International Journal of Plant & Soil Science

Volume 38, Issue 2, Page 150-166, 2026; Article no.IJPSS.150897
ISSN: 2320-7035

Occurrence, Distribution and Molecular Identification of Candidatus Phytoplasma Strain Causing CLYD in Coastal Region of Kenya

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/ijpss/2026/v38i25973

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://pr.sdiarticle5.com/review-history/150897

Original Research Article

Received: 20/11/2025
Published: 17/02/2026

Abstract

Coconut lethal yellowing disease (CLYD) caused by phytoplasma is a destructive disease affecting coconut and other palm species. The disease has been reported in Mozambique, Tanzania, and some West African countries, where millions of coconut trees have been affected. Although the disease was first reported in 1999 in Northern Coast of Kenya, no further studies have been conducted, leaving the giant coconut industry at risk of destruction. It is for this reason that a surveillance was undertaken to study the extent of the disease spread and the specific *Candidatus phytoplasma* strain causing the disease. A total of 125 coconut tree samples were collected in Kwale, Kilifi, Lamu, and Tana River counties by drilling the tree trunks aseptically. DNA extractions

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Cite as: *Muli, John Muithya, Simon Muti Mbuvi, and Rose Kigathi. 2026. "Occurrence, Distribution and Molecular Identification of Candidatus Phytoplasma Strain Causing CLYD in Coastal Region of Kenya ". International Journal of Plant & Soil Science 38 (2):150-66. https://doi.org/10.9734/ijpss/2026/v38i25973.*

were done, followed by PCR process done using P1/P7 universal primers, gel electrophoresis and amplicon sequencing. For the coconut tree drillings results, the BLAST comparison with existing data in the NCBI database revealed it to be C*andidatus phytoplasma palmae* strain isolate Tanz08, under gene accession No. GU952107.1. Further phylogenetic analysis using the maximum likelihood method indicated that the strain is closely related to those reported in Tanzania. The CLYD positive samples were identified from Lamu and Kwale counties, with 6 cases from each County. Although there were no positive cases reported in Kilifi and Tana River counties, the presence of the vectors suggests that more studies need to be done on the disease. Further studies should be done on the alternative hosts, varieties prone to the disease, characterization and diversity of the vectors, the incubation period and the host pathogen relationship.

Keywords: Coconut lethal yellowing disease; phytoplasma; Candidatus phytoplasma palmae.

1. Introduction

1.1 Background Information

In Kenya, coconut palm (*Cocos nucifera*) is an important perennial oil crop that supports livelihood of majority of farmers in the coastal region by providing food, shelter, employment, and income. Over 150,000 households depend directly on coconuts while several thousand benefits indirectly from the coconut and its by-products (Agriculture and Food Authority (AFA), 2021). These benefits range from, but not limited to, medicinal, food, furniture, coconut milk, alcohol, construction, oil, and fiber. Almost every part of the coconut plant can be processed to produce a variety of commercial products (Pham, 2016).

In the recent past, Coconut Lethal Yellowing Disease (CLYD), initially reported in Tanzania and Mozambique (where it reportedly killed over 40% of the total coconut population), is suspected to have spread and crossed over into Kenya as a result of germplasm exchange between the two countries (Bila et al*.,* 2017; Mpunami et al*.,* 2000; Hemmati et al*.,* 2020; Mpunami et al., 2021). This disease is reportedly one of the most detrimental phytoplasma diseases in the world (Harrison et al., 2014; Solomon et al., 2019). It inhabits the plant phloem tissue and is fast-spreading, affecting over 38 palm species globally (Rosete et al., 2017; Pilet et al*.,* 2019). It is majorly transmitted through vectors and vegetative propagation (Bila, 2016; Oropeza- Salin *et al* 2020). It is thus a threat and a major limiting factor in coconut production (Bertaccini et al*.,* 2014; Rosete et al., 2017). The occurrence of this disease in the Coastal region of Kenya threatens over 84,000 ha of coconut, which translates to the death of over 15 million trees which will likely lead to collapse of this giant coconut industry along the Coastal region (Eziashi and Omamor 2010; AFA, 2021). Kilifi and Kwale counties are the leading coconut production zones, contributing respectively, 48.8% and 36.9%, of the total coconut production in Kenya. Therefore, the collapse of the industry will adversely affect the economy and livelihoods of the local communities especially the subsistence and small holder farmers due to their over reliance on coconut for their nutrition and economy (Pole et al., 2014; Hemmati et al., 2021). Thus, this study was conceived to investigate the extent, occurrence and identification of *Candidatus phytoplasma* strain causing CLYD in Coastal region of Kenya.

2. Methodology

2.1 Study Site

The study was carried out along the Coastal strip in the main coconut growing areas of the Coastal region of Kenya namely, Kwale, Kilifi, Lamu, and Tana River counties which lie in the lower Coastal region within the latitudes 38˚E - 41.5˚E, longitudes 0˚ - 4.5˚S and altitude of between 0 m above sea level (ASL) and less than 300 m ASL (Fig. 1). The study excluded Taita Taveta County that lies at higher elevations of more than 500 m ASL (Jaezold *et al*, 2012).

The region experiences bimodal type of rainfall and is characterized by hot and humid type of climate with temperatures ranging between 29℃ and 32℃ for most part of the year except during rainy season in the months of May and June when low temperatures of 23℃ to 28℃ are experienced. Long rains occur during months of March to July, and short rains from September to December, with annual totals of 1100 mm (Gogo et al., 2020). This Coastal strip is part of the Coconut–Cassava belt of Agro-ecological zone 3, namely, the Coastal Lowland two and three

(CL2 and CL3) which has the highest density of coconut trees and highest number of coconut farmers in the region. This Coconut–Cassava zone forms a strip of coconut belt that runs parallel to the coastline and stretches from Vanga in South of Kwale County, at the borders of Kenya and Tanzania, and extends northward up to Lamu and Tana River Counties (Muti et al., 2017) (Fig. 1). The soils in the region are predominantly sandy loam with pockets of clayey soil (Omuto, 2013). The climatic conditions in this coconut belt are the most suitable for coconut growing and characterize much of the study area. Most farmers in the region grow annual crops such as maize, cassava and cowpeas that are compatible and tolerant to some level of shading, beneath the coconut canopies. Varieties of coconuts grown include the dwarf, medium tall and the East African Tall varieties and these trees benefit from management practices conducted on the annual crops such as weeding, fertilization, among others.

2.2 Study design and sampling

A detection surveillance study was used where stratified random sampling of coconut trees was done based on levels of infestation (Hernández et al. 2020). This design was used because it is most preferred in circumstances where pest distribution was presumed (Intergovernmental Panel on Climate Change (IPPC), 2008). Thus, the study area was delineated into high incidence ("hot spots") areas, namely Kwale and Lamu counties and low incidence areas, namely Tana River and Kilifi counties. Kwale County was considered hot spot because it borders Tanzania to the South, where the disease was first reported as early as 1905 and also known to have caused great damage. Lamu, on the other hand was considered hot spot area because the disease was reported in 1999 on three East African Tall (EAT) varieties (Mpunami et al., 1999; Martinez et al., 2010). Further stratification was done in each selected County based on administrative Wards that had highest number of coconut plants. It is from these wards that farmers with coconut trees were identified for sampling. Based on this premise and using equations (1) and (2), the number of samples and therefore sampling intensity for each County was determined as per the International standards for phytosanitary measures (ISPM) 31 guidelines using the KEPHIS (Kenya Plant Health Inspectorate Service) surveillance protocol (Table 1) (KEPHIS, 2019; International Plant Protection Convention 2018).

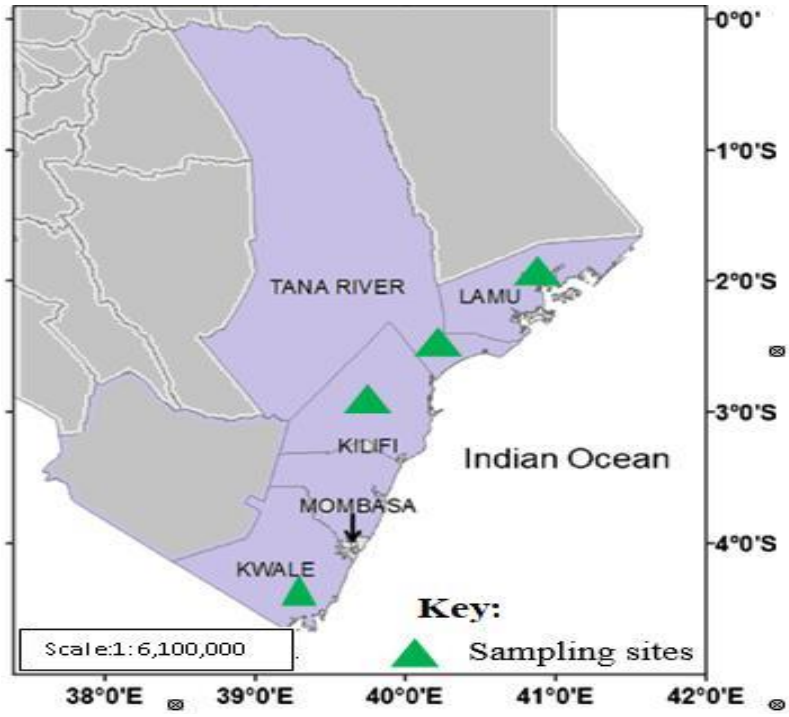

Fig. 1. Map of the study area showing counties where sampling was done in Coastal region of Kenya

Level of confidence interval = 1 – (1 – design prevalence) sample size (Eq. 1)

$$\text{Where sample size} = \frac{\log(1-\text{confidence level})}{\text{Log}(1-\text{design prevalence})} \quad \text{(Eq. 2)}$$

Where: actual prevalence referred to the true proportion of infested units in a population (infested by one or more pests/disease) and design prevalence was based on a pre-survey estimate of the likely actual prevalence of the pest/disease in the field (IPPC, 2008; Parnell, et al., 2017).

In Kwale (bordering Tanzania) sampling was done in: Vanga, Mwereni, Dzombo and Pongwe wards, while in Lamu County, sampling was done in Lamu West sub-County in Majembeni, Hongwe, Mkunumbi and Bahari wards.

After sampling the coconut trees and obtaining the drillings, County percentage infection rate by CLYD was given by: Number of CLYD positive trees against the total sampled in the County multiplied by 100. Also regional percentage CLYD infection rate was given by: Number of CLYD positive trees in the County against the total sampled in the whole Coast region.

2.3 Determination of the Extent and Occurrence of CLYD in Coastal region of Kenyan

2.3.1 Sample Collection for DNA Extraction

The study involved drilling symptomatic coconut trunks and collecting insects for DNA extractions and identification as shown in Table 1.

2.4 Data Collection

This study adopted stratified sampling and allocated the highest sampling intensity to areas perceived as high incidence “hot spots”, namely Kwale and Lamu, while Kilifi and Tana River counties were allocated lower sampling rates. Thus, coconut drilling samples collected from these counties were then subjected to DNA extraction, PCR processes, and Gel electrophoresis (Contaldo et al., 2019). During sampling, 4 disease scales were developed and applied, based on the physical symptoms observed: 0 = no symptoms; 1 = leaf chlorosis; 2 = drooping leaves with skirt-like appearance; and 3 = dead crown and trunk, referred to as “telephone poles”. During the survey, an online questionnaire via ODK (Open Data Kit) was administered to capture various information regarding the Name of the farmer, GPS (global positioning system) location of the farm and sampled tree, symptoms expressed, the contacts of the interviewee, Sub-County, ward, village and the CLYD symptoms observed.

2.4.1 Materials and Methods of Collecting the Coconut Drillings

The sampling tools used in the study included a rechargeable drill (Bosch GSR 1800-LI Professional), drilling bits, 10 mm in diameter (Bila et al., 2015), machete, cooler box with ice, and vials. The consumables used included Silica gel desiccant and sodium hypochlorite. The sources of DNA material were either the coconut tree trunks or from the source tissues such as the leaves and inflorescence. However, in this study coconut tree trunks were preferred rather than the source tissues because the sink tissues, namely the trunk phloem had been found to yield more DNA than the source tissues (Rosete et al., 2017).

A machete was first used to slice the tree trunk bark, followed by a non-destructive procedure of boring 10 - 15cm depth holes in the trunk as described by Gurr et al. (2016) using the drilling machine and drilling bits. The coconut tree drillings collected and sampled in the fields were placed in 50 ml vials, then carefully stored in the cooler box. The sampling tools were disinfected

Table 1. Sampling intensities in the four counties of the study area

County	Coconut tree trunks drilled per County	Sampling intensity per County (%)	Insect vectors collected per County	Sampling intensity per County (%)
Kwale	52	41.6	510	57.3
Lamu	43	34.4	225	25.2
Kilifi	23	18.4	135	15.2
Tana River	7	5.6	20	2.2
Region Totals	125	100	890	100

using 10 % sodium hypochlorite before any sampling began. Silica gel particles were used to absorb the moisture and dampness from the drillings (Štorchová et al., 2000). Drilling was done in a clockwise direction and removed in the same direction since anticlockwise drilling would leave the wood carvings inside the tree. All personnel involved in collecting samples were dressed in personal protective clothing, gloves, and masks to minimize contamination of the samples with human DNA. The gloves and masks were changed every time a new sample was being taken.

2.4.2 DNA Extraction

DNA extractions were performed using Cetyl Trimethylammonium Bromide (CTAB) extraction method with modifications, as described by Novaes et al. (2009) and Myrie et al. (2011). The two modifications had a different range of concentration from 2% to 3% to increase the yield of nucleic acid. The trunk drillings were crushed into a fine powder using pestle and mortar and added 2 ml of CTAB buffer [2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA (Ethylene diamine tetra acetic acid), pH 8.0, PVP 2%], 2% (20 µL) 2-mercaptoethanol and 35 µL proteinase K (1 mg/m). The mixture was ground to a fine mix and allowed to settle for 10 minutes. The liquid from each sample was then decanted off to a clean 1.5 ml microcentrifuge tube. The samples were then centrifuged at 22,000 x g for exactly 10 minutes at room temperature (21℃). Subsequently, 700 µl of the supernatant was transferred into a clean 1.5 ml microcentrifuge tube. An equal volume of chloroform: Isoamyl (24:1) was added and mixed thoroughly and centrifuged at 16,000 x g for 5 minutes at 4℃. The supernatant was transferred to clean 1.5 ml microcentrifuge tube and 500 µl of ice-cold isopropanol was added and mixed carefully but thoroughly by inversion. The mixture was then left at room temperature for approximately 10 minutes and centrifuged at 16,000 x g for 10 min at 4℃. After the centrifugation, the supernatant was decanted while taking care so that the pellet remained within the tube. The DNA pellet was then washed with 100 µl of 70% ethanol and centrifuged at 16,000 x g for 5 minutes discarding the supernatant material. The pellet was then air dried for approximately 30 minutes and 100 µl of TBE buffer was then used to suspend the nucleic acid. Quantification by spectrophotometre was done using a Nano drop and quality checked by conducting electrophoresis on a 0.8% (w/v) agarose gels to confirm. Good quality samples were stored at -20℃.

2.4.3 The PCR Process

PCR was done following the protocol described by Smart et al. (1996). Two primers were used in this process; P1 (forward): 5′-AAG AGT TTG ATC CTG GCT CAG GAT T-3′ (reverse): 5′-CGT CCT TCA TCG GCT CTT-3′ and R16F2n (forward): 5′-GAA ACG ACT GCT AAG ACT GG-3′, R16R2 (reverse): 5′-TGA CGG GCG GTG TGT ACA AAC CCC G-3′ (Deng and Hiruki, 1991, AFA. 2017).

After preparation of the reaction mixtures, all samples were transferred to the thermocycler and a nested PCR amplification was done. Nested PCR involves combining two sets of primers that are used in two runs of the PCR (Ramjegathesh et al., 2019). The next set of primer amplifies a secondary with the product of the first run. The process allows a low number of runs in the first process thus limiting non-specific products. Using primers P1, nested 1 was achieved by setting the initial denaturation temperature to 94℃ for 5 minutes for one cycle, followed by the denaturation step at 95℃ for 30 seconds. The annealing temperature was set at 55℃ for 1 minute and elongation at 72℃ for 2 minutes and final elongation at 72℃ for 10minutes. A total of 35cycles were employed for this step. The final holding temperature was set at 4℃. Nested 2 was achieved by setting the initial denaturation temperature to 94℃ for 40 seconds, followed by an annealing temperature set at 55℃ for 1 minute. Elongation temperature was set at 72℃ for 2minutes and the final elongation was set at 72℃ for 10 minutes. A total of 35 cycles were set for this step and the final holding temperature was set at 4℃.

2.4.4 Gel Electrophoresis

DNA samples were loaded into wells at the positive end of a gel**,** and an electric current was applied to pull them through the gel. Loading dye was used to give illumination of color that indicates the movement and intensity of the DNA, hence settling it at the bottom of the well (Voytas, 2001, Oyoo, et al., 2015). A 1kb ladder was used in the procedure. The concentration of 1% was prepared by having 1gm of agarose which was measured and dissolved in 100 milligrams of sodium dodecyl sulfate (SDS). Approximately 5 µl of the sample was mixed in 3 µl of loading dye and cautiously added in the

wells. Gel electrophoresis was run at 100V for 1 hour, then observed on normal electrophoresis dock and a photo taken.

2.4.5 Sanger Sequencing

The PCR product was run into a 0.8% gel via electrophoresis. The single DNA kit was extracted using the Qiagen gel (QG) extraction kit procedure. The DNA fragment was excised from the Agarose Gel using a sharp clean scalpel and its weight was taken using a weighing balance in a colorless tube on the lab bench. Three volumes of the buffer QG were added to one volume of the Gel. This was then incubated to allow the gel slice to dissolve at 50℃ for 10 minutes. The tube was vortexed after every 2-3 minutes to help dissolve the gel. Upon completion of the dissolving process of the gel slice, a yellow color was noted that was similar to buffer QG which was not dissolved in agarose. One gel volume of isopropanol was added to the sample and mixed well. A QIAquick spin column was placed in a 2 ml collection tube and a sample was applied to the QIAquick column. This was followed by centrifugation for one minute. The flow-through was removed, then the QIAquick column was reinserted into the same tube. A 500 µl buffer QG was added to the QIAquick column and centrifuged for another 1minute. The flow-through was then poured off and the QIAquick column was placed back into the same tube. To wash, 750 µl Buffer PE was added to the QIAquick column and centrifuged for 1 minute. The flow-through was then discarded and the QIAquick column was placed back into the same tube. The QIAquick column was then placed into a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 ul Buffer EB was added to the center of the QIAquick membrane and centrifuged for 1 minute. For high DNA concentration, 30 µl Buffer EB was added to the center of the QIAquick membrane column and left to stand for 1 minute, then centrifuged for 1 minute. Since the purified DNA was to be analyzed on a Gel, 1 volume of loading dye to 5 volumes of purified DNA was added. The solution was mixed by pipetting up and down before loading the gel. Fifteen microliters of the gel extracted PCR product was aliquoted into two tubes and labeled with the unique sample ID. Into one 15 µl aliquot, 4 µl Forward primer was added for the purpose of DNA amplification. Into the second 15 µl aliquot, 4 µl Reverse Primer was added. Labeling of the tubes was done as either forward or reverse primer. Approximately 11 µl of distilled water was added into each of the DNA-primer mix bringing the total volume to 30 µl. The final concentration of the primer was 10 pmol/µl (10 µM). This was then forwarded to the Laboratory for Sanger sequencing.

Sanger sequencing was undertaken and the 16S rRNA gene sequences obtained were likened with those of known phytoplasmas using Basic Local Alignment Search (BLAST) searches from the National Centre for Biotechnology Information (NCBI) (Bila et al., 2017). This was done to ascertain the specific *Candidatus phytoplasma* strain causing CLYD in the region. Phylogenetic analyses were performed with software *ngphylogeny.fr* using the maximum likelihood methods and evaluated with 1000 bootstrap replicates (Mpunami et al., 2021, Arocha et al., 2016).

3. Results

3.1 Occurrence of CLYD in the Lower Coastal Region of Kenya

The results indicated that coconut lethal yellowing disease (CLYD) positive samples were only found in Kwale and Lamu counties although symptomatic trees were spotted in Kilifi and Tana River (Fig. 2). The presence of the disease in the sampled trees was confirmed using the Sanger sequencing method and the results are summarized in Tables 2 and 3. Of the large number of sampled trees, six trees in Kwale County, responded positive for CLYD, while also six trees responded positive for CLYD in Lamu, giving a regional infection rate of 9.6% and a County infection rate of 25.49%. Kwale County accounted for 11.54% infection rates and Lamu County accounted for 13.95% (Table 2). In Kwale, the CLYD positive trees were found in areas of Lunga lunga Sub-County bordering Tanzania (Fig. 2).

The results of Sanger sequencing and PCR indicated that the CLYD strain identified in Lamu and Kwale counties was found to be CLYD Tanz08-01 and was similar across the counties (Table 3).

Of the four wards in Lunga Lunga sub-County, two wards, namely Vanga and Pongwe had trees positive for CLYD. Out of the four wards in the Msambweni Sub-County, one ward namely, Kinondo recorded positive for CLYD. Of the seven wards in Lamu west sub-County, four wards recorded positive for CLYD.

Despite the low infection rates of 9.6% recorded using sequencing method, a large proportion of trees that were sampled in this study showed that coconut trees in coastal counties were showing symptoms very similar to those of coconut lethal yellowing disease (Plate 1) (Córdova et al., 2014, Wang & Wang, 2012).

The results also indicated that Kilifi and Tana River counties had symptomatic trees although they tested negative for CLYD (Figs. 3 and 4). Thus, not all the trees that showed visual CLYD symptoms tested positive for CLYD disease. This suggests that either the disease was yet to show up after the incubation period, or the sensitivity of the test primers was low (Mazivele et al., 2018).

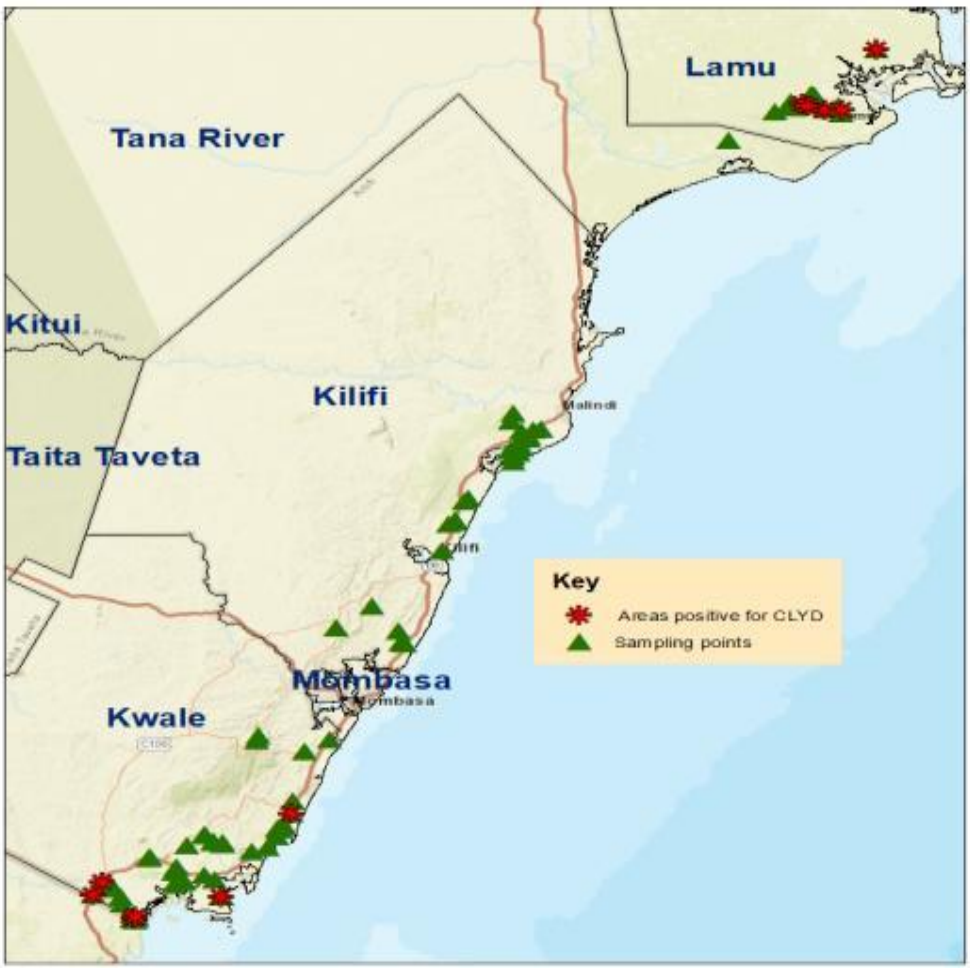

Fig. 2. Map of study area showing sites where coconut trees were sampled for CLYD testing (Green triangles) and where sampled coconut trees tested positive for CLYD (Red asterisks) following Sanger sequencing

Table 2. CLYD positive (trees) samples and % CLYD infection rates in the study area

County	Coconut tree trunks drilled per County	CLYD Positive trees	County % CLYD Infection rate	Region % CLYD Infection rate
Kwale	52	6	11.54	4.8
Lamu	43	6	13.95	4.8
Kilifi	23	6	0	0
Tana River	7	0	0	0
Totals	125	12	25.49	9.6

Table 3. Sanger sequencing results confirming CLYD positive samples in the study area

County	Sub-County	Ward	Sample No	Sanger (16S: F2/R2) results (Forward /Reverse)	Gene bank Accession No.
Kwale	Lunga lunga	Vanga	4	CLY iso Tanz08	GU952107.1
Kwale	Lunga lunga	Pongwe	21	CLY iso Tanz08	GU952107.1
Kwale	Lunga lunga	Vanga	31	CLY iso Tanz08	GU952107.1
Kwale	Lunga lunga	Vanga	31F2	CLY iso Tanz08	GU952107.1
Kwale	Lunga lunga	Vanga	39	CLY iso Tanz08	GU952107.1
Kwale	Msambweni	Kinondo	43	CLY iso Tanz08	GU952107.1
Lamu	Lamu West	Hongwe	123	CLY iso Tanz08	GU952107.1
Lamu	Lamu West	Hongwe	129	CLY iso Tanz08	GU952107.1
Lamu	Lamu West	Bahari	130	CLY iso Tanz08	GU952107.1
Lamu	Lamu West	Mkunumbi	133	CLY iso Tanz08	GU952107.1
Lamu	Lamu West	Hongwe	149	CLY iso Tanz08	GU952107.1
Lamu	Lamu West	Majembeni	154	CLY iso Tanz08	GU952107.1

Plate 1. Coconut Lethal Yellowing Disease (CLYD) symptoms observed during sample collection in study sites. A) Skirt-like features around the trunk, B) Leaf chlorosis, C) Leaf chlorosis and drooping leaves observed in Majembeni ward in Lamu west Sub, D) Dead crown referred to as "telephone poles", signaling death of the plant in late stages of the disease development, observed in Lunga lunga Sub-County of Kwale at the Kenya-Tanzania border

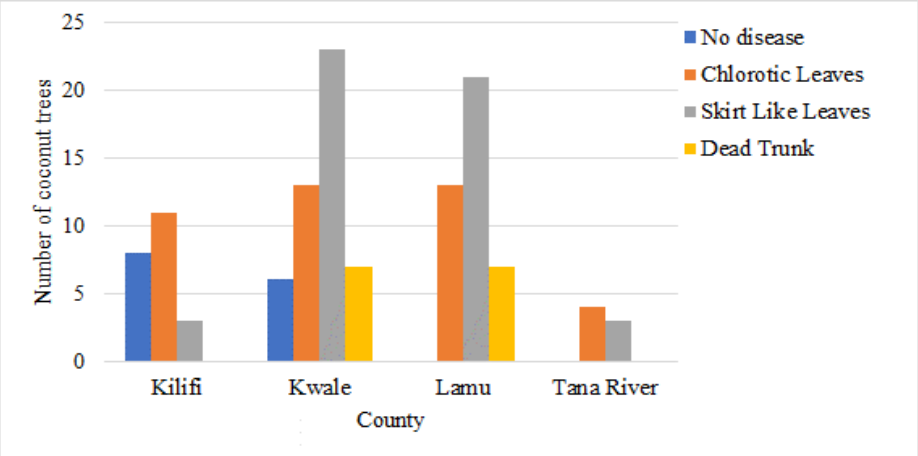

Fig. 3. Number of coconut trees showing different disease scales of coconut lethal disease-like symptoms in the different counties

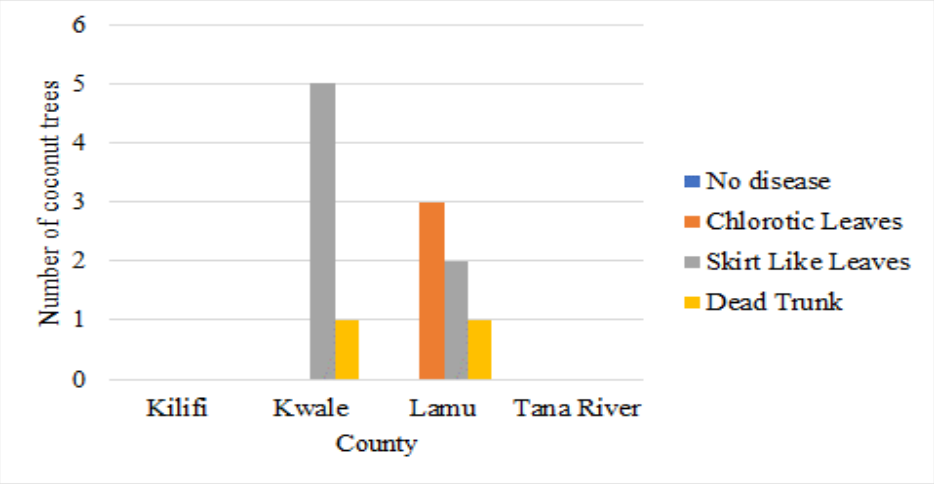

Fig. 4. Number of coconut trees that tested positive and the disease symptoms they showed in the different counties. All twelve trees showed symptoms but at different damage scales.

3.2 Identification of the *Candidatus phytoplasma* strain causing CLYD in Coastal Region of Kenya

3.2.1 PCR and Sanger Sequencing Results

The PCR amplification of phytoplasma DNA using P1/P7 universal primers resulted in several samples that showed the ideal band for the phytoplasma at 1250bp (Plate 2).

From Sanger sequencing results, a multiple sequence alignment of positively identified CLYD phytoplasma 16S rRNA genes was observed which indicated that there was a high level of gene conservation across the sequences with few mutations noted in a few samples, namely 4F; 43F, and 129F (Figs. 4 and 5). These were sample numbers S21-F2; S31-F2; 4-F; 31-F and 39-F (from Lunga lunga, Kwale); 43-F (from Msambweni, Kwale); 133-F (from Mkunumbi, Lamu); 130-F (from Bahari, Lamu); 129-F; 149-F and 123 (from Hongwe, Lamu) and 154-F (from Majembeni, Lamu).

A phylogenetic tree was constructed and from Phylogenetic analysis, it became evident that of the samples that tested positive in this study, they clustered closely with existing Tanzania Isolates Tanz08-43, Tanz08-05, Tanz08-16, and Tanz 08-01 (Fig. 6).

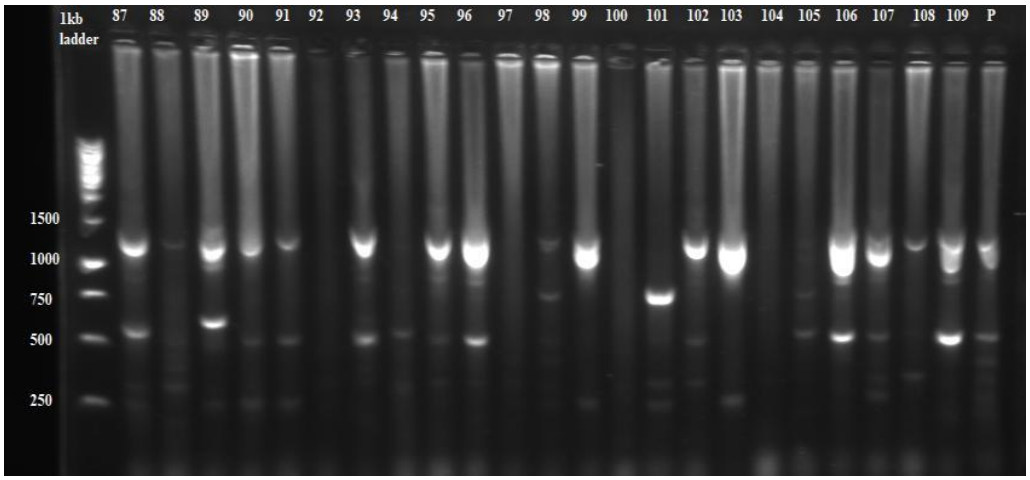

Plate 2. PCR Amplification of phytoplasma DNA using universal primer pairs for samples collected in Kwale County. The ideal band for the phytoplasma was 1250bp. Sample numbers 87, 91, 96, 102, 89, 90, 93, 95, 98, 99, 103, 106, 107, and 109 tested positive for the band 1250bp. However only samples 87, 91, 96, 102 tested positive in the Sanger sequencing results. Samples 92, 94, 97,100, and 104,105 all tested negative for CLYD in both PCR and Sanger sequencing tests

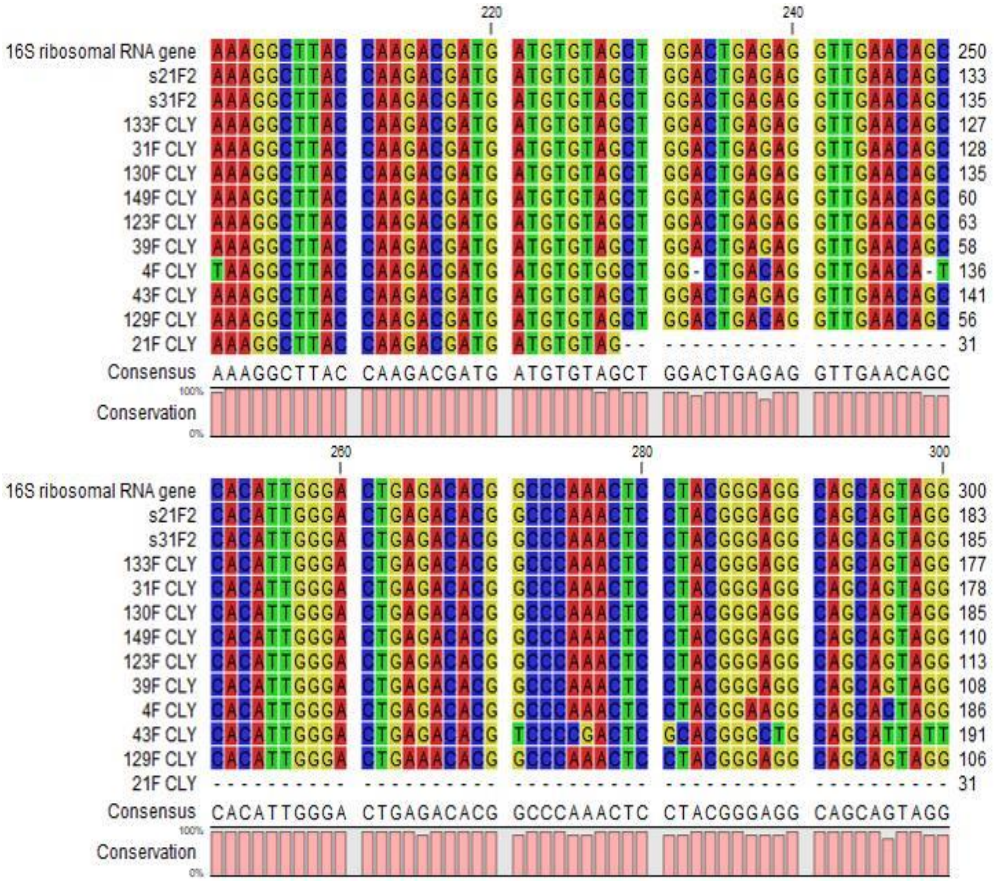

Fig. 5. Multiple sequence alignment of positively identified CLY phytoplasma 16S rRNA gene. There was high conservation across the sequences with few mutations seen in sample numbers 4FCLY; 43F CLY and 129F CLY

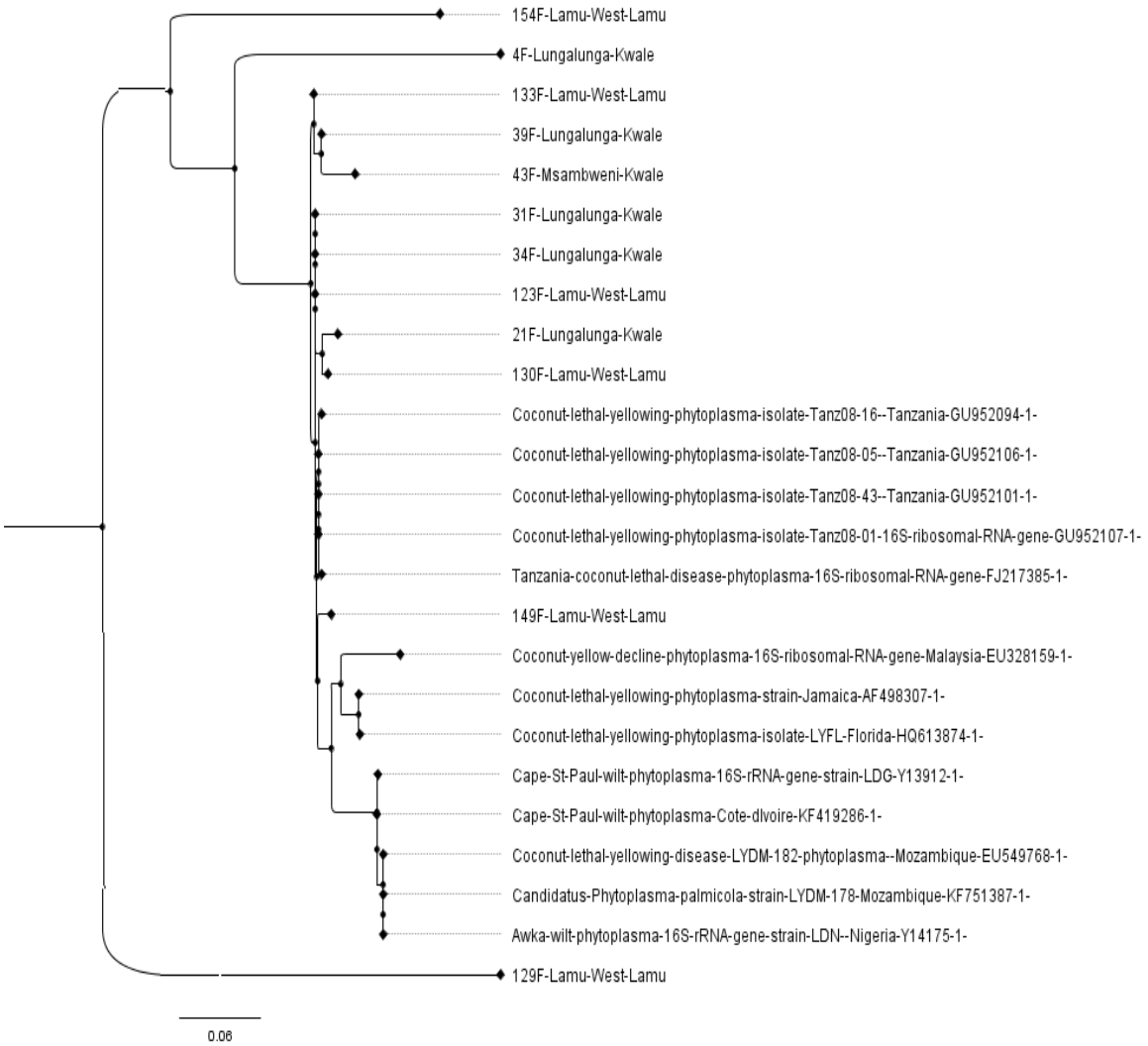

Fig. 6. A Phylogenetic tree constructed using the maximum likelihood method showing the phylogenetic relationship among the *Candidatus phytoplasma* strain detected in Kwale and Lamu compared with existing representatives from other different 16sr groups

Although the 16sr sequences in Kwale and Lamu were similar, there were a few mutations that distinguished sample numbers 129F (from Hongwe, Lamu), 4F (from Lunga lunga), 154F (Lamu West, Lamu), and 43F (from Msambweni, Kwale) from the rest of the Tanz08-01 16 ribosomal gene as depicted on the phylogenetic tree (Figs. 4 and 5).

4. Discussion

4.1 Occurrence and Distribution of CLYD in Coastal Region of Kenya

The Sanger sequencing results confirmed that CLYD is present in Kwale and Lamu counties. This was further affirmed by the visual symptoms observed in the field where both counties had the highest number of dead coconut tree trunks. However, despite the low infection rates, many of the coconut trees across the counties that tested negative for CLYD disease exhibited visual CLYD like symptoms (Córdova et al., 2014). Kwale County borders Tanzania at its southern side. It ranks second after Kilifi County in terms of coconut production with 31,384 ha under coconut production that accounts for 36.9% of the total coconuts trees grown in Kenya. Although not confirmed, it is speculated that the robust trade between the two countries and transboundary exchange of germplasm may have attributed the presence of the disease in the County. Majority of the detected CLYD positive trees were found in Vanga ward (in

Lunga lunga Sub-County), which is just at the border of Kenya and Tanzania. Thus, since it has been established that the disease is already in the coastal region of Kenya (Mpunami et al., 2000), and that although the infection rates are low, (11.54% for Kwale and 13.95% for Lamu), a total of 3.9 million trees are in danger of death from the disease in the region under study. These findings are in agreement with those reported by Hemmati et al. (2020), who observed that about 38% and 86% of the coconut trees in Tanzania and Jamaica were wiped out by the disease between 1960's to 1983, with several other millions destroyed in Mozambique. Since it appears that the disease kills progressively over a period of time, the annual rate of disappearance of the coconut trees due to the disease can be determined.

While the occurrence of the disease in Kwale County can be explained by its neighborhood and bordering Tanzania, the occurrence of the disease in Lamu County which is far removed and in the middle of non-infected counties is a bit of a puzzle. However, one possible (unpublished) explanation, as narrated by the elders in both Lamu and Vanga is hereby put forward. Historically, Lamu Island is known to have been inhabited by migrants from Central and Eastern regions of Kenya majority of whom were settled there by the Government of Kenya between 1950's and late 1970's, hence the occurrence of Lamu settlement scheme. These settlers are said to have been successful farmers and traders. This explains the successful cultivation of such crops as cotton, (hence Bura cotton irrigation scheme), cashew nuts (supplying the then Cashew nut factory, in Kilifi), coconut (supplying coconut oil milling factories then, in Mombasa and Kilifi), among others in Lamu County. Through government sponsored land and crop development programs to settlement schemes (such as Magarini, Tezo, Lamu, among others) in the 1970's through to 1980's, these areas were supplied with initial seedlings of coconut, cashew nut and mangoes planting materials initially sourced from the government sponsored tree nurseries, and also from then well-developed coconut and cashew nut industries in Tanga, Tanzania, at the behest of the then, the East African Community. Being heavy and bulky, these planting materials were transported using dhows (the cheapest and most convenient transportation method, then) to Lamu by sea. Long after the programs ended, exchange of these materials among the neighboring border communities still continue to date. This perhaps explains the concurrent occurrence of CLYD disease in Lamu and Kwale counties as being historical.

Kilifi County which has the highest coconut population of over seven million trees is sandwiched between the infected counties of Lamu and Kwale, with Mombasa and Tana River counties intervening in between them. However, while none of them tested positive for CLYD disease, quite a number of trees sampled in the field exhibited visual symptoms of leaf chlorosis, but none had skirt-like drooping leaves or dead trunks, the true symptoms of CLYD. This observation leads the following postulation. That, the coconut trees that tested negative but had some of the visual symptoms associated with CLYD disease may have been having the disease developing but at incubation or latent stage. Since the incubation period for the disease lasts between 112-844 days from the detection of the disease to the onset of the symptoms, then as Nipah et al. (2007), puts it, it would just be a matter of time before the disease shows up or is expressed. None of the coconut varieties grown in the region have been reported to be completely resistant to the disease (Gurr et al. 2016). The second postulation is that, either the primers used in the PCR process were not sensitive or specific enough to detect even very low levels of the disease in the collected DNA samples, which suggests the need to further research on this.

4.2 Identification of the *Candidatus phytoplasma* strain causing CLYD in Coastal Region of Kenya

CLYD phytoplasma is generally classified into the 16SrIV group, which are then subdivided into sub-groups that varies depending on the vectors transmitting the disease, the expressed symptoms, and the host range (Bahder et al., 2018; Hemmati et al., 2020). The 16sr subgroup reported in Kenya is the 16srIV-C, with coconut as the host. The presence of the same phytoplasma strain in Kenya and Tanzania may be associated either with the material exchange between the two countries, occasioned by mutual relationship and friendly neighborhood, or some common vectors in the two countries. Although Mozambique borders Tanzania to the South, it was reported that Mozambique has three phytoplasma strains (Bila et al., 2015). From the three strains reported, one is related to the Tanzanian, *Candidatus Phytoplasma Cocostanzaniae*; West African – *Candidatus*

Phytoplasma palmicola, and the Mozambique *Candidatus Phytoplasma palmicola* 16SrXXII-A (CABI, 2012). In summary, Mozambique has the 3 sub-groups of the 16sr group i.e. 16srIV-B, 16sr IV-C, and 16sr XXII-A, while Kenya and Tanzania have the 16sr IV–C group (Hemmati et al., 2020).

In Kwale and Lamu, the Phytoplasma strain identified was CLYD isolate Tanz08-01 of Genbank accession No. GU952107.1. The total CLYD positive samples identified were twelve, evenly distributed in Lamu and Kwale Counties. Three of the samples showed some divergence from the Tanz08-01 16 ribosomal RNA gene, probably due to mutation, while the rest had similarities indicating that they all belonged to the same Tanzanian isolate. These were from Lamu West, in Lamu County, Msambweni, and Lunga lunga sub-counties in Kwale County. This suggests that the phytoplasma strain affecting the coconut trees in all these counties is similar, as there were minimal mutations distinguishing these three samples from the rest.

During the study, eleven samples had previously been identified as positive during the PCR stage but were confirmed to belong to different bacillus species with different accession numbers from the NCBI during the sequencing stage. This was attributed to the universal primers used during the study. Also, seven of the samples which had tested positive at the PCR level failed to yield any result at the sequencing level, which was attributed partially to the poor quality of the DNA.

5. Conclusion and Recommendation

5.1 Conclusion

This study was mainly a detection surveillance study whose aim, as defined by IPPC, (2021) was to discover or establish the presence or absence of an invasive species (namely the CLYD disease) while its populations are statistically small and geographically confined. The study has re-established and confirmed the existence and occurrence of CLYD disease in the Coastal region of Kenya. This is the first study to confirm establishment and occurrence of this disease ever since it was first reported in 1999 by Mpunami et al. (1999) who observed that there was evidence of genetic variation and variability on the phytoplasma affecting coconuts in Kenya and Tanzania.

The causal agent responsible for the disease has been identified as *Candidatus Phytoplasma palmae* strain Isolate Tanz-08, mainly transmitted by vectors which also inhabit in other alternate host plants in the region.

5.2 Recommendation

From the study, it is evident that some areas or issues needed further clarification or investigation. However, due to the limitation of time, funding, and scope of the study, this was not feasible. Therefore, further studies are suggested to seek clarification on the following issues: There is a need to determine the varieties most affected by the CLYD disease as well as the vectors transmitting the disease, their characterization, and genetic diversity. To effectively understand the disease and the possible control measures, further studies should be done on the alternative hosts and the incubation period. There is also a need to undertake social-economic surveys and studies to ascertain the social-economic impact of the disease and the estimated coconut trees which have been destroyed by the disease in Kenya since 1999 when it was first reported in Lamu Kenya. There is also a need to develop more specific primers to ensure better diagnostics when using the PCR approach.

This study provides a baseline information that concerned authorities and stakeholders can use to manage and curtail any further spread of the disease. Their swift action can secure the livelihoods of the millions of Kenyans who depend on the coconut industry for their survival. Given the fact that the CLYD disease has great potential for widespread destruction of the coconut industry, prevention measures must be taken early enough to either quarantine or eliminate the disease or its vectors at its earliest stage or curtail its further spread through the adoption of various mitigation measures. Among these measures, include: increasing surveillance, application of stringent quarantine practices, rapid detection and destruction of symptomatic coconut trees, controlling the exchange of planting materials among the farmers and replanting with varieties that are less susceptible to the disease. Others measures include proper weed control to get rid of the alternate hosts and possible control of the alternate hosts, for which further studies are required. Regular scouting and delimiting surveillance should be done to avoid further spread of the disease.

Disclaimer (Artificial Intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

Competing Interests

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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