



Trends in Molecular Sciences

ISSN 1994-5469

science
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DNA Molecular Analysis between Lethal Yellowing Disease and Non-lethal Yellowing Disease of Coconut Palms (*Cocos nucifera* L.) in Nigeria

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ABSTRACT

The aim of this study was to establish phylogenetic similarity between lethal yellowing susceptible and apparently healthy Coconut palm varieties in Nigeria. Lethal Yellowing Disease (LYD) of coconut palm is caused by phytoplasma, the organism is not detectable in culture media. It has practically wiped off great majority of the palms in South East, South-South and is now extending towards the South West. Crownless coconut palms are a common sight in these areas. Despite the wide geographical occurrence, no report is available on phylogenetic similarity between LYD and non-LYD palms. In this study, Random Amplified Polymorphic DNA (RAPD) was used to detect the phylogenetic similarity of 16 coconut varieties representing 4 species. A total of 30 polymorphic RAPD bands were detected. Similarity Coefficient (SC) between varieties ranged from 0.75 to 1.00 showing high level of similarity. Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendrogram clearly separated the LYD and non-LYD varieties. At SC value of 0.75, RAPD markers separated the hybrid and Malayan dwarf green from Malayan dwarf yellow and Malayan dwarf red varieties. At the value of 0.79, LYD and non-LYD varieties were grouped into four major clusters. First and second clusters distinguished themselves separately while the third and fourth, clustered together. Result indicated that hybrid (5S) and Malayan dwarf green (4S) varieties exhibited better non-LYD with Distant Coefficient (DC) from LYD infected varieties screened. This result could be exploited in breeding program.

Key words: Dendrogram, phytoplasma, phylogenetic, RAPD, similarity

INTRODUCTION

Coconut palm (*Cocos nucifera* L.) has long been of concerned because of the economic importance of coconut in Nigeria. Lethal yellowing is a highly destructive disease of the coconut palm (McCoy *et al.*, 1983). About 10 million families rely on coconuts as their main source of food and incomes (IPGRI, 2004). Lethal Disease (LD), a destructive Lethal Yellowing-type Disease (LYD) has become one of the major factors limiting coconut production in Nigeria. Symptoms of LD are similar to those caused by LYD in West Africa and the Caribbean (Schuiling *et al.*, 1981). Since, this disease was found around the 19th century in the Caribbean regions (Cordova *et al.*, 2003), it has destroyed several thousands hectares of coconut fields (Dery and Philippe, 1995). Coconut LY and related diseases are believed to be caused by phytoplasma as this cell wall-less prokaryotes

are consistently found in the phloem of diseased palms but not in healthy palms (McCoy *et al.*, 1983). Detection of phytoplasma for diagnostic purposes has been complicated by several factors including an inability to culture these pathogens in culture media, their small size and presence in low numbers in plant tissues (Thomas and Norris, 1980). The difficulty with LYD diagnosis is that symptoms vary according to the palm species (Harrison and Jones, 2004). Recently there has been reports in which LY DNA has been detected in coconut embryos (Cordova *et al.*, 2003) along with indications of phytoplasma seed transmission in alfalfa (Khan *et al.*, 2002), tomato, oilseed rape and lime (Botti and Bertaccini, 2006). Phytoplasma cause systemic infections. Electron microscope analysis of tissue from affected palms reveals phloem cells packed with the phytoplasma. This is assumed to cause a physical obstruction to the flow of nutrients, which eventually kills the tree (Smith, 2000).

Phytoplasmas are difficult to isolate and visualize from the plant species and therefore DNA-based molecular techniques for detection and diagnosis were applied (Harrison *et al.*, 2002). Increased sensitivity in phytoplasma detection has been attained through amplification of phytoplasma genomic DNA sequences by the use of Polymerase Chain Reaction (PCR) assays (Ahrens and Seemuller, 1992; Deng and Hiruki, 1991). In recent years, molecular markers have been applied to a wide number of genetic and breeding studies. One of the most extensively used molecular markers is RAPDs (Williams *et al.*, 1990) which have been applied to address genetic diversity issues in plants (Vilanova *et al.*, 2001; Gichuki *et al.*, 2003). The objective of the study was to establish the genetic similarity/distance between LY susceptible and apparently healthy coconut palm varieties in Nigeria.

MATERIALS AND METHODS

Plant sample for DNA isolation: Total DNA was extracted from 1 g of leaflet/coconut palm collected from coconut seed garden in NIFOR, in 2008 and 2009, using a metab protocol. They were Malayan dwarf red, 127 (8); dwarf red, 129 (9); dwarf red, 127 (10) and dwarf red 129 (11) are LYDs. The Malayan dwarf yellow 1650 (12); dwarf yellow 1654 (13); dwarf green 1721 (14); dwarf red 565 (15); dwarf green 1721 (16) are non-LYDs. Young leave tissues were also collected from coconut palms at NIFOR sub-station, Badagry Lagos State also in 2008 and 2009. They were Malayan dwarf red (1); dwarf green (2); dwarf yellow (3); dwarf green (4) ; hybrid (5); vanatu parent stock (6) and VTT×VTT = vanatu hybrid (7) are non-LYDs. One gram of fresh leaves of LYD and non-LYD were harvested and placed separately in each labeled mortar. Two milliliter of Dellaporta extraction buffer which was pre-heated at 65°C and 0.2 mL 20% SDS was added to each of the mortars. The leaves were then ground with a pestle and thoroughly mixed until the tissue became dispersed in the buffer. The mixture was then poured into correspondingly labeled falcon tubes. 0.5 mL of ice-cold 5 M Potassium acetate (CH₃COOK) was then added to the mixture and gently mixed 5-6 times by inverting the tubes. The tubes were place in ice for 30 min and then centrifuged at 4000 rpm for 20 min. The supernatant was carefully transferred to tubes.

DNA quantification using spectrophotometric analysis: DNA quantification was done using spectrophotometer measurement of UV absorption at wavelength 260, 280 and 320 nm. The DNA shows a clear absorption peak at A₂₆₀.

DNA quality check using gel electrophoresis: Gel electrophoresis tanks were available in different volumes usually 10-300 mL and this determine the amount of agarose to be measured.

Similarly, the concentration of agarose depend on the size of the DNA sample to be checked 0.7-1% concentration of agarose is required for DNA checking, while 2-3% concentration of agarose is required for PCR products.

Gel electrophoresis of DNA: The dissolved agarose gel mixture was cooled to a temperature of 50-55°C for 5 min before pouring the gel into a tray. The combs were properly fitted into the gel tray before the gel was poured into the tray and allowed to solidify for 20-30 min. The tray was then placed in the tank with 0.5× TAE buffer. The samples were then allowed to run into the gel at a constant voltage of 60-90 volts for 1.5 h. It was visualized under ultra-violet trans-illumination and expected bands were observed and recorded using a Polaroid camera.

PCR amplification of DNA sequence: The cocktail mix consisted of 10X buffer (Biorad) 1.25 mL, MgCl₂ (Bioline) 1.0 mL, 5% Tween 20 at 1.25 mL, 2.5 mM dNTPs (Sigma) 0.5 mL, Taq polymerase (Biorad) 0.2 mL, RAPD primers (Operon) 0.5 mL, Distilled water 5.25 mL and Template DNA 2.5 mL, totaling 12.5 mL, were required for Random Amplified Polymorphic DNA (RAPD) reaction, this was put into the PCR tube. The PCR buffer, dNTPS, MgCl₂ solution and primer solution were thawed from frozen stocks and mixed by vortexing and placed on ice. The thawed DNA was also mixed gently. The assembled reaction was sealed, vortexed, centrifuged and placed in the PCR machine for DNA amplification.

DNA amplification in thermocycler: A fast thermocycler (TECHNE TC3000) was used in carrying out this project. Profile for PCR in thermocycler consisted of 94°C for 3 min, 94°C for 20 sec, 37°C for 40 sec, 72°C for 1 min, repeat stage 2-4 for 44 cycles, 72°C for 7 min extension time and 4°C for ever.

Agarose gel electrophoresis of PCR products: 1.5% agarose gel concentration was used for the PCR products using 0.5×TAE electrophoresis running buffer solution. The gel was placed in the electrophoresis tank and allowed to run for about 2.5 h. The gel was visualized under ultra-violet trans-illuminator and the expected bands were observed using Polaroid camera.

Data analysis: Data were subjected to cluster analysis using pair wise distance (similarity) matrices and was compared with sequential and nested (SAHN) clustering analysis option of the NTSYS-pc version 2.02j software package (Rohlf, 1993). The program generated dendrogram, which grouped the test lines on the basis of Nei genetic distance (Nei, 1972) using Unweighted Pair Group Method with Arithmetic (UPGMA) average cluster analysis (Sneath and Sokal, 1973).

RESULTS

All samples showed smooth absorbance scan with symmetric peak at 260 nm, indicating high purity. The quality of the extracted bands indicated the presence of quality DNA observed (Fig. 1). Out of 80 primers evaluated, 76 were monomorphic while 4 were polymorphic. The 4 polymorphic primers were selected based on their quality and reliability of their amplification A total of 30 fragments were scored from 4 primers with most bands ranging in size from 6 to 9 (Table 1). Of the total bands scored, 30 (100%) were polymorphic (Fig. 2). The OPERON OPT-12 produced the greatest number of polymorphic fragments (9 bands) while OPERON OPV-06 produced the lowest number of polymorphic fragments (6 bands). None of the DNA bands shared or was identical to DNA marker band indicating the collection did not duplicate.

Table 1: No. of polymorphic bands obtained from four operon universal primers among 16 coconut varieties

Primer	Polymorphic bands	Monomorphic bands	Polymorphism (%)
OPERON ODI-14	7.0	-	100
OPERON OPS-10	8.0	-	100
OPERON OPT-12	9.0	-	100
OPERON OPV-06	6.0	-	100
Total	30.0	-	400
Mean	7.5	-	100

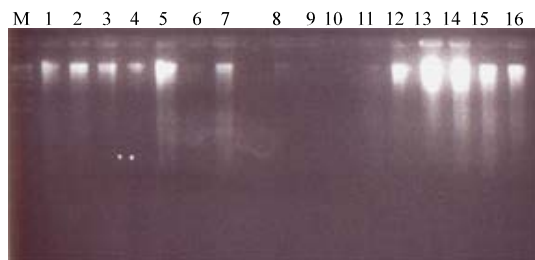


Fig. 1: DNA quality of 16 Coconut varieties from Badagry and NIFOR. Badagry: Lane M: Marker, Lane 1: (non-LYD) Malayan dwarf red, Lane 2: (non-LYD) Malayan dwarf green, Lane 3: (non-LYD) Malayan dwarf yellow, Lane 4: (non-LYD) Malayan dwarf-green, Lane 5: (non-LYD) hybrid, Lane 6: (non-LYD) Vanatu parent stock, Lane 7: (non-LYD) Vanatu hybrid. NIFOR: Lane 8: (LYD) Malayan dwarf red 127, Lane 9: (LYD) Malayan dwarf red 129, Lane 10: (LYD) Malayan dwarf red 127, Lane 11: (LYD) Malayan dwarf red 129, Lane 12: (non-LYD) Malayan dwarf yellow 1659, Lane 13: (non LYD) Malayan dwarf yellow 1654, Lane 14: (non-LYD) Malayan dwarf green 1721, Lane 15: (non-LYD) Malayan dwarf red 565 and Lane 16: (non-LYD) Malayan dwarf green 1721

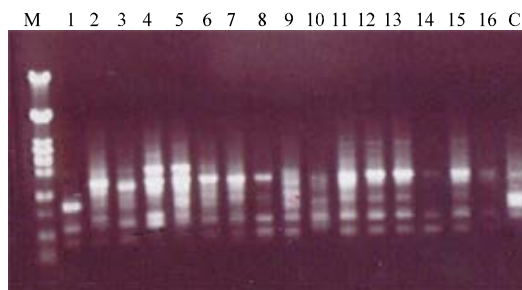


Fig. 2: DNA detection of 16 coconut varieties from Badagry and NIFOR in PCR using OPERON OPT 12 universal primers

Genetic Similarity Coefficients (SC) and Distance Coefficients (DC) were based on RAPD markers.

However, an Unweighted Paired Group using Mathematical Average (UPGMA) dendrogram grouping of Badagry and NIFOR coconut varieties together formed four major clusters (Fig. 3). The first major cluster Malayan dwarf green (4) was grouped with second major cluster hybrid (5), the third major cluster (14 H) Malayan dwarf green was grouped with the fourth cluster of different varieties from Badagry and NIFOR (Fig. 3). The first and second major clusters distinguished

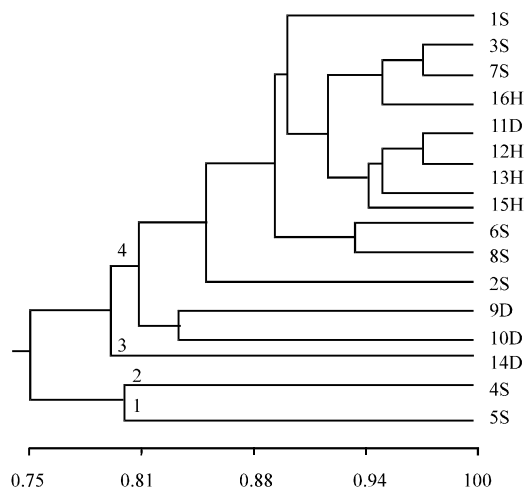


Fig. 3: UPGMA dendrogram of the 16 coconut varieties from Badagry and NIFOR based on RAPD markers. Badagry: 1S (non-LYD) Malayan dwarf red; 2S (non-LYD) Malayan dwarf green; 3S (non-LYD) Malayan dwarf yellow; 4S (non-LYD) Malayan dwarf-green; 5S (non-LYD) hybrid; 6S (non-LYD) Vanatu parent stock; 7S (non-LYD) Vanatu hybrid. NIFOR: 8D (LYD) Malayan dwarf red 127; 9D (LYD) Malayan dwarf red 129; 10D (LYD) Malayan dwarf red 127; 11D (LYD) Malayan dwarf red 129; 12H (non-LYD) Malayan dwarf yellow 1659; 13H (non LYD) Malayan dwarf yellow 1654; 14H (non-LYD) Malayan dwarf green 1721; 15H (non-LYD) Malayan dwarf red 565; 16H (non-LYD) Malayan dwarf green 1721

themselves clearly from the third and fourth major clusters. Out of 7 non-LYDs coconut varieties screened from Badagry, 5 varieties clustered with both LYDs and non-LYDs from NIFOR coconut garden while the remaining 2 varieties (4S and 5S) did not cluster with any of the varieties from NIFOR. Moreover, all the 9 varieties from NIFOR which were LYDs and non-LYDs clustered among themselves (Fig. 3).

DISCUSSION

It is essential to understand the extent and distribution of lethal yellowing disease causing premature nut-falls in coconut growing regions in Nigeria. Furthermore, information regarding DNA molecular analysis could be used to help identify and develop resistant cultivars. The RAPD method allowed us to access phylogenetic similarity between susceptible/tolerant coconut varieties. This agreed with Momeni and Razmjoo (2006), reported the use of RAPD method to access genetic diversity between and within *Mentha* species.

The percentage of polymorphic RAPD fragments detected in this collection of sixteen coconut varieties was 100%. This supported Khanuja *et al.* (2000) which reported 93.5% for a collection of diverse *Mentha* accessions. Genetic similarity estimates based on molecular marker data have been described as a direct measure of genetic similarity (Graner *et al.*, 1994; Russell *et al.*, 1997). The accuracy of phylogenetic similarity is based upon the number of polymorphic bands obtained from the primers. This agreed with Schut *et al.* (1997), reported the accuracy of genetic similarity estimates based on molecular data depends on several variable factors such as the number of markers analyzed, their distribution over the genome and the accuracy of scoring the markers. Analysis of these RAPD profiles for band similarity indices clearly differentiated all the LYDs and

non-LYDs coconut varieties from Badagry and NIFOR. This result based on molecular markers showed that the seven coconut varieties from Badagry are closely related with the nine varieties from NIFOR. Moreso, the presently non-infected (non-LYDs) varieties such as 12H, 13H and 15H clustered with 11D which was infected variety (LYDs), all from NIFOR. Further more, 6S non-LYDs from Badagry, clustered with 8D LYD from NIFOR. Both 1S and 2S also non-LYDs from Badagry clustered with 12 H, 13H and 15H from NIFOR (non-LYDs). This agrees with Mpunami *et al.* (1995), reported that LD can be detected in symptom-less palms. Only 4S and 5S (non-LYDs) from Badagry clearly distinguished themselves by forming one major cluster each that did not cluster or sub-cluster with the other varieties. The phylogenetic similarity among the coconut varieties can be exploited in breeding program. This supported Gobert *et al.* (2002), reported genetic variability could in future be exploited through molecular approaches for gene introgression in breeding programs to produce desire genotypes. However, this study was not able to detect phytoplasma causing LYD. This supported Lee *et al.* (1993), reported that development of specific DNA probes has provided a reliable means for identification number of different phytoplasmas, but Cordova *et al.* (2003) successfully used RAPD to identify markers associated with resistance to coconut lethal yellowing disease.

This study has shown that RAPD analysis has the potential to group LYDs and non-LYDs coconut varieties based on phylogenetic similarities. The study also demonstrated that presently infected LYD coconut varieties in NIFOR are phylogenetically similar with non-LYDs from NIFOR and Badagry coconut gardens. The Malayan dwarf green (4) and hybrid (5) varieties which were non-LYD from Badagry proved to be the best when compared with the other coconut varieties screened. However, more screening should be pursued before a decisive recommendation can be made.

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