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Genetic diversity in the coconut lethal yellowing disease phytoplasmas of East Africa

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DNA primers, based on the ribosomal sequences of lethal yellowing-type disease (LYD) phytoplasmas, were used to analyse genetic variation within the lethal yellowing-type diseases of coconut in East Africa. Samples were collected from palms in Kenya, Mozambique and high, medium and low disease incidence areas of Tanzania. The mollicute-specific primer pair P1 and P6 amplified a 1·5 kbp product from all diseased palms and no product from symptomless palms, indicating that phytoplasmas were associated with all of these diseases. However, the Rohde forward and Rohde reverse primers (a second rRNA primer pair designed to detect East African LYD-associated phytoplasmas) only amplified products from Tanzanian and Kenyan diseased palms and not from those of Mozambique. Conversely, primers Ghana 813 and AK-SR, designed for specific detection of coconut-associated phytoplasmas in West Africa, amplified products only from the Mozambique palms, indicating that the phytoplasma associated with LYD in Mozambique is more closely related to those from West Africa. This was supported by restriction enzyme digestion of PCR products. DNA sequencing of PCR products from phytoplasmas within Tanzania revealed no detectable differences in the rDNA sequences of isolates from high, medium and low incidence areas.

Keywords: coconut, genetic diversity, lethal yellows, PCR, phytoplasma, rDNA

Introduction

Coconut palm (*Cocos nucifera*) is an important perennial oil crop that supports the livelihood of most farmers in coastal areas of Tanzania, providing food, building materials and also conservation of the environment, as an agroforestry crop. A total of 22 million palms grow on 240 000 ha along the coastal belt of mainland Tanzania and the islands of Zanzibar, Pemba and Mafia. However, a destructive lethal yellowing-like disease known as Lethal Disease (LD), first reported affecting coconuts near Bagamoyo early in this century (Stein, 1905), has caused extensive damage to plantations on the mainland for more than 30 years, and is now present on the island of Mafia.

The incidence of disease differs significantly among the affected regions on the mainland. It is widespread in the southern regions, where it has killed about 56% of palms since 1965, while only 8·5% have been affected in the northern regions (Schuiling *et al*., 1992a). These differences are difficult to reconcile because environmental conditions, including moisture, temperature, soils, flora and insect fauna on palms, appear to be similar. Schuiling *et al*. (1992a) suggested that genotypic differences within the local coconut populations in relation to the origin of the coconut groves might be responsible. Another possible explanation could be that different strains of the pathogen or different insect vectors are involved.

Symptoms of LD are premature nutfall, bronzing of successively younger leaves, blackening of young emergent inflorescences, drying of older inflorescences, rot and collapse of the spear leaves, and decay of the root system, with subsequent sudden death (Schuiling *et al*., 1992a). Affected palms die within 4–6 months of the onset of symptoms, and the disease affects palms at all ages, including transplants as young as 18 months. Similar symptoms have been reported for other yellowing diseases of coconut associated with phytoplasmas. These include Lethal Yellowing (LY) in the Caribbean, Florida and Mexico (Plavsic-Banjac *et al*., 1972; Thomas, 1979; Robert & Zizumbo, 1990), Cape St. Paul Wilt (CSPW) in Ghana (Dabek *et al*., 1976), Kaincopé disease in Togo (Nienhaus & Steiner, 1976), Kribi disease in Cameroon (Dollet *et al*., 1977), and Awka or bronze leaf wilt in Nigeria (Bull, 1955; Ekpo & Ojomo, 1990). The yellowing diseases also occur in Kenya (Nienhaus, 1984) and Mozambique (Mpunami & Seguni, unpublished observations). All these yellowing diseases, including LD, are collectively referred to as lethal yellowing-type diseases (LYD).

Despite symptomatological similarities with LY, LD

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differs with respect to epidemiology, susceptibility of coconut varieties and insect vectors. Whereas LY is characterized by a 'rapid jump spread' pattern in the Caribbean (McCoy, 1976), jump spread is rare with LD (Schuiling *et al.*, 1992a). The vector of LY has been reported as the planthopper, *Myndus crudus* (Howard *et al.*, 1983), but this insect has not been found in association with coconuts in Tanzania (Schuiling & Mpunami, 1990). Furthermore, while some coconut varieties, such as the Malayan and Sri Lanka Green dwarfs, are considered highly resistant to the Caribbean LY (Been, 1981), all are susceptible to LD in Tanzania (Schuiling *et al.*, 1992b). These differences led to speculation that different pathogens might be involved for each disease (Schuiling *et al.*, 1992b), and this was recently confirmed by genomic studies of the respective phytoplasmas (Harrison *et al.*, 1994; Tymon, 1995).

A recent approach to phytoplasma detection and classification has been the analysis of the highly conserved genes coding for the 16S rRNA, and the spacer region between 16S and 23S rRNA (Lee *et al.*, 1993; Schneider *et al.*, 1993). By using a primer pair based on the 16S rRNA gene, selective amplification of phytoplasma DNA from mixtures with host DNA has been achieved (Deng & Hiruki, 1991; Ahrens & Seemüller, 1992; Lee *et al.*, 1993; Namba *et al.*, 1993). Lee *et al.* (1993) amplified about 80% of the gene sequence of 40 different phytoplasma strains from different continents. The amplified sequences were compared by RFLP analyses and used to classify the phytoplasmas into distinct 16S ribosomal RNA (16Sr) groups and subgroups.

In the present study, primers based on the 16S ribosomal RNA gene were used to look for genetic variation in the LD phytoplasmas from different areas within Tanzania, to determine whether there is any detectable strain variation that might account for the difference in disease incidence. The Tanzanian LD was also compared with LYD isolates from neighbouring Kenya to the north and Mozambique to the south.

Materials and methods

Plant material

Palms from Tanzania for extraction of DNA were selected from National Coconut Development Programme (ARI Mikocheni) trial sites. Crowns were excised from East African Tall (EAT) palms from Sotele and Kifumangao in southern Tanzania (high incidence), Chambezi in central Tanzania (moderate incidence) and Kigombe in the north (low incidence). Three EAT palms were also sampled at Mpeketoni on the northern Kenya coast, and three from Mocimboa da Praia and Pemba in northern Mozambique. The excised palm heart tissues were brought to the laboratory at NCDP, trimmed, and immediately processed for DNA extraction. Where immediate extraction was not possible, they were kept at 4°C and processed within three days. From each site, symptomless palms were also selected for comparison. DNA from Ghanaian and Nigerian samples were from the collection held at IACR-Rothamsted.

Extraction of DNA

DNA was prepared essentially by the miniprep procedure of Doyle & Doyle (1990), except that tissue was not frozen in liquid nitrogen for extraction. Instead, fresh meristematic tissue (5 g) was diced and directly ground to a paste with a mortar and pestle in the presence of acid washed sea sand and 15 mL preheated (65°C) CTAB buffer (2% (w/v) CTAB, 1·4 M NaCl, 0·2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8·0). After incubation at 60°C for 30 min, the lysate was cooled to room temperature, then extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The mixture was centrifuged for 10 min at 20,000 × g and the aqueous phase re-extracted with chloroform/isoamyl alcohol. Precipitation of nucleic acids from the aqueous phase was by addition of 0·1 volumes of 3 M sodium acetate, pH 5·2, and 1 volume of isopropanol. Large cobwebs of nucleic acid were then spooled out with a glass hook. Alternatively, the mixture was chilled at – 20°C for at least 2 h, then centrifuged at 20,000 × g for 10 min to pellet the nucleic acid. Pellets were each rinsed in 0·5 mL of 80% (v/v) ethanol, allowed to air dry, and dissolved in 2 mL of 1 × TE buffer, pH 8·0, containing RNAase A at a concentration of 10 μg mL^{-1}, and incubated at 37°C for 1 h. A 0·25 volume of 5 M NaCl and 2·5 volumes of cold 95% (v/v) ethanol were added to each tube to precipitate the DNA as described above. Pellets were again rinsed and dried as described, then dissolved in 1 mL of 1 × TE buffer, pH 8·0, and stored at 4°C, until required for amplification.

Detection of phytoplasma DNA in palm tissue by PCR

Three different 16S rRNA primer combinations were used to amplify phytoplasma 16S rDNA from LD-infected palms. These were Rohde forward primer (5′-GAG TAC TAA GTG TCG GGG CAA-3′) with Rohde reverse primer (5′-AAA AAC TCG CGT TTC AGC TAC-3′) (Rohde *et al.*, 1993); forward primer P1 (5′-AAG AGT TTG ATC CTG GCT CAG GAT T-3′) with reverse primer P6 (5′-TGG TAG GGA TAC CTT GTT ACG ACT TA-3′) (Deng & Hiruki, 1991); and Ghana 813 (5′-CTA AGT GTC GGG GGT TTC C-3′) with AKSR (5′-TTG AAT AAG AGG AAT GTG G-3′) (Tymon, 1995).

PCR conditions were optimized for each of the combinations used, and for each PCR a 25 μL reaction mixture contained about 50 ng template DNA extracted from LD-infected tissue, 150 μM mixed deoxynucleotide triphosphates (dNTPs), 50 ng of each primer, 1 Unit of Taq Polymerase (Promega Corporation), and PCR buffer (100 mM Tris-HCl pH 8·3, 500 mM KCl, 15 mM $MgCl_2$,

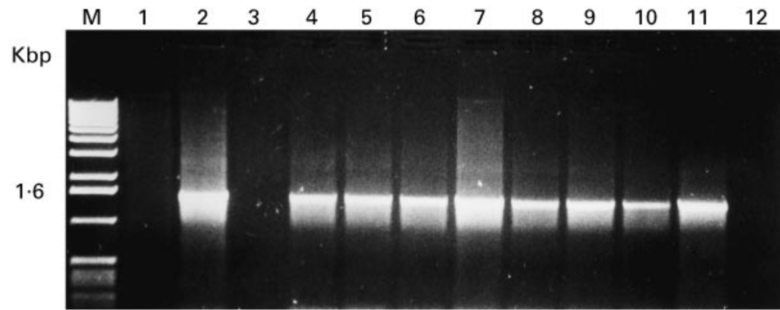


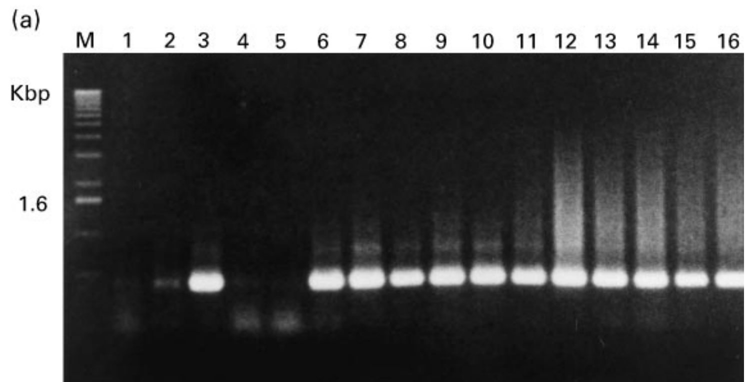
Figure 1 Amplification of phytoplasma DNA using the P1 and P6 universal primers. PCR products were analysed on a 1% agarose gel. Lanes: M, 1 kbp ladder marker; 1, healthy palm (Tanzania); 2, LD-infected palm (Tanzania); 3, healthy palm (Mozambique) 4–11, LYD-infected palms (Mozambique); 12, water control.

0·01% gelatine, 0·5% (v/v) Nonidet P40, and 0·5% (v/v) Tween (20). The mixture was overlaid with 25 μL of mineral oil, and subjected to 36 cycles in an automated thermocycler (Biometra, UNO Thermoblock) using the following parameters: 1 min (2 min for the first cycle) denaturation at 94°C, 1 min 20 s of annealing at 57°C, and 2 min 10 s (5 min for last cycle) extension at 72°C. Reaction mixtures containing DNA template from symptomless palms, and/or water substituted for DNA served as negative controls in each experiment. The annealing temperature depended on the primer combination: 57°C for Rohde forward/Rohde reverse, and 52°C for the other primers.

PCR products were analysed by electrophoresis through a 1% (w/v) agarose gel and visualized in the gel by UV transillumination after staining with ethidium bromide.

Cloning of PCR products and DNA sequencing

PCR bands were excised from gels and purified using the Geneclean II kit (Bio 101, Anachem) according to the manufacturer's instructions. They were ligated into the pTAg vector (R & D Systems, Europe Ltd, UK) and transformed into *E. coli* DH1-derived competent cells, according to the manufacturer's instructions. Transformed colonies containing cloned inserts were identified as white colonies by blue-white screening, and single white colonies picked from culture plates were added to 2 mL LB medium with 0·1 mg mL^{-1} ampicillin, and grown overnight at 37°C. Plasmid DNA containing the cloned insert was prepared for sequencing essentially as described in Sambrook *et al.* (1989) and 200 ng DNA was sequenced on an ABI 373 automated sequencer at



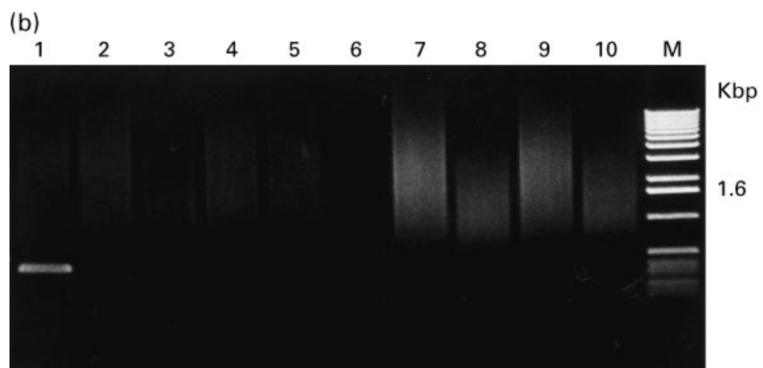


Figure 2 Amplification using Rohde forward and reverse primers. (a). Lanes: M, 1 kbp ladder marker; 1, symptomless palm (Chambezi); 2, symptomless palm (Kifumangao); 3 infected palm (Chambezi); 4, symptomless palm (Chambezi); 5, symptomless palm (Kifumangao); 6, infected palm (Chambezi); 7–8, infected palms (Kifumangao); 9, infected palm (Kenya); 10, infected palm (Sotele); 11, infected palm (Chambezi); 12–13, infected palms (Kifumangao); 14–16, infected palms (Kenya). The faint bands in lanes 1, 2, 4 and 5 may be caused by low levels of latent infection in these palms, as no such bands were present in symptomless palms. (b). Lanes: 1, infected palm (Tanzania); 2, healthy palm (Tanzania); 3–10, infected palms (as used in Fig. 1) (Mozambique); M, 1 kbp ladder marker.

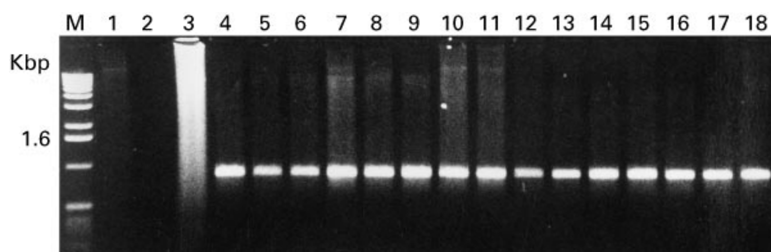


Figure 3 Amplification using primers Ghana 813 and AKSR. Lanes: M, 1 kbp ladder marker; 1, healthy palm (Tanzania); 2 infected palm (Tanzania); 3, healthy palm (Mozambique); 4–18, infected palms (Mozambique).

the University of Nottingham. Primers for plasmid sequencing were either M13 forward primer or M13 reverse primer, and sequence data was analysed using DNASTAR and on-line databases.

Results

Detection of LYD phytoplasmas using the P1/P6 universal primers

To confirm that phytoplasmas were associated with LYD in Mozambique, Kenya and Tanzania, PCR incorporating the P1/P6 primer pair, designed by Deng & Hiruki (1991) as mollicute-specific 16 s rDNA primers, was used to analyse DNA derived from symptomless and diseased palms. A 1·5 kbp product was amplified from all diseased samples tested from Mozambique and Tanzania (Fig. 1) and from Kenya (results not shown). No product was obtained from symptomless palm tissue.

Analysis of variation between isolates

Having confirmed that phytoplasmas were consistently associated with the LYD throughout East Africa, the Rohde forward and reverse primers were used to look for variation between isolates both from different countries and from the high, medium and low incidence areas within Tanzania. A 560 bp DNA band was resolved by agarose gel electrophoresis from all LD-infected palms in Tanzania and Kenya but not from symptomless coconuts (Fig. 2a). However, no such band was detected from any of the Mozambique palms from which bands had previously been amplified with the P1 and P6 primers (Fig. 2b).

To analyse variation within the Tanzanian phytoplasmas, the PCR products obtained with Rohde forward and reverse primers from 3 palms in Kifumangao (high incidence), 3 palms in Chambezi (medium incidence) and 3 in Tanga (low incidence) were each cloned into the pTAg plasmid vector. All nine clones were sequenced using the Universal M13 forward and reverse primers and compared. There were no detectable differences in the sequences between any of the isolates throughout the entire 560 bp of the cloned regions (results not shown), or between these and the sequence deposited in the EMBL database by Tymon A.M. (accession X80117).

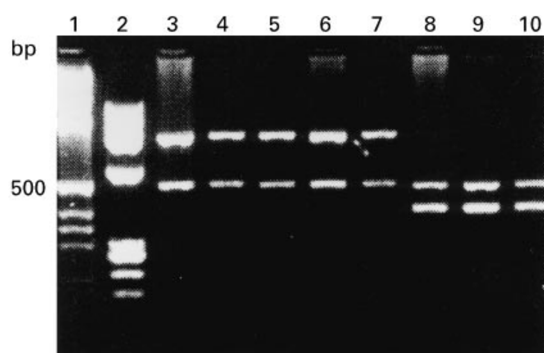


Figure 4 *Rsa* I restriction digest of 16S rDNA amplified from palms infected with LYD using primers P1 and P6. The different LYD isolates are from Ghana (lanes 3–4), Nigeria (5), Mozambique (6–7), Kenya (8) and Tanzania (9–10). Lanes 1 and 2 are size markers, 1 kbp ladder and ϕX174, respectively.

The origin of the Mozambique LYD phytoplasma

Failure to amplify an rDNA product from DNAs of LYD-affected Mozambique isolates by PCR incorporating the Rohde primer pair suggested that phytoplasmas associated with the latter palms were dissimilar from the pathogen commonly associated with East African LYD. However, upon further analysis of coconut samples by PCR employing primer pair Ghana 813 and AKSR, a 1·0 kbp rDNA product was amplified from all LYD-affected Mozambique palms but not from Tanzanian LD-affected palms (Fig. 3). Positive detections resulting from the latter assay indicated that phytoplasmas infecting Mozambique palms are quite similar to those previously implicated with LYD in West Africa.

To substantiate relationships between coconut-associated phytoplasmas in Mozambique and West Africa, the 1·5 kbp PCR products amplified from LD DNA, Kenyan LYD DNA, Mozambican LYD DNA, CSPW DNA and Awka disease DNA using the P1/P6 primer pair were digested with the restriction enzyme *Rsa* I (Fig. 4). The pattern of restriction fragments was the same for LD and Kenyan LYD, giving rise to a band of 600 bp and a presumed doublet at 450 bp. This was in contrast to the pattern shown by Mozambique, CSPWD and Awka phytoplasmas, in which there was

the same band of 600 bp and a larger band of 900 bp. The patterns for these phytoplasmas were, however, identical to each other, strongly supporting the evidence that the Mozambique phytoplasma was of the West African LYD type. These results were supported further by digestion using a second enzyme, *Alu* I (results not shown).

Discussion

With the increasing use and development of PCR-based diagnostic techniques, analysis of the causal agents for many plant diseases is becoming increasingly robust and capable of discriminating between pathogens that induce similar host plant symptoms. In this study, the lethal yellowing-type diseases of coconut in East Africa were analysed. Despite very similar syndromes, previous studies on coconut LYD in East and West Africa and the Caribbean have shown that genetically distinct phytoplasmas are associated with each of these diseases (Harrison *et al*., 1994; Tymon, 1995). The results from the current study show that primers that specifically detect the LD phytoplasma can be used to differentiate it from other closely related but genetically different organisms. The causal agent of LYD in northern Mozambique could not be amplified with the Rohde forward/reverse primer combinations, whereas the Tanzanian and Kenyan samples could. However, universal mollicute primer combinations were able to amplify bands from both Mozambique and Tanzanian samples, confirming that all these diseases were associated with phytoplasmas. Thus, the Rohde forward/reverse primer pair proved very useful for differentiating the LD phytoplasma from strains of coconut LYD phytoplasmas, which were similar but not genetically identical.

Tests using a third set of primers specifically designed to amplify West African LYD phytoplasmas, combined with restriction enzyme digestion of PCR products, provided evidence that the Mozambique phytoplasma is more closely related to the West African LYD than to LD. The origin of the Mozambique disease is unclear, and there remains the possibility that it has been spread from West Africa to Mozambique (or vice versa) in contaminated palms. There is no evidence to suggest that the phytoplasmas causing LD in Tanzania and LYD in Kenya are different.

Within Tanzania, the incidence of LD varies between the north and south of the country (Schuiling *et al*., 1992a). Possible explanations for this are genetic variability within the palm populations; different insect vectors; or different strains of the pathogen. In this study, a comparison was made of the sequences of the PCR fragments amplified by the Rohde forward and reverse primers from isolates obtained in low, medium and high incidence areas. Sequence data from the amplified ribosomal DNA fragments showed no indication of strain differences, although this could be because the amplified region is short and based on the highly conserved region of the 16S ribosomal gene. Sequencing and analysis of fragments amplified from the more variable 16S/23S intergenic region may possibly show differences, if they exist.

One possible alternative explanation to account for the higher disease incidence in the south of Tanzania is that both the Tanzanian LD and the LYD from Mozambique are affecting palms in this region, and that the Mozambique LYD has as yet not spread to northern Tanzania. The current studies did not rule out this possibility, because the Ghana 813 and AKSR primers were not used in a detailed survey of palms from within Tanzania. This study confirmed the existence of different phytoplasma strains associated with LY-type disease in East Africa. With the development of primers capable of distinguishing these strains, studies on aspects of disease epidemiology such as the relationships between strain differences and disease incidence, or vector identification, are now possible.

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