Published online November 21, 2006

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Population Genetics, Lethal Yellowing Disease, and Relationships among Mexican and Imported Coconut Ecotypes

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ABSTRACT

Lethal Yellowing (LY) is one the main diseases affecting coconut worldwide, making the research for resistant germplasm vital to production. Study objectives were to: (i) estimate diversity and genetic structure within four commercial Mexican ecotypes and five imported ecotypes; (ii) analyze the genetic relationships between Mexican ecotypes and the main coconut gene pools identified worldwide; and (iii) measure the correlation between the genetic distance among these ecotypes and Malayan Yellow Dwarf and their percentage mortality due to LY. The Mexican pool had high genetic diversity ($pl = 94$; $H_T = 0.34 \pm 0.02$) similar to that of the populations imported from the world's main gene pools ($pl = 94$; $H_T = 0.36 \pm 0.01$). Both molecular variance and Wright's index of differentiation indicated strong differentiation among Mexican ecotypes ($F_{ST} = 0.32$) despite significant gene flow ($Nm = 1.4$ to 5.6). UPGMA analysis and exact tests of differentiation suggested that the Indo-African gene pool is found along the Caribbean and Gulf of Mexico coasts, while the Asian-Pacific pool is found on the Pacific coast. High positive correlations were found between genetic distance and LY mortality percentages under severe incidence conditions during 9- and 14-yr periods ($r^2 = 0.80$; $P = 0.02$; $r^2 = 0.78$; $P = 0.04$), suggesting that genetic distance may be useful for the estimation of the potential LY mortality in regions as yet unaffected and identification of potential parents for LY resistance breeding.

The coconut (*Cocos nucifera* L. $2n = 2x = 32$) (Uhl and Dransfield, 1987) is grown extensively in the humid tropics worldwide, including Mexico. This palm belongs to the Arecaceae family, subfamily Cocoideae, and it is the only species in the *Cocos* genus. The morphological variation patterns observed worldwide in coconut are the product of four main factors: (i) natural selection, for sea-born seed dissemination between islands, and colonization of isolated coastal locations; (ii) direct and indirect selection during domestication, resulting in higher fruit water content, cultivation at inland locations and exposure to epidemic disease; (iii) introgressive hybridization when domestic forms were dispersed by humans to localities where the wild forma already existed; and (iv) genetic drift in natural and introduced populations (Harries, 1978, 1990).

The wild gene pool, represented by the *Niu kafa* type, has fruit characteristics related to its sea-born dispersal mode: elongate fruit with three triangular ridges that reflect the three carpels forming the pistillate flower, characteristics that permit a stable position while floating in the sea and landing in the beach; high mesocarp and low liquid endosperm content, which favor that embryo is not immersed in the water while floating; and relatively slow germination that increases the dispersion time. This wild gene pool is characterized by its susceptibility to windstorms and epidemic diseases such as LY (Harries, 1978). The domesticated gene pool, represented by the *Niu vai* type, has characteristics that make dispersal less effective by floating and more dependent on humans: spherical fruit; low mesocarp content, high liquid endosperm content; and precocious germination. This gene pool has some tolerance to windstorms and resistance to epidemic disease (Harries, 1978). Introgression occurs wherever the two pools coincide, *Niu kafa* types predominate on the coasts and islands of Africa and the Indian Ocean as well as isolated Pacific islands such as Palmyra, while *Niu vai* types predominate in the Far East and the Pacific islands, particularly those settled by Polynesians and other early voyagers (Harries, 1978; Ashburner et al., 1997; Lebrun et al., 1998).

Existence of these two pools has been confirmed in different studies using molecular markers: ITSRs (Rohde et al., 1995; Duran et al., 1997); RAPDs (Ashburner et al., 1997; Manimekalai and Nagarajan, 2006a); RFLPs and AFLPs (Lebrun et al., 1998; Teulat et al., 2000); SSRs (Rivera et al., 1999; Perera et al., 2003); and ISSR (Manimekalai and Nagarajan, 2006b). These studies have also supported suggestions (Harries, 1978) for possible dissemination routes, largely by means of European voyagers, of *Niu kafa* types from the Indian Ocean to the Atlantic coasts of Africa and the Americas, and of *Niu vai* types from Pacific Ocean locations to the Pacific coast of the Americas. Paleontological considerations indicate that coconut may have originated on the coasts of the ancient Tethys Sea, remnants of which correspond to the current coasts and islands of the Indian Ocean (Harries, 1990; Leach et al., 2003). Archaeological and ethnobotanical evidence suggests that domestication of coconut may have taken place in a region of Malesia that was subsequently submerged (Smith and Briden, 1977; Harries, 1990).

Initial studies of fruit and leaf morphological variation patterns, both in situ and ex situ, indicate that coconut

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Abbreviations: AFLP, Amplified fragment length polymorphism; AMOVA, analysis of molecular variance; ANOVA, one-way analysis of variance; *D*, genetic distance; F_{ST}, Wright index of population differentiation; H_E, expected heterozygosity; H_S, intrapopulation heterozygosity; H_T, total heterozygosity; HW, Hardy-Weinberg equilibrium; *I*, genetic identity; ISSR, Inter simple sequence repeat; ITSR, Inverse sequence-tagget repeat; *pl*, percentage of polymorphic loci; LY, Lethal Yellowing disease; MXAT, Mexican Atlantic Tall; MXPT, Mexican Pacific Tall; MYD, Malayan Yellow Dwarf; NJ, Neighbor-Joining analysis; *Nm*, number of migrants; PNT, Panama Tall; RAPD, Random amplified polimorphism; RFLP, Restriction fragment length polymorphism; RIT, Rennell Island Tall; SSR, Simple Sequence Repeat; TAT, Tahiti Island Tall; UPGMA, un-weighted pair group method with arithmetic mean; WAT, West African Tall.

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Published in Crop Sci. 46:2509–2516 (2006).
Plant Genetic Resources
doi:10.2135/cropsci2005.12-0462
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populations in Mexico exhibit characteristics of both pools: *Niu kafa* on the Gulf of Mexico coast and *Niu vai* on the Pacific coast (Zizumbo-Villarreal and Piñero, 1998; Zizumbo-Villarreal et al., 2005a). In addition, four different ecotypes have been documented: Mexican Atlantic Tall (MXAT) with populations on the Caribbean and Gulf of Mexico coasts; Mexican Pacific Tall 1 (MXPT1); Mexican Pacific Tall 2 (MXPT2); and Mexican Pacific Tall 3 (MXPT3), all on the Pacific coast (Zizumbo-Villarreal and Colunga-GarcíaMarín, 2001). Use of isozyme markers has confirmed the presence of the two pools (*Niu kafa* and *Niu vai*) in Mexico and of the differentiation among the four ecotypes (Zizumbo-Villarreal et al., 2002).

Coconut did not exist in Mexico before 1539 (Bruman, 1947), when it was possibly introduced to the Pacific coast at Tehuantepec or Colima from the south coast of Panama (Bruman, 1945; Zizumbo-Villarreal and Quero, 1998). In 1549, coconut was probably introduced to the ports of Veracruz and Campeche on the Gulf of Mexico from India or East Africa, via the Cape Verde islands (West Africa), Puerto Rico, or Hispaniola (Dominican Republic) (Harries, 1977; Zizumbo-Villarreal, 1996). There was another introduction opportunity to the Pacific cost in 1569 at Santiago in Colima from the Solomon Islands (Sevilla del Río, 1977) and many more from the Philippines between 1571 and 1815 during annual journeys along the Manila-Acapulco route (Harries, 1978; Zizumbo-Villarreal, 1996).

Coconut cultivation in Mexico subsequently extended along both coasts, peaking at 200 000 ha in 1980 (Zizumbo-Villarreal et al., 1993). Over the last two decades of the 20th Century, the LY epidemic disease attacked coconut plantations in the Caribbean and Gulf Coast states and low international vegetable oil prices reduced profitability. This led to a drop in cultivated area to 140 000 ha, and a decrease in cultivation density from 100 trees per hectare to just 60. Coconut growers all over Mexico have been adversely affected.

Mortality due to LY among the Mexican ecotypes is known to differ, as has been reported by Zizumbo-Villarreal et al. (1999, 2006) in studies done over 9 and 14 yr in populations suffering high and severe LY incidence on the north coast of the state of Yucatan, Mexico. Tackling LY and reviving coconut's profitability as a crop requires integrated disease management including measures such as genetic improvement; replanting with resistant, high-yielding varieties; introduction of new agricultural technology in coconut cultivation; associating coconut with other crops; product diversification; improving post-harvest processing; and developing new uses and processes to increase its value. Genetic improvement requires identification of resistant and genetically diverse parental combinations, including hybridization with the Malayan Yellow Dwarf (MYD) variety that has shown high resistance to LY (Been, 1981). The present study aims to contribute to genetic improvement of coconut by (i) estimating diversity and genetic structure in the four commercial Mexican tall ecotypes and five imported ecotypes using ISSR markers; (ii) analyzing these ecotypes' genetic relationships with the main coconut gene pools identified worldwide; and (iii) measuring the correlation between the genetic distance among these ecotypes and Malayan Yellow Dwarf and their percentage mortality due to LY.

MATERIALS AND METHODS

Plant Material and DNA Extraction

On the basis of previous studies (Zizumbo-Villarreal and Colunga-GarcíaMarín, 2001; Zizumbo-Villarreal et al., 2005a), a representative population of each commercial Mexican Tall ecotype (Fig. 1) and five imported ecotypes from the main gene pools identified worldwide were selected (Table 1). The imported ecotypes were previously introduced to the Marc Delorne Research Station in Ivory Coast (Bourdeix, 1999; Bourdeix et al., 2005) and then to the Benito Juárez Research Station on the west coast of Mexico in 1977 (Manciot, 1978). Mexican ecotype samples were taken from the germplasm collection at the Centro de Investigación Científica de Yucatán (CICY) in Mexico and samples of the imported ecotypes were obtained from the Benito Juárez Research Station. The passport and priority characterization data are included in the international Coconut Genetic Resources Network (COGENT) database (www.ipgri.cgiar.org/networks/cogent/CGRDatabase.htm; verified 20 July 2006). An average of 20 individuals per ecotype were analyzed, resulting in a total of 180 individuals. Genomic DNA was extracted from the first opened leaf of adult plants by the CTAB method (Doyle and Doyle, 1987).

ISSR Analysis

ISSR is a multilocus molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, provides Mendelian segregation, and has been successfully applied in genetic and evolutionary studies of many species, including coconut (Zietkiewics et al., 1994; Wolfe et al., 1998; Camacho and Liston, 2001; Zizumbo-Villarreal et al., 2005b; Manimekalai and Nagarajan, 2006b).

Amplification reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) with seven ISSR primers: MS-4: $(GACA)_4$; MS-15: $(GACAC)_3$ RG; MS-17: $(GACAC)_2$; MS-22: $(GA)_8$ RG; MS-30: $(GACAC)_3$AG; MS-31: $(GACAC)_3$GG; and MS-32: $(GACAC)_3$RG (Primer's characteristics shown in Table 1s Supplementary Data). Each 20-μL amplification reaction consisted of 10 mM Tris-HC1 (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton X-100, 2 mM $MgC1_2$, 200 μM each dNTPs, 1 μM of primer, 1 unit of Taq polymerase (Promega, Madison, WI) and 50 ng of template DNA. Amplification was done under the following conditions: 4 min at 94°C for 1 cycle, followed by 2 min at 94°C, 1 min at 42 to 56°C and 2 min at 70°C for 35 cycles, and 5 min at 72°C for final extension (Table 2).

The generated DNA fragments were separated by electrophoresis (SQ3 Sequencer, Hoeffer Scientific Instruments, San Francisco, CA) on 320 × 380 × 0.4 mm, 6% (w/v) nondenaturing 30:1 bisacrylamide gels containing 3 M urea and 1× TBE buffer (Zietkiewics et al., 1994). A 123-bp molecular marker standard was included in each gel. Gels were run at 300 V and PCR products were detected by silver nitrate staining.

Data Analysis

Genetic Diversity

Data were recorded as the presence or absence of bands (Examples of ISSR profiles shown in Fig. 1s, 2s and 3s Sup-

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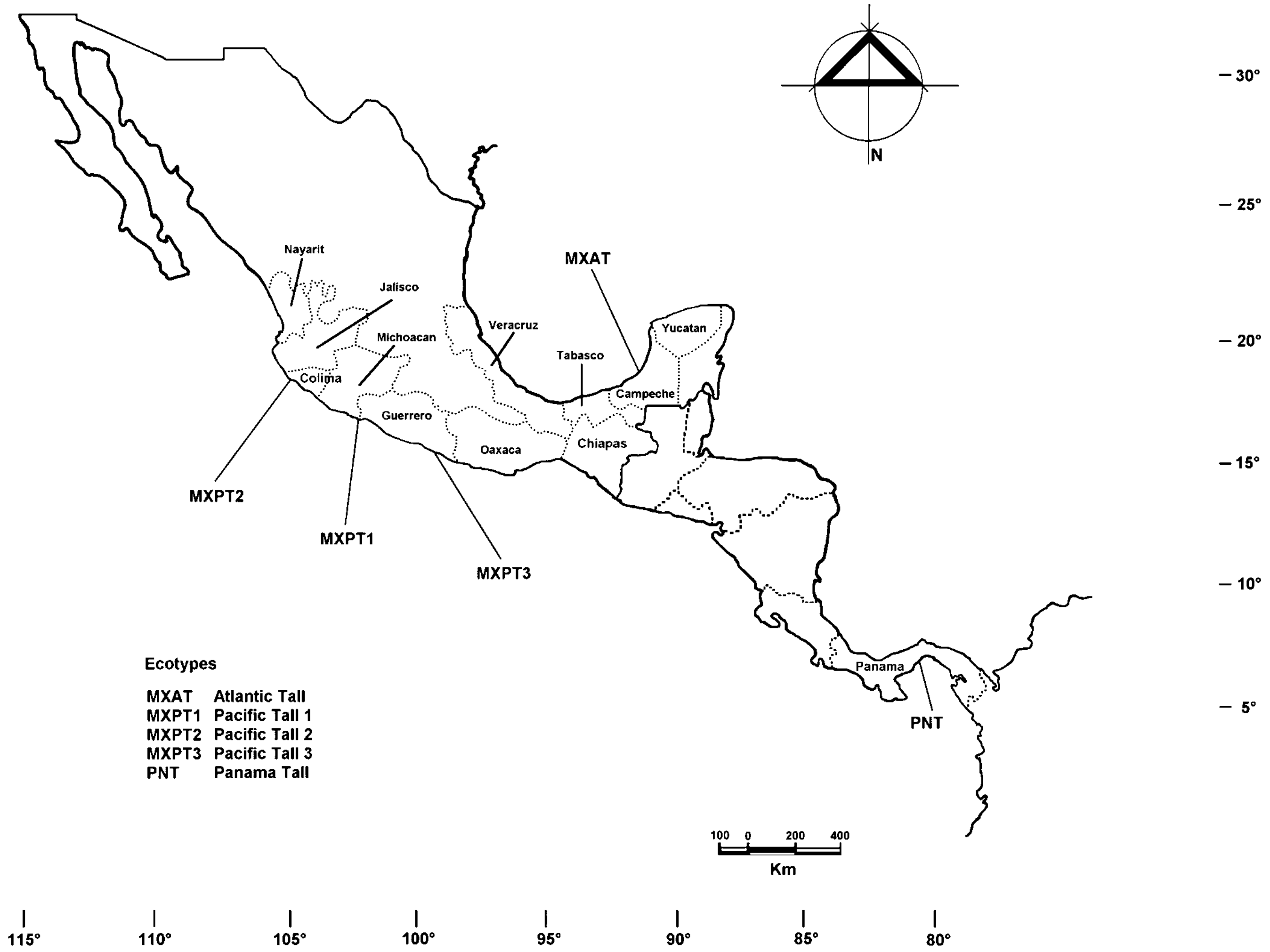

Fig. 1. Distribution of Mexican and Panama ecotypes.

plementary Data). Allelic frequencies were calculated from individuals with no band, which were considered as recessive homozygous, assuming Hardy-Weinberg equilibrium (HWE), where (q) allelic frequency was $q = x^{0.5}$ and (p) allelic frequency was $p = 1 - q$. Diversity estimates for the ecotypes were (i) percentage of polymorphic loci (pl), where $pl = x/m$, x is the number of polymorphic loci in the sample, and m the overall number of polymorphic loci under a 95% criterion; and (ii) expected heterozygosity (H_E) under HW. H_E in a particular locus was calculated as $H_E = 1 - \sum p^2 i$, where p^2 is the homozygous genotype by allele i. Lynch and Milligan's (1994) correction for dominant markers was used. For a locus i: $H_E = 2\,q\,(j)\,[1 - q\,(j)] + 2\,\mathrm{Var}\,[q\,(j)]$, where $\mathrm{Var}\,(q) = (1 - x)/4N$, $q = x^{1/2}\,[1 - \mathrm{Var}(x)/8x^2]^{-1}$ and $\mathrm{Var}\,(x) = x(1 - x)/N$.

When a number of loci were simultaneously analyzed, the formula used was $\overline{H}_E = 1 - \frac{1}{m}\sum_{j=1}^{m} Hj$, where Hj is the heterozygosity of locus j and m is the total number of polymorphic loci. The TFPGA 1.3 (Miller, 1997) program was used to calculate pl and H_E.

Total expected heterozygosity (H_T) and expected heterozygosity within ecotypes (H_S) under HW was calculated with the TFPGA 1.3 program in four levels or pools: Mexican

Table 1. International code, name, country, and geographical region of the studied ecotypes or cultivars.

International Code	Name	Country of origin	Geographical region
MXAT	**Mexican Atlantic Tall**	**Mexico**	**America**
MXPT1	**Mexican Pacific Tall 1**	**Mexico**	**America**
MXPT2	**Mexican Pacific Tall 2**	**Mexico**	**America**
MXPT3	**Mexican Pacific Tall 3**	**Mexico**	**America**
WAT	**West African Tall**	**Ivory Coast**	**West Africa**
PNT	**Panama Tall**	**Panama**	**America**
RIT	**Rennel Island Tall**	**Salomon Islands**	**Southwest Pacific**
TAT	**Tahiti Island Tall**	**Tahiti**	**Polynesia**
MYD	**Malayan Yellow Dwarf**	**Ivory Coast**	**Malaysia**

Table 2. Ecotype, number of plants (*n*), polymorphic loci (*l*), percentage of polymorphic loci under 95% criterion (*pl*) and expected heterozygosity ± Standard Deviation (H_E ± *SD*) under Hardy-Weinberg equilibrium (Nie, 1978; Lynch and Milligan, 1994) of nine studied *Cocos nucifera* ecotypes, standard deviation (SD). Different letters in column *P* indicate significant difference ($P < 0.05$).

Ecotype	*n*	l	*pl*	H_E ± SD	*P*
MXAT	**19**	**29**	**84.8**	**0.31 ± 0.18**	**A**
MXPT1	**19**	**23**	**66.6**	**0.24 ± 0.19**	**AB**
MXPT2	**21**	**26**	**57.6**	**0.21 ± 0.17**	**B**
MXPT3	**21**	**25**	**66.6**	**0.27 ± 0.20**	**AB**
WAT	**20**	**26**	**75.8**	**0.25 ± 0.18**	**AB**
PNT	**19**	**30**	**90.1**	**0.33 ± 0.19**	**A**
RIT	**20**	**27**	**78.8**	**0.30 ± 0.17**	**AB**
TAT	**20**	**30**	**75.8**	**0.29 ± 0.18**	**AB**
MYD	**21**	**13**	**21**	**0.07 ± 0.11**	**C**
Total	**180**	**33**	**97**	**0.34 ± 0.20**	

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ecotypes; imported tall ecotypes; Mexican Pacific ecotypes; and overall (four Mexican tall ecotypes and four imported tall ecotypes).

Comparison of H_E among ecotypes and of H_T and H_S between Mexican and imported pools was done with an ANOVA using heterozygosity per allele and the general linear models procedure and Duncan's multiple comparison of means tests ($\alpha = 0.05$) with the SAS program (1997). The assumption of HWE among and between ecotypic groups was evaluated comparing the results of the molecular variance partition (MANOVA) (Excoffier et al., 1992) with the values of the Wright's differentiation index $F_{ST} = (H_T - H_S/H_T)$ (Wright, 1978), estimated as described in the next section.

Genetic Differentiation

Differentiation between ecotypes was analyzed by tree statistical procedures: (i) genetic variance components, calculated by MANOVA (Excoffier et al., 1992) using the Arlequin Program (Schneider et al., 2000); (ii) Wright's differentiation index (F_{ST}) estimated as in Weir and Cockerham (1984) using TFPGA 1.3 under the assumption of HWE because we are using diploid/dominant data. Values were averaged across polymorphic loci for each population using a jacknife procedure to produce variances estimates and generate 95% confidence intervals with 1000 bootstraps; (iii) exact tests of differentiation for diploid/dominant data (Raymond and Rousset, 1995), done at the ecotype level using TFPGA 1.3 with 1000 dememorization steps, 20 batches, 2000 permutations per batch and a 95% confidence level.

Gene Flow

Long-term gene flow was indirectly estimated for each Atlantic–Pacific and Pacific–Pacific Mexican ecotype with the indirect method based on Wright's F_{ST} statistic (1951), which is a measure of correlation between the genes of a subpopulation versus the entire population. To calculate gene flow, we used the model $Nm = 1/4\ (1/F_{ST}-1)$ (Wright, 1969) corrected by Hedrick (2000): $Nm = 1/4\ (1/F_{ST} - 1)\alpha$, where $\alpha = (n/n - 1)^2$ as a correction for sample size, where n is the number of populations. F_{ST} was calculated as in Weir and Cockerman's (1984) method for diploid/dominant data under HWE. Cockerman and Weir (1993) showed that F_{ST} values provide much more accurate gene flow (Nm) estimators than those generated by Slatkin and Barton (1989) with their G_{ST} estimator (Slatkin, 1994).

Genetic Distance and Relationships between Individuals and Ecotypes

The genetic relationships between all studied individuals were inferred from two cluster procedures. Neighbor-Joining (NJ) using the Jaccard similarity coefficient (Saitou and Nei, 1987) and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on the Nei's minimum distance (Nei, 1972). Both analyses were performed by Free Tree freeware (Pavlicek et al., 1999). The genetic relationships between the nine ecotypes were inferred from an UPGMA done with the TFPGA program, based on the Nei's unbiased distance (Nei, 1978). Topology robustness was evaluated by selecting the bootstrapping option with 1000 random resamplings with replacement over loci (Felsenstein, 1985).

Correlation between Genetic Distance and LY Mortality Percentage

A simple linear model was applied using SAS (1997) to test the correlation between the Nei's genetic distance (1978) between the Mexican ecotypes and MYD and the LY mortality percentage of each ecotype after 9 and 14 yr of experiencing high incidence (57 and 63% of controls dead) or severe incidence (79 and 86% of controls dead) of LY. Mortality data were obtained from Zizumbo-Villarreal et al. (1999, 2006). Mortality percentage data were transformed with an arcsine function.

RESULTS

Genetic Diversity

The MS-17, MS-30, and MS-32 primers exhibited the optimum resolution and sensitivity for detection of polymorphisms in coconut. A total of 33 polymorphic bands were recorded as putative loci: 8 bands generated with the MS-17 primer; 18 with the MS-30 primer; and 7 with the MS-32 primer. The MYD ecotype had less genetic diversity ($pl = 21$; $H_E = 0.07$) than all the tall ecotypes ($pl = 58$–90%; $H_E = 0.20$–0.32), and PNT and MXAT had more genetic diversity ($pl = 85$ and 90%; $H_E = 0.31$ and 0.32) than MXPT2 ($pl = 58\%$; $H_E = 0.20$). The remaining ecotypes had no significant differences with PNT, MXAT, and MXPT2 ($P < 0.05$) (Table 2). The Mexican pool H_T was high ($H_T = 0.34 \pm 0.02$) and similar to that of the imported pool ($H_T = 0.36 \pm 0.01$). Diversity within Mexican ecotypes was high ($H_S = 0.27 \pm 0.02$) and not different from the imported ecotypes ($H_S = 0.29 \pm 0.02$) ($P < 0.05$) (Table 3).

Genetic Differentiation

The partition of the molecular variance among and between ecotypic groups obtained with the MANOVA resulted within the confidence limits for F_{ST} values, meaning that it is valid to assume HWE for the genetic analysis with the populations and the molecular markers used (Tables 3 and 4). Genetic differentiation was high at all three levels. In the overall pool, 33% of the variation was found to occur between ecotypes ($F_{ST} =$

Table 3. Ecotype groups, number of plants (*n*), percentage of polymorphic loci under 95% criterion (*pl*); total diversity ± Standard Deviation ($H_T \pm SD$), intra-ecotype diversity ± Standard Deviation ($H_S \pm SD$), genetic variance among ecotypes groups (*V*) (Excoffier et al., 1992), inter-ecotype groups coefficient differentiation ± Standard Deviation ($F_{ST} \pm SD$) (Weir and Cockerham, 1984), its confidence intervals for F_{ST}: (upper and lower) of ecotypes groups studied of *Cocos nucifera* L.

Ecotype groups	*n*	*pl*	$H_T \pm SD$	$H_S \pm SD$	V	$F_{ST} \pm SD$	upper	lower
Mexican Tall	**80**	**94**	**0.34 ± 0.02**	**0.26 ± 0.02**	**0.38**	**0.32 ± 0.05**	**0.41**	**0.16**
Imported Tall	**80**	**94**	**0.36 ± 0.02**	**0.29 ± 0.02**	**0.28**	**0.26 ± 0.05**	**0.37**	**0.16**
Mexican Pacific Tall	**60**	**85**	**0.31 ± 0.02**	**0.24 ± 0.02**	**0.29**	**0.24 ± 0.05**	**0.33**	**0.14**
Mexican/Imported	**160**	**97**	**0.37 ± 0.2**	**0.37 ± 0.2**	**0.03**	**0.027 ± 0.05**	**0.04**	**0.01**
Global	**160**	**97**	**0.36 ± 0.02**	**0.27 ± 0.01**	**0.31**	**0.33 ± 0.003**	**0.39**	**0.26**

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Table 4. Number of plants (*n*), inter-ecotype genetic variance (*V*) (Excoffier et al., 1992), inter-ecotype genetic differentiation ± Standard Deviation ($F_{ST} \pm SD$) (Weir and Cockerham, 1984), its confidence (intervals upper and lower) and genetic flow (*Nm*) (Slatkin and Barton, 1989; Slatkin, 1994; Hedrick, 2000) between the studied Mexican *Cocos nucifera* ecotypes.

Mexican ecotypes	*n*	V	$F_{ST} \pm SD$	Upper	Lower	Nm
MXAT-MXPT1	38	0.38	0.35 ± 0.07	0.48	0.19	1.8
MXAT-MXPT2	40	0.49	0.43 ± 0.07	0.55	0.29	1.4
MXAT-MXPT3	40	0.39	0.30 ± 0.06	0.39	0.19	2.3
MXPT1-MXPT2	40	0.32	0.35 ± 0.09	0.50	0.19	1.8
MXPT1-MXPT3	40	0.21	0.16 ± 0.09	0.25	0.07	5.6
MXPT2-MXPT3	42	0.34	0.33 ± 0.09	0.49	0.15	2.1

0.33 ± 0.03) and 67% within ecotypes. In the Mexican pool, 32% of total variation was found to occur between ecotypes ($F_{ST} = 0.32 \pm 0.05$) and 68% within ecotypes. In the imported ecotypes, 26% of total variation was found to occur between ecotypes ($F_{ST} = 0.26 \pm 0.05$) and 74% within ecotypes. Differentiation between the Mexican and imported pools was lower than 3% ($F_{ST} = 0.027 \pm 0.005$) and was 97% within each pool. The Fisher' combined probability test (data shown in Table 2s. Supplementary Data) showed that MXAT and WAT had no genetic differentiation ($P < 0.05$) since 82% of their loci exhibited no differences in their allelic frequencies ($P < 0.05$). In the MXPT2-TAT, MXPT3-TAT, MXPT3-RIT, PNT-RIT and RIT-TAT pairs, 75 to 80% of their loci had no differences in allelic frequencies, indicating a low level of genetic differentiation. By contrast, in the MXAT-MXPT2, MXAT-MYD, and WAT-MYD pairs, 79 to 82% of their loci did have differences, indicating a high level of genetic differentiation.

Gene Flow

Gene flow between MXAT and MXPT3 ($Nm = 2.3$) was observed despite geographical isolation between the two coasts. As was expected, high genetic flow ($Nm = 5.6$) was observed between MXPT3 and MXPT1, resulting from their spatial proximity and the lack of any geographical barrier between them (Table 4). Genetic distance between the Mexican Pacific ecotypes and WAT and MXAT was high but low between the Mexican Pacific ecotypes and those imported from Malaysia, Southwest Pacific, Polynesia, and Panama. Genetic distance between MXAT and WAT was low ($D = 0.06$), though it was high between MXAT and MYD ($D = 0.37$). Between the MXAT and MXPT2 ecotypes, genetic distance values were high ($D = 0.37$), but they were low between MXPT1 and MXPT3 ($D = 0.06$). MXPT2 had the smallest genetic distance from MYD ($D = 0.06$) (Table 5).

Genetic Relationships between Individuals and Ecotypes

The NJ and UPGMA analysis considering all the individual (Fig. 4s and 5s. Supplementary Data), resulted in a general clustering of individual in their ecotypes. There was a remarkable difference between the two topologies. In the first differentiation level, NJ separated all MYD individuals from the rest of the tall ecotypes, indicating that the main phenetic differentiation is between dwarf and tall ecotypes, as has been shown in previous morphological and physiological studies (Zizumbo-Villarreal and Piñero, 1998; Zizumbo-Villarreal and Colunga-GarcíaMarín, 2001; Zizumbo-Villarreal et al., 2005a). The topology of the UPGMA dendrogram, separated, in the first differentiation level, all the WAT individuals and most of the MXAT individuals from the rest of the ecotypes, suggesting that the main genetic differentiation is between the ecotypes that belong to the *Niu kafa* pool and the *Niu vai* pool. The first one geographically distributed in West Africa and, in Mexico, in the Atlantic coast, and the second in the coasts of Asia and, in Mexico, in the Pacific coast. This second group includes all the MYD individuals. Similar results have been reported with other molecular markers (Zizumbo-Villarreal et al., 2002; Rohde et al., 1995; Duran et al., 1997; Lebrun et al., 1998; Teulat et al., 2000; Rivera et al., 1999; Perera et al., 2003).

The UPGMA analysis, considering the nine studied ecotypes (Fig. 2), separated them, in the first step, into two groups. The first group corresponded to the *Niu kafa* pool, including WAT and MXAT [support: 98% of the replicates and 42% of the loci (14)], while the second group corresponded to the *Niu vai* pool, including the remaining tall ecotypes and MYD, [support: 84% of the replicates and 24% of the loci (8)]. The second step showed there to be two groups within the *Niu vai* pool: one formed by the MYD and MXPT2 ecotypes [support: 74% of the replicates and 27% of the loci (9)] and a second group consisting of the remaining tall ecotypes (support: 59% of the replicates but none from loci). The third step also showed there to be two groups: one consisting of the MXPT1 and MXPT3 ecotypes [support 52% support of the replicates and 9% of the loci (3)] and another containing all the imported ecotypes [support 40% of the replicates and 3% of the loci (1)].

The greatest genetic distance between a Mexican ecotype and MYD occurred with MXAT, while the smallest genetic distance was with MXPT2 (Table 5). This suggests a positive correlation between genetic distance and

Table 5. Genetic distance (Nei, 1978) between nine studied *Cocos nucifera* ecotypes.

Ecotype	MXAT	MXPT1	MXPT2	MXPT3	WAT	PNT	RIT	TAT	MYD
MXAT	****								
MXPT1	0.230	****							
MXPT2	0.291	0.174	****						
MXPT3	0.198	0.070	0.164	****					
WAT	0.056	0.312	0.394	0.322	****				
PNT	0.121	0.078	0.141	0.095	0.201	****			
RIT	0.200	0.065	0.116	0.065	0.294	0.040	****		
TAT	0.257	0.120	0.084	0.123	0.344	0.086	0.032	****	
MYD	0.370	0.296	0.064	0.260	0.478	0.169	0.172	0.096	****

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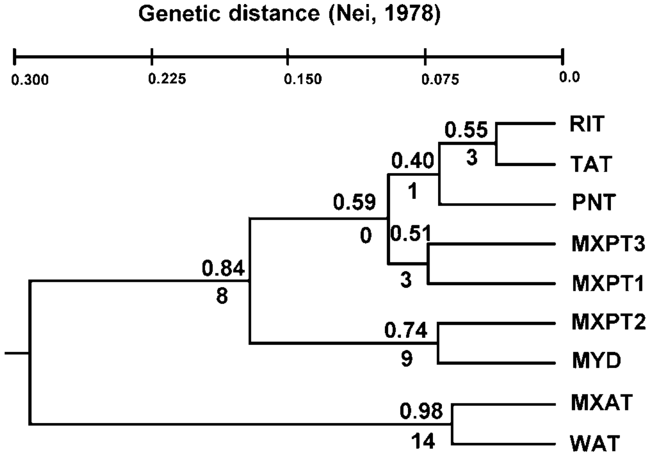

Fig. 2. Dendrogram (UPGMA) based on Nei's unbiased distance (Nei, 1978) of nine *C. nucifera* ecotypes. Numbers above the lines are the proportions of similar replicates supporting the node, and below the lines are the percentages of loci supporting the node. MXAT: Mexican Atlantic Tall; MXPT1: Mexican Pacific Tall1; MXPT2: Mexican Pacific Tall 2; MXPT3: Mexican Pacific Tall3; WAT: West African Tall; PNT: Panama Tall; RIT: Rennell Island Tall; TAT: Tahiti Island Tall; MYD: Malayan Yellow Dwarf.

LY mortality, since MYD and MXPT2 had low mortality levels, and MXAT had the highest levels (Table 6) (Zizumbo et al., 1999, 2006). Under high incidence conditions, at 9 and 14 yr, no significant correlation was observed between genetic distance and mortality ($r^2 = 0.6$; $P = 0.08$ y $r^2 = 0.74$ $P = 0.06$). However, under severe incidence conditions a significant correlation was observed at 9 yr ($r^2 = 0.8$; $P = 0.02$) and 14 yr ($r^2 = 0.78$; $P = 0.04$).

DISCUSSION

Previous research has shown that greater polymorphism is detected by ISSR molecular markers than by isozymes in same coconut populations (Zizumbo-Villarreal et al., 2002). This coincides with reports for other species, meaning molecular markers can be considered more useful for characterizing germplasm and estimating levels of diversity, structure, and genetic relationships between populations. Nonetheless, the total diversity levels detected here were lower ($H_T = 0.37 \pm 0.02$) than those reported in studies using SSR microsatellites ($H = 0.7 \pm 0.01$ in Perera et al., 2003; $H = 0.57 \pm 0.2$ in Meerow et al., 2003), suggesting that multi-allelic markers such as SSR are more sensitive, even though ISSR identifies DNA sequence insertions and deletions. Another reason for the lower diversity detected in the present study may be that the above studies were done with a larger number of ecotypes (94 in Perera et al., 2003; 18 in Meerow et al., 2003).

The low diversity in MYD detected here suggests that high self-pollination in this ecotype has led to endogamy, as mentioned in previous morphological and molecular studies (Zizumbo-Villarreal and Piñero, 1998; Perera et al., 2003). The similarly high genetic diversity levels and low allelic differentiation among the Mexican ecotypes and among the imported ecotypes suggest that introductions of coconut into Mexico involved varied and diverse sources of germplasm from a wide geographical range. This hypothesis is supported by the fact that almost 30% of the genetic variation in the Mexican pool was explained by genetic differences among ecotypes, despite significant genetic flow between them, which would be expected to historically operate against differentiation.

Low diversity within the MXPT2 population from Colima, on the Pacific coast ($H_E = 0.20$), versus MXAT ($Hs = 0.31$) suggests that introduction of the former ecotype may have consisted of a limited number of seeds. Early records of introductions into Colima indicate that as few as a dozen seeds may have been introduced from Panama in 1539 (Bruman, 1947) and an equally limited number of fruits from the Solomon Islands in 1569 (Sevilla del Río, 1977; Zizumbo-Villarreal, 1996). These low seed numbers and the relative geographical isolation of Colima may have created a bottleneck, although significant genetic flow was observed between Pacific coast ecotypes: MXPT1-MXPT2 ($Nm = 1.8$), MXPT2-MXPT3 ($Nm = 2.1$), MXPT1-MXPT3 ($Nm = 5.6$), probably enough to avoid endogamy. The fact that trading occurred between the ports of Colima, Zacatula, and Acapulco throughout the Spanish Colonial period may have favored movement of seeds from different ecotypes between sites and resulted in subsequent hybridization and genetic infiltration in areas near these ports.

Movement of seeds along the Pacific coast increased as the area under coconut production rose in the period between the world wars, particularly on the Pacific Guerrero and Atlantic Tabasco coasts, the main production areas in Mexico (Zizumbo-Villarreal et al., 1993). Seeds were moved from the areas of Lázaro Cardenas and Acapulco, Guerrero (where mainly MXPT1 is distributed), to Costa Chica, Guerrero, and the state of Oaxaca (where mainly MXPT3 is distributed) (Zizumbo-Villarreal et al., 1993). Seed movement favored contact, infiltration, and possibly counterbalancing the effects of low seed number. This is supported by the low dif-

Table 6. Genetic distance (Nei, 1978) between Mexican ecotypes and MYD, and lethal yellowing mortality percentage in: experiment A = 1990–1999 under high incidence (56% mortality in test ecotype); experiment B = 1990–99 under severe incidence (79% mortality in test ecotype); experiment C = 1999–2006 under high incidence (63% mortality in test ecotype); and experiment D = 1999–2006 under severe incidence (86% mortality in test ecotype) (Zizumbo-Villarreal et al., 1999, 2006).

Ecotypes	Genetic distance	Experiment A	Experiment B	Experiment C	Experiment D
MYD-MYD	0.0	6	6	6	17
MXAT-MYD	0.39	56	79	63	86
MXPT1-MYD	0.30	17	38	19	39
MXPT2-MYD	0.064	12	23	14	23
MXPT3-MYD	0.270	32	55	34	55

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ferentiation between the MXPT1 and MXPT3 ecotypes, which have differences in just 18% of the loci in their allelic frequencies.

The smaller differentiation observed between MXAT-MXPT3 (F_{ST} = 0.30) than between MXAT-MXPT1 (F_{ST} = 0.35) and MXAT-MXPT2 (F_{ST} = 0.43) can be at least partially explained by the high genetic flow among them (Nm = 2.3). However, the Atlantic and Pacific coasts of Mexico are too geographically isolated for pollen to move readily between them, suggesting that this genetic flow has been the result of seed movement. Introduction and genetic infiltration of MXAT with MXPT3 is also supported by the higher genetic diversity values in MXAT (H_E = 0.31) compared with WAT (H_E = 0.25), despite their possible common ancestry.

The UPGMA suggests that MXAT may be an introduction from Cape Verde, West Africa, since MXAT groups with WAT. Using SSR, Baudouin and Lebrun (2002) reported that the MXAT ecotype did group with Indo-Atlantic region ecotypes, further supporting its hypothetical East African or Indian origin. The UPGMA also suggested that the MYD ecotype forms part of the Asian-Pacific pool. The UPGMA grouped MYD with MXPT2. Like Baudouin and Lebrun (2002), the present study supports a South Pacific origin for MYD, though other studies have indicated it may have originated in Southeast Asia (Lebrun et al., 1998; Teulat et al., 2000). All four studies are consistent with the suggested center of domestication, Malesia, the region between continental Southeast Asia and the oceanic islands of the Southwest Pacific (Harries, 1990).

A close genetic relationship was shown in the UPGMA between MXPT1 and MXPT3, separating them from RIT, TAT, and PNT. The close genetic relationship between MXPT1 and MXPT3 may be explained by the high genetic flow (Nm = 5.6) between them. Baudouin and Lebrun (2002) reported a close genetic relationship between Philippine germplasm and MXPT1 and PNT; Philippine data were not included in the present study.

The high correlation found between the genetic distance to MYD and the levels of mortality observed, indicate that this distance could be used as a tool in the estimation of the potential mortality levels from LY in regions as yet unaffected and in the localization of populations that might show a low mortality from this disease and which could therefore be selected as pollen or seed producers, allowing us to take pertinent measures against LY.

The high morphological and physiological differentiation and the low mortality observed in MYD and MXPT2 suggest the convenience of using the hybrids between them in the fight against LY. However, it is important to develop other strategies against this disease, since a massive use of F1 hybrids must be done with great care because of the fact that the cultivation of their offspring by the producers could lead to a reduction in productivity in the medium term, instead of an increase. It would be convenient therefore, to also establish an improvement program through the selection of elite plants in populations of the tall ecotype MXPT2, where the ISSR could be of great help in the characterization of the ecotypes and in the estimation of their genetic distance with MYD. Thus, the established ISSR markers can be a useful tool both for estimating the level of potential impact of LY in areas as yet free of the disease and in the programs for selecting progenitors to be used in the initiation of improvement and replanting programs.

ACKNOWLEDGMENTS

This study was done at the Plant Genetic Resources Diversity and Molecular Evolution Laboratory of the Natural Resources Department, Centro de Investigación Científica de Yucatán (CICY). The authors thank the Benito Juárez Experimental Station, Guerrero, and the San Crisanto Ejido and Hacienda Oxtapacab, Yucatan, for plant samples. Thanks are also due Patricia Flores, Miguel Fernández, Nelson Torres and Julián Coello for their technical support. MRR thanks the Consejo Nacional de Ciencia y Tecnología (CONACYT)-México for her bachelor's thesis scholarship. This research was financed by CONACYT- Mexico, Project G34540-B.

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Table S1. Characteristics of primers used in Intersimple Sequence Repeat (ISSR) analysis of the nine studied *Cocos nucifera* ecotypes.

Primer Code	Repeat Motif (R = AG)	Sequence (5′-3′)	Annealing temperature
MS4	$GACA_4$	GACAGACAGACAGACA	48°C
MS15	$(GACA)_3RG$	GACAGACAGACARG	48°C
MS17	$(GACAC)_2$	GACACGACAC	42°C
MS22	$(GA)_8RG$	GAGGAGGAGGAGGAGGAGGAGGAG	56°C
MS30	$(GACAC)_3AG$	GACACGACACGACAC	54°C
MS31	$(GACAC)_3GG$	GACACGACACGACAC	56°C
MS32	$(GACAC)_3RG$	GACACGACACGACAC	54°C

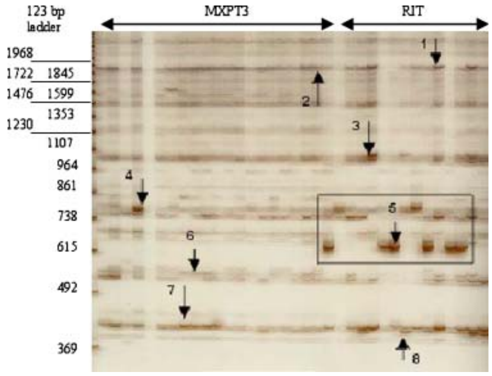

Fig. S1. Examples of ISSR profiles for the MS-17 primer of the Mexican Pacific Tall 3 (MXPT3) and Rennell Tall (RIT) ecotypes. Scored bands (pb): 1(1968), 2(1845), 3(1080), 4(810), 5(620), 6(560), 7(455), and 8(390).

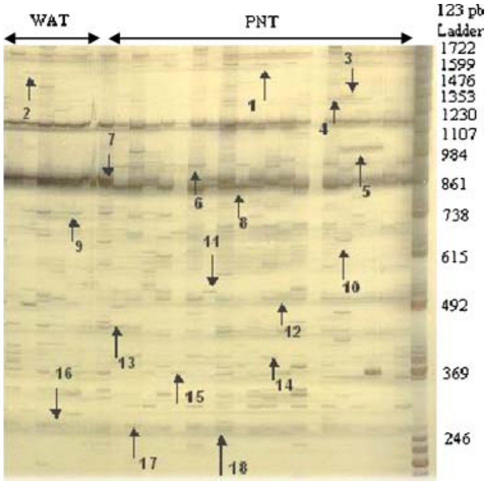

Fig. S2. Examples of ISSR profiles for the MS-30 primer of the ecotypes West African Tall (WAT) and Panama Tall (PNT). Scored bands (pb): 1(1650), 2(1580), 3(1353), 4(1343), 5(1035), 6(912), 7(879), 8(861), 9(738), 10(656), 11(510), 12(500), 13(438), 14(394), 15(369), 16(290), 17(285), and 18(280).

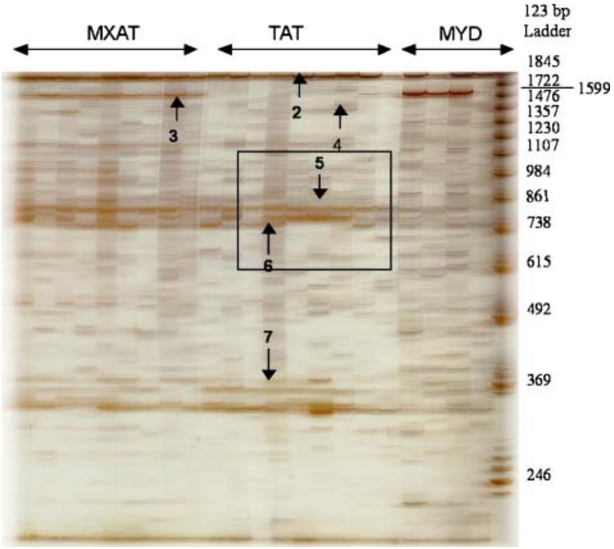

Fig. S3. Examples of ISSR profiles for the MS-32 primer of the of Mexican Atlantic Tall (MXAT), TAT and Malayan Yellow Dwarf (MYD) ecotypes. Scored bands (bp): 1(2249), 2(1845), 3(1661), 4(1425), 5(781), 6(759), and 7(352).

Table S2. Exact test for differentiation among ecotypes (Raymond and Rousset 1995), above significance in 33 loci, below percentage of loci with different alleic frequencies ($p < 0.05$) between.

	MXAT	MXPT1	MXPT2	MXPT3	WAT	PNT	RIT	TAT	MYD
MXAT	****	0.0000	0.0000	0.0000	0.0569	0.0000	0.0000	0.0000	0.0000
MXPT1	55	****	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000
MXPT2	79	37	****	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000
MXPT3	48	21	42	****	0.0000	0.0002	0.0000	0.0000	0.0000
WAT	18	52	70	55	****	0.0000	0.0000	0.0000	0.0000
PNT	55	34	45	24	40	****	0.0000	0.0000	0.0000
RIT	61	27	33	24	48	21	****	0.0186	0.0000
TAT	58	39	21	27	40	34	21	****	0.0000
MYD	82	67	30	64	82	67	58	52	****

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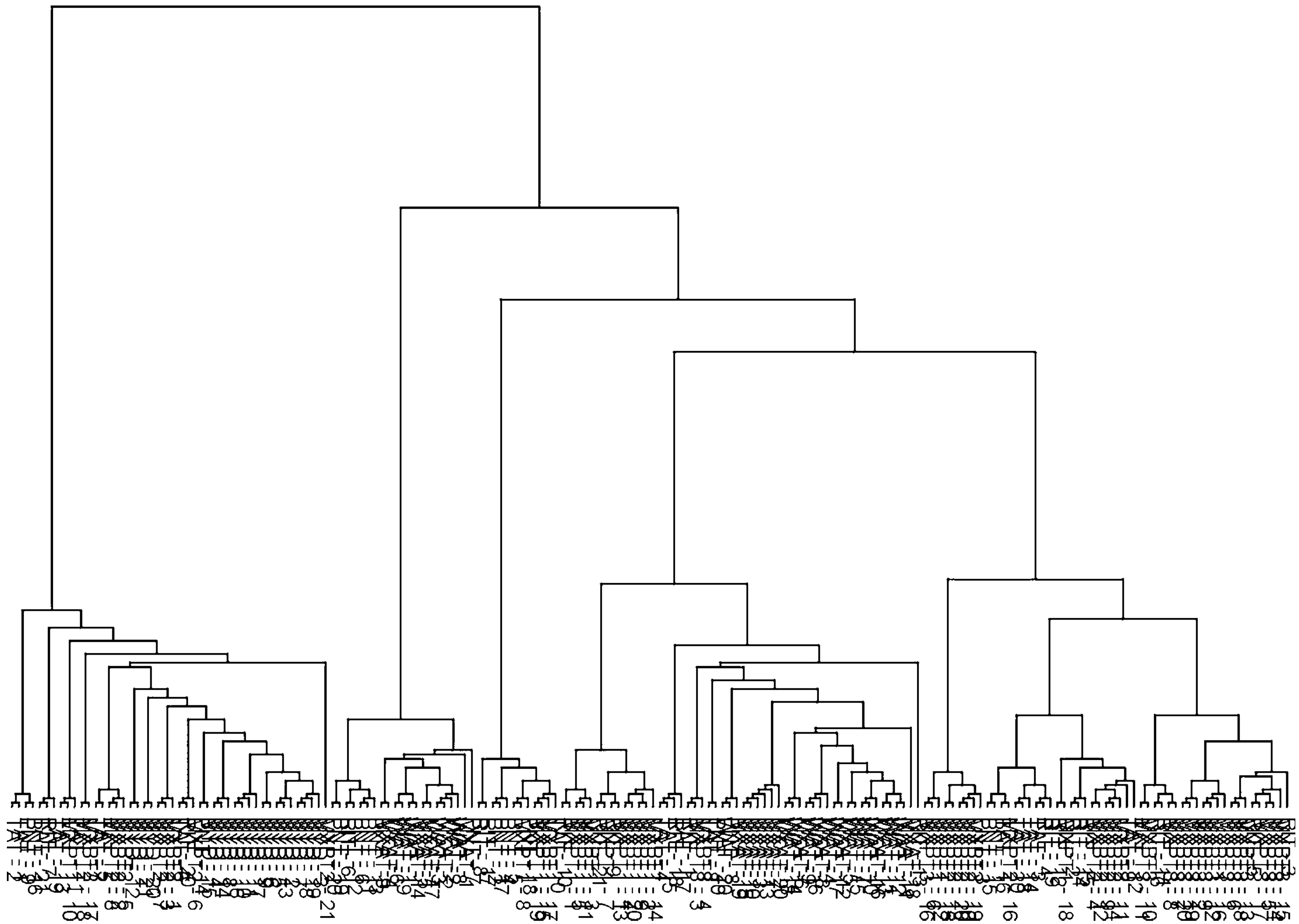

Fig. S4. Dendrogram of Neighbor-Joining analysis with Jaccard similarity coefficient (Saitou and Nei, 1987) with 180 individuals of nine *Cocos nucifera* ecotypes studied: MXAT: Mexican Atlantic Tall; MXPT1: Mexican Pacific Tall1; MXPT2: Mexican Pacific Tall 2; MXPT3: Mexican Pacific Tall3, WAT: West African Tall; PNT: Panama Tall; RIT: Rennell Island Tall; TAT: Tahiti Island Tall; MYD: Malayan Yellow Dwarf.

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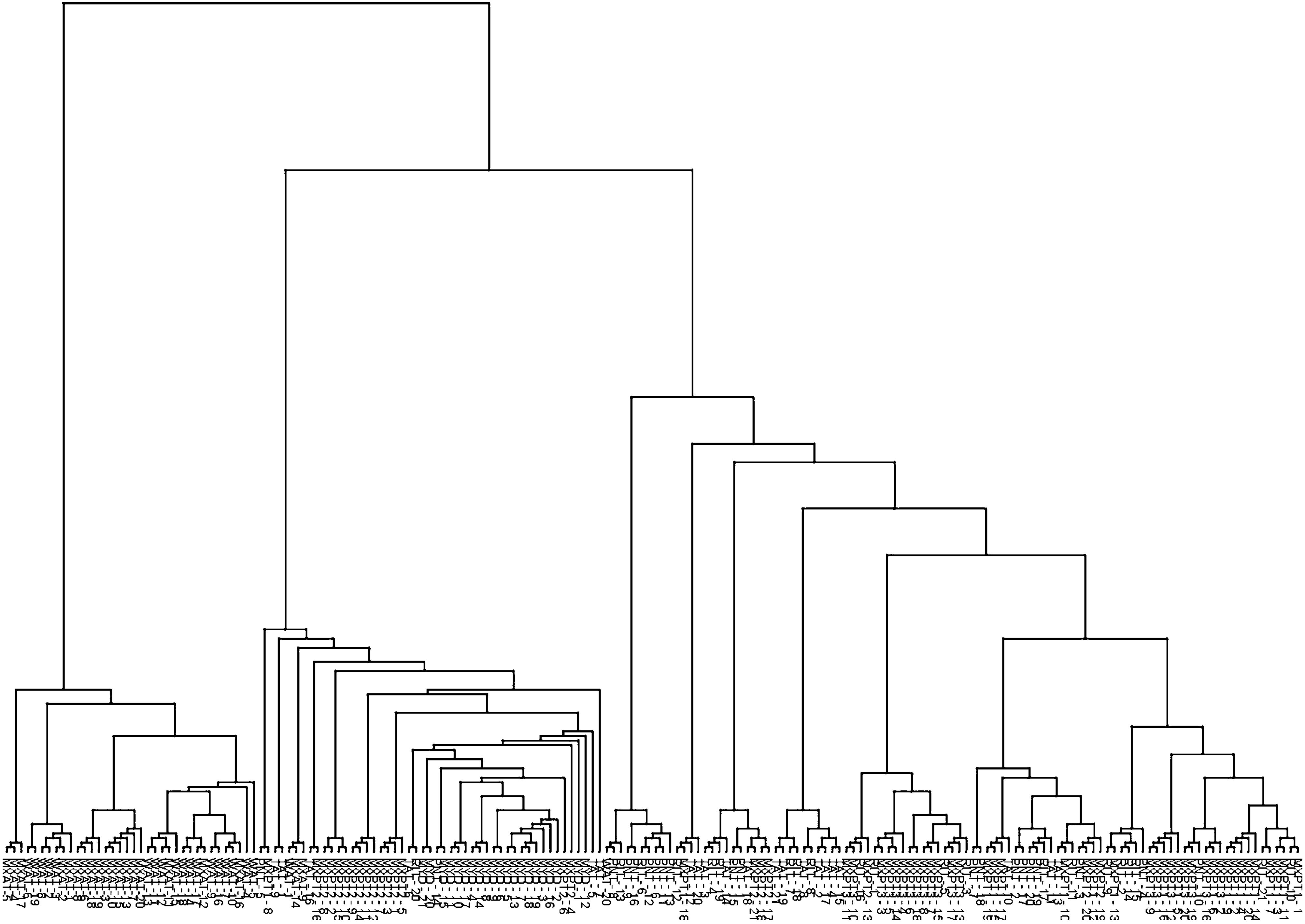

Fig. S5. **Dendrogram UPGMA based on the Nei's unbiased distance (Nei, 1978) with 180 individuals of nine *Cocos nucifera* ecotypes studied. MXAT: Mexican Atlantic Tall; MXPT1: Mexican Pacific Tall1; MXPT2: Mexican Pacific Tall 2; MXPT3: Mexican Pacific Tall3, WAT: West African Tall; PNT: Panama Tall; RIT: Rennell Island Tall; TAT: Tahiti Island Tall; MYD: Malayan Yellow Dwarf.**