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DIFFERENTIATION OF TWO *PLEUROTUS* SPECIES BASED ON THE RESTRICTIVE DIGESTION PROFILE OF THE INTERNAL TRANSCRIBED SPACER REGION

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ABSTRACT

Two oyster mushrooms (*Pleurotus eous* P-31 and *P. ostreatus* EM-1) are under either cottage industry or semi-commercial cultivation in Ghana. The latter (*P. ostreatus*) is already well known to the public and on the shelf of some leading supermarkets. There is morphological resemblance between the two species making it difficult for the untrained eye to distinguish between them except for the colour difference. In this study, molecular methods were employed to differentiate among the two species. The Internal Transcribed Spacer ITS 1 and ITS 4 regions of the rDNA of the two oyster species were amplified by the conventional PCR using the universal primer pair, ITS 1 and ITS 4 followed by restrictive digestion with enzymes, (Hh I, Hinf I, Rsa I and Hae III). The two species could not be separated based on the amplified bands only, as both produced a characteristic band size of 650 bp. Gel profiling showing restrictive patterns generated by the four enzymes indicated that only the Hae III restrictive enzyme was effective in separating *P. eous* P-31 and *P. ostreatus* EM-1. This is the first record of the separation of the Ghanaian *Pleurotus* species by molecular methods indicating their genetic differences.

Keywords: PCR amplification, ITS region, rDNA, restrictive enzymes, *Pleurotus eous* P-31, *P. ostreatus* EM-1

Introduction

Mushrooms have been an important food item in human health, nutritional and disease prevention (Chang, 1999). Dietary mushrooms provide a wide variety of medicinal properties and they are effective against certain life-threatening diseases. The major medicinal properties attributed to mushrooms include anticancer, antibiotic, antiviral activities, immunity, and blood lipid lowering properties, antidiabetic, antihypertensive, etc., (Wong *et al.,* 2010; Kim *et al*., 2011; Patel, Narian & Singh, 2012; Roupas, *et al.,* 2012; Jesenak *et al.,* 2015 etc.).

The term mushroom applies to fungi with distinct fruiting body, fleshy in nature having tough umbrella-like structure called cap (pileus), carried on stipe (stem). The cap bear basidia (spore-bearing structures) on the surface of gills or plate (lamellae). The distinct fruiting bodies can either occur above the soil (epigeous) or below (hypogenous) soil or on decaying wood (cespitose or caestipose) (Chang & Miles, 2004).

Oyster mushrooms (*Pleurotus* spp.) are useful medicinally and are cultivated in many countries including China, Japan,

India, South East Asia, USA, the Netherlands and recently introduced to Africa (Nigeria, Ghana, Bénin, Burkina Faso, Cameroon, Cote d'Ivoire, Egypt, Ethiopia etc.) (Obodai, 1992; Atikpo *et al*., 2008; Osarenkhoe *et al.,* 2014; Jongman, Khare & Loeto., 2018). They are edible mushroom belonging to the family Pleurotaceae (Randive, 2012). There are several strains of *Pleurotus* (*P. pulmonarius*= *Lentinus sajor-caju*, *P. sajor-caju, P. eryngii, P. eousmus, P. ostreatus, P. tuber-regium=Lentinus tuber-regium*, *P. cystidiosus, P. cornucopiae, P. florida, P. citrinopileatus, P. eous, P. djamor* etc.) (Bresinsky *et al*., 1987; Obodai & Vowotor, 2002; Kang, 2004; OECD, 2005; Patil *et al.,* 2010; Shukla & Jaitly, 2011; Obodai *et al*., 2014, Piska, Sułkowska-Ziaja, & Muszyńska, 2017 etc.). Oyster mushrooms (*Pleurotus* spp.) are very effective in reducing total plasma cholesterol and triglyceride levels in humans (Nuhu *et al*., 2008) and thus reduce the chance of atherosclerosis and other cardiovascular and artery related disorders.

Pleurotus species also have antitumour activity and antitumor activities (Nayana & Janardhanan, 2000; Manpret *et al*., 2004). Chemical analyses of fruiting body show that *P. ostreatus* contains Ascorbic acid (Vitamin C), Thiamine (Vitamin B1); Folic Acid (Vitamin B), Niacin (Vitamin B3), Riboflavin (Vitamin B2) (Patil *et al*., 2010; Kortei *et al*., 2016; Nasiruddin *et al*., 2018; Obina-Echem & Churunda, 2018) as well as essential amino acids such as leucine, valine, lysine, isoleucine, threonine, tyrosine, methionine and phenylalanine (Oyetayo *et al*., 2007; Patil *et al.,* 2010; Khan & Tania, 2012; Obodai et *al*., 2014). Appreciable levels of energy, carbohydrates, crude protein, lipids, fibre, ash, sugar alcohol, glycogen, chitin have been detected in *P. ostreatus* (Bhatti *et al*., 2007; Daba *et al*., 2008; Hung & Nhi, 2012).

The oyster mushroom *P. ostreatus* cultivated in Ghana contained 49.93% total carbohydrates, 20.02% crude protein, 2% lipids, 15.8% crude fibre, 7.62% ash, 279.92 Kcal/100g, Ca (43.06mg/100g), P (939.0mg/100g), K (3334mg/100g), Fe (42.65mg/100g), and Ascorbic Acid (Obodai, 1992; Obodai *et al*., 2014; Kortei *et al*., 2016; Wiafe-Kwagyan *et al*., 2016). Studies in Ghana by Wiafe-Kwagyan (2014) and Wiafe-Kwagyan *et al*. (2016) have shown the fruit bodies of *P. eous* contained Ca, Fe, K, Mg, Na, P as well as Cu, Zn, Pb, Mn, in varying quantities depending on the substrate formulation. In most instances, the levels of nutrients and elements detected in *P. eous* was higher than what existed in *P. ostreatus* (Wiafe-Kwagyan, 2014; Kortei *et al*., 2016; Wiafe-Kwagyan *et al*., 2016, 2017). This may imply genetic difference between the two *Pleurotus* species.

Geographical and climatic difference between tropical areas do not always allow the extension of results of knowledge of research into new regions. It is therefore necessary to investigate, using local conditions in order to arrive at data which will provide clearly defined techniques for the cultivation of a particular species without extra cost. Although morphological features and colour characteristics are important in the elucidation of the Biological Efficiency (BE) and identification of mushrooms, this may be elusive in some instances. The current trend is to use molecular and biochemical techniques to complement observed conventional taxonomic features.

Recent molecular polygenic studies have demonstrated that the ITS (Internal Transcribed Spacer) region of genomic DNA is very useful in assessing polygenetic relationship at lower taxonomic levels. The

variations of the ITS of rDNA of organisms is considered as sufficient for distinguishing among species and even among strains (Vilgalys *et al*., 1993; Zervakis *et al*., 1994; Iracabal et. al., 1995).

In this paper the ITS 1 and ITS 4 regions of the rDNA of *P. eous* strain P-31 and *P. ostreatus* strain EM-1 were amplified by PCR techniques using universal primers (Hh I, Hindf I, RSa I and Hae III) with the view to ascertaining their primary taxonomic status and also obtain an ancillary information to elucidate their comparative growth performance, Biological Efficiency and the differential level of elemental composition and nutrient status.

Experimental

Morphological and cultural differences between P. eous and P. ostreatus

Cultures of *P. eous* P-31 and *P. ostreatus* EM-1 were raised on Potato Dextrose Agar and Malt Extract Agar and incubated at 28 – 30 ºC for 7 days. *P. eous* appears whiter than *P. ostreatus* with profuse mycelia on Potato Dextrose Agar and Malt Extract Agar. Photograph of the cultures were taken after the incubation period. The pictures of mature fruiting bodies of both *Pleurotus* species from the cropping house were taken for record purposes and the gill regions were captured by camera as well as the gross morphology.

Extraction of DNA from dry mushroom sample

Total genomic DNA was isolated and purified using E.Z.N.A. ™ SP Plant DNA Mini Kit. 0.3g of dried ground mushroom samples was weighed into a microfuge tube. 700 µL of Buffer SP1 was added followed by 5 µL of RNase A after which samples were incubated at 65 °C for 15 minutes. Two hundred and ten micro litres of buffer SP2 was added and the samples were mixed vigorously by vortex followed by incubating samples on ice for 5 minute and centrifuged at 14000 rpm for 10 minutes. The supernatant obtained was carefully aspirated into an Omega® Homogenizer Column placed in 2 mL collection tube and was then centrifuged at 14000 rpm for 2 minutes. 500 µL of the clear lysate that resulted was transferred into to a 1.5 mL tube. Binding conditions of the sample was adjusted by pipetting 750 µL of buffer sp3/ ethanol mixture directly on to cleared lysate. 650 µL of the resulting mixture 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 2mL collection tube and 650µL of SPW Wash Buffer diluted with ethanol was 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 1.5mL tube and 100µL of pre-warmed (65°C) elution buffer was added. This was then centrifuged at 14000 rpm for 1 minute to elute DNA based on the manufacturer's instructions.

Polymerase Chain Reaction (PCR) amplification

PCR was performed with ITS 1 and ITS 4 primer to characterize the ITS region of mushroom samples. ITS amplification was carried out in a 25 µL PCR reaction composed of 1X GoTaq PCR master mix, 0.2 µM each of ITS 1 and ITS 4 primers and 1 µL of extracted DNA. The thermal cycling conditions were as follows; 94 for 5min followed by 35 cycles of 94 for 1 min, 55 for 1 min and 72 for 2 min, and a final extension at 72 for 6min. Products were run and visualized on a 1.5% agarose matrix stained with ethidium bromide as prescribed by manufacturers.

Restriction digestion

Restriction enzymes were used to segregate the two mushroom species based on the generated restriction patterns of ITS 1 and ITS 4 amplicons following the manufacturer's guidelines. Final volumes were made with deionized water.

Results and discussion

Morphological differences of the two species were hard to distinguish except for the size and length of the stipe and colour of pileus (cap). The pileus of *P. eous* strain P-31 was whitish (Fig. 1) while that of *P. ostreatus* strain EM-1 was greyish brown to dark brown (Fig. 2). The emergence of the fruiting body was cespitose (or caestipose) and similar for both *P. eous* and *P. ostreatus*. The photograph of the gill regions (under surface or adaxial side) are shown in Fig. 3 (*P. eous*) and Fig 4 (*P. ostreatus*). They were indistinguishable for the untrained eye. This finding underscores the difficulty of using morphological parameters for separation of species. Thus, needs a molecular approach to distinguish between the two species.

Fig. 1: Emergence and appearance of *Pleurotus eous* P-31 fruiting bodies in the cropping house (Mg. x0.05).

Fig. 2: Emergence and appearance of *Pleurotus ostreatus* EM-1 fruiting bodies in the cropping house (Mg. x0.05).

Fig. 3: Gills of *P. eous* P-31 (Mg. x1/2).

Fig. 4: Gills regions of *P. ostreatus* EM-1 (Mg. x1/2).

On the other hand, the culture of *P. eous* on agar plate PDA was clearly whitish and cottony in contrast with buff grey ochre for *P. ostreatus* (Plate 5). The other distinguishing features was

the slow growing *P. ostreatus* which could not cover the plate for the same incubation period of 7 days at 28 – 30ºC. Clearly this could only offer partial clues to distinguish between the two species.

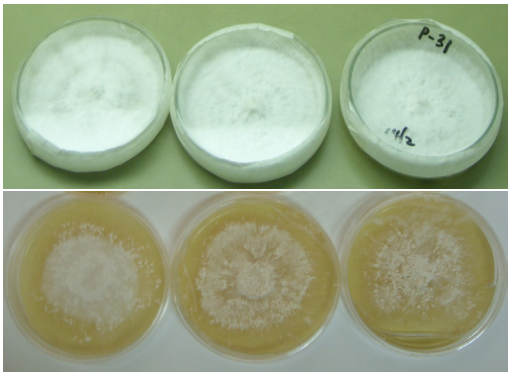

Fig. 5: Vegetative radial growth of *Pleurotus* species used in the investigation on Potato Dextrose Agar Top: *Pleurotus eous* P-31; Bottom: *P. ostreatus* EM-1 (Mg. x1/2).

PCR performed with ITS I and ITS 4 primers to amplify their ITS region of specimens grown in different substrates showed that the two *Pleurotus* species could not be separated as they both produced a characteristic band size of 650bp (Fig. 6) when run with ITS I and ITS 4 primer pairs along Kapa Universal DNA ladder M. Therefore, Hha I, Hinf I and RSa I restrictive enzymes were unable to distinguish between the two mushroom species. Restrictive digestive patterns generated by four restrictive enzymes Hha I, Hinf I, RSa I and Hae III showed that only one restrictive enzyme Hae III was the most effective in segregating *P. eous* P-31 and *P. ostreatus* EM-1 (Fig. 7).

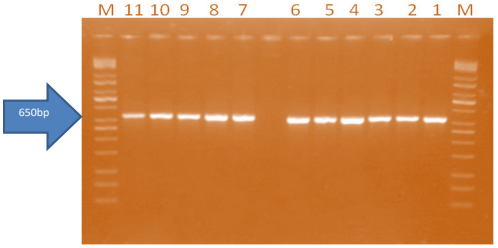

Fig. 6: Gel profile showing ITS 1 and ITS 4 amplification of Mushroom isolates (1 - 11) run alongside Kapa universal DNA ladder (M) i.e., left; Strain EM-1 and right; Strain P-31 respectively.

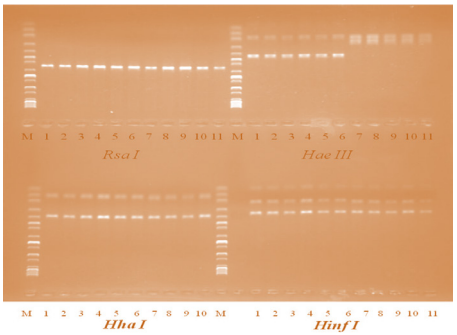

Fig. 7: Gel profile showing restriction patterns generated by four restriction enzymes; Hha I (**C**), Hinf I (**D**), Rsa I (**A**) and Hae III (**B**) on the samples.

It can be concluded that the two *Pleurotus* species are different genetically presumably leading the varying to physiological capabilities of bioconversion of same substrate as observed in previous studies by (Wiafe-Kwagyan, 2014; Wiafe-Kwagyan *et al*., 2016).

Conclusion

This is the first record of separation of Ghanaian *Pleurotus* species on molecular basis. It was clear from a previous preliminary study (Wiafe-Kwagyan, 2014; Wiafe-Kwagyan *et al*., 2016) that two distinct species of *Pleurotus* (*P. eous* and *P. ostreatus*) on the market used in these investigations on varying substrates have differential bioconversion abilities. It remains to show, using DNA gene mapping, dendrograms and enzyme production/induction why *P. eous* was a superior utilizer of substrate to produce nutrients from lignocellulose rice waste.

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