)/G0	0.085		0.092		0.025	

MZT, Mozambique tall; GPT, Gazelle Peninsula tall; THT, Tahitian tall; P, probability value of Mann–Whitney U test; ns, not significant at level of 5%; G0, parental population of coconut palm; G1, first cycle of regenerated population of the coconut palm; (G0-G1)/G0, reduction rate of gene diversity from G0 to G1 generation.

Table 4. Frequencies of the private alleles identified in parental (G0) and regenerated (G1) populations of tall coconut palm studied.

Population	Generation	Locus	Allele	Frequency
MZT	G0	CnZ40	158	0.041
	G1	CnCirA3	248	0.019
		CnCirA9	089	0.019
		CnCirB12	163	0.011
			173	0.011
		CnCirC5	082	0.009
			094	0.009
		CnCirH7	113	0.010
		CnZ40	136	0.020
GPT	G0	CnCirC12	167	0.041
		CnCirG11	198	0.033
	G1	CnCirA3	242	0.011
		CnCirA9	099	0.010
		CnCirE10	244	0.019
THT	G0	CnCirC5	102	0.071
	G1	CnCirB12	151	0.011
		CnCirC7	169	0.021
		CnCirC12	163	0.033
		CnCirF2	197	0.034
		CnCirH7	149	0.042

G0, Parental population of coconut palm; G1, first cycle of regenerated population of the coconut palm; MZT, Mozambique tall; GPT, Gazelle Peninsula tall; THT, Tahitian tall.

respectively. Likewise, the degree of genetic differentiation (G_{ST}) between regenerated and parental accessions was less than 2% of the total genetic diversity (Table 5). The FAC revealed a very great genetic proximity between regenerated and parental genotypes of MZT, GPT and THT. However, within each population of coconut palm, the structuring showed particular individuals at the sight of their isolation from the homogeneous group constituted by regenerated and parental individuals (Figure 1). The mean genetic dis-similarity index between generations of the tall coconut populations was 0.11, ranging from 0.072 to 0.133. Results of the hierarchical cluster analysis showed that the microsatellites markers distinguished the three tall coconut populations. However, G1 and G0 ge-

Table 5. Index of Nei's genetic diversity of parental (G0) and regenerated (G1) populations of tall coconut palm studied.

Population	Gene diversity between accession generations (D_{ST})	Degree of genetic differentiation (G_{ST})
MZT	0.007	0.011
GPT	0.005	0.009
THT	0.006	0.009

MZT, Mozambique tall; GPT, Gazelle Peninsula tall; THT, Tahitian tall.

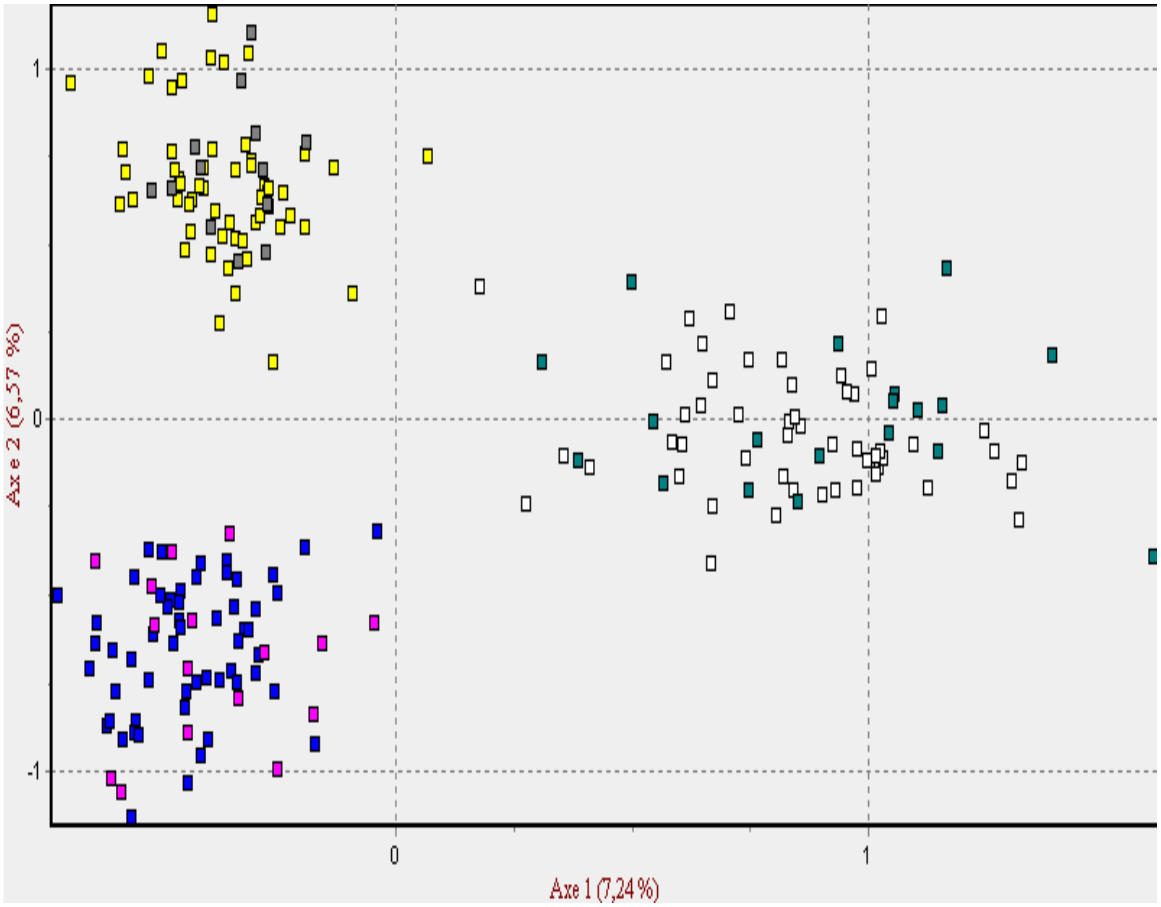

Figure 1. First plane of the FAC performed on 212 genotypes from six populations of tall coconut palm including three parental (G0) and three regenerated (G1) populations. Dark square, parental genotypes from coconut population MZT G0; yellow square, regenerated genotypes from coconut population MZT G1; rose square, parental genotypes from coconut population GPT G0; blue square, regenerated genotypes from coconut population; GPT G1; green square, parental genotypes from coconut population THT G0; white square, regenerated genotypes from coconut population THT G1.

nerations of each tall coconut population were more genetically close (Figure 2).

DISCUSSION

The mean number of allele per locus (8.2) and the mean gene diversity (0.771) of all the studied tall coconut populations were high. These results confirm a high level of polymorphism using SSR markers for coconut diversity analysis. Similar results were reported by Teulat et al. (2000) about polymorphism of 39 microsatellites markers with 14 coconut populations. These authors reported 9.14 alleles per locus and a diversity index varying from 0.47 to 0.90. Earlier, 7.35 allele/locus with He = 0.66 (Rajesh et al., 2008) and 6.3 allele/locus with He = 0.65 (Perera et al., 2000) were reported also in tall populations of coco-

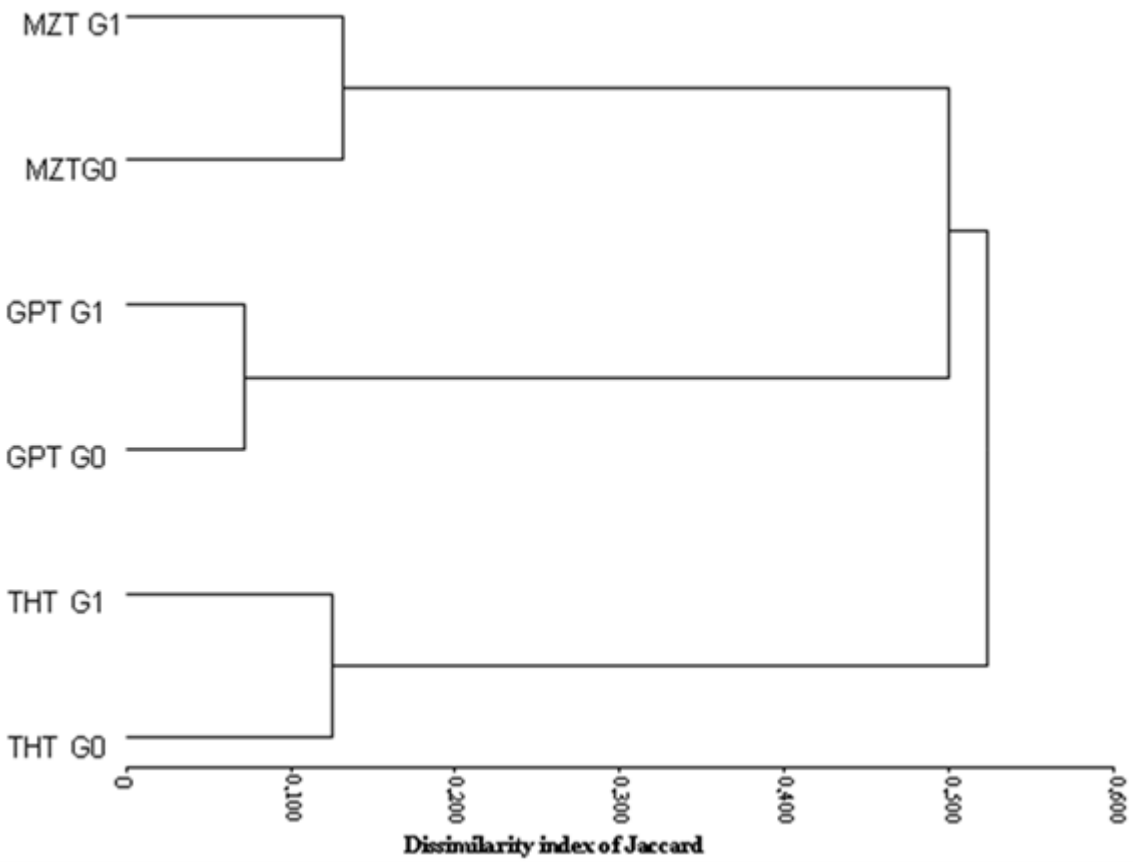

Figure 2. Dendrogram derived from cluster analysis of SSRs markers based on genetic dissimilarity matrix showing the genetic relationships between six populations of tall coconut palm including three parental (G0) and three regenerated (G1) populations.

nut palm genetic diversity studies with SSRs markers.

Allelic richness varied from 4.33 to 6.13. These values were higher than those of Konan (2008) who evaluated the genetic diversity of 39 tall coconut accessions within Côte d'Ivoire genebank. This author reported that each accession involved an average number of alleles ranging from 1.92 to 4.08. The high values of allelic richness recorded in this study could be explained by the high number of individuals sampled (13 to 57) per accession. Indeed, the low size of sampling could underestimate the genetic diversity of the assessed coconut populations (Herrera et al., 2010). It is notified that the tall coconuts are preferentially cross-pollinated and have high level of genetic diversity within populations (Konan et al., 2007). After one cycle of regeneration, the controlled pollination method induced a loss of parental gene diversity from 2.5 to 9.2%. Compared with study reported by Elizabeth et al. (2006) about resemblance between corn populations preserved *in situ* and *ex situ* with a gap of 14.2% of the gene diversity (0.7 vs 0.6), the decreasing of gene diversity values in the regenerated populations of tall coconut palm could be considered as low. This result is in agreement with the values found from Mann–Whitney U test probability ($p > 0.05$), D_{ST}, G_{ST} and Jaccard's dis-similarity index. Diversity between accession generations (D_{ST}) varied from 0.005 to 0.007. Only 0.9 to 1.1 % of total gene diversity is due to the difference between studied regenerated and parental tall coconut accessions. These results could translate a low genetic differentiation between founder parents and first generation of regenerated coconut accessions. Likewise, with degree of genetic differentiation (G_{ST}) value of 0.62%, Kumar et al. (2011) concluded lower genetic differentiation between Andaman coconut populations. In the same way, dissimilarity index between regenerated and parental populations of tall coconut ranged from 0.072 to 0.133. So, genetic similarity coefficients between regenerated and parental generations of accessions which vary from 0.867 to 0.928 were close to unit. Based on study reported by Kumar et al. (2011) about molecular resemblance between South Pacific coconut populations with a similarity index varying from 0.867 to 0.93, the studied parental and regenerated populations of tall coconut could be considered as identical.

However, a relative reduction of gene diversity was observed from G0 to G1 coconut palm generations. The evo-

lutionary of genetic diversity for the coconut during regeneration would support the homozygote individuals. Similar results were found from wild G0 to wild x domestic G3 populations of sunflower (Noryazdan, 2009). The lost of genetic diversity could be explained by the sampling effect which has been described by Noryazdan (2009) as the inbreeding effect due to genetic proximity of selected parents. Indeed, the sequential sampling of parents or seed batches for the constitution of the following generation could reduce the genetic base within initial population and involves inbreed crossings. Populations of coconut palm introduced into the Côte d'Ivoire genebank were collected from the origin area with mass selection of the seeds provided by the high-yielding coconut palms (De Nucé and Wuidart, 1981). Thus, parental individuals of MZT population were collected from Mozambique plantation Company, the Companhia do Boror whose seeds probably originated in India (De Nucé and Wuidart, 1979). Originally, individuals of THT and GPT came from seeds selected respectively at Tahiti (Polynesia) and New Guinea (De Nucé and Wuidart, 1979; Bourdeix, 1990). This sampling of parental individuals which are narrow genetic base could explain the reducing of heterozygote genotypes within the rege-nerated populations of coconut palm studied. It was clearly established that sequential rejuvenations of genebank involve significant fluctuations of allelic frequencies in regenerated populations (Johnson et al., 2002; Johnson et al., 2004). Also, this fact can be explained by the unbalance number of allele between G0 and G1 accession generations. The non inheritance of some alleles into the regenerated accessions could be due to the high pressure in parent palms sampling as mentioned by Harlen (1992).

This study reveals that regenerated accessions can contain off-types or illegitimate individuals. Their detection was carried out; thanks to molecular markers such as SSRs. As the G1 genotypes come from crossings between G0 genotypes, the private alleles identified within regenerated accessions could come from off-types. Indeed, during regeneration planning, six to 10 parental accessions were treated at the same time. Consequently, several factors can affect the purity of the original accessions. The sources of contamination could come from the adulteration by foreign pollen during fertilization, seeds adulteration during harvesting and packaging (Breese, 1989). Also, the errors of the seedlings positioning could occur during establishment of regenerated accession at field.

In conclusion, the results show that the regenerated and parental populations of tall coconut palm studied are very close genetically. The genetic diversity level of the initial population remains no more statical during regeneration. For an important operation like the coconut genebank regeneration, a light change of the initial genetic diversity must alert the curators on the risks of gene losses during sequential regenerations. For the first generation, the controlled pollination technical preserved the genetic diversity level of the original tall coconut accessions.

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Abbreviations

ICG-AIO, International Coconut Genebank for Africa and Indian Ocean; **MZT,** Mozambique tall; **GPT,** Gazelle Peninsula tall; **THT,** Tahitian tall.

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