MORPHOLOGICAL AND RESTRICTION ANALYSIS OF THREE SPECIES OF *Agaricus* foung GROWING IN NORTHERN GUINEA SAVANNA OF NIGERIA

Supported by tetfund TERTIARY EDUCATION TRUST FUND

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Received: January 18, 2019 **Accepted:** March 26, 2019

Abstract: Mushrooms vary according to species, strain, environment and growth conditions. This research highlights the morphological and restriction analysis of three wild *Agaricus* species in Northern, Nigeria. A survey of mushrooms in Zaria, Nigeria was carried out within the months of May through October 2018. During the survey, *Agaricus Silvicola*, *Agaricus alphitochrous* and *Agaricus diminitivus* were encountered and their photographs were taken in their habitats using digital camera. Morphological descriptions and identifications of the three *Agaricus* species were carried out using mushroom field guides. Spore prints were collected and pure mycelia cultures were developed for genotyping. The genomic DNA of the *Agaricus* mushroom was extracted. Restriction digests using Mbo1, Saq 1 and Taq 1 showed different bandings patterns across the *Agaricus* species that revealed variation in the morphological characters of wild *Agaricus* species and this confirmed that the *Agaricus* species are new additions to the Nigerian mushroom biodiversity. Morphological and restriction digest of wild *Agaricus* have showed genetic relatedness that revealed taxonomic characters in the study that placed them in different species. The study may serve as baseline information for further studies on the taxonomy of other genera of mushrooms in Nigeria.

Keywords: *Agaricus*, enzymes, macroscopic, mushrooms, taxonomy

Introduction

Mushrooms belong in the kingdom Fungi (Kenderick, 2000) and they form the largest group of Agaricomycetes, which are also known as Homobasidiomycetes (Hibbert and Thorn, 2001). The largest genus, *Agaricus* L. consists of more than 200-250 species that are mostly mycorrhizal and saprophytic (Piepenbring, 2015).

Mushrooms are widely used for various purposes. Species in the Agaricaceae are widely recognized due to their medicinal and nutritional properties (Avian *et al*., 2012). Nutritional profile of mushrooms include total carbohydrate content ranging from 26 to 82%, largely made of carbon, starches, pentoses, hexoses, disaccharides, amino sugars, sugar alcohols and sugar acids (Adewusi *et al*., 1993). This is in addition to their high fibre content, low lipid levels that is made up of polyunsaturated fatty acids and absence of cholesterol. Others include dyeing of wool and other natural fibers (Peintner *et al*., 2001), medicine (Chen *et al*., 2015), food (Adewusi *et al*., 1993), dietary supplement (Chang, 1999; Musa *et al*., 2015), among others. For example, several species of mushrooms such as *A. campestris* and *A. micromegatuus* are being collected for consumption, while *A. subrufescens* is used for medicinal purposes (Bodensteiner *et al*., 2001). Ancient Egyptians believed that mushrooms like *Agaricus augustus* are plants of immortality (Dijk *et al*., 2003), while in other nations such as the Latin America, China, Russia and Mexico were the Fairy ring mushroom (*Amanita muscaria*) were used for rituals, as they were believed to have properties that could produce super- human strength and also help in finding lost objects (Kuo, 2004). The poisonous species that grow along with the edible *Agaricus* species such as *Amanita phylloides* are used as sources of mycotoxins and produce enzymes such as disulfiram which inhibits aldehyde dehydrogenase (ALDH) (Kuo, 2007). Due to the nutritional importance of mushrooms, identification of wild species is required in order to recognized and separate edible species from toxic ones. The conventional method of mushroom identification is the use of macro- and micro-morphological features (Raper, 1985). However, this method is not accurate and reliable due to phenotypic plasticity and intraspecific variability among the mushrooms, which could arise from mutations, or substrate and growth effects. Barros *et al*. (2007) noted that, standard methods of identification of *Agaricus* species is lacking; while the use of nomenclatural keys developed by Arora (1986) and Kendrick (2000) are limited by varying environmental conditions that affect mushroom growth in different geographical locations, therefore current classification of Agaricales needs to be reviewed (Kuo, 2007). Inaccurate identification of mushrooms has a serious implication on the species diversity of *Agaricus* in the tropical and subtropical regions. Similarly, little is known about the taxonomy of wild species of mushrooms in Nigeria in spite of reported species diversity and habitat diversification (Zoberi, 1973). However, Sanqiao *et al*. (2009) gave insight into the transitional state of mushroom taxonomy for more accurate identification (using molecular methods).

Molecular techniques have shown to be more reliable the identities of wild collection and are helpful in mushroom taxonomy (Tang *et al*., 2005; Meudt and Clarke, 2007). Molecular systematic studies of the Agaricales have radically transformed our interpretations of the evolution and classification of gilled mushrooms and their relatives (Mathany and Bougher, 2006; Thomas and Isabella, 2012). In Europe and some parts of the United States of America, progress have been made in recent times regarding fungal classification facilitated by molecular characterization and phylogeny (Vos *et al*., 1995; John and Ralph, 2009). In African countries including Nigeria, there is paucity of reports in molecular systematics of fungi. Worse still, most of molecular systematic studies of mushrooms are focused on cultivated species. The study was to determine the morphological and molecular characterization of three wild *Agaricus* species growing in northern savanna of Nigeria.

Materials and Methods

Study area

The research work was carried out in the Department of Biological Sciences and Department of Microbiology of Ahmadu Bello University, Zaria, Nigeria on latitude

11°13''North, Longitude 7°12" East and on Altitude 630 meters above sea level (Kafoi, 2008) and Institute of Biomedical Research (IBR) Laboratory, KIU, Uganda.

Field survey and collection of mushroom samples

A survey was conducted to collect wild mushrooms from four different locations in the northern savanna, Nigeria. The area was demarcated into the sampling sites as shown below:

Location (sampling site):

Area A		($11^0$08'56.58"N; $7^0$39'35.62"E)
	Botanical Garden	($11^0$08'44.60"N; $7^0$39'19.34"E)
	Institute of Agricultural Research	($11^0$09'55.08"N; $7^0$38'02.57"E)
B	ABU Kongo Campus	($11^0$04'59.82"N; $7^0$43'27.69"E)
C	Kufena	($11^0$04'24.98"N; $7^0$40'11.70"E)

Data collection

The method of collection of data was based on participatory method of field study. Field trips were undertaken during rainy season when the fruiting bodies are formed from May to October. Random Collection of wild mushrooms was carried out seven times in a month to all the locations and was identified using the standard descriptions of Arora (1986) and Kendrick (2000). Photographs of encountered mushrooms were taken using digital camera to aid in morphological study for the purpose of identification and naming. A pocket pH meter (Hanna instrument) was used to take the pH of the soil. Plastic baskets were used to convey the mushrooms to the laboratory for further studies (Kuo, 2004).

Preservation of collected mushrooms

Collected mushrooms were preserved in Formalin Acetic Acid (3:5:7) to 85 cm^3 of distilled water inside clean specimen bottles as soon as they were brought from the field (Piepenbring, 2015).

Morphological studies

The morphometric of the collected mushrooms were critically observed using table lens in the laboratory according to the methods of Kendrick (2000) and Kuo (2007).

Macroscopic studies

Characteristic features of stipe such as stipe length, colour, texture, density and configuration were recorded. Characteristic features of Pileus such as size in diameter, colour, texture, shape and edge (margin) were examined and recorded. Pileus and stipe were bruised to record any changes of colour (Smith, 1973; Pegler and Spooner, 1997). Annulus and veil was observed to note their presence or absences and were recorded. Lamellae were also observed to note their colour, attachment to pileus, texture, and edge using magnifying lens.

Microscopic studies

Spore prints from the collected mushrooms were obtained using the methods of Musa *et al*. (2018). Colours of the spore print were observed and recorded. Slides were prepared from collected spores and were mounted in Meltzer's reagent. They characters were observed and recorded using the method of Kuo (2007). Shapes of basidia, cystidia, arrangement and number of basidiospores on basidia were recorded (Ahmad and Gucel *et al.*, 2009). After recording the morphological features of the collected mushrooms, they were compared with documented species using the keys of Kendrick (2000) to ascertain their identity.

Molecular studies of Agaricus species

Production of active mycelia spawn of Agaricus species

Mycelia cultures of the wild *Agaricus* mushroom species were prepared using the spores obtained from the spore prints for genotyping (Kadiri *et al*., 2009). The basidiospores were inoculated onto PDA medium (15 g/L pollard extract, 2 g/L peptone, 2 g/L yeast, 20 g/L glucose, 1g/L KH2PO4, 0.5 g/LMgSO4 and 2.1% agar) and incubated at 25°C for a week in order to prepare the culture for molecular analyses overseas. Strains were inoculated in a liquid medium (glucose 40 g, peptone 10 g, NaCl 2.5 g KH_2PO4 1 g, MgSO4 0.5 g, bringing the total volume to 1000 mL with distilled water) and incubated at 26°C for 3-5 days. The mycelium was dried with filter paper and was stored at -20°C for molecular analysis. This was carried out at the institute for Biomedical Research of the Kampala Internationl University, Westerrn Campus Ishiaka, Bushenyi, Uganda.

DNA extraction and restriction with Mbo1, Sac1 and Taq1

DNA from pure cultures of the mushroom samples was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research Corp.) according to the manufacturer's instruction. Preparation of mycelium for DNA extraction was done using the methods of Sanqiao *et al*. (2009). DNA extraction was done using the modified CTAB (Zhou *et al*., 2011) protocol. DNA concentrations was estimated using the spectrophotometer and was standardized against a known concentration of DNA on 1.5% (w/v) agarose gels. 10.0 µl of purified DNA was added to a mix of 7.5 µl water, 2.5 µl restriction buffers and 5.0 µl restriction enzyme. The digestion was carried out at 37°C for 3 h and the digest terminated at 65°C for 15 minutes. The enzymes used were *Mbo I* (5' ...^GATC ... 3') and *Taq I* (5' ...T^CGA ... 3'). The fragments were separated by gel electrophoresis on a 2% agarose gel run at 100 volts for 1 h.

Results and Discussion

Agaricus silvicola or (or sylvicola; Woodward Agaricus)

Habitat: Solitary, scattered or in small groups under trees; widely and commonly distributed, often called Woodward *Agaricus* because it was commonly found growing under the trees in the wild but rarely fruiting in large numbers and fruiting in months of July to August (Plate Ia).

Soil PH: 6.10

Cap: Broad, convex becoming plane. Dry surface, smooth or silky-fibrillose, may be fibrillose-scaly with age; pileus is white and becomes yellowish with age, especially at the center, or staining at least slightly yellow when bruised, particularly at margins. Flesh is firm, white, unchanging or slightly yellowing when crushed and has a sweet mushroom odor when young, 5.0-12.0 cm diameter (Plate Ia). It has Convergent interwoven lamellae trama (Plate Ib). Cap staining yellow in KOH.

Gills: Lamellae close, free at maturity, white becoming grey or pinkish-grey, then brown and finally chocolate-brown or darker (Plate Ia)

Stalk (Stipe) stem: usually enlarged below, hollow or stuffed, smooth or with small cottony scales, below the ring; white or pinkish at apex, white below, but often aging or bruising yellowish; base not staining bright yellow when cut. 5.0-14.0 cm long (Plate Ia)

Veil: Membranous, white or stained yellow, with patches on underside that sometimes forms a cogwheel pattern, forming a prominent, superior, skirt-like ring on stalk (Plate Ia)

Spore print: There was no spore print of this collection because it was in an over-matured stage and probably the

spores have already been released before collections were done. Spores 5.0-6.5×3.5-4.5 µ, elliptical and smooth (Plate Ic-d)

Agaricus alphitochrous (Berk and Broome)

Habitat: Solitary or in groups, troops on lawns, along paths, on ground, grassy sandy soils under trees (shade). They are widely distributed. Fruiting in early months of rains from June, July to August (Plate IIa)

Soil PH: 6.80

Cap: Pileus is broad, convex to plane; surface dry; fibrillose scales that are minute; umbonate, vicacious with pink-colored to pale salmon colored to purplish-pink to reddish-brown fibrils at the center; margin often pale. Flesh is thin, very firm when young but soft in age bruising yellowish with a filamentous cuticle; odor mushroomy. 2.0-5.0 cm diameter (Plate IIa and c). Cap staining yellow in KOH.

Gills: Close, free at maturity; grayish to pinkish when young; to chocolate-brown or darker at maturity with interwoven lamellae trama (Plate IIa and b)

Stalk (Stipe) stem: The stipe stains yellow to orange when handling, hollow and fragile at maturity. Equal or slightly enlarged at base, 2.0-6.0 cm long (Plate IIa)

Veil: Membranous; thin, forming a fragile, superior to median ring on stalk, or disappearing entirely; ring skirt-like or intermediate (Plate IIa).

Spore print: No spore print in this collection because it was over matured when collection was done. Spore 4.5-6.0×3.5-4.5 µ, broadly elliptical and smooth (Plate IId-e)

Agaricus diminitivus (Diminutive Agaricus)

Habitat: Solitary, widely scattered or in small groups on field, woods, widely distributed. The fruiting is common during the rains, June to September (Plate IIIa).

Soil PH: 5.80

Cap: Pileus is broad oval or convex, becoming plane or slightly umbonate; surface dry, with flattened pink to purplish-pink to amytyst-gray to reddish-brown fibrils at the centre, margin often pale. Flesh thin, white, not staining, odor faintly fragrant, mild (like anise). 1.0-4.0 cm diameter (Plate IIIa). Cystidia and hymenium were also observed (Plate IIIb). Cap surface staining yellow in KOH.

Gills: Lamellae free at maturity, but close when young; phallid pink becoming reddish-brown, chocolate brown or darker (Plate Iva and c).

Stalk (Stipe) stem: equal or with small basal bulb, white or phallid, but in age, often staining yellow or orange below the ring; hollow or stuffed, fragile. 2.0-7.0 cm (Plate IIIa)

Veil: Membranous, thin, white, forming a fragile, superior to median skirt-like ring or stalk which often disappears in age (Plate IIIa).

Spore print: spore print of *Agaricus diminitivus* was not collected in this study because it was in an over-matured stage and probably the spores have already been released before collections were done. Spores 4.5-6×3.5-4.5 µ broadly elliptical, smooth (Plate III d and e).

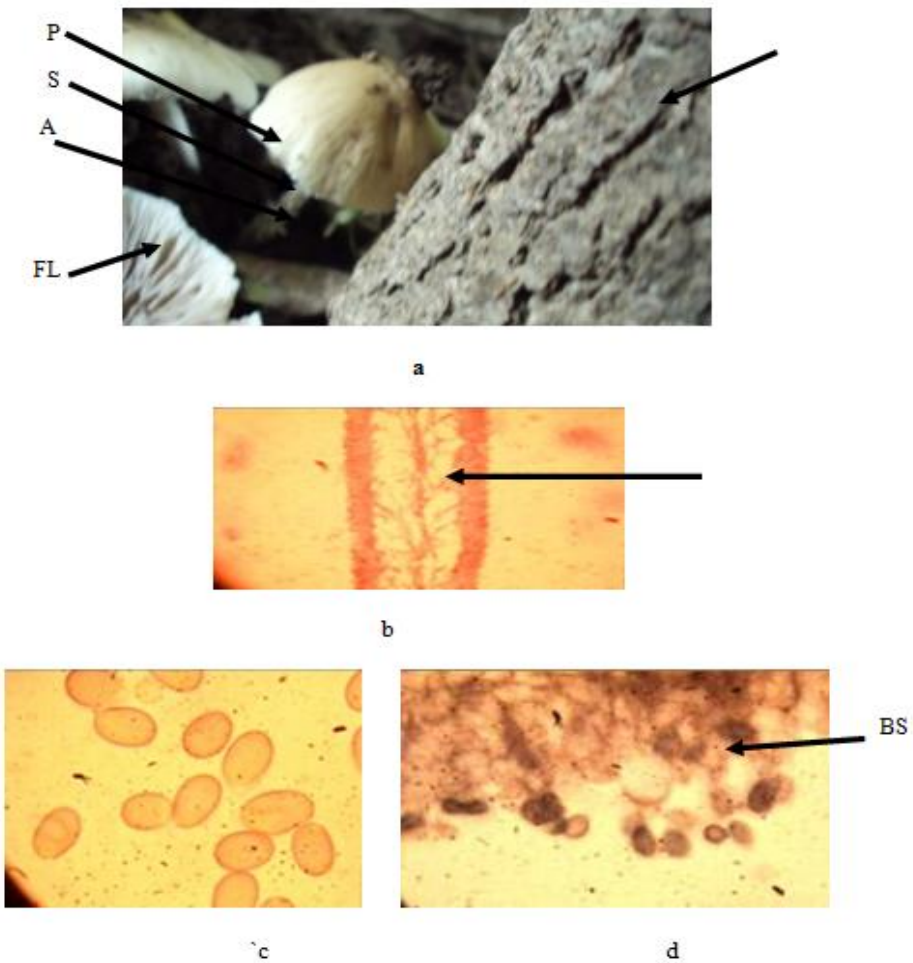

Plate I (a-e): Photomacrograph and Photomicrograph of *Agaricus silvicola* (a- free lamellae (FL); pileus (P), stipe (S), and a ring (A). It is growing on a living tree trunk (T), b- a convergent interwoven lamellae trama (CT) X400, c- basidiospores in Melzer's reagent x1000, d- basidiospores (BS) released from basidia x1000)

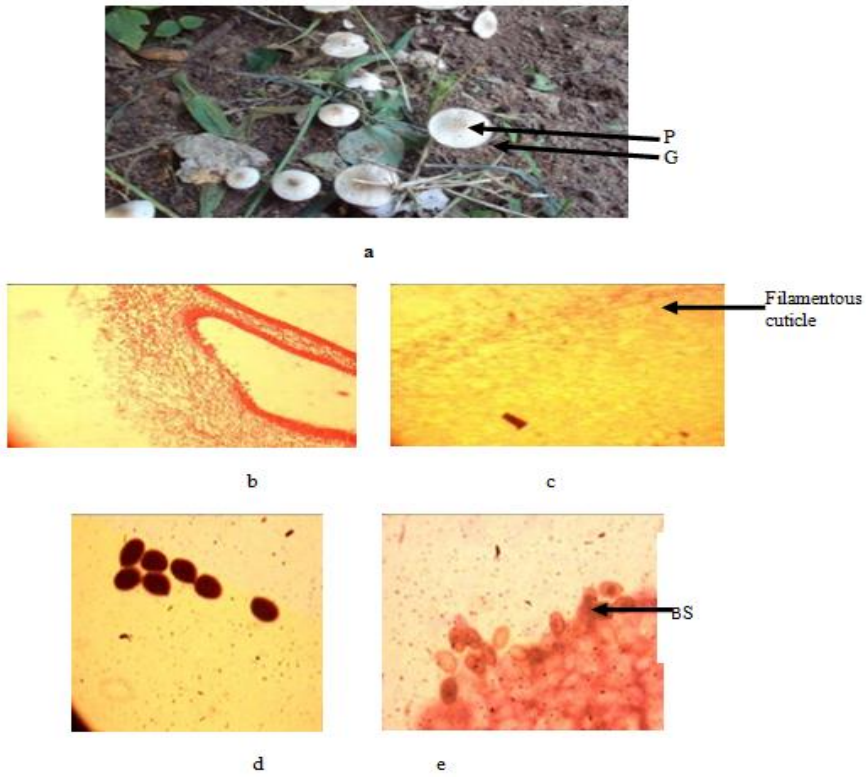

Plate II (a-e): Photomacrograph and Photomicrograph of *Agaricus alphitochrous* (a- umbonated pileus (P) growing on soil with grasses and herbs with gills (G), b- an interwoven lamellae trama x400, c- filamentous pileus cuticle x400, d- basidiospores in Melzer's reagent x1000, e- basidiospores (BS) released from basidia x1000)

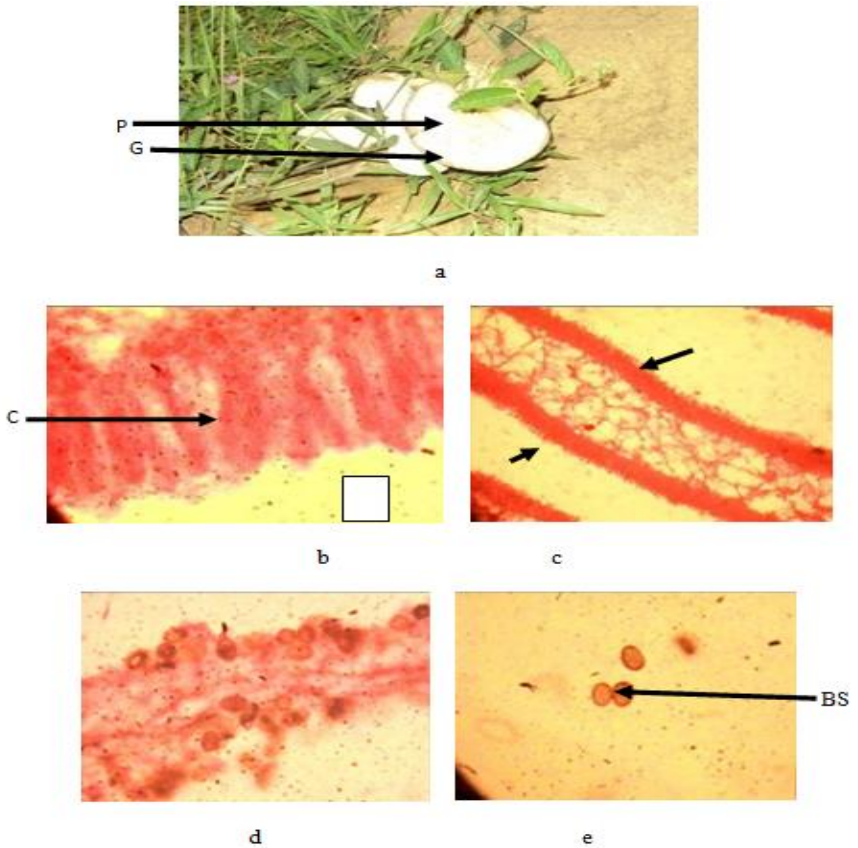

Plate III(a-e): Photomacrograph and Photomicrograph of *Agaricus diminitivus* (a- mbonate pileus with gills under the cap (G) growing on field with grasses and herbs, b- cystidium (C) and hymenium (H) x400, c- convergent interwoven trama x400, d- basidiospores release from basidia x1000, e- smooth oval basidiospores (BS) x1000)

Agaricus silvicola

Macroscopic and microscopic details in this collection conform to those of Arora (1986) and Callac (2005). However, this collection differs by possessing fibrillose scales in age usually becoming yellowish or purplish at the centre of the cap and has a sweet mushroom odour when it was crushed at its young stage. *Agaricus sylvicola* is also known as *Agaricus silvicola* and similar in morphological characters with *Agaricus silvaticus* but did not cluster together in the same clade. This finding agrees with those of Challen *et al*. (2003) who reported that *Agaricus silvicola* differs from *Agaricus silvaticus* by being tall and slender in stature with longer spores but was not found to cluster together in the same clade rather was found to cluster with *Agaricus albolutescens, Agaricus summosasis* and *Agaricus abruptibulbous*. This may be due to competition of growth factors with mycorrhizal fungi growing with the mushrooms as reported by Kerrigan (1986), Parra (2008) and (Kuo, 2007).

Agaricus alphitochorus (Berk and Broome)

Both morphological and phylogenetic identification of *Agaricus alphitochrous* (Berk and Broome) were in agreement with the details provided by Smith (1973) and Karunarathna *et al*. (2014). However, the collection differs in having a tan, fragile, superior to median ring on stalk and disappearing entirely with age. The stipe stains yellow to orange when handling, hollow and fragile at maturity. In addition, the pileus has fibrillose scales that are minute and clustered together in the same group with *Agaricus porphorocephalus* that showed similarities in their genotyping. This work is supported by the reports of Parra (2008) who reported that fibrillose scales are veinacious in nature and possess umbonate vicacious pink-coloured to pale salmon coloured to reddish-brown fibrils at the pileus centre and also has interwoven trama, hymenium and cystidia with few spores broadly elliptical and smooth. This collection was similar to *Agaricus semotus, Agarcus diminitivus* and *Agaricus comptulus* as reported by Kerrigan (2011).

Agaricus diminitivus

This collection was compared with the description given by Arora (1986) and was found to be similar both macroscopically and microscopically. However, the thin, membranous, superior to median ring on stalk which often disappears in age was observed to be similar with the reports of Kendrick (2000) and differs by being short and less broad with reddish-brown fibrils scales at the centre of the pileus. This is in conformity with the findings of Petrova *et al*. (2007) who reported that species of *Agaricus* are genetically similar both macroscopically and microscopically. This collection description by possessing the amethyst- tinged cap resembles those of *Agaricus purpurellus, Agaricus dulcidulus* and *Agaricus amethystina* that were supported by the reports of Callac *et al.* (1993).

The morphological descriptions of the collection in this study are in conformity with those provided by Kendrick (2000). However, it differs in the pileus color that is whitish to clingy-grey with a violet-grey background and its name is called purpled veiled *Agaricus.* The veil does not freely break from the cap until cap has completely expanded forming an apical fragile skirt-like, sterile, striate ring on the stipe. This finding is supported by the reports of Kuo (2004) who reported that pileus and stalk of species of *Agaricus* varies in colors may be due to habitat and substrate influence and the membraneous veil may not breaks freely from the pileus until is matured and almost decayed. Kendrick (2000) and Arora (1986) also reported that this collection resembles *Agaricus silvaticus* and *Agaricus placomyces*.

Molecular studies of wild Agaricus species in Zaria

The product of amplification using restriction digest (DNA RFLPs) from the mycelial cultures of *Agaricus* species, showed an approximately 680 base pairs fragments of the rDNA-ITS as represented in Fig. 1

DNA Extraction and Restriction digest of DNA using Mbo1, Sac1 and Taq 1

The genomic DNA of the wild *Agaricus* species was extracted and restriction analysis using the enzymes (Mbo1 and Taq 1) revealed the restriction patterns of *Agaricus* species (Fig 1). In all the samples, the fragment size of the restricted DNA appears to be 680bp (Fig. 2).

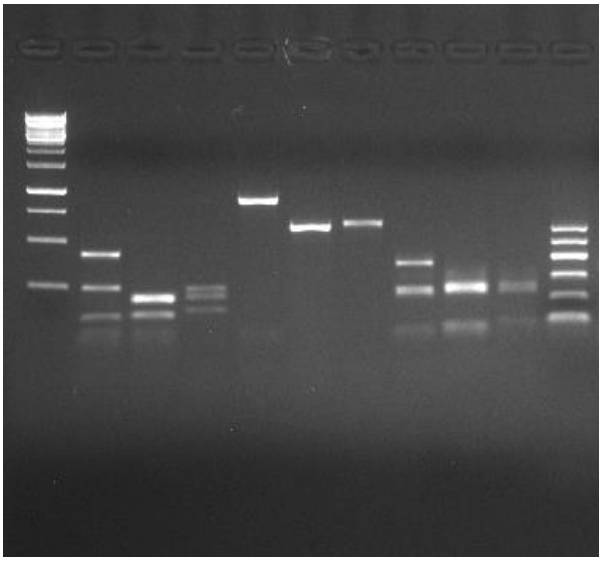

Fig. 1: Restriction of DNA with Mbo1, Sac1 and Taq1

Key: Lane 1:100bp ladder marker; Lane 2-4: Mbo1+DNA *Agaricus* species (*Agaricus silvicola; Agaricus alphitichorus* and *Agaricus dimintivus* (respectively); Lane 5-7: Sac1+DNA *Agaricus* species (*Agaricus silvicola; Agaricus alphitichorus* and *Agaricus diminitius* (respectively); Lane 8-10: Taq1+DNA *Agaricus* species (*Agaricus silvicola; Agaricus alphitichorus* and *Agaricus diminitivus* (respectively); Lane 11: 1kb Ladder

Identification of species of Agaricus using restriction digests

DNA extraction was done using the mycelia cultures collected from the spore prints. The makers used showed polymorphism between and among the *Agaricus* species. The species of *Agaricus* in the study showed the relatedness amongst the species in the genera due to their unique banding patterns. This work was supported by the reports of Williams *et al*. (1991 and Zhao *et al.* (2011) who reported that DNA polymorphisms amplified by primers are useful genetic markers that help in revealing variations between the species of tropical mushrooms. However, using Taq 1 and Sac1 in restriction digest of the same *Agaricus* species it also revealed bands in the digestion that also showed some differences and relatedness in their morphological features. This also indicates that there were morphological variations on *Agaricus* species and DNA polymorphism reveals similarities and differences among the genera of *Agaricus.* This finding was supported by the works of Zabeau and Vos (1993) and Tang *et al*. (2005) who reported that restriction fragment amplification is a selective general method for DNA finger printing that helps in producing similarity in restricted fragments patterns of species of mushrooms.

Conclusion

Agaricus silvicola cap diameter measured 5.0 – 12.0 cm. However, their basidiospores shapes were both smooth and elliptical; cuticles were filamentous, divergent and convergent interwoven trama. Restriction analysis using Mbo1, Sac 1 and Taq 1 showed different banding patterns across the isolates of the species of *Agaricus* and thus revealed some variations in the morphological characters of wild *Agaricus* species in diameter of pileus; length of stalk and size of basidiospores.

The morphometric result obtained in this study may be needed to distinguish the different species for selection of strains of these *Agaricus* species adapted for efficient protoplast mycelia growth. Molecular study of more species in the genera can also be carried out in other parts of Nigeria using the molecular makers to enable us compare and establish the taxonomy of mushrooms in Nigeria.

Conflict of Interest

The authors declare that there is no conflict of interest related to this study.

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