



Morphological and Molecular Identification of the Fungus Associated with Pink Disease of Cocoa (*Theobroma cacao* L) in the Eastern Region of Ghana

F. G. Kwarteng^{1*}, E. Cornelius², K. K. Acquah² and E. K. Asare³

¹Oil Palm Research Institute, P.O.Box 74, Kusi-Kade, Ghana.

²Department of Crop Science, University of Ghana, Legon, Ghana.

³Cocoa Research Institute of Ghana, P.O.Box 8, New Tafo-Akim, Ghana.

Authors' contributions

This work was carried out in collaboration between all authors. Author FGK designed the study, wrote the first draft of the manuscript and managed the literature searches. Author EC wrote the protocol. Authors KKA and EKA managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

The Pink Disease is caused by the fungus *Erythricium salmonicolor* (Berk & Broome) Burdsall. It attacks broad hosts such as cocoa, coffee, citrus *Eucalyptus* spp. and *Acacia* spp. An outbreak of similar disease symptoms on cocoa trees in the Eastern region of Ghana has been a threat to the cocoa industry. However, the organism causing the disease in the Eastern region of Ghana has not been properly identified. Therefore, objectives of this study were to identify the causal organism of the pink disease of cocoa in the Eastern Region of Ghana and to also determine the genetic variability among the isolates collected. All isolates produced salmon-pink fluffy mycelia with concentric zones and regular margins on (Potatoes Dextrose Agar) PDA and Malt Extract Agar (MEA). The hyphae were hyaline, thin-walled, joined to each other and with clamp-connections.

*Corresponding author: Email: fg.kwarteng@oilpalmresearch.org;

Amplification of the ITS region of isolates of *E. salmonicolor* using primers produced a 750 bp which is the expected fragment size. The isolates varied genetically with mean similarity of 55%. Isolates from Saamang, Bunso and Osino communities related by 78% whiles Isolates from Osino and Bunso communities clustered together at 88% making them the most related among all the isolates.

Keywords: Cocoa; encrustation; *Erythricium salmonicolor*; pink disease; salmon-pink.

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.), the main source of raw material for chocolate, is the most important cash crop for most of the countries of the West and Central African sub-region (mainly, Cote d'Ivoire, Ghana, Nigeria and Cameroon). It provides livelihoods for millions of people in this region [1,2]. Ghana was the world's second largest producer of cocoa after La Cote d'Ivoire in 2010/2011 crop year [3]. The crop occupies a key position in Ghana's economy as a foreign exchange earner, domestic income, and source of revenue for the provision of socio-economic infrastructure [4,5]. Ghana's cocoa beans are richer in Theobromine and Flavonoids which render the beans a unique, mild and rounded flavour and has become the world's standard against which all cocoa is measured [6].

Eastern region is an important cocoa production area in Ghana because climatic conditions favour cocoa and it was the first place of introduction of the crop in 1878 [7]. The region, together with Brong Ahafo Region accounts for about 19 percent of total production of cocoa beans in Ghana [3].

The production of the crop is faced with many phytosanitary problems, including more than seven fungal diseases (black pod, frosty pod rot, white threat blight, cushion gall, *Phytophthora* root rot, *Lasiodiplodia* rot, vascular streak dieback) among which is the pink disease, caused by the fungus, *Erythricium salmonicolor* (Berk. & Broome) Burdsall [8]. *E. salmonicolor*, a basidiomycetous fungus which attacks many woody perennials mostly in the tropics was first described by Berkeley and Broome at Ceylon in 1873 [9]. Since then, the same fungus has been known to have had several synonyms including *Pellicularia* [10], *Botryobasidium* [11], *Phanerochaete* [12] and recently *Erythricium* [13].

The fungus is able to penetrate intact bark of plants, usually through lenticels [14] where it may

then kill the cambium or the cambium may be infected directly through wounds. It spreads longitudinally through the vessels and radially through the ray parenchyma [15].

When the disease becomes established, it is characterised by production of salmon-pink encrustation on branches and stems of the tree, which cause twig and branch injuries, stem canker and eventual host plant death [16,14]. The infection starts with white mycelial growth on the surface of infected branches and stems. In severe cases, the salmon-pink encrust cracks and falls off causing a decrease of plant growth and production [17]. Even though pink disease of cocoa is reported to have occurred several years ago in Ghana [18], the knowledge about the pathogen is very limited [8].

However, in recent times, the use of molecular techniques has enhanced the characterization and diagnosis of microbial population, thereby improving disease surveillance and management strategies. The aim of this study was therefore to identify the causal organism of the disease in the region using both morphological and molecular characteristics and to also determine strain diversity if any.

2. MATERIALS AND METHODS

2.1 Sample Collection and Isolation of Causal Organism of Pink Disease

Disease samples for analysis were randomly taken from four farms across three cocoa growing districts (Atiwa, East-Akim and Fanteakwa) in the Eastern Region of Ghana where pink disease has been reported. Samples were collected from cocoa branches with salmon-colour encrustations using sharp machete and placed in sterilized polythene bags and sent to the mycology laboratory of Cocoa Research Institute of Ghana, New Tafo-Akim. A flamed scalpel was used to cut about 0.25 cm² of the advancing margin of the disease tissue, surface sterilized in 70% ethanol for one minute,

rinsed in three changes of sterile distilled water and blot-dried between sterile tissue papers. The sterilized disease tissues were placed on Water Agar (WA) in Petri dishes containing 1 mL/L stock solution of penicillin G (0.5 g/10 ml) to inhibit the growth of bacteria. Plates were incubated at $25 \pm 2^\circ\text{C}$ for three days. Pure cultures were obtained by sub-culturing hyphal tips on fresh Potato Dextrose Agar (PDA— 4 g of Potato extract, 20 g of Dextrose and 15 g Agar powder) and Malt Extract Agar (MEA -10 g of Malt Extract Broth [Oxoid] and 10 g of agar powder [Titan Biotech Ltd.]) plates [19].

2.2 Morphological Characteristics of the Fungus Associated with Pink Disease

Slides of 14 days old pure cultures of the isolated pathogen were prepared for microscopic observation and identification. The slides were prepared by mounting mycelial bits in a drop of distilled water, teased with flamed pins and covered with slips and examined under a compound microscope at x400 magnification. Identification of the isolates was based on colour, morphology of mycelia, conidia and sporulating structures as described by [20] and [21]. The isolates were coded according to the communities they were collected from and stored in a refrigerator at 5°C until used.

2.3 Molecular Identification of the Fungus Associated with Pink Disease

2.3.1 DNA extraction

Total genomic DNA was extracted and purified from isolates (Table 1) grown on MEA for six days using E.Z.N.A.™ SP Plant DNA mini Kit as follows: about 0.1 g of freeze dried mycelia was ground in a microfuge tube. Buffer SP1 700 μL was immediately added followed by 5 μL of RNase after which samples were incubated at 65°C for 15 minutes.

Buffer SP2 210 μL was added and samples mixed vigorously by vortexing. The samples were incubated on ice for 5 minute and centrifuged (Jouan MR23i, Thermo scientific inc., 81 Wyman Street, Waltham, MA 02454-9046, USA) at 14000 rpm for 10 minutes.

The supernatant was carefully aspirated into an Omega® Homogenizer Column placed in a 2 mL

collection tube. This was then centrifuged at 14000 rpm for 2 minutes. The clear lysate (about 500 μL) was then transferred into to a 1.5 mL tube. Buffer SP3/ ethanol mixture 750 μL was added directly to the lysate. The resulting mixture (650 μL) was transferred into a Hiband® DNA Mini Column placed in a 2 mL collection tube and centrifuged for 1 minute at 14000 rpm after which the flow through was discarded. This was repeated for the remaining mixture.

The columns were placed into a new 2 mL collection tube and 650 μL of SPW Wash Buffer diluted with ethanol added. This was centrifuged at 14000 rpm for 1 minute and the flow through discarded. This step was repeated with the sample volume of buffer SPW wash buffer. The empty column was centrifuged at 14000 rpm for 2 minutes. The Hiband® Mini column was then transferred into a sterile 1.5 mL tube and 100 μL of pre-warmed (65°C) elution buffer added. This was then centrifuged at 14000 rpm for 1 minute to elute the DNA.

2.3.2 Polymerase chain reaction (PCR) amplification

To characterize the fungal isolates, PCRs were performed using primers ITS 1 and ITS 4 (Table 2). Eight random amplified polymorphic DNA (RAPD) primers were used to assess diversity of the isolates.

PCR amplification of the fungal ITS region was carried out in a 25 μL PCR reaction mix composed of 1X GoTaq PCR master mix, 0.2 μM each of ITS 1 and ITS 4 primers and 1 μL of the DNA template. The thermo cycling conditions were as follows: 94°C for 5 minutes followed by 35 cycles of 94°C for 1 min, 55°C for 1 minute and 72°C for 2 minutes, and a final extension at 72°C for 6 minutes. PCR products were run and visualized on a 1.5% agarose matrix stained with ethidium bromide to achieve a final concentration of 0.5 $\mu\text{g}/\text{ml}$.

RAPD PCR reaction was performed in a 25 μL reaction volume containing 1 μL of genomic DNA template, 1.6 μM of each RAPD primer, 0.1 mM of each dNTPs, 1X Taq buffer, 2 mM MgCl_2 , and 1U of Taq DNA polymerase. The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 minutes followed by 35 cycles of 1 minute each at 94°C and 35°C , followed by 5 minutes at 72°C , and a final extension for 5 minutes at 72°C . PCR products were run on either 1.5% (in the case of ITS amplification) or a

3% (in the case of RAPD amplification) agarose gel stained with ethidium bromide.

Table 1. List of fungal isolates used in this study

Isolate	District
Os (Osino)	Fanteakwa
Sa (Saamang)	Fanteakwa
As (Asamama)	Atiwa
Bu (Bunso)	East-Akim

Table 2. Primers used in PCR and their corresponding flanking sequences

Primer name	Flanking sequence (5' – 3')
ITS 1	TCCGTAGGTGAACCTGCGG
ITS 4	TCCTCCGCTTATTGATATGC
OPE-10	CACCAGGTGA
OPA-3	AGTCAGCCAC
OPX-4	CCGCTACCGA
OPX-13	ACGGGAGCAA
OPW-8	GACTGCCTCT
OPF-20	GGTCTAGAGG
OPC-04	CCGCATCTAC

3. RESULTS AND DISCUSSION

The fungus isolated from infected cocoa samples from Atiwa, East Akim and Fanteakwa districts consistently produced salmon-pink fluffy mycelium with concentric zones and regular margins at a maximum growth and filled a 9 cm Petri dish in six days on MEA and nine days on PDA at 22 - 30°C and 65 - 70% relative humidity

(Plate 1). These features are consistent with the characteristics of *E. salmonicolor* documented in literature [20,22,23].

3.1 Morphological Characteristics of *E. salmonicolor*

Hyphae were hyaline, thin-walled, joined with each other and with clamp-connections (Plate 2) as described by [20] and [21], however, no conidia or sporulating structures (eg. basidiospores) were present in any of the isolates in culture. This is consistent with the findings of previous study [16] which found that two *E. salmonicolor* isolates picked from different geographic locations in Ghana failed to sporulate on selective medium. This occurrence may be because the media used, PDA and MEA do not support sporulation of the organism. Although this study failed to get the microorganism to sporulate, efforts to stimulate sporulation using different culture media, probably natural media prepared from plants parts need to continue. In addition, consideration to alter environmental conditions such as aeration, light, moisture, temperature etc. that facilitate sporulation should be considered in future studies.

3.2 Molecular Identification and Diversity of *E. salmonicolor*

Amplification of the ITS regions from all the fungal isolates produced a 750 bp fragment (Plate 3) characteristic of *E. salmonicolor*. Random Amplified Polymorphic DNA (RAPD) analysis of the four fungal isolates was able to resolve the isolates as quite diverse (Plate 4).

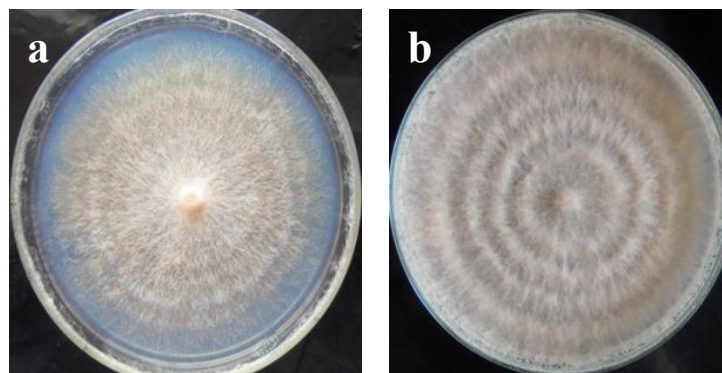


Plate 1. Six-day old cultures of microorganism associated with pink disease of cocoa on PDA (a) and on MEA (b)

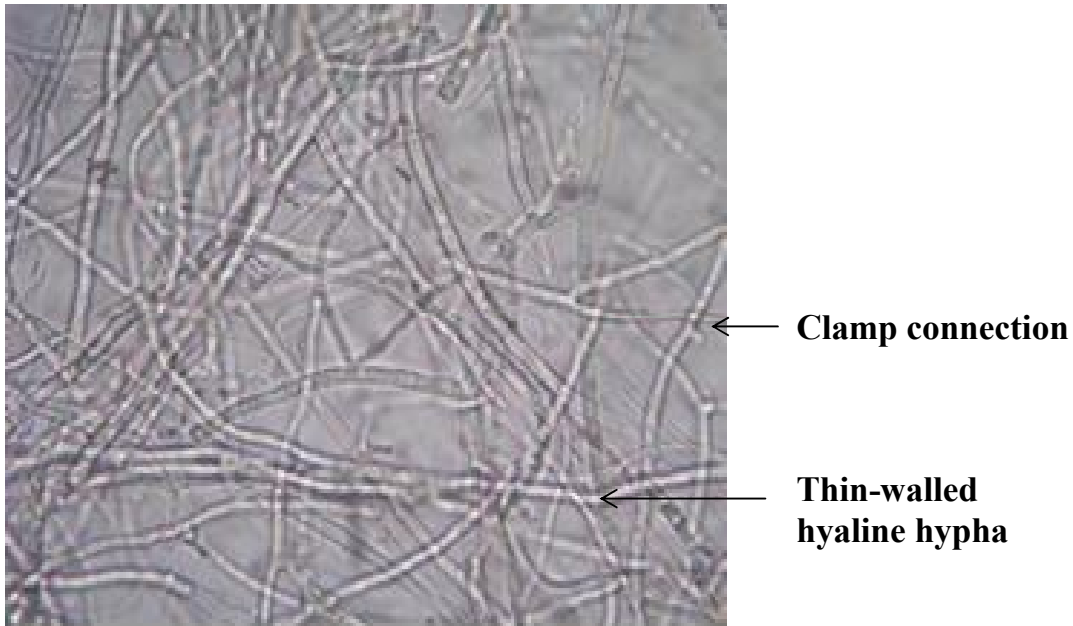


Plate 2. Morphological characteristics of *E. salmonicolor* (x400)

The dendrogram (Plate 5) shows the genetic variability among isolates, with mean similarity among the four isolates around 55% whereas isolates from Saamang, Bunso and Osino related by as much as 78%. Isolates from Osino and Bunso clustered together at 88% and were the most closely related among the four isolate (Plate 5). A similarity coefficient of 78% or more is likely to indicate a common source of origin [24] probably through the transfer of infected plant material from one site to the next.

In addition, Bunso and Osino are in close proximity to each other and therefore, it is likely that basidiospores and conidia of the necator stage of the pathogen were dispersed by wind from either of the communities to cause the disease [25]. As observed by [26], the variability may predict a greater difficulty of the control of this disease. Future studies about the genetic recombination in this pathogenic fungus are necessary for a better understanding of the genetic variability of this species.

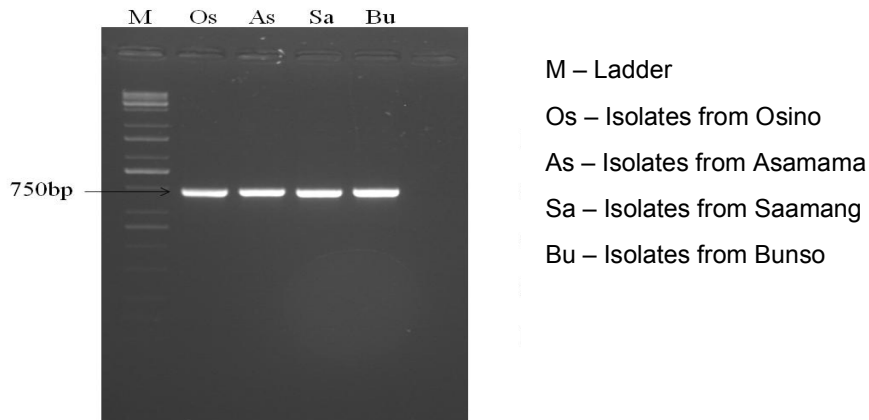


Plate 3. ITS amplification of *Erythricium salmonicolor* isolates from Osino, Asamama, Saamang and Bunso

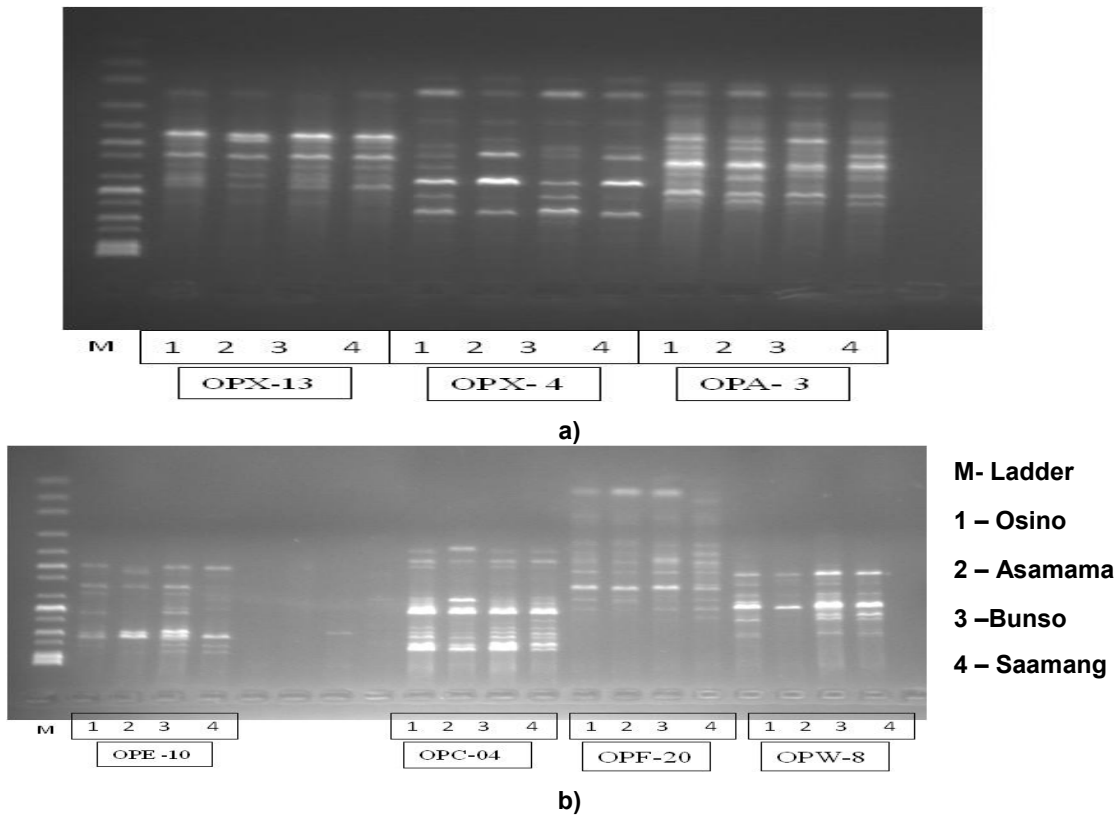


Plate 4. PCR amplification of isolates from Osino, Asamama, Bunso and Saamang with RAPD primers: a OPX-13, OPX-4 OPA-3; and b OPE-10, OPC-04, OPF-20, and OPW-8

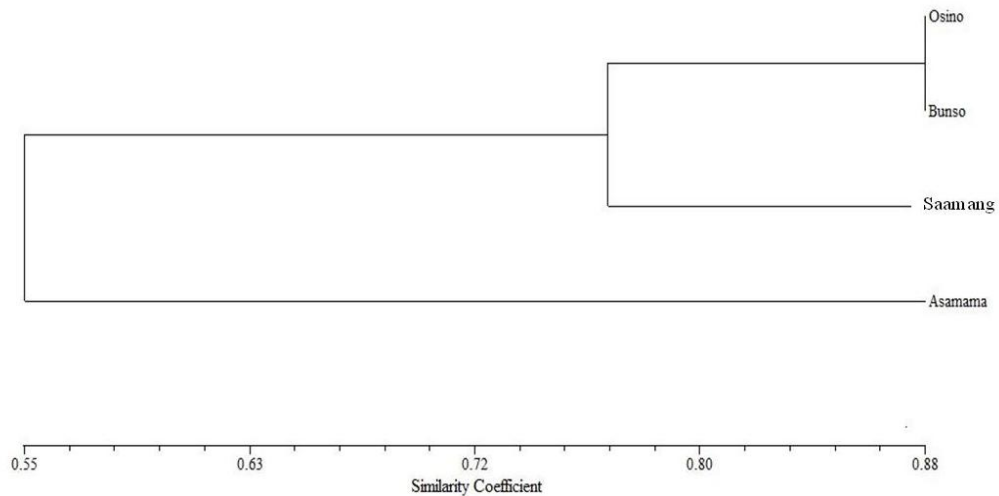


Plate 5. Dendrogram (UPGMA) showing the relatedness of four *Erythricium salmonicolor* isolates from Osino, Saamang, Asamama and Bunso

4. CONCLUSION AND RECOMMENDATION

The causal agent of the pink disease of cocoa in the three districts was identified to be *E.*

salmonicolor. RAPD analysis showed that the isolates are not uniform and that different strains are involved with Bunso and Osino isolates being most related. It is therefore recommended that further studies should be conducted on this

pathogenic fungus for a better understanding of the genetic diversity and the virulence levels of the various strains.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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