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Coconut Microsatellite Gene Diversity Analysis Technology Transfer to Côte D'ivoire

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Abstract: This study aims to develop a molecular method easily applicable in Côte d'Ivoire for the International Coconut Genebank for Africa and Indian Ocean (ICG-AIO) finest characterization. A total of 62 individuals from eleven coconut accessions were used for microsatellite gene diversity analysis experienced. The mean number of alleles and the mean gene diversity within accession were 3.08±1.11 and 0.536±0.218, respectively for all accessions studied. By cluster analysis, accessions were structured into two main gene pools. Coconut accessions originated to south pacific were clustered together with the Sri-Lanka Green Dwarf accession and those from Africa grouped in a second cluster. Within the two groups, it was observed divergences between some accessions. These first results evidently indicated that microsatellite markers can be successfully applied in Côte d'Ivoire to study coconut resources of the ICG-AIO, as it's important for ongoing efforts to facilitate local coconut resources characterization and conservation.

Key words: Coconut, microsatellite, technology transfer, Côte d'Ivoire

INTRODUCTION

Cocos nucifera L is a tropical tree crop originating from the indo-Pacific region (Zizumbo *et al*., 2002; Lebrun *et al*., 1998a). Because of its various utilisations, coconut tree is playing a very important role in the economy of many tropical countries where it's cultivated. About 10 million families rely on coconuts as their main source of food and incomes (Moore and Batugal, 2004).

Most of the coconut traditional cultivars or varieties are relatively low productive and due to the strong competition from the other oil crops and phytopathologic constraints generated specially by the lethal yellowing disease, coconut cultivation is declining in many countries. To face these difficulties, coconut breeding programs were developed through the world. Their efforts aim to select new coconut cultivars with high yield and tolerance to phytopathologic stresses. The selection scheme is mainly based on crossing dwarf X tall or tall X tall coconut cultivars from various geographical ranges (Baudouin *et al*., 1997). In general, identification of genetic diverse parental combinations is a prerequisite to increase heterosis in any crop. Teulat *et al*. (2000) reported that the International Coconut Genetic Resources Network (COGENT) recognised to date about 900 coconut accessions. The first step of a better use in selection of the whole coconut resources is to conduct an extensive survey of the genetic diversity.

Traditional approach of coconut population characterization and evaluation based on morphological and agronomic traits is constrained by environment plasticity of the traits under consideration and provides a simplified image of diversity (Sugimura *et al*., 1997). Recently, the development of various molecular marker techniques such as ISTR (Rohde *et al*., 1995), RFLP (Lebrun *et al*., 1998a, b), RAPD (Ashburner *et al*., 1997; Upadhyay *et al*., 2004), AFLP (Perera *et al*., 1998; Teulat *et al*., 2000) and SSR (Perera *et al*., 2003; Meerow *et al*., 2003) offers powerful alternative tools for analysing coconut resources. But, only a few methods are useful in networked efforts across several continents consistent with a cogent strategy. Results must be reproducible and the analysis using standardised scoring and analytical methods.

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Microsatellites are one of the promising molecular markers which meet conditions indicated. The genome of all eukaryotes contain a group of mono-, di-, tri-or tetranucleotid repeats, termed microsatellites or simple sequence repeats. These sequences are known to be abundant and high polymorphic. A moment ago, a microsatellite kit, financially supported by the International Plant Genetic Resources Institute (IPGRI) and the COGENT, was developed at Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) Montpellier, France (Baudouin and Lebrun, 2002). Since the workshop which took place at Cirad in Montpellier, France on April 2002, the microsatellite markers still disposed to the countries members of the Cogent network for coconut genetic resources analysis.

The objectives of this study were to transfer the microsatellite gene diversity analysis technology to Côte d'Ivoire and to follow its application for the local International Coconut Genebank for Africa and Indian Ocean (ICGAIO) resources characterization.

MATERIALS AND METHODS

The present investigation was conducted from March to June 2004 at Laboratoire Central de Biotechnologies (LCB) of Centre National de Recherche Agronomique (CNRA), Côte d'Ivoire.

Plant material: The International Coconut Genebank for African and Indian Ocean relevant to coconut breeding program in Côte d'Ivoire was made available by CNRA. In this research, seven tall coconut accessions introduced from south pacific region and two accessions from Africa were used. In addition to these accessions, two accessions were originated from a screening trial located to Lethal Yellowing Disease (LYD) endemic area in Ghana. These last accessions are reported to be tolerant to lethal yellow disease, since they were exposed to LYD more than twenty year ago. A total of eleven accessions were used for the microsatellite analysis technology experienced (Table 1). Letters have been used to indicate cultivar types and the numbers to specify the accessions or populations analyzed.

Methods

Sampling and DNA extraction: A green leaflet, not damage, was taken from the youngest leaves of the coconut palm. Total genomic DNA was extracted from 1 g freeze leaflet tissue per coconut palm using a matab protocol as described by Risterucci *et al.* (2000). DNA was then quantified comparing the fluorescence intensities of the ethidium bromide treated samples to those of reference standard diluted DNA excerpts in 1% agarose gel electrophoresis under UV light.

PCR: PCR amplification was performed in 10 μL reaction mixture contained 25 ng template DNA, 10 mM Tris, 50 mM KCl, 2.25 mM $MgCl_2$, 0.001% glycerol, 200 μM of each dNTPs, 0.2 μM of each primer and 1 unit taq polymerase. Reactions were overlayed with one drop of mineral oil. PTC 100 thermal cycler was used for amplification. The PCR regime consisted of an initial denaturation (94°C) for 5 min, 35 cycles each consisting of 30 sec denaturation (94°C), 1 min annealing (51°C) and 1 min elongation (72°C). At the end of the final run, an extension period of 30 min at 72°C was observed. The primers used were developed by the Center of International cooperation in Agronomic Research for the Development (Cirad), Montpellier France and described by Baudouin and Lebrun (2002).

The PCR products were separated on 5% acrylamide gels. The gels were run at constant power of 55 W for 2 h in 1X TBE buffer. Then the products were revealed using a silver staining method as described by Creste *et al.* (2001).

Statistical analysis: For each individual X marker combination, microsatellite bands were scored using allele size. The genetic polymorphism for each accession was thus estimated as the mean number of alleles per locus and Nei's (1973) gene diversity, also know as expected

Table 1: Coconut cultivars and accessions of the International Coconut Genebank for Africa and Indian Ocean used for the SSR study initiation

Cultivar	Accession	Site	Region	Country	No.
Kar kar tall	KKT'75	ICGAIO	Pacific	Papua N.G.	5
Markham v. tall	MVT'84	ICGAIO	Pacific	Papua N.G.	7
Gazelle tall	GPT'84	ICGAIO	Pacific	Papua N.G.	7
Rotuma tall	RTMT'70	ICGAIO	Pacific	Fiji	6
Tonga tall	TONT'70	ICGAIO	Pacific	Tonga	5
Rennel Island tall	RIT'88	ICGAIO	Pacific	Salomons	6
Salomons I. tall	SIT'88	ICGAIO	Pacific	Salomons	6
Vanuatu tall	VTT'82	Ghana	Pacific	Vanuatu	8
Sri-Lanka Green Dwarf	SGD'82	Ghana	Indian Oc.	Sri-Lanka	6
West African tall	WAT'82	ICGAIO	Africa	Côte d'Ivoire	6
Kiribi tall	CKT'81	ICGAIO	Africa	Cameroon	4

heterozygosity ($D = 1-\Sigma Pi^2$). These analyses were performed using molecular diversity indices module of the software Arlequin vs 3.1. The genetic relationship between the accessions tested was estimated using cluster analysis. These last analyses were performed using the software Xlstat vs 7.5. The analyses were applied on pairwise FST differentiation index between the accessions.

RESULTS

Leaflet samples were collected from the middle part of leaf No. 1 or No. 2 and labelled correctly (Fig. 1a). DNAs extracted from the samples collected were tested and quantified on 1% agarose gel (Fig. 1b). In general, satisfactory DNAs were obtained and were used for PCR reactions (Fig. 1c). Twelve microsatellite markers were used for PCR amplification. The whole PCR products showed polymorphism as exemplified in Fig. 1d. A total of 95 different alleles were scored. The mean number of alleles and the average gene diversity in each accession are indicated in Table 2. The Sri-Lanka Green Dwarf showed the least allelic diversity with a mean gene diversity of 0.198±0.218, while that with the highest was Tonga tall with mean 0.678±0.229 gene diversity. On average, genetic diversity was lower amongst the African coconut accessions compared to the accessions from Pacific region (Table 2). The mean number of allele per locus and the mean gene diversity for the 62 genotypes were, respectively 3.08±1.11 and 0.536±0.218.

The First pairwise index was estimated to evaluate difference between the genotypes. The Fst pairwise differentiation index ranged from 0.036 between Rotuma tall accession and Salomons Island tall accession to

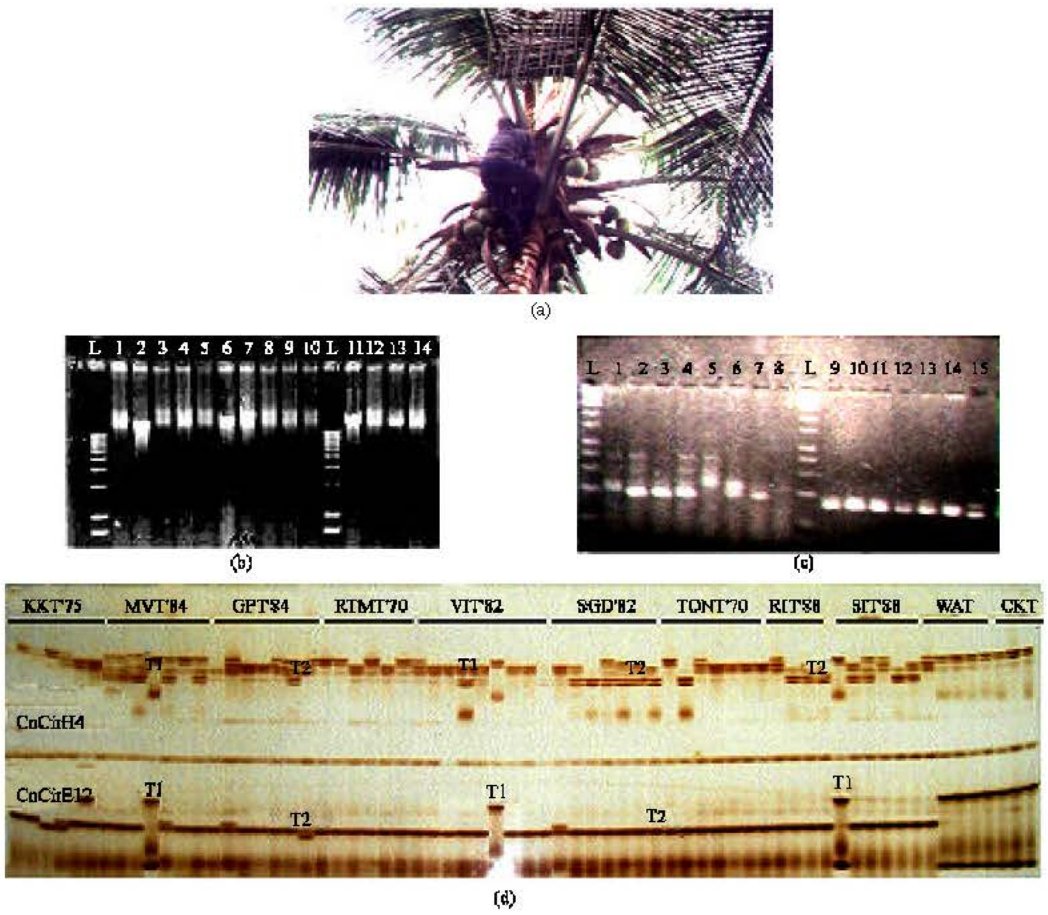

Fig. 1: Coconut microsatellite gene diversity analysis: (a) Leaf sample collection. (b) DNA extracted from different coconut trees. (c) example of PCR products separated on 4% agarose gel and (d) example of PCR product sequencing on 5% acrylamide gel (primers CnCirH4 and CnCirE12 multiplex separation), L = 1 kb ladder; T1 and T2 are standard samples used for allele size determination

Table 2: Genetic diversity within eleven coconut accessions

Accession	Allele No.	Allele/locus ±SD	Gene diversity ±SD
Accessions from Pacific			
KKT'75	39	3.25±1.30	0.618±0.181
MVT'84	46	3.83±1.28	0.638±0.183
GPT'84	46	3.83±1.46	0.639±0.181
RTMT'70	41	3.42±1.11	0.635±0.237
TONT'70	42	3.50±1.04	0.678±0.229
RIT'88	34	2.83±1.07	0.491±0.271
SIT'88	48	4.00±1.47	0.659±0.213
	Mean	3.52±1.25	0.623±0.214
Accessions from Africa			
WAT'82	25	2.08±0.86	0.377±0.246
CKT'81	24	2.00±0.71	0.439±0.275
	Mean	2.04±0.78	0.408±0.260
Reference accessions			
VTT'82	42	3.50±1.12	0.533±0.164
SGD'82	20	1.67±0.74	0.193±0.218
	Mean	2.58±0.93	0.363±0.191

Table 3: Pairwise genetic differentiation (Fst below diagonal) between accessions and p-value (above diagonal). The p-value is the probability of obtaining an equal or more extreme value by chance alone, estimated from 1023 permutations

	KKT'75	MVT'84	GPT'84	RTMT'70	TONT'70	RIT'88	SIT'88	VTT'82	SGD'82	WAT'82	CKT'81
KKT'75	-	0.225	0.0088	0.0605	0.0078	0.0059	0.0312	0.0000	0.0000	0.0029	0.0098
MVT'84	0.0668*	-	0.023	0.0000	0.0019	0.0010	0.0556	0.0000	0.0029	0.0000	0.0000
GPT'84	0.0823	0.0593*	-	0.0019	0.0010	0.0010	0.0039	0.0000	0.0000	0.0000	0.0000
RTMT'70	0.0796*	0.1244	0.1267	-	0.2422	0.0049	0.3525	0.0010	0.0010	0.0000	0.0000
TONT'70	0.1251	0.1343	0.1464	0.0487*	-	0.0166	0.1504	0.0000	0.0039	0.0010	0.0000
RIT'88	0.1734	0.1597	0.2440	0.1249	0.1548*	-	0.1123	0.0000	0.0010	0.0010	0.0000
SIT'88	0.0767*	0.0471*	0.1027	0.0360*	0.0529*	0.0703*	-	0.0000	0.0019	0.0010	0.0000
VTT'82	0.1253	0.1679	0.1751	0.1249	0.1723	0.1966	0.1420	-	0.0000	0.0000	0.0000
SGD'82	0.4444	0.3623	0.4477	0.4270	0.4487	0.4649	0.3733	0.4324	-	0.0019	0.0059
WAT'82	0.4830	0.4486	0.4530	0.4525	0.4404	0.5479	0.4485	0.5264	0.7113	-	0.0000
CKT'81	0.3944	0.2834	0.3034	0.3719	0.2952	0.3869	0.2987	0.3502	0.5947	0.3653	-

* Fst not significant, $\alpha = 1\%$

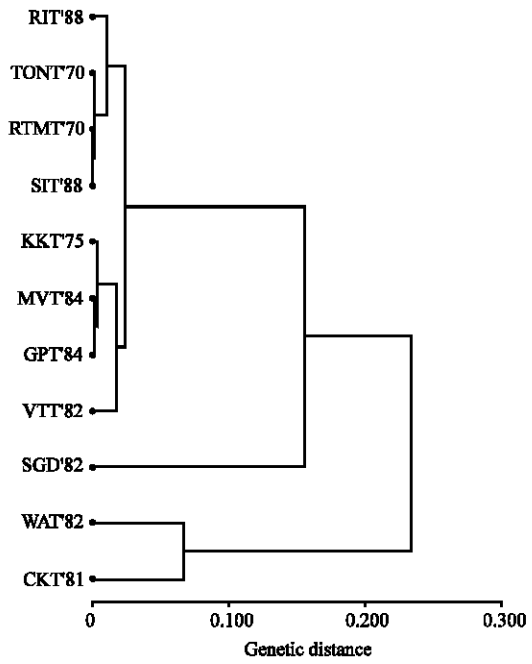

Fig. 2: Genetic relationships among eleven coconut accessions in Côte d'Ivoire based on scoring twelve microsatellite loci

0.7113 between West African tall accession and Sri-Lanka Green dwarf accession (Table 3). The genetic relationships (Fig. 2) constructed from the population pairwise FSTs showed two main clusters of accession. The first cluster included accessions originated to south pacific region and the two reference accessions supposed to be tolerant to lethal yellowing disease. In the second cluster were grouped together the West African tall and Cameroon kiribi tall. These results indicated that genetic structuring of the accessions analysed is correlated with their geographic region.

In the first group, accessions are grouped into three subgroups. The first subgroup concerned RIT'88, SIT'88, TONT'70 and RTMT'70. In the second subgroup were clustered VTT'82, KKT'75, MVT'84 and GPT'84. The SGD'82 was isolated in the third subgroup.

DISCUSSION

The mean number of alleles per locus (3.08±1.11) and the mean gene diversity (0.536±0.218) for all the coconut accessions studied were high suggesting a high level of genetic diversity within the coconut populations analysed. It is not an agreement with the results obtained by Ashburner *et al.* (1997). Those authors reported, for RAPD analysis of south pacific coconut palm

populations, a lower level of genetic diversity. So, present results corroborated that a high polymorphism can be detected by using microsatellite markers for coconut accessions genetic diversity analysis. However, it was notified that the mean number of alleles per locus and the gene diversity values were excessively lower for the Sri-Lanka Green dwarf accession compared to the others accessions. The self pollination of this accession certainly contributed to this unbalance. The Tall accessions are preferentially cross-pollination.

The genetic relationships among the eleven analysed coconut accessions were estimated by a cluster analysis of the Fst pairwise genetic differentiation between accessions matrix based on the microsatellite derived data. The result indicated that it's easy to distinguish coconut accessions experienced, particularly accessions originated to Africa from those collected across south pacific region. This indicated that the genetic relationships between these coconut accessions, based on microsatellite markers, are correlated with the geographic distribution. It's an agreement with the results observed in coconut genetic diversity analysis using RFLPs markers by Lebrun *et al*. (1998a). Those authors reported a clear partition of Far East and South Pacific coconut cultivars or varieties from those of Africa and Indian Ocean.

In present study, the two reference accessions, VTT'82 and SGD'82, identified to be tolerant to lethal yellowing disease, were mainly genetically closed to those from south pacific and clearly different to African ones. The Vanuatu tall accession (VTT'82) used in an experimental trial in Ghana was introduced in the International Coconut Genebank for Africa and Indian Ocean established in Côte d'Ivoire from south pacific too. Concerning the Sri-Lanka Green Dwarf, previous study reported that all dwarf coconut cultivars are originated from Far East and south pacific region and were more genetically similar to cultivars from these regions (Lebrun *et al.*, 1998b). The apparent genetic divergence of the two African accessions from the tolerant accessions certainly explained their comparatively susceptible to the lethal yellowing disease as reported by Eden-Green (1995). So, it is thought that a new lethal yellowing tolerance gene source could probably be identified with coconut resources from this region. Consequently, we think that possible new sources of tolerance to LYD should be explored among south pacific coconut genotypes and comprehensively in the neighbouring zones.

Moreover, if the two reference accessions were belonged to the same genetic group, it was notified a difference between these accessions. They were clustered into two subgroups. This suggests that relatively extensive gene diversity is present in the tolerant materials. It's evidently attested with the high within genetic diversity of the accession VTT'82, measured as mean number of alleles (3.50±1.12) and Nei's gene diversity (0.533±0.164).

These first results support two separate origins for the coconut accessions analysed, one through the south pacific and another through Africa. In addition, these results were a good example of a successful technology transfer. Local biotechnology laboratory can fully carry out applied coconut microsatellite gene diversity analysis research. Consequently, these results are useful for ongoing efforts to characterize the genebank ICG-AIO in order to facilitate resources utilization and conservation.

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