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Research Article
Molecular Characterization of Phytophthora Pod Rot of Cocoa (*Theobroma cacao* L.) in Southwestern Nigeria

[1]E.B. Rotimi, [1]B. Ikotun and [2]S.O. Agbeniyi

[1]Department of Crop Protection and Environmental Biology (Phytopathology Unit), Faculty of Agriculture, University of Ibadan, Ibadan, Nigeria
[2]Training Unit Crop Protection Division, Cocoa Research Institute of Nigeria, Idi-Ayunre, Ibadan, Nigeria

Abstract

Background and Objective: *Theobroma cacao* L., is an important economic tree in Nigeria but is limited by *Phytophthora* pod rot (PPR). Efficient management of PPR requires identification and characterisation of the causal pathogen (CP). However, there is a dearth of information on the CP of PPR on the study site. Therefore, this study examined the characterization of PPR of cocoa in Southwestern Nigeria. **Material and Methods:** Fungal isolates were obtained from cocoa pods and pure cultures were assessed for pathogenicity following standard procedures. Morphological characteristics were determined by measuring the sporangial pedicel length (SPL), sporangial breath (SB), SPL: SB and the sporangial shape (SS) with the shape of the mycelial growth. The molecular analysis was carried out among PPR isolates and compared with CP genomes within the *Phytophthora* database. Data were analyzed using descriptive statistics and ANOVA at $\alpha_{0.05}$. **Results:** A total of 135 fungal isolates were identified and 45 were pathogenic in all the states. The mycelial growth of all isolates was cottony in appearance with a colony pattern slightly papillate. The SPL ranged from 29.9±7.89 to 38.8±8.65, while SB ranged from 24.01±5.42 to 30.8±5.35 and the SPL: BR was from 1.2±0.13 to 1.4±0.09. The SS was ellipsoid in all isolates. Thus, established the characteristic CP of PPR of cacao as *Phytophthora megakarya*. Extracted DNA and PCR amplification of the pathogen at ITS1 and ITS4 yielded an estimated 550 bp product. **Conclusion:** Results concluded that the *Phytophthora megakarya* is the causal organism of *Phytophthora* pod rot of cocoa in Southwestern Nigeria.

Key words: *Theobroma cacao*, *Phytophthora megakarya*, *Phytophthora* sporangial morphology

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Corresponding Author: E.B. Rotimi, Department of Crop Protection and Environmental Biology (Phytopathology Unit), Faculty of Agriculture, University of Ibadan, Ibadan, Nigeria Tel: +2348033824272

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a major economic tropical rainforest tree native to the Amazon region of South America[1]. The tree is commonly referred to as cacao, while the products made from fermented and dried seeds are referred to as cocoa. There are over 20 species in the *Theobroma* family, but the *Theobroma cacao* tree is the only one commonly grown. International cocoa output is largely concentrated in regions between 10° North and 10° South of Ecuador, whereas, Western Africa accounts for 65% of world production[2].

Cacao is a significant crop with more than 20 M Nigerians solely dependent on it for their economic security[3]. The explanation for its popularity around the world could be attributed to its value as a commercial crop generating foreign exchange for producing countries.

The cocoa bean, a partly fermented and dried seed from the cocoa tree is a major component of chocolate and other beverages. Cocoa is believed to be produced principally in developing countries, having West Africa as their foot, particularly in areas along the equatorial region.

A significant population of Nigerians makes their livelihood from the production of cocoa, thereby creating explicit or implicit job opportunities[3].

Theobroma cacao is produced in 14 federal states of Nigeria, where Southwestern states are considered Nigeria's cocoa belt. In 2008, the region accounted for over 160,000 metric tons of Nigeria's 242,000 metric tons of annual cocoa production.

Meanwhile, more than 98% of the produce is exported, providing livelihood and employment for many people[3].

The identification of the presence of pathogens amongst various cocoa-growing regions within the population of *Phytophthora* will be useful for the implementation of schemes for the management of the cocoa agrosystem. An important question about which of the *Phytophthora* spp., will be in that area? Which is common to recognize in Nigeria's agro-ecological growing area? Which is more virulent? The observed organisms, pathogenicity and their possible molecular diversity?

There is a dearth of information on the characterization of pathogen diversity for *Phytophthora* spp., infecting cocoa in Southwestern Nigeria. In addition, there is no comprehensive inventory of all *Phytophthora* spp.'s for disease control caused by the pathogen in the agro-ecological region. This has persistently negated the creation of successful black pod disease control interventions and poses a serious threat to all potential control strategies[4-6].

An elucidating comprehension of the genetic characteristics of the pathogens within the environment or region is an important requirement in the creation of a systematic and effective method for handling PPR disease. In the present work efforts were made for isolation, morphological and molecular characterization of the causal organism while screening for resistance to *Phytophthora* species among the six newly released cocoa hybrids available at Cocoa Research Institute of Nigeria was carried out.

MATERIALS AND METHODS

Study site: The field research was conducted from 2015-2017. The research was conducted on selected cocoa plantations in the South-Western States of Nigeria and laboratory screening at the Cocoa Research Institute of Nigeria (CRIN), Ibadan, in addition to the Crop Protection and Environmental Biology (CPEB) Plant Pathology Laboratory, University of Ibadan. The molecular analysis was conducted by the Bioscience Department of the International Institute of Tropical Agriculture (IITA), Ibadan.

The study was executed in five states that were Southwestern Nigeria's primary cacao development centres. The regions were the states of Ekiti, Ogun, Ondo, Oyo and Osun. From the study, it was observed that all states had relatively similar effects and distribution of cocoa black pod diseases. This condition became a justification to choose Southwestern Nigeria as a major cocoa producer in Nigeria (up to 70% of total annual production).

Collection of infected samples: Infected cocoa pods have been collected from strategic farm locations across all states in the Southwestern Region of Nigeria. These were packaged aseptically, numbered and transferred using Koch's postulate for treatment, separation, processing, classification and further pathogenicity screening at the Cocoa Research Institute of Nigeria. Fungal isolates were obtained from cocoa pods and pure cultures were assessed for pathogenicity following standard procedures. *Phytophthora* species isolation was performed using suitable media cultivation (carrot dextrose agar and V8 agar) for growth[4], classification and possible morphological diversity among the isolates from the five states being studied[3,5,6].

Morphology and characterization protocol: Morphological characteristics were determined by measuring the sporangial pedicel length (SPL), sporangial breath (SB), SPL: SB and the sporangial shape (SS) with the shape of the mycelial growth. DNA extraction and Polymerase Chain Reaction (PCR) were

performed in a small plastic pipe containing all the biochemicals required for the synthesis of new DNAs. When amplification was introduced, sequences specific to each species of *Phytophthora* were used to delineate the various isolates following the standard procedures[7].

Statistical analysis: Data were evaluated using Genstat® 11th Version of Variance (ANOVA) (Genstat, 2008). The importance of the variations in genotyping for each phenotype was checked through the F-test. While the ANOVA showed significant genotypic variations, the least substantial difference (LSD) was at $p = \alpha_{0.05}$.

RESULTS

The survey showed that cocoa was successfully grown between longitude 3.43-5.0°E and latitude 7.00-7.43°N, which comes between 10°E and 10°N of the equator for the cultivation of the crop. The outcome of this research confirmed the existence of *Phytophthora megakarya* in the area through the initial sampling and analysis of specific sites where samples were collected. Provided clean, stable and mature cocoa (*Theobroma cacao*) pods that separated it from the heap of *Phytophthora megakarya* contaminated pods filled with plentiful sporangial. Stages and spread of infection on pods that are unique to *Phytophthora megakarya* were reported indicating distal and lateral infection, pod filled with sporangial to further assess the similarity of the causal organism's infection symptoms. It provides a clear description of the propagation of the fungus after the inoculum/inocula may have found its way to the host cell. Infection can start at different parts of the pod or tree crop depending on the pathogen's entry point.

All surveyed local government areas and all cacao plantation clusters visited had the infection. The compilation of samples further confirmed the fact that the crop is still considered lucrative, taking into account the area covered by the farmland and the development of new plantations. It was also observed that most farmers across the state are getting older (±60 years) and polygamous although inadequacies in good farming practices introduced in some of the state-wide plantations were the major causes of a substantial or total loss to the region's *Phytophthora* pod rot. Figure 1a-f showed the colony morphology of *Phytophthora megakarya* isolates

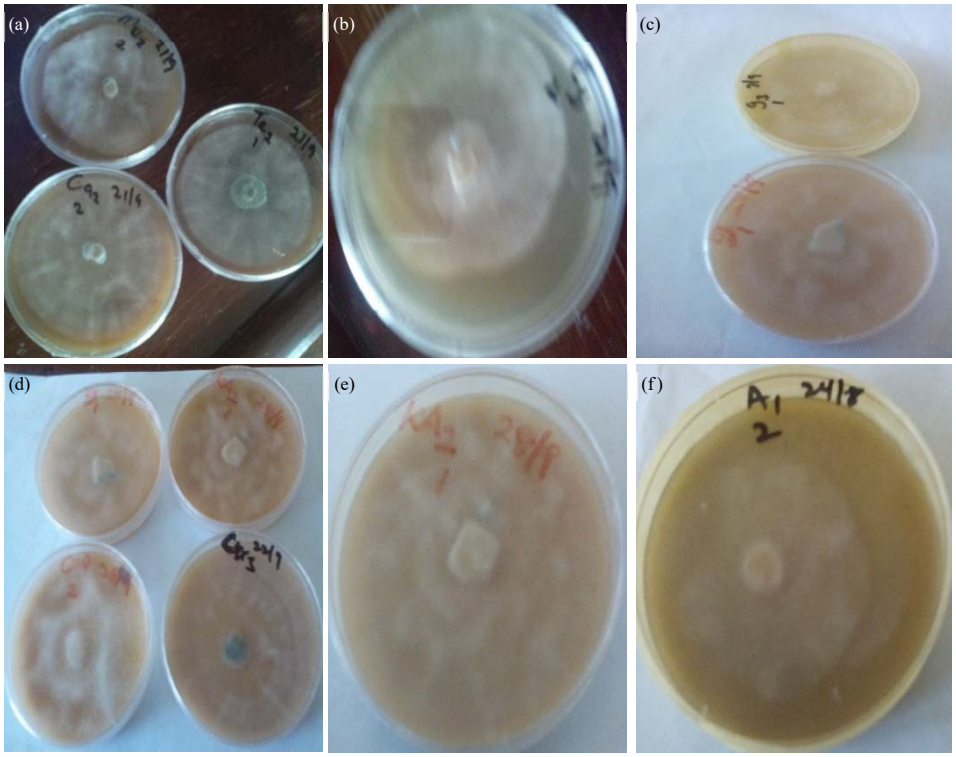

Fig. 1(a-f): Colony morphology of *Phytophthora megakarya* isolate10 days growth at 20-25°C on carrot agar and V8-agar from infected matured fruits of cocoa plantations across the Western States of Nigeria, (a) Ekiti State: Ab_2, Ogun State: Ta_2 and Oyo State: Ca_2, (b) Oyo State: C_3, (c) Ogun State: g_1 and g_2, (d) Ogun State: g_1, Osun State: Op_2, Ekiti State: C_2 and Oyo State: Ca_3, (e) Ondo State: Ka_2 and (f) Ondo State = A_1

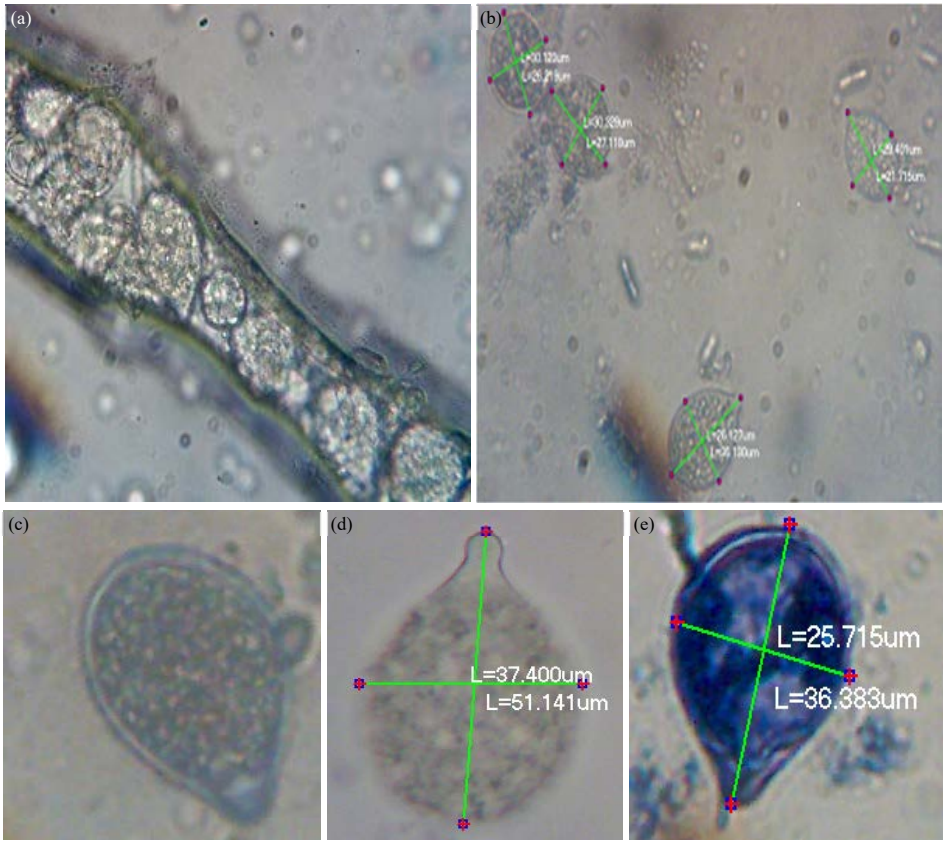

Fig. 2(a-e): *Phytophthora megakarya* sporangial morphology using a digital compound microscope. Structures were produced after 10 days of growth in V8 and carrot agar. (a) Hypha containing *Phytophthora megakarya* sporangial, (b) Few *Phytophthora megakarya* sporangial measured with a digital microscope, (c) 100× magnified single *Phytophthora megakarya* sporangial under the digital microscope, (d) 100× single *Phytophthora megakarya* sporangial measured with the digital microscope and (e) 100× single coloured *Phytophthora megakarya* sporangial measured with a digital microscope

growth within 10 days at 20-25°C on carrot agar and V8-agar from infected matured fruits of cocoa plantations across the Western States of Nigeria. *Phytophthora megakarya* developed very well after 7 days on the selected media with strong sporulation. It has been shown that all isolates from the five Southwestern States of Nigeria have similar media growth expressing a cotton appearance on carrot and V8 agar with aerial mycelia and coralloid hyphae.

On carrot agar, all *P. megakarya* isolates examined produced colonies with patterns of finely radiating growth. When grown on V8 agar, there have been slight variations in growth rates and trends among isolates. On V8, all isolates developed most abundantly. On V8, all *P. megakarya* isolates from across-the-board cocoa samples developed identical thick hyphae rosettes that were very close to those produced by *P. megakarya* isolates from other previous studies reports. The colony pattern was cotton-like and slightly papillate for *Phytophthora megakarya* in all the samples. Figure 2 revealed the distinctive presence of *P. megakarya* isolates on slides as seen under the optical ×40 mm microscope. The hyphae in Fig. 2a were full of sporangia that were about to burst open for spores to be released. Slightly papillate and ellipsoid in form was the sporangial colony pattern in Fig. 2b-e which are the complexes of spores on the slide of the causal organism. They all shared common sporangial characteristics.

Table 1 described the product of the digital microscopic view of the morphological characteristic pattern of *P. megakarya* in the samples. Using Duncan's Multiple Range Test, the morphological growth trend on V8 agar and carrot agar was important at p = 0.05 across the column with separate superscripts. In all the states, the colony pattern (slightly papillate) was identical while the mean length to width (l:b) was between 1.24-1.36. The mean pedicel length always comes between 4.01-5.13 µm. The colony growth rate varies from 4.5-6.2 mm/day for all the isolates. The sporangial form throughout the states was ellipsoid.

Table 1: Morphological properties of *Phytophthora megakarya* observed in the study

State	Colony pattern	Growth rate (mm/day)	Sporangial shape	Mean l/b ratio	Mean pedicel length
Ekiti	Slightly papillate	5.5 ±0.8	Ellipsoid	1.26±0.18[a]	5.13±0.89[a]
Ogun	Slightly papillate	4.5±0.8	Ellipsoid	1.24±0.13[a]	4.01±0.90[b]
Ondo	Slightly papillate	5.8±0.5	Ellipsoid	1.25±0.10[a]	4.37±0.70[ab]
Osun	Slightly papillate	5.2±0.8	Ellipsoid	1.36±0.09[a]	4.35±0.59[ab]
Oyo	Slightly papillate	6.2±0.6	Ellipsoid	1.29±0.12[a]	4.59±0.87[ab]

Mean±SD across the column of various superscripts is significant at 5% with a>ab>b. Mean differentiation achieved by the Duncan's Multiple Scope Check

Table 2: Mean±SD sporangial character and variation of *Phytophthora megakarya* disease isolates across the cocoa (*Theobroma cacao* L.) growing states of Southwestern Nigeria

	Mean±Standard Deviation			
State	Sporangial length (l) (µm)	Sporangial breath (b) (µm)	(l:b) ratio	Pedicel length (pl) (µm)
Ekiti	38.82±8.65[a]	30.80±5.35[a]	1.26±0.18[a]	5.13±0.89[a]
Ogun	29.97±7.89[a]	24.09±5.42[b]	1.24±0.13[a]	4.01±0.90[b]
Ondo	32.99±6.26[a]	26.21±4.21[ab]	1.25±0.10[a]	4.37±0.70[ab]
Osun	35.41±4.98[a]	26.12±3.57[ab]	1.36±0.09[a]	4.35±0.59[ab]
Oyo	35.58±8.23[a]	27.55±5.21[ab]	1.29±0.12[a]	4.59±0.87[ab]

Mean±SD across the column with different superscripts is significant at a 5% level with a>ab>b. Mean separation done by Duncan's Multiple Range Test

Table 3: Characteristics and properties of partial internal transcribed spacer region of *Phytophthora* in five states in Southwestern Nigeria using nBLAST on GenBank

Isolate	Number of nucleotides	Highest nBLAST identity (%)	e-value	Alignment score	Highest query coverage (%)
OY	849	99	0.0	>200	96 (JX315261)
OG	765	99	0.0	>200	100 (KR818206)
OD	777	100	0.0	>200	99 (KR818142)
OS	761	99	0.0	>200	99 (MG865534)
EK	781	99	0.0	>200	99 (MH620121)

OY: Oyo State, OS: Osun State, EK: Ekiti State, OD: Ondo State and OG: Ogun State

Whereas, Table 2 gave the sporangial character and variation of *Phytophthora megakarya* in (µm) data, which was significantly throughout the column at a p range of 0.05, in the five states in which the study was conducted. Their sporangial length ranges from 29.97-38.82 µm from Ogun State samples 29.97 µm to Ondo 32.99 µm, Osun 35.41 µm, Oyo 35.58 µm and the Ekiti States 38.82 µm in that order. The same difference occurred in all the samples for their sporangial breadth. The length to breadth ratio gave a slight difference where Ogun State samples 1.24, which was the least followed by Ondo at 1.25, Ekiti at 1.26, Oyo at 1.29 and Osun the highest with 1.36 in the order of occurrence. This was also reported for the significant length to breadth ratio at p = 0.05. In sporangial width and pedicel length across the states, the degree of significance was much smaller. The study also showed that there was no significant difference in the device character parameter. This is in respect of samples collected across the cocoa (*Theobroma cacao* L.) growing states of Southwestern Nigeria.

The characteristic variations in the colony between *Phytophthora palmivora* and *Phytophthora megakarya* pure colonies on V8 were shown in Fig. 3a and b, *P. palmivora* shows the appearance of a stellate mycelial growth while *P. megakarya* shows the appearance of cotton mycelial development. This is a clear difference in development in the same medium when normal laboratory monitoring during each of the experiments has been observed.

Integrity test for extracted DNA samples with PCR amplification: *Phytophthora megakarya* derived DNA has been amplified with ITS1 and ITS4 main pairs in Fig. 4 and 5. *Phytophthora* oomycete, *P. megakarya* ITS1 and ITS4 PCR amplification yielded an estimated 500 and 550 bp samples, respectively. The isolates developed a substance of the same size.

From this study, pure fungal cultures developed a similar peak as the photo gel of DNA and PCR. This displays the genomes of the area similar to those shown in the plates. The test revealed consensus traces of the presumed fungal DNA.

The agarose gel electrophoresis of the internal spacer area base pair (bp) isolates products from five states where the samples were obtained as shown in Fig. 6. The PCR extension of the ITS regions with the ITS1 and ITS4 primers culminated in an estimated 500 bp in Fig. 4, while, Fig. 5 showed the integrity test for extracted DNA samples.

The characteristic properties of *Phytophthora megakarya* isolates collected from five Southwestern Nigerian states using nBLAST on GenBank were shown in Table 3. It showed that all the isolates are pathogens linked to evolution. In this analysis, the number of nucleotides sequenced from isolates of *Phytophthora* differed. Isolate 'OY' had the highest number of sequenced nucleotides (849 nt), while it had the lowest number of nucleotides (761 nt). Using BLAST on NCBI, all isolates had an identity of 99-100% with isolates in the sample.

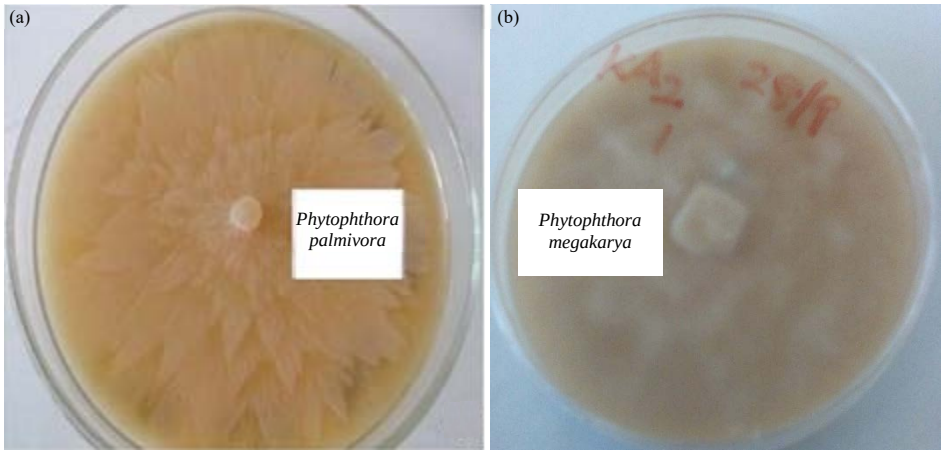

Fig. 3(a-b): Characteristic colony morphology of *Phytophthora* species growth media, (a) *Phytophthora palmivora* showing the stellate appearance in V8 juice agar medium and (b) *Phytophthora megakarya* showing cotton-like appearance in V8 juice agar medium

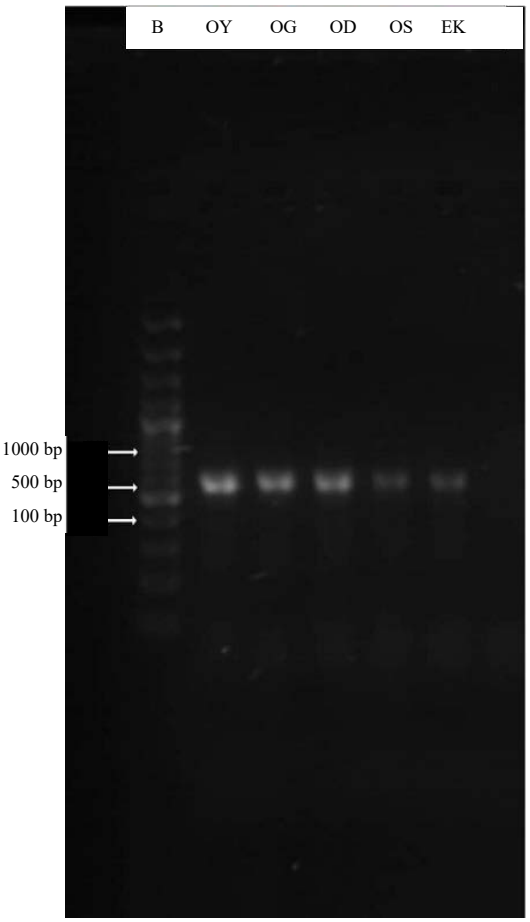

Fig. 4: Extracted DNA of *P. megakarya* amplified with primer pairs ITS1 and ITS4

An oomycete in the genus *Phytophthora*, PCR amplification of *P. megakarya* ITS1 and ITS4 yielded an estimated 500 bp product for all the states. OY: Oyo State, OS: Osun State, EK: Ekiti State, OD: Ondo State, OG: Ogun State and B: Buffer

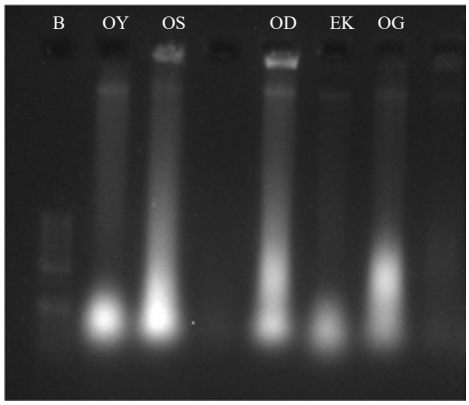

Fig. 5: Integrity test for extracted DNA samples

Phytophthora megakarya isolated DNA was amplified with ITS and ITS4 primer pairs at 550 bp. OY: Oyo State, OS: Osun State, EK: Ekiti State, OD: Ondo State and OG: Ogun State

In addition, the highest possible query coverage (100%) for isolate 'OG' was registered, providing a perfect match with Cameroon origin accession number KR818206. For isolate 'OY' at 96% coverage with isolate JX315261, also sequenced from Cameroon, the lowest percentage coverage was reported.

A pair sequence alignment (PSA) was shown in Fig. 6. This indicates the structural and developmental association between one state and the other of the pathogen of molecular diagnosed samples. In comparison, the similarities and evolutionary sequence among the states show that Ekiti State had 99% of nucleotide in nBlast identity which compared closely with that of Ogun State 99%, Osun State 99% and Oyon State also 99%. Ondo State had 100%. While the pathogen of Ekiti State samples was well compared to that

of Oyo and Ogun State, which is true because PSA samples of Oyo State were produced with the pathogenic PSA samples of Ekiti and Ogun State and Ogun State. There was a pair correlation of sequences between Ondo State samples and Osun State samples. This is also confirmed in the phylogenetic tree in Fig. 7, which shows the evolutionary relationship of the isolates compared with other cocoa-growing countries in the West and Central Africa.

The available phylogenetic tree by analysis showed the branching diagram that represents the evolutionary history or relationship between the species of *Phytophthora* that are commonly discovered in the growing regions of *Theobroma cacao* in Africa. The phylogenetic species of *Phytophthora* was based on the pattern of character distributions, which is consistent with the full range of possible evolutionary processes that contribute to species formation through both biotic and environmental factors. This is by the sequence of samples that have been molecularly identified that was displayed in Fig. 7. This highlighted the evolutionary relatedness of *Phytophthora megakarya* established through the analysis and compared among West Africa and Central Africa, cacao rising countries. Furthermore, the figure shows that *P. megakarya* was evolutionally traced as the origin of *P. palmivora*, thereby identifying the possible genetic variation/mutation that could allow the pathogen to evolve and develop.

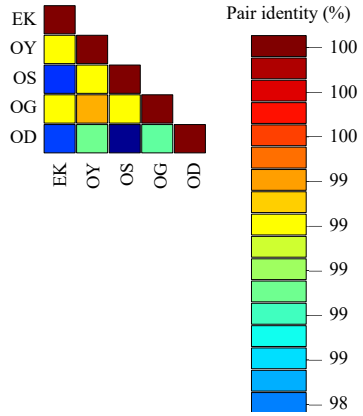

Fig. 6: Pairwise sequence identities of partial internal transcribed spacer region from *Phytophthora* isolates obtained from five states in Southwestern
OY: Oyo State, OS: Osun State, EK: Ekiti State, OD: Ondo State and OG: Ogun State

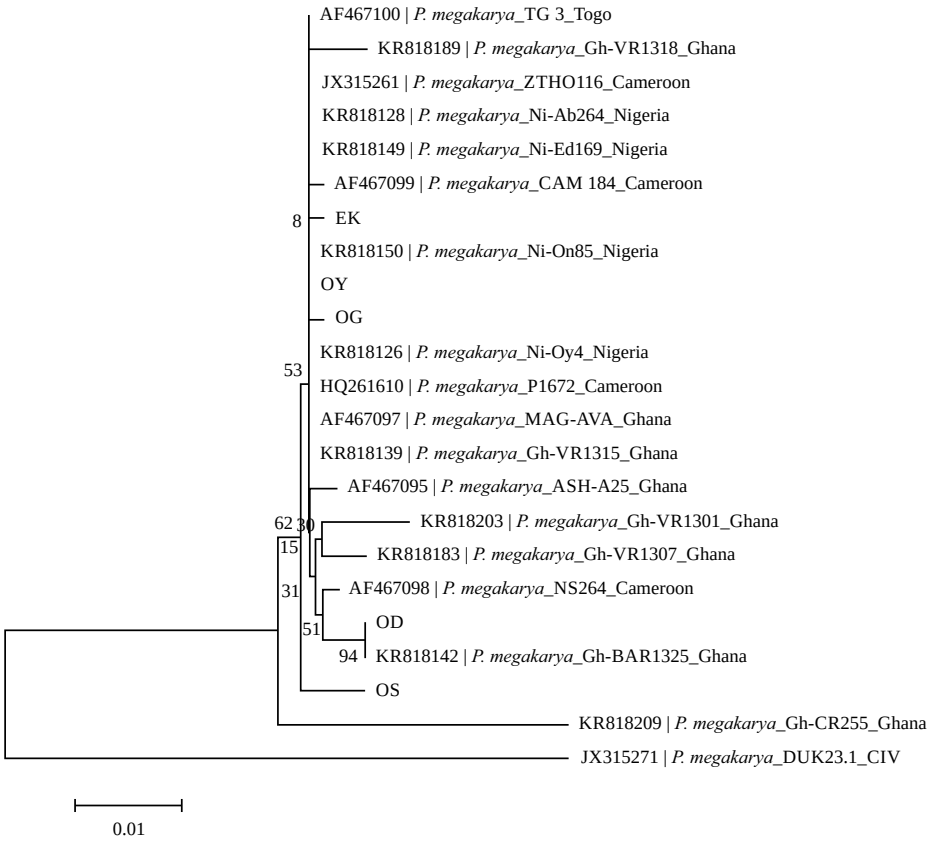

Fig. 7: Phylogenetic tree showing relationships among partial internal transcribed spacer region of five *Phytophthora* isolates in Southwestern Nigeria with others across West and Central Africa

Theobroma cacao was mostly influenced by *P. megakarya* in West Africa, especially in Ghana and Nigeria, cacao grown in Cameroon and Togo was not left out of this same *P. megakarya* devastation. Samples from Ekiti, Oyo and the Ogun States were found to be on the same lane or main trunk of the tree while Ondo and Osun were still further away from sources from the main trunk of the phylogenetic tree than Ekiti, Ondo and the Ogun States were seen on the evolutionary tree.

DISCUSSION

This study provides morphological and molecular data to identify *Phytophthora megakarya* as a distinct mycological genus that targets *Theobroma cacao* in Southwestern Nigeria[1]. This classification is based on a 3 year analysis of a group of isolates from Ekiti, Ogun, Ondo, Osun and Oyo States' 15 local governments. Based on analyses of the DNA sequence, *P. megakarya* was found to be in the same clade (clade 4) as *P. palmivora* that morphologically identified papillate sporangia with the topology of clad relationships according to previous results and studies[7,8].

While *P. palmivora* is similar to *P. megakarya* in classification, a distinction is seen in the arrangement of sporangia. Similarly, *P. palmivora* infects multiple hosts while *P. megakarya* infects only cacao. This agreed with the previous finding in the growing regions in Africa[7].

Therefore, it was necessary to utilize taxonomic articulate keys and information about the pathogen's host range[9]. This was primarily recognized in the morphological and molecular study of the *Phytophthora* pod rot (PPR) in Southwestern Nigeria. The time required to identify and evaluate the potential risk of the isolated pathogen requires frequent use of sequence-based DNA analysis to improve and supplement morphological data.

The results of this study reveal that the media used to grow the causal organism, namely, V8 agar and carrot agar, as opposed, are rich and favourable for organism growth due to the preferential growth of *Phytophthora* species.

The relatively large sporangia with several spores supported the name of the "super" genus, meaning big *P. megakarya* which could also inform of its virulent nature. This is also consistent with previous research in the *Phytophthora* plant production laboratory on specific media[4].

This study showed that there is a characteristic colony difference between *Phytophthora palmivora* and *Phytophthora megakarya* pure colonies on V8. *Phytophthora palmivora* displays a stellate mycelial growth appearance where it occurs as a causal organism, *P. megakarya* shows a cotton-like mycelial growth appearance in all isolates from the five Southwest Nigerian States. This is a clear difference in the production of the same medium as standard laboratory testing has been observed in each of the experiments. *Phytophthora palmivora* showed an ovoid micrograph of sporangium while *P. megakarya* showed an ellipsoid micrograph of the sporangium. In all the sample isolates obtained in Southwestern Nigeria in the five states, the ellipsoid sporangial feature was found. This also clarified the fact that the causal organism is *P. megakarya* in the area being investigated[9-13].

The findings of this work researching PPR disease in Southwestern Nigeria clearly showed that the pathogen was of the same morphological type across the provinces. There was no record of *Phytophthora palmivora* in any of the five states in which the study was conducted. This further identified the fact that the causative organism is *Phytophthora megakarya* with the help of a coherent key for identifying species of *Phytophthora* as predicted earlier[10,14].

The DNA and PCR methods used in this work established the testing tool for plant disease and eventually could be used to identify other species in the genus. The DNA derived from *P. megakarya* augmented by the primary ITS1 and ITS4 pairs. *P. megakarya* ITS1 and ITS4 PCR amplification produced an average product of 500 and 550 bp, respectively widely used for oomycete in the *Phytophthora* genus. The isolates have produced the same size object. This further enhances the similarity of the pathogen across the region, where the research is being conducted in southwestern Nigeria. Pure fungi cultures created a similar peak from this study as shown in the DNA and PCR photo gel. This shows the area's genomes similar to those shown on the plates. The result shows the suspected fungal DNA's consensus remains. This further clarifies the fact that the fungus is widespread across the states of South-West Nigeria in all places where the samples were obtained. *Phytophthora megakarya*, however, may have shared a common ancestor with *P. palmivora* or as a result of interspecific hybridization[10,13].

Sporangia from all isolates in this study were ellipsoid with rounded bases, ranging from 29.97-38.82 to 24.09-30.8 µm, with a length-to-breadth ratio of 1.24:1.36, resulting in sympod. Sporangia are caduceus, with pedicels that are 4-01-5.13 µm long. *Phytophthora megakarya* is, as described above, a member of the *Phytophthora* group II. It grew caduceus papillate sporangia heterothallic and amphigynous. Regarding the molecular analysis of the pure culture obtained from the isolates, a Basic Local Alignment Search Tool (BLAST) showed that ITS PCR products from five states in which *P. megakarya* was reported to have exhibited 96-100% homology with the associated sequences in the GenBank[6,7,13].

An NCBI GenBank search reveals that Oyo State samples with gene accession number (GAN)-JX3I5261 and GAN-KR818206 Ogun State has an evolutionary relationship with strain from Cameroon, Ondo State with strain from Ghana that has the gene accession number GAN-KR818142. While Osun State samples with GAN-MG865534 were well associated with Ajayi *et al.*[11], findings predicting their evolutionary relationship with strain from Cameroon also, Ekiti State samples GAN-MH620121 accession number with Cameroon strain based on sequence analysis of previous studies in the region[2]. Although the number of nucleotides from all five isolates varies, nBLAST was used to report similar percentages of identity. This indicated high accuracy and matching already contained in *Phytophthora* isolates' regional repository. The e-values of zero and very high alignment scores indicate just high match performance. The strong query coverage recorded in the matches also confirms the reliability of the *Phytophthora* sequences, especially as a sequenced match with isolates from Cameroon and Ghana[1,9].

The pair sequence alignment (PSA) showing the structural and developmental relationship of pathogenic molecular samples from one state and the other revealed that pathogenic samples from Ekiti State were well associated with pathogenic samples from Oyo and Ogun State, which is valid because Oyo State PSA samples were produced from pathogenic PSA samples from Ekiti and Ogun State. There is a pair sequence similarity between samples from Ondo State and the one showing Osun State's evolutionary association. In addition, there is a significant multiple sequence alignment (MSA) of all samples from the five Southwestern States of Nigeria understudy in the experiment with sequences of similar length. The consequence of the sequence correlation suggested homological and developmental associations between the observed sequences[9,13].

CONCLUSION

Cacao infestation of *Phytophthora megakarya* is a threat to countries' economies in Southwestern Nigeria. It spreads rapidly in the sub-region, it has displaced the less severe *P. palmivora* original populations. This research partly identified the current molecular strategies and common analytical methods used to study fungal species in a specific environment. The clear trend in implementing both morphological and molecular methods is to explain the observations and make them scientifically valid.

Phytophthora megakarya was found to be the common pathogen in the area under study based on isolated surveys. The results indicate that the pathogen has severe potential for cocoa fruits and more work is needed to determine its distribution in the rest of the country where the crop is grown. Through this research, it has been shown that the fungal disease *Phytophthora megakarya* is pathogenic to the survival of the crop.

SIGNIFICANCE STATEMENT

This study discovered the pathogenic fungus *Phytophthora megakarya* causing *Phytophthora* pod rot of cocoa in Southwestern Nigeria as against the previous species *Phytophthora palmivora*. This can be beneficial for the control of the pathogen to avoid annual losses due to the disease infection caused by the organism.

This study will help the researchers in further investigations to uncover the critical areas of the causal organism that many researchers were not able to explore. Thus, integrated disease management could be employed to control the disease for better quantity and quality harvest of the crop.

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REFERENCES

1. Akrofi, A.Y., I. Amoako-Atta, M. Assuah and E.K. Asare, 2015. Black pod disease on cacao (*Theobroma cacao*, L) in Ghana: Spread of *Phytophthora megakarya* and role of economic plants in the disease epidemiology. Crop Prot., 72: 66-75.
2. Zhang, D. and L. Motilal, 2016. Origin, Dispersal, and Current Global Distribution of Cacao Genetic Diversity. In: Cacao Diseases. Bailey, B.A. and L.W. Meinhardt (Eds.), Springer, Cham, Switzerland, ISBN: 978-3-319-24787-8, pp: 3-31.

3. Nkang, M., A. Ajah, O. Abang and O. Edet, 2007. Investment in cocoa production in Nigeria: A cost and return analysis of three cocoa production management systems in the cross river state cocoa belt. J. Cent. Eur. Agric., 8: 81-90.
4. Basu, S., A. Pal and P.K. Desai, 2005. Quality control of culture media in a microbiology laboratory. Indian J. Med. Microbiol., 23: 159-163.
5. Bartley, B.G.D., 2005. The Genetic Diversity of Cacao and Its Utilization. CABI Publishing, Wallingford, ISBN: 9781845930240, Pages: 356.
6. Saville, A.C., M.D. Martin and J.B. Ristaino, 2016. Historic late blight outbreaks caused by a widespread dominant lineage of *Phytophthora infestans* (Mont.) de Bary. PLoS ONE, Vol. 11. 10.1371/journal.pone.0168381.
7. Drenth, A., G. Wagels, B. Smith, B. Sendall, C. O'Dwyer, G. Irvine and J.A.G. Irwin, 2006. Development of a DNA-based method for detection and identification of *Phytophthora* species. Australas. Plant Pathol., 35: 147-159.
8. Yang, X. and C. Hong, 2018. Differential usefulness of nine commonly used genetic markers for identifying *Phytophthora* species. Front. Microbiol., Vol. 9. 10.3389/fmicb.2018.02334.
9. Tahi, G.M., B.I. Kébé, A. Sangare, C. Cilas and A.B. Eskes, 2007. Foliar resistance of cacao (*Theobroma cacao*) to *Phytophthora palmivora* as an indicator of pod resistance in the field: The effect of light intensity and time of day of leaf collection. Plant Pathol., 56: 219-226.
10. Martin, F.N., Z.G. Abad, Y. Balci and K. Ivors, 2012. Identification and detection of *Phytophthora*: Reviewing our progress, identifying our needs. Plant Dis., 96: 1080-1103.
11. Ajayi, R., M.O. Afolabi, E.F. Ogunbodede and A.G. Sunday, 2010. Modeling rainfall as a constraining factor for cocoa yield in Ondo State. Am. J. Sci. Ind. Res., 1: 127-134.
12. Capote, N., A.M. Pastrana, A. Aguado and P. Snchez-Torres, 2012. Molecular Tools for Detection of Plant Pathogenic Fungi and Fungicide Resistance. In: Plant Pathology, Cumagun, C.J.R. (Ed.), Intech Open, Vienna, Austria, ISBN: 978-953-51-0489-6, pp: 152-202.
13. Meszka, B. and M. Michalecka, 2016. Identification of *Phytophthora* spp. isolated from plants and soil samples on strawberry plantations in Poland. J. Plant Dis. Prot., 123: 29-36.
14. Guest, D., 2007. Black pod: Diverse pathogens with a global impact on cocoa yield. Phytopathology, 97: 1650-1653.