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Full Length Research Paper

Microsatellite gene diversity in coconut (*Cocos nucifera* L.) accessions resistants to lethal yellowing disease

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One of the problems faced in coconut cultivation is the lethal yellowing disease. Experimental trials, conducted in endemic region, showed that the Vanuatu Tall and Sri-Lanka Green Dwarf genotypes were tolerant while the West African Tall appeared susceptible to the lethal yellowing disease. Genetic differences between these tolerant genotypes and the susceptible ones were evaluated using twelve microsatellite markers. This work aimed to use identified materials as reference to select suitable parents for gene mapping studies. A total of 58 alleles were detected at the 12 microsatellite loci. The number of alleles varied from 3 to 7, with an average of 4.83 alleles. The F_{st} index revealed that 59.70% of the total allele variability explained differences between the three accessions. Genotypes of West African Tall, susceptible to the lethal yellowing disease, were less genetically clustered to the genotypes of the two tolerant accessions. This differentiation was based on specific alleles and frequency variation of shared allele in the three accessions. This molecular typology was useful as reference for large molecular screening of coconut genetic resources and the identification of suitable parents for the development of mapping populations for tagging the lethal yellowing resistance genes.

Key words: Coconut tree, lethal yellowing disease, microsatellite, tolerance.

INTRODUCTION

Cocos nucifera L. is a perennial crop plant widely cultivated in tropical humid areas. Most parts of this plant can be used (Persly et al., 1992). About 10 million families rely on coconuts as their main source of food and incomes (IPGRI, 2004). To day, coconut cultivation faces a strong phytopathologic constraint generated by the lethal yellowing disease. Since this disease was found around the 19th century in the Caribbean regions (Eden-Green et al., 1997, mentioned by Cordova et al., 2003), it has destroyed several thousands hectares of coconut fields (Rohde et al., 1993; Dery et al., 1995). Its incidence differs among the affected regions and referred to as Cape St. Paul Wilt in Ghana (Dabek et al., 1976), Kaincopé disease in Togo (Nienhaus and Steiner, 1976), Kribi disease in Cameroon (Dollet et al., 1977) and Awka disease in Nigeria (Ekpo and Ojomo, 1990). General symptoms are premature nuts fall, bronzing of successively younger leaves and blackening of inflorescences. Infected trees often die 4 to 6 months after appearance of symptoms (Mpunami, 1999). In spite of research efforts, no efficient methods have been identified yet to control this disease. The more explored solution is the use of tolerant coconut tree for replanting infested areas.

C. nucifera L. is the only species of the genus *Cocos*. It includes more than 300 cultivars or varieties (Lebrun et al., 1998). About 53 cultivars were introduced in collection in Côte d'Ivoire. Since 1980, screening for lethal yellowing tolerance was conducted in endemic area in Ghana by the National Center of Agronomic Research (Cnra),

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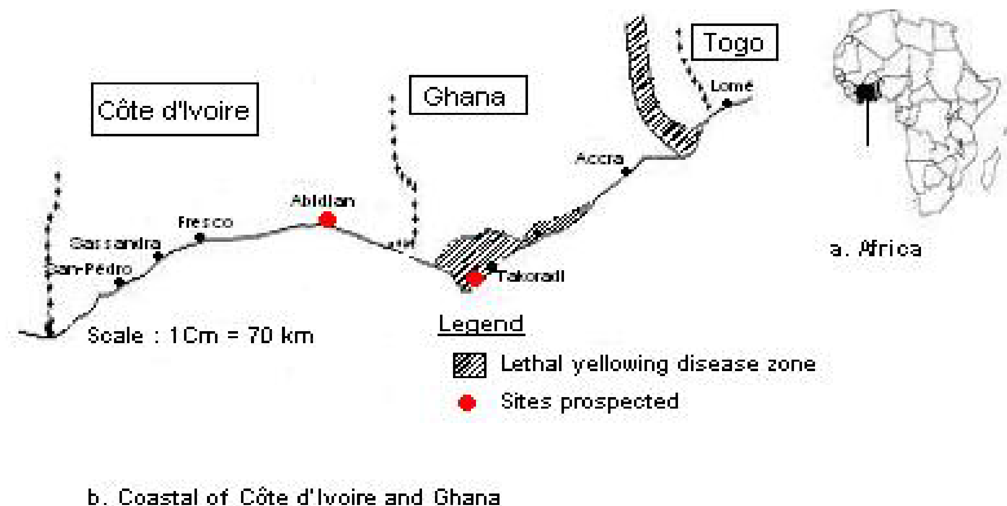

Figure 1. Sites from which coconut plants were collected.

Table 1. Coconut plants used in the study.

Accession	Localization	Sensibility	Number
Vanuatu Tall (VTT'82)	OPRI / Ghana	Tolerant	8
Sri-Lanka Green Dwarf (SGD'82)	OPRI / Ghana	Tolerant	6
West African Tall (WAT'82)	Cnra/ Côte d'Ivoire	Susceptible	7

in collaboration with the Coconut Research Program of the Oil Palm Research Institute (OPRI) of Ghana. Genotypes of Sri-Lanka Green Dwarf and Vanuatu Tall appeared mainly tolerant to the lethal yellowing disease. These materials are not genetically analysed.

Microsatellites markers have been developed in *C. nucifera* L. (Perera et al., 2001, 2003; Rivera et al., 1999; Teulat et al., 2000). Rivera et al. (1999) tested primers for 41 microsatellites loci on 20 coconuts and then used eight of these on a larger sample. Teulat et al. (2000) used 37 of the SSR loci developed by Rivera et al. (1999) to measure genetic diversity in 31 individual plants representing 14 populations of diverse geographic origin. Perera et al. (2001, 2003) used eight microsatellites to study the levels and distribution of genetic variation across 33 typical coconut populations from Sri-Lanka and twelve other primers to study genetic diversity and genetic relationships among 94 coconut varieties / populations. In all cases, microsatellites were very successful in distinguishing between coconut genotypes.

In this paper we used 12 microsatellite markers (i) to evaluate genetic characteristics of the identified tolerant materials, (ii) to quantify their genetic similarity, (iii) to identify the possible markers associated to characters and (iv) to establish a reference molecular typology for an assessment of the international coconut collection established in Côte d'Ivoire.

MATERIALS AND METHODS

Plant materials

Three accessions were analysed. Two of them were tolerant and obtained from the screening trials of the Coconut Research Programm of OPRI Ghana, located in endemic area (Figure 1). These have been exposed to lethal yellowing disease since 1982. The two tolerant accessions evaluated are Vanuatu Tall and Sri-Lanka Green Dwarf. Eight genotypes of Vanuatu tall and six genotypes of Sri-Lanka Green dwarf were analysed. The third accession, the West African Tall susceptible to the lethal yellowing disease, originated from the International Coconut Germplasm for Africa and Indian Ocean at Marc Delorme Port-Bouët, Côte d'Ivoire. Seven genotypes composed the accession of West African tall. Letters have been used to indicate coconut cultivars and the numbers to specify the accessions or populations analyzed (Table 1).

Samples collection and DNA extraction

A green leaflet, not damage, was taken from the youngest leaves of the coconut palm. Due to distance between the collecting sites and the laboratories, sampling was done in the morning and DNA extraction in the afternoon. Total DNA was extracted from 1 g of leaflet/coconut palm using a matab protocol as described by Risterucci et al. (2000). DNA extractions were done at the OPRI molecular lab in Ghana for the tolerant accessions VTT'82 and SGD'82. DNA extracted was transferred to Côte d'Ivoire, at Central Laboratory of Biotechnology (LCB) of CNRA, for molecular analyses. The DNA extraction of the West African Tall accession was done in our Laboratory.

Table 2. Number of alleles and gene diversity values for the microsatellite markers used.

Locus	T_a (°C)	T_i (s)	Allele size (bp)	Allele number	Gene diversity
CnCirA3	51	60	228 - 248	6	0.662
CnCirA9	51	60	089 - 109	4	0.702
CnCirB6	51	60	196 - 228	5	0.730
CnCirC7	51	60	147 - 189	7	0.832
CnCirC12	51	60	161 - 185	4	0.712
CnCirE2	51	60	115 - 177	6	0.652
CnCirE10	51	60	226 - 246	4	0.666
CnCirE12	51	60	162 - 174	3	0.475
CnCirF2	51	60	187 - 215	5	0.678
CnCirG11	51	60	186 - 212	5	0.702
CnCirH4	51	60	218 - 236	3	0.652
CnCirH7	51	60	127 - 149	6	0.768
Mean				**4.83**	**0.686**

T_a (°C), annealing temperature; T_i (s), annealing time.

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 (Table 2) 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 s 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 "genotype X marker" combination, microsatellite bands were scored as present (1) or absent (0) among individuals. A similarity matrix was created between all individuals with the obtained binary matrix using Jaccard's coefficient. The similarity matrix was then used for hierarchical cluster analysis by the mean link strategy. The hierarchical cluster analysis was examined in order to estimate genetic relationship between the genotypes. Analyses were performed using the XLSTAT software v.7.5.3. For each microsatellite, gene diversity values ($D = 1-\sum Pi^2$), as employed by Wei et al. (2005), were estimated according to Masatoshi (1973). Allele frequencies and distribution among accessions were analysed using the programme, Microsoft Excel 2003. The genetic polymorphism for each accession was then examined calculating the total number of allele per accession, the mean number allele per locus and accession and the observed heterozygosis. F-statistics fixation index (F_{is}, F_{st}, F_{it}) were also computed to quantify the genetic variability at the level of individuals (I), accessions (S), and the total population (T). The last analyses were conducted using information on the allelic size variance and the software Genetic v.4.05.2.

RESULTS

Microsatellites polymorphism

For the total of individuals analysed, the twelve microsatellites marker used were polymorphic. An example of bands pattern variation is shown in Figure 2. A total of 58 different microsatellite alleles were detected. Allele number ranged from 3 for microsatellites CnCirE12 and CnCirH4 to 7 for the microsatellite CnCirC7 (Table 2). The mean number of allele was 4.83. Genetic diversity at the loci varied from 0.475 to 0.832, with an average of 0.686. These results indicated that a high level of polymorphism was detected at the microsatellite loci.

Intra-accession gene diversity

The F-statistic index show that 59.70% (F_{st}) of allele variations traduced genetic differences between accessions and 40.30% intra-accession variability. These results showed that genetic divergences were more important between accessions compared to intra-accession variability. These results were attested to by the lower number of alleles per locus and observed heterozygosis per accession (Table 3). But, it was noticed that Tall accessions were relatively more polymorphic compared to the dwarf one.

High differences in allelic frequency distribution were observed among accessions following the microsatellite (Figure 3). Variations were maximal in CnCirC7 with 7 alleles. The three accessions were characterized by the presence of specific alleles and striking differences in frequencies of shared alleles (Figure 3). Specific alleles were particularly observed at all the loci. For the two tolerant accessions, VTT'82 and SGD'82, 10 specific alleles were conjointly observed. These alleles were absent

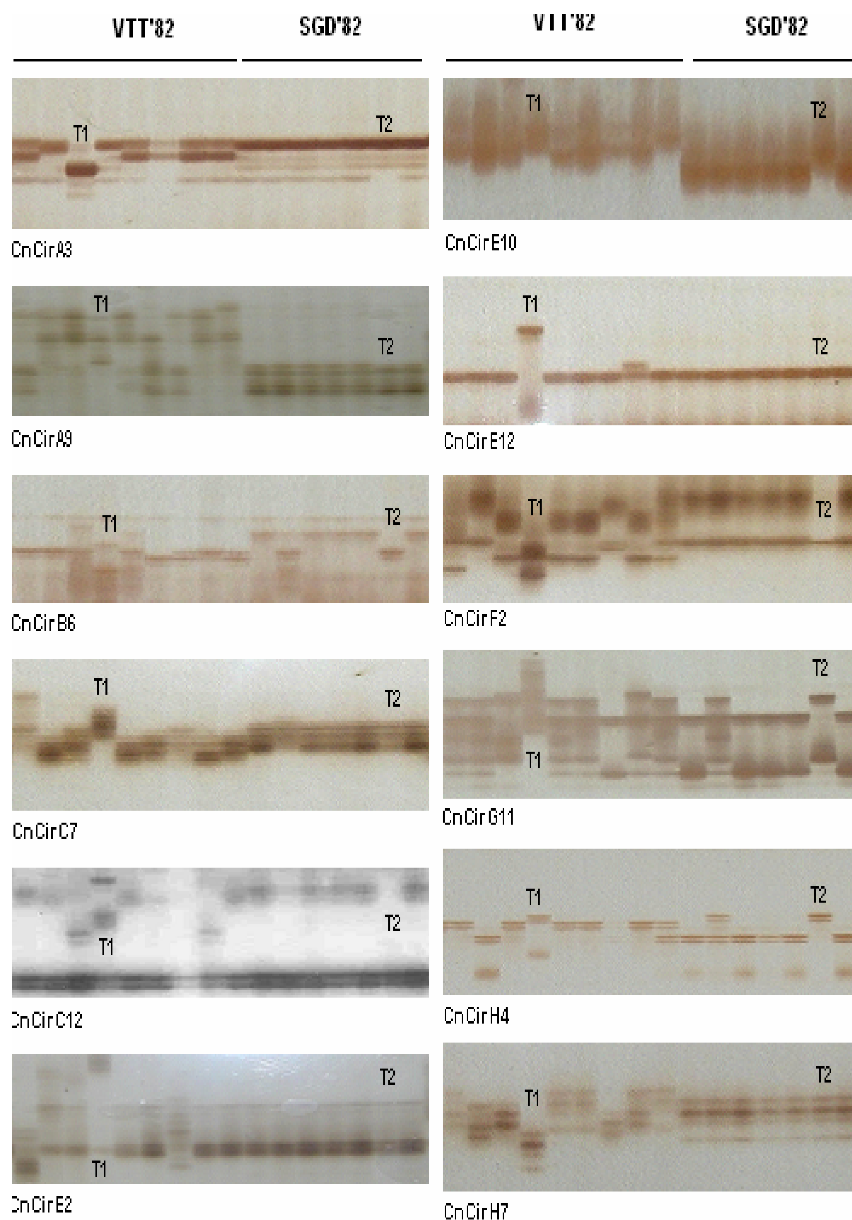

Figure 2. 5% acrylamide gels showing the 12 microsatellite markers polymorphism among 14 tolerant genotypes of coconut (T1 and T2 are standard samples used for allele size determination).

in the susceptible accession (WAT'82). In this accession, 16 particular alleles, absent in tolerant ones, were also detected. Therefore, the two groups of sensibility were specified by 26 alleles (Figure 3). Additional specific alleles were detected in each tolerant accession, VTT'82 (21 alleles) and SGD'82 (2). The common alleles observed were 4 for the three accessions, 4 for VTT'82 and WAT'82 and 1 for SGD'82 and WAT'82.

Accessions relationships

The mean genetic similarity index for the 21 genotypes was 0.239, ranging from 0 to 1. The highest index similarities were found between four tolerant genotypes of Sri-Lanka Green Dwarf. The lowest ones were observed between Sri-Lanka Green Dwarf tolerant genotypes and the susceptible genotypes of West African tall. For the 14 genotypes tolerant to lethal yellowing disease, the mean gene similarity index was 0.400. This was traduced an extensive genetic diversity in tolerant genotypes. The results of the hierarchical cluster analysis showed that the microsatellite markers distinguished the three accessions analysed. However, the tolerant genotypes were more genetically close and less related to the susceptible ones. Among the 8 tolerant genotypes of Vanuatu Tall, one was less related to the others and clustered with the Sri-Lanka

Table 3. Number of alleles per accession, mean number of allele per locus per accession and observed heterozygosity (H_o) in the three analysed accessions.

Accession	Allele number	Allele per locus	Observed heterozygosity (H_o)
VTT'82	39	3.25	0.469
SGD'82	16	1.33	0.097
WAT'82	23	1.92	0.179
Mean	**26**	**2.17**	**0.248**

F-statistics index: FIS = 0.244, FIT = 0.695 and FST = 0.597.

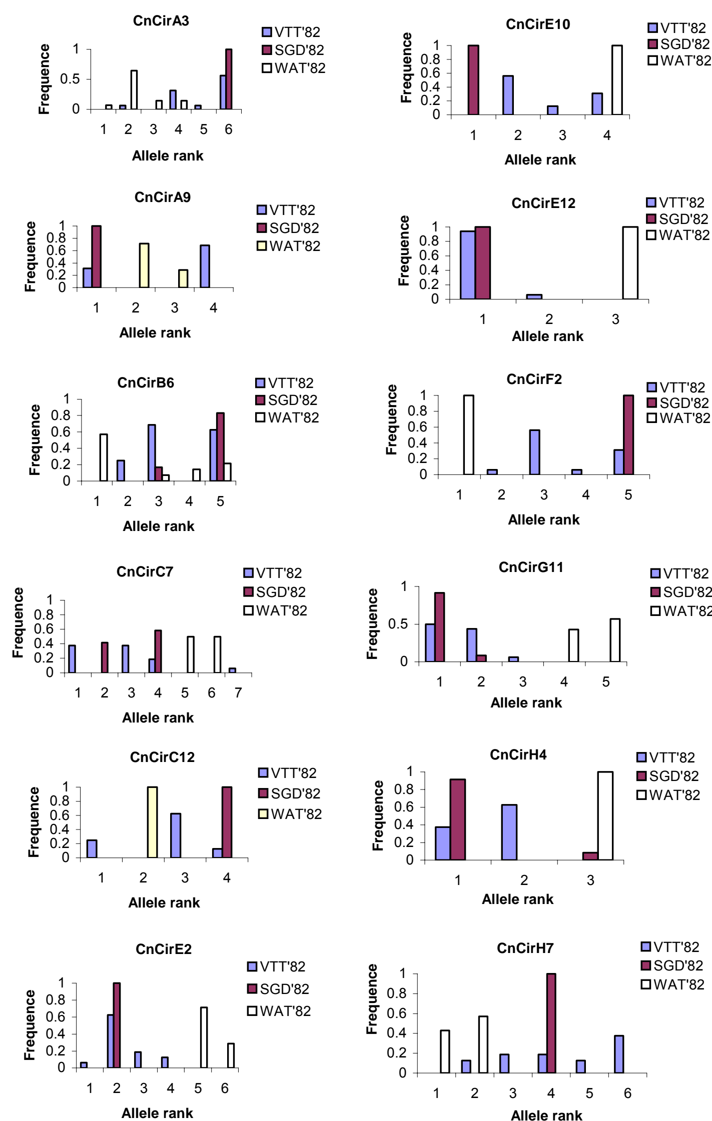

Figure 3. Distribution and allele frequencies at the 12 microsatellite loci in the three accessions.

Green Dwarf genotypes (Figure 4).

DISCUSSION

In the study, we detected 58 alleles at the 12 loci among the 21 coconut genotypes. The mean allele number per locus was 4.83 and the mean gene diversity at all the loci was 0.690, with 0.828 as maximum gene diversity value. These results show that a high polymorphism can be detected by using microsatellite markers for coconut acessions genetic diversity analysis. Microsatellite success in polymorphism detection has been justified with studies in

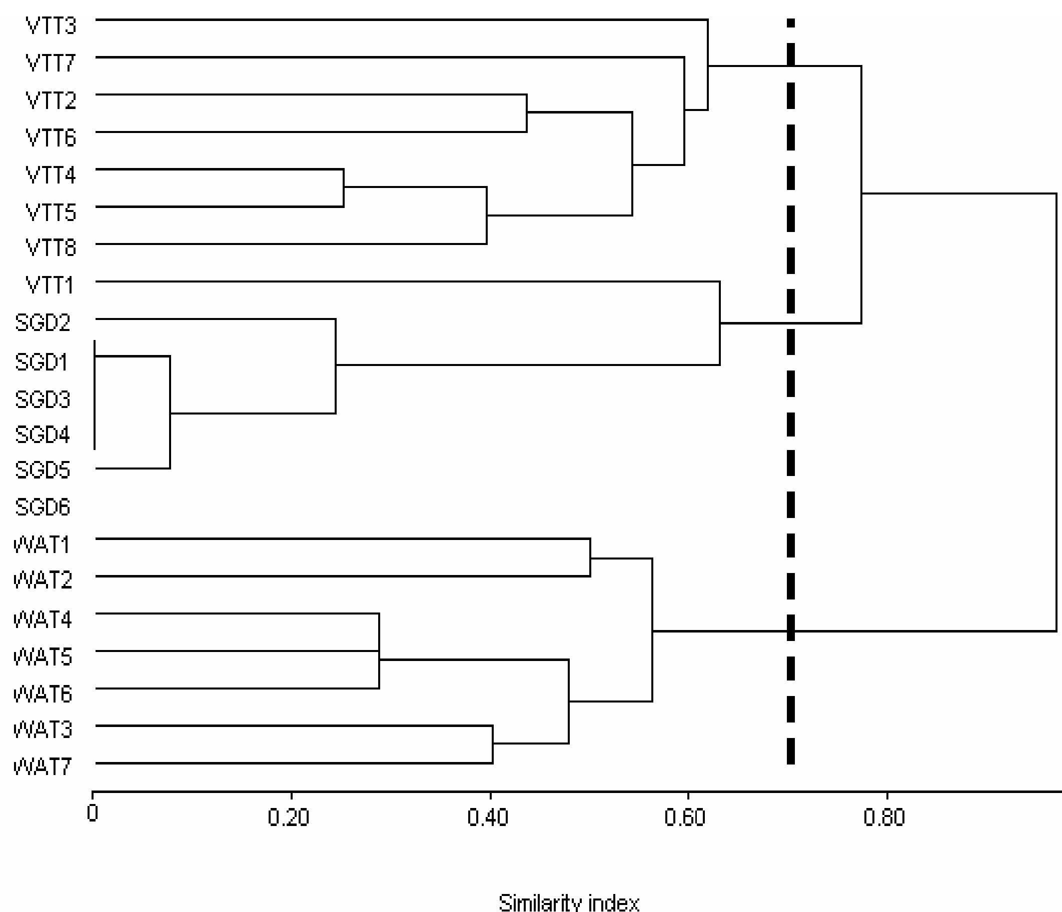

Figure 4. Dendogram resulting from cluster analysis of microsatellites based on genetic similarity matrix for 21 coconut genotypes.

other crops (Bernatchez et al., 2002; Ferguson et al., 2004; Zhao et al., 2005). The percentage of polymorphic markers in the three accessions analysed was comparable to that found by Meerow et al. (2003) who observed a gene diversity ratio of 0.574 among eight coconut accessions using 15 microsatellite markers. However, a lower intra accession genetic variability was observed. This relatively low within population diversity in the three acessions can be explained by the genetic origin of the studied material. In reality, seeds used for the experimental trials in Ghana were from a few number of coconut trees of the International Collection established in Côte d'Ivoire. This restricted resource variability was observed in the analyzed accessions. For the three studied accessions, the Sri-Lanka Green Dwarf appeared more homogeneous than the two tall accessions. The self pollination of the dwarf coconut trees contributed to this unbalance polymorphism between the Sri-Lanka Green Dwarf's and the two Talla accessions. The Tall accessions are preferentially cross-pollinations (Sangaré et al., 1978).

The relatively high between population diversity in the three coconut accessions indicate a lower mean genetic similarity index between them. For the 14 tolerant genotypes analysed, the genetic index similarity was 0.400. By cluster analysis, we found that tolerant coconut genotypes were clustered into two subgroups. This suggested extensive gene diversity is present in the analysed tolerant materials. We are of the opinion that new genes exist which can not be associated to the microsatellite markers used. Cardeña et al. (2003) successfully used RAPD to identify markers associated with resistance to coconut lethal yellowing disease. Also, the few number of microsatellite markers used did not permit us to identify more alleles associated with the tolerance to coconut lethal yellowing disease. This has to be done by more microsatellite markers and tolerant materials evaluation.

However, it was clearly established that tolerant genotypes can be differentiated from susceptible ones. These results are useful as reference for screening coconut resources and in the identification of suitable parents for the development of mapping populations and for tagging the lethal yellowing resistance genes.

In conclusion, the results showed that molecular polymorphism can be detected among the coconut genotypes. The Sri-Lanka Green Dwarf and Vanuatu Tall, tolerant to the lethal yellowing disease, showed an important

genetic divergence from the West African Tall, which is susceptible to the disease. These results showed that it is easily to separate tolerant genotypes from susceptible ones. Therefore, microsatellite markers are useful in the identification of other tolerance sources among the *C. nucifera* L species and the development of suitable population for tagging the lethal yellowing resistance genes.

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