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First report of '*Candidatus* Phytoplasma palmicola' detection in the planthopper *Diostrombus mkurangai* in Mozambique

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Abstract

Knowledge of putative insect species vectors of the coconut lethal yellowing disease (CLYD) in Mozambique is crucial to develop an effective disease management plan. Hemiptera specimens from the families Derbidae and Pentatomidae were collected in the Inhambane and Zambezia provinces of the coastal region of Mozambique in 2014, covering the two main growing seasons. Sequence analyses of the 16S rRNA gene were used for phytoplasma clustering. Polymerase chain reaction (PCR) amplification was performed employing three different primer sets specific for phytoplasma. BLAST sequence comparison and phylogenetic analysis of the 16S rDNA PCR products revealed that collected specimens of Derbidae *Diostrombus mkurangai* Wilson were carrying the CLYD phytoplasma. Virtual RFLP analyses of the obtained sequences confirmed this assigning the detected phytoplasmas to the 16SrXX-A subgroup, confirming that they are '*Candidatus* Phytoplasma palmicola'-related strains. This is the first detection of a '*Candidatus* Phytoplasma palmicola'-related strain in *D. mkurangai*.

Key words: coconut palm (*Cocos nucifera*), coconut lethal yellowing phytoplasma, insect vector, phylogeny.

Introduction

The coconut palm (*Cocos nucifera* L.) is a major cash crop in Mozambique, widely grown in coastal regions. It contributes to the livelihood, income, nutrition and food security of millions of rural inhabitants. Epidemic outbreaks of a coconut lethal yellowing disease (CLYD) associated with the presence of phytoplasmas have killed more than eight million coconut trees, and are threatening the industry and the livelihood of over 14% of the people in Mozambique. CLYD is impacting on coconut production worldwide (Oropeza *et al.*, 2005). Similar devastating lethal yellowing-like diseases (LYD) of coconut palms have occurred in Africa (Eden-Green, 1997; Tymon *et al*., 1998; Mpunami *et al*., 1999). Tymon *et al*. (1998) showed that there are molecular differences between African and American lethal yellowing (LY) phytoplasmas and between strains from the East and West African coasts. Bila *et al*. (2014) observed the existence of three different types of phytoplasmas in Mozambique coconut palms: '*Candidatus* Phytoplasma palmicola'-related strains, Tanzanian Lethal Disease (LD), and a '*Candidatus* Phytoplasma pini'-related phytoplasma. When controlling phytoplasma diseases, the primary concern is prevention rather than treatment, which includes control of the insect vectors and alternative plant hosts, and removal of the infected palms (Brown *et al*., 2007). The known phytoplasma insect vectors are leafhoppers (Membracidae, Cicadellidae), planthoppers (Delphacidae, Cixiidae, Derbidae, among others) and psillids (Psyllidae) (Philippe *et al*., 2007). In the Caribbean region and Florida, LY disease is vectored by a cixiid *Haplaxius crudus* Van Duzee (previously known as *Myndus crudus*), and potentially by *Cedusa* species (Derbidae) (Brown *et al*., 2006). Mpunami *et al*. (2000) associated LD transmission in Tanzania with the derbid planthopper *Diostrombus mkurangai* Wilson (Wilson, 1987) and *Meenoplus* sp. (family Meenoplidae). In the Cabo Delgado province, northern Mozambique, pentatomid specimens of *Platacantha lutea* Westwood were found carrying the same phytoplasmas as those identified in the diseased coconut from which they were collected (Dollet *et al*., 2011). Because of the diversity of phytoplasmas involved in CLYD in Mozambique (Bila *et al*., 2014) as well as of the existing entomofauna in the different regions where CLYD occurs, a variety of insect vectors could be involved in the transmission of the different CLYD-type diseases in Mozambique. The naturalized African fan palm (*Borassus aethiopum* Mart.) and oil palm (*Elaeis guineensis* Jacq.) species were recently recorded as CLYD alternative hosts in Mozambique (Bila *et al*., 2015). The recurrence of CLYD in replanted devastated coconut farms, in addition to the detection of LY-type phytoplasmas in certain sucking insect species (Mpunami *et al*., 2000; Brown *et al*., 2006; Dollet *et al*., 2011) and in alternative host plants, support the idea that some other Hemiptera species could be vectoring this phytoplasma in Mozambique. In this study, the hypothesis that Hemiptera specimens of the families Derbidae and Pentatomidae are potential vectors of phytoplasmas associated with LYD in Mozambique was tested.

Materials and methods

The survey was conducted in the coastal areas of the Inhambane and Zambezia provinces in Mozambique, during 2014, covering both the cold-dry and warm-rainy seasons. Hemiptera specimens were collected from the plant canopy of palms showing typical CLYD symptoms, or growing in the vicinity of symptomatic coconut palms, mainly from the inflorescences and the underside surface of leaves. In order to prevent damaging specimens needed for taxonomical identification, these were

collected using a mouth aspirator or large conical flasks, immediately preserved in 96% alcohol and maintained at room temperature until DNA extraction. Prior to DNA extractions, specimens were morphologically identified. Insects were sorted by the first and last authors and species identifications made by M. R. Wilson using keys of Synave (1973) and Wilson (1987). DNA extractions were performed using a CTAB method or the DNeasy Blood® and Tissue Kit (Qiagen). Direct PCR was carried out with primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by nested PCR with primers G813/AwkaSR (Tymon, 1997) and LY16Sf/LY16Sr (Harrison *et al.*, 2002). Specimens were tested either as single or sets of five individuals. In total 1190 insect specimens were PCR phytoplasma screened. The PCR positive products with primer pairs G813/AwkaSR and LY16Sf/LY16Sr were purified using spin columns (Cycle-Pure Spin PCR purification kit, Omega Bio-tek Inc.) and directly sequenced using ABI sequencing (Macrogen Europe, Netherland). The sequences were compared using the Basic local alignment search tool (BLAST) search (Altschul *et al*., 1990) at the National Centre for Biotechnology Information (NCBI) and aligned using CLUSTAL-W (Larkin *et al.*, 2007). Phylogenetic analyses were performed with MEGA v. 6.06 (Tamura *et al*., 2013) using the neighbour-joining (NJ) and maximum likelihood (ML) methods, evaluated with 1,200 bootstrap replicates. The phytoplasma 16S ribosomal group was assigned using the *i*PhyClassifier online interactive software tool (Zhao *et al*., 2009).

Results

Hemiptera specimens collected belonged to the families Derbidae (*D. mkurangai*, *Diostrombus abdominalis* Distant (Distant, 1907), *Lyddastrombus* sp. and *Zoraida* sp.) and Pentatomidae. *Diostrombus* spp. were by far the most abundant taxa. PCR bands were only detected from *D. mkurangai*, *D. abdominalis* and *Lyddastrombus* sp. Phytoplasma sequences were retrieved from two *D. mkurangai* specimens deposited in GenBank, under the accession numbers KU853993 and KU853994, respectively. In the other PCR-positive specimens Gram positive bacteria were identified. All retrieved 16S rDNA phytoplasma sequences were used in a phylogenetic analysis along with other phytoplasma sequences, where the NJ and ML trees showed similar topologies (data not shown). BLAST comparison and phylogenetic analysis of the phytoplasma 16S rDNA sequences revealed that *D. mkurangai* is carrying phytoplasmas showing 99% identity on the 16S rRNA gene with coconut phytoplasmas detected in Mozambique and clustering with the phytoplasma strains detected in palm in this country (figure 1). Based on the *i*PhyClassifier online software tool, the phytoplasma detected on the *D. mkurangai* specimens were identical to the '*Ca.* P. palmicola' reference strain (GenBank accession: KF751387) and were therefore assigned to the 16Sr group XXII-A.

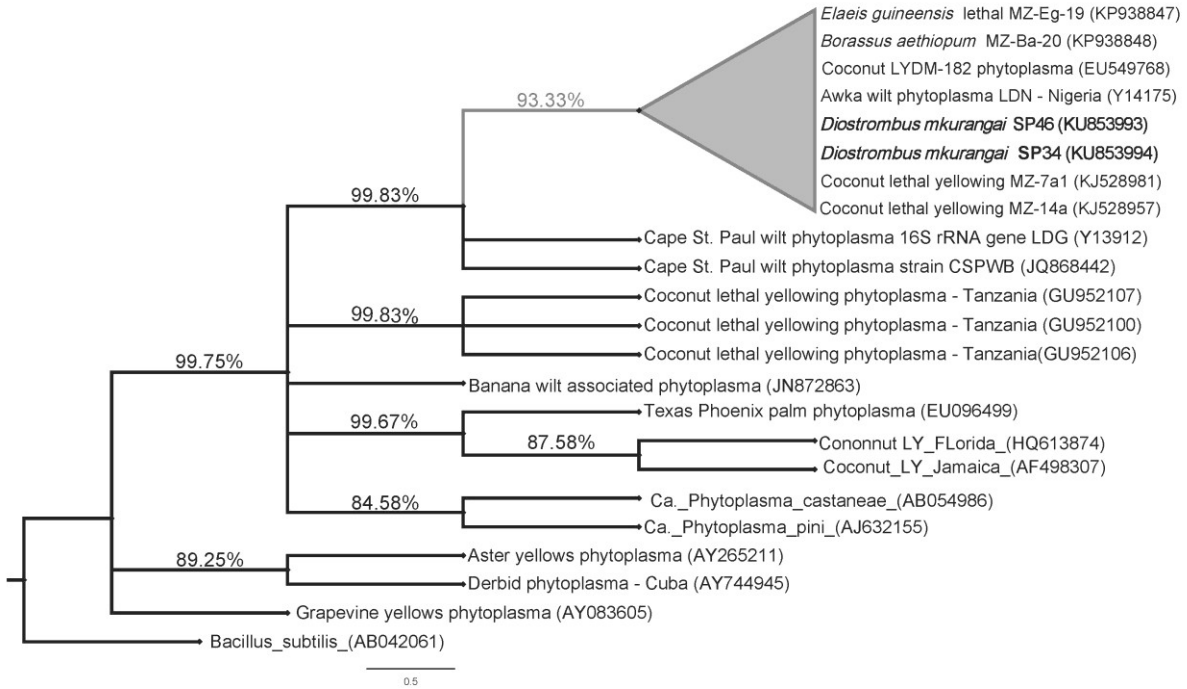

Figure 1. Dendrogram constructed by the maximum likelihood method showing the phylogenetic relationships among the *D. mkurangai* phytoplasma from Mozambique compared with those detected in Mozambican palm (MZ-Eg-19, MZ-Ba-20, MZ-7a1 and MZ-14a) and representatives from different 16Sr groups. The Mozambican insect samples in the present study are indicated by *D. mkurangai*_SP46 and *D. mkurangai*_SP34. The GenBank accession numbers are shown in parentheses. Bootstrap values greater than 80% based on 1,200 replicates are shown.

Discussion

This is the first molecular detection of '*Ca.* P. palmicola' in *D. mkurangai*. Based on PCR screening the LD phytoplasmas in Tanzania were earlier been associated with *D. mkurangai* and *Meenoplus* sp. (Mpunami *et al*., 2000). Philippe *et al*. (2007) also suspected *Diostrombus* sp. as potential CSPWD vector in Ghana. Moreover, in this study, the specimens collected were predominantly *D. mkurangai* and *D. abdominalis*. This result is in line with the Mpunami *et al*. (2000) study, which found that Derbidae insects were more abundant than other insect families in infected coconut plantations. As such, it is plausible that *D. mkurangai* occurring in Mozambique may also carry the Tanzanian LD phytoplasma type. Dollet *et al*. (2011) identified a pentatomid bug, *P. lutea*, as a potential CLYD vector in Mozambique; however none of the pentatomids tested in this study was confirmed to contain phytoplasma, and hence they were not identified up to the species level. In line with this study, Philippe *et al*. (2007) also failed to confirm pentatomid bugs as potential vectors of CSPWD in Ghana. Besides the planthopper *H. crudus* (Cixiidae), which is the only vector confirmed by transmission experiments, other potential vector(s) of different LY palm phytoplasmas have not yet been conclusively identified. The presence of a phytoplasma in an insect does not necessarily prove its capacity to transmit the disease, but this fact will help investigators to focus on the most probable vectors for transmission experiments. The search for putative insect vectors for the '*Ca.* P. palmicola' related strain in Ghana, using derbids and *Haplaxius* spp. proved to be negative through both molecular screening and transmission tests (Philippe *et al*., 2007). In Mozambique, the most common CLYD management strategy is the cutting and burning of symptomatic coconut palms, but this strategy alone, is by far not sustainable. Removal and destruction of coconut palms is very power demanding and must be done with chain saw machine, which maybe a limiting factor among small scale farmers. Successful management of palm LY is usually through an integrated approach involving strict disease surveillance, immediate removal and destruction of LY infected trees, replanting with LY resistant varieties, proper weed of plant hosts and control of the insect vector (Brown *et al*., 2007; Eziashi and Omar, 2010; Myrie *et al*., 2011). Further research is underway to confirm the phytoplasma vector transmission capacity of *D. mkurangai* and its epidemiological role in the CLYD epidemic in Mozambique.

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