



Molecular characterization of wood ear mushrooms [*Auricularia* sp.] from Kakamega Forest in Western Kenya

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Onyango BO, Mbaluto CM, Mutuku CS, Otieno DO 2016 – Molecular characterization of wood ear mushrooms [*Auricularia* sp.] from Kakamega Forest in Western Kenya. Current Research in Environmental & Applied Mycology 6(1), 51–60, Doi 10.5943/cream/6/1/6

Abstract

Mushrooms of the genus *Auricularia*, generally termed wood ear mushrooms are in high demand in Western Kenya due to their numerous medicinal and nutritional properties. Interventions to characterize and conserve the native wood ear mushrooms are necessary to mitigate possible extinction of this valuable bio-resource. Currently, the species richness and bio-geographical relatedness of the Kenyan native wood ears is not fully elucidated. This study used molecular sequence analysis of the internal transcribed spacer (ITS) and the 28S nuclear ribosomal large subunit (nLSU) genes in species delimitation of six strains of wood ear mushrooms native to Kakamega Forest. Phylogeny of both the ITS and nLSU gene regions showed that three strains clustered with *Auricularia delicata* while the other three strains clustered with *Auricularia polytricha* at bootstrap support values of above 97%. An intragenomic dichotomy appeared to occur in the *Auricularia delicata* strains based on the genetic distance of the nLSU gene sequences. The wood ear mushrooms identified from the Kakamega Forest strains were *Auricularia delicata* and *Auricularia polytricha* and not *Auricularia auricula* as previously reported. This rich biodiversity needs further exploration to widen the nutritional and medicinal base of the rural populace who depend on the mushrooms through conservation, cultivation and commercialization activities.

Key words – Mushrooms – morphology – phylogeny – taxonomy

Introduction

Research on edible fungi in the world has concentrated on a few species that are commercially cultivated (FAO 2015). The consequence of this is that the biology and ecology of some edible species collected from the wild, particularly in developing countries are poorly known. In Western Kenya, mushrooms of the genus *Auricularia*, generally termed wood ear mushrooms are in high demand due to their numerous medicinal and nutritional properties (Palapala 2006). Communities residing around Kakamega Forest indiscriminately harvest the wood ear mushrooms for food and medicine, which threatens to deplete this valuable bio-resource (Onyango et al. 2011). Interventions to characterize and conserve these native mushrooms are necessary to mitigate possible extinction due overharvesting and the rapid destruction of its forest habitat. Taxonomic characterization of the wood ear mushroom will enhance its commercialization potential and provide information for its utilization and management.

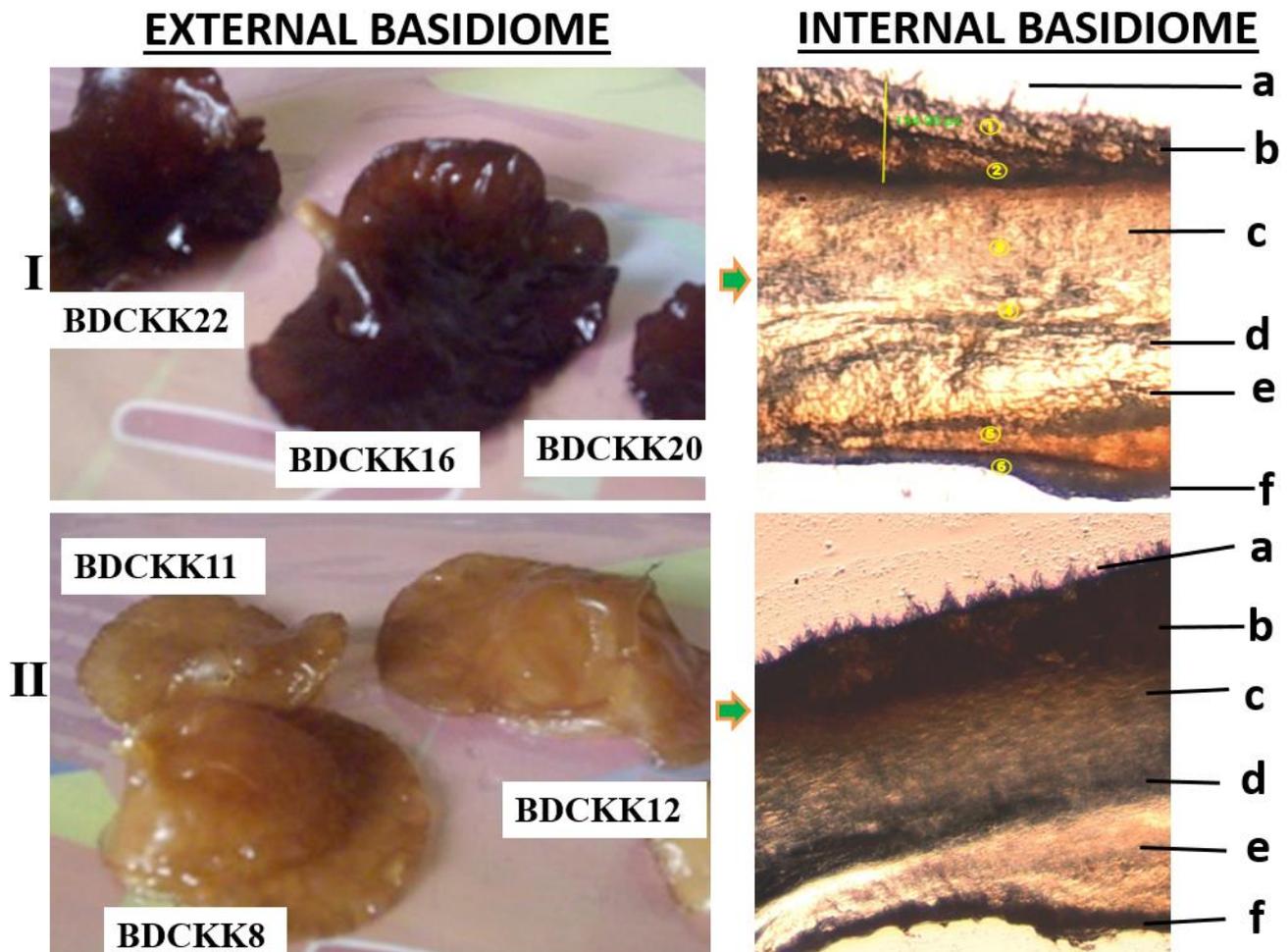


Fig. 1 – External morphology of wood ear basidiomes collected from Kakamega Forest and the cross sections showing hyphal stratification and abhymenial hairs. The scale of the cross sections is 134 μm . Group I – Black strains, Group II – Brown strains. Note the prominent stipe on sample BDCKK16. a. Abhymenial hairs b. *Zona compacta* c. *Zona laxa superioris* d. Medulla e. *Zona laxa inferioris* f. Hymenium

Macro and micro-morphological characterization of the genus *Auricularia* has proven inconclusive in species delineation due to numerous convergent morphologies (Martin et al. 2004, Tang et al. 2010). For instance, macroscopic features of *Auricularia* vary with age of the specimen, exposure to light, availability of moisture and other environmental factors (Bandara et al. 2015). The taxonomic scheme of *Auricularia* has for a long period of time considered Lowy's monograph (Lowy 1951) which emphasised a strongly or weakly differentiated medulla in the internal basidiome structure. In recent times however, a new taxonomic character within the internal basidiome called a schizomedulla led to re-classification of two novel species; *Auricularia subglabra* and *Auricularia scissa* (Looney et al. 2013). Such morphological plasticity and absence of clearly distinguishable features has limited adequate discrimination of the genus *Auricularia* with clear genetic lineages. Onyango et al. (2011) analysed *Auricularia* mushrooms of Kakamega Forest using external and internal features of the basidiome and indicate that *A. auricularia* species are abundant in the Forest. However, a low dissimilarity index of 0.318 was observed indicating the limitation of morphological markers in determining genetic diversity. Thus, the species richness and bio-geographical relatedness of the Kenyan native wood ears is not fully elucidated. A diagnostic diversity study using more accurate molecular markers is therefore necessary.

Table 1 Accession numbers and countries of origin of sequences used for phylogenetic analyses. Sequences newly produced in this study are shown in bold.

Identity of strain	ITS gene	nLSU gene	Country
<i>Auricularia polytricha</i> BDCKK16	KR605640	KR605636	Kenya
<i>Auricularia polytricha</i> BDCKK22	KR605642	KR605638	Kenya
<i>Auricularia polytricha</i> BDCKK20	KR605643	KR605641	Kenya
<i>Auricularia delicata</i> BDCKK8	KR605639	KR605644	Kenya
<i>Auricularia delicata</i> BDCKK11	KR605637	KR605645	Kenya
<i>Auricularia delicata</i> BDCKK12	KR605645	KR605646	Kenya
<i>Auricularia polytricha</i> AP3/ AP910	KF297984	KM267736	China
<i>Auricularia polytricha</i> AP10158 /HN4076	KF297985	KF298021	China/US
<i>Auricularia polytricha</i> M E7069/JMH43	AJ537388	KF298017	Australia/Tanzania
<i>Auricularia delicata</i> HNSD	KF297965	KF298000	China
<i>Auricularia delicata</i> GIM5.424	KF297964	KF297999	China
<i>Auricularia delicata</i> GIM5.177	KF297963	KF297998	China
<i>Auricularia fuscosuccinea</i> TFB3463/PR1496	JX065148	KM396828	US/China
<i>Auricularia fuscosuccinea</i> PR 1378	AF291270	KM396827	China
<i>Auricularia fuscosuccinea</i> TFB11532	JX065150	KF298002	US/China
<i>Auricularia auricula-judae</i> AFTOL-ID 1681	DQ520099	DQ520099	Germany
<i>Auricularia auricula-judae</i> TFB4296/MW 447	JX065174	FJ644518	US/Gemany
<i>Auricularia auricula-judae</i> Dai 13210	KM396769	KM396824	China
<i>Auricularia nigricans</i> E3	KP942816	-	Nigeria
<i>Auricularia nigricans</i> TENN 056825	NR_120153	-	US
<i>Auricularia nigricans</i> TFB11410	JX065176	-	US
<i>Exidia recisa</i>	KF297985	-	Germany
<i>Ramaria rubella</i>	-	JX287493	United States

Molecular characterization of fungal genera provides more reliable taxonomic information; which is not influenced by environmental factors (Bandara et al. 2015). Markers such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Amplified Polymorphism (RFLP) and Sequence Related Amplified Polymorphism (SRAP) have revolutionized fungal taxonomy (Park *et al.*, 2014). Potential barcodes targeted by these markers include the nuclear and ribosomal DNA (rDNA) cistrons. Sequence analysis of the (ITS) gene region of the rDNA has been proposed as the primary barcode of fungi (Schoch et al. 2012). The ITS gene region consists of ITS1-5.8S-ITS2; with the 5.8S rDNA gene being highly conserved and is therefore not very informative at the specie level of the taxonomy of fungi (Sharma *et al.*, 2015). The intervening ITS1 and ITS2 regions show variation between fungal species with different hypervariable domains and high probabilities of successful intra and inter-specific variation (Korabecna et al. 2003). Due to these qualities, the two ITS gene regions can possibly be used to delimit closely related genera by a measure of their genetic distances (Martin et al. 2004).

The 28S nLSU gene has also proven to be a useful marker in species delimitation. In addition to being easy to amplify, sequence and align, the nLSU gene has been shown to improve phylogenetic resolution (Park et al. 2014). The nLSU gene can be used alone or in combination with the ITS region to discriminate fungal species (Vellinga 2004). Therefore, inter-specific variations of ITS and nLSU gene sequences is a crucial step in construction of the phylogeny of native wood ear mushrooms of Kakamega Forest as a prerequisite for their conservation and domestication.

Materials and Methods

Sources of germplasm

Wood ear mushroom basidiomes were obtained from Isecheno forest reserve of Kakamega Forest in Western Kenya between January and April 2013. The forest reserve is located between longitudes of 34°32'0"E, 34°57'0"W and latitudes of 0°7'30"N, 0°10'15"S (Wambua 2004). Sixteen fully mature basidiomes were excised from their fallen and decaying wood habitats using a sharp knife and put in polythene zip-lock bags. Care was taken to ensure the collected samples were at different

stages of growth for proper representation. Diseased parts were avoided to eliminate contamination. Sixteen fruiting bodies were collected and assigned accession numbers then transferred to the laboratory for characterization.

Morphological characterization

Morphological analysis was done to determine differences between the external and internal features of the collected wood ear mushrooms based on Looney's monograph (Looney et al. 2013). External characters such as colour, shape, and stipe presence were evaluated by eye observation. Based on these characters, the samples were classified into two Groups I and II for further characterization. Six samples representative of the two groups with accession numbers BDCKK16, BDCKK22, BDCKK20, BDCKK8, BDCKK11 and BDCKK12 were selected for further morphological and molecular analysis. Thin cross sections of the basidiomes were obtained using sterile scalpel blades and immersed in a diluted solution of methyl blue stain for 10 minutes. The sections were mounted on 5% KOH and viewed using a Nikon Eclipse 80i light microscope. Cross sections were photographed at $\times 4$ magnification for comparative analysis of the internal basidiome structures.

DNA extraction

Total genomic DNA was extracted from dried fruiting bodies of the selected six wood ear mushroom accessions by the Cetyl trimethyl-ammonium bromide (CTAB) method (White et al. 1990) with some modifications. Approximately 5 g was sliced from each fruiting body using a sterile scalpel blade and transferred into 1.2 ml stripe tubes containing a stainless steel grinding balls. Strip tubes were cooled by immersing in liquid nitrogen previously stored at -4°C . Cooled mycelium was subsequently ground into fine powder using Geno grinder-2010 at $1750 \text{ strokes min}^{-1}$ for 5 min. The samples were then centrifuged at 2000 rpm for 1 min and the resultant powder at bottom of strip tubes was collected. Seven hundred micro-litres of CTAB extraction buffer was pre-warmed at 65°C for 1 min then 200 μL of 2% polyvinylpyrrolidone was added to the samples and homogenized by gently inverting the tubes. The samples were incubated in a gently roasting water bath at 65°C for 1 h. The tubes were removed and allowed to cool at room temperature, followed by addition of 700 μL Chloroform Isoamyl Alcohol (24:1). The contents were mixed by gently inverting the tubes 10 times. Centrifugation followed at 4000 rpm for 20 min and the aqueous phase transferred into freshly labelled strip tubes. Thereafter, 600 μL of ice cold 40% isopropanol was added and incubated in a freezer at -20°C overnight. DNA was pelleted by centrifugation at 4000 rpm for 20 min and then Ethano: Sodium acetate (25:1) was added and the mixture incubated at -20°C for 45 min. The supernatant was removed and the DNA washed twice with 500 μL of 70% ethanol. The DNA was left to air dry for about 15 min and re-suspended in 100 μL $\text{TE}_{0.1}$. Two microlitres of RNase solution was added to each tube and incubated at 37°C for 30 min. The concentration and purity of DNA was estimated using a NanodropTM Lite Spectrophotometer (Thermo-Scientific Inc, USA) at 260-280 nm. Horizontal gel electrophoresis (Thistle Scientific Ltd, USA) was done on a 0.8% (w/v) agarose gel at 100 V for 30 min and visualized under UV after staining with 2 μL of GelRedTM (Thermo-Scientific, USA).

Molecular characterization

Polymerase chain reaction (PCR) amplification of the ITS region and 28S rDNA gene was performed in a programmable Mastercycler thermocycler (C1000-BioRad, USA) using the PCR conditions described by Vellinga *et al.*, (2003). The primers used for the ITS region were adopted from White et al. (1990) and included; forward primer ITS5 and the reverse primer ITS4. The primers used to amplify the nLSU region were derived from Gardes & Bruns (1993) and included; forward primer LROR which aligns in the domain 26-42 and the reverse primer LR16 which aligns in the domain 1081-1065. The amplified products were separated by horizontal gel electrophoresis on 1.5 % (w/v) agarose gel on 0.5X TBE at 70V for 60 min and visualized under UV after staining with 2 μL GelRedTM (Thermo Scientific). The PCR amplicons were purified using a Thermo Scientific[®] GeneJET Purification Kit (EU, Lithuania) according to the manufacturer's specifications.

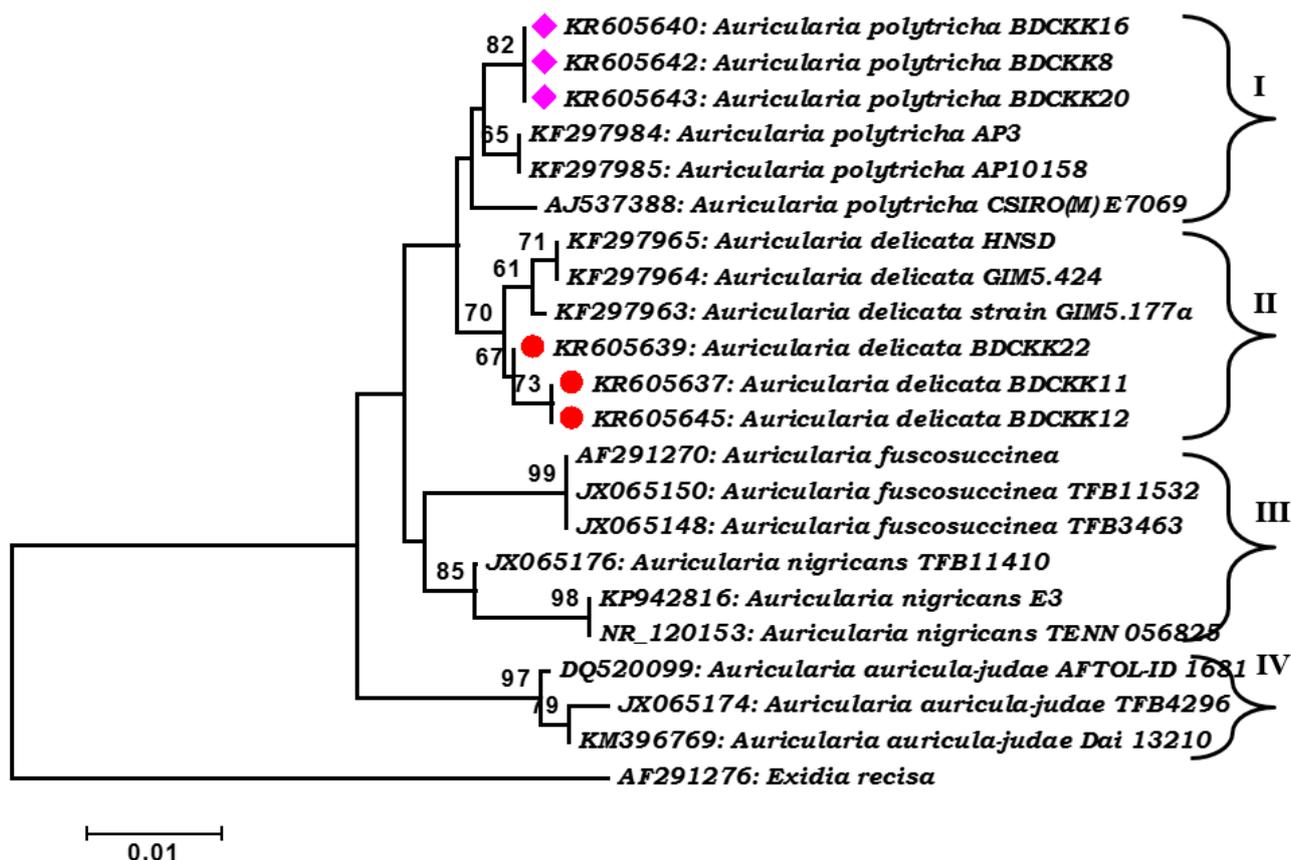


Fig. 2 – A Neighbour-Joining phylogenetic tree showing evolutionary relationships of the ITS gene sequences. The strains from this study are shown in different colours. The tree was constructed based on evolutionary distances computed using the Maximum-Composite Likelihood Method. The optimal tree with the sum of branch length = 0.10599626 is shown. The tree is drawn to scale and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (if >50%).

Sequencing and data analysis

The purified PCR products were pipetted into 10 µl Eppendorf tubes and submitted to the Segolip Sequencing Unit, BecA-ILRI Hub, for capillary sequencing on a 3730xl DNA Analyzer (Thermo Fisher Scientific Inc. USA). Forward and reverse sequences were assembled and trimmed on CLC Main Workbench (CLC Bio Version 6.8.3). Assembled sequences were transferred to MEGA Version 6.0 and aligned using CLUSTAL W according to Tamura et al. (2011). Individual consensus sequences of the ITS and nLSU gene regions were used to evaluate closely related sequences at the NCBI GenBank (www.ncbi.nlm.nih.gov) using Basic Local Alignment Tool (BLAST Query).

Nine sequences that recorded maximum identity hits of greater than 97% similarity to both the ITS and nLSU gene regions on the BLAST query were retrieved and used to supplement construction of phylogenetic trees on MEGA6.0. Three sequences of *Auricularia auricula-judae* for both the ITS and nLSU gene regions were also retrieved and used in the comparative phylogenetic analysis. Evolutionary histories were inferred using the Neighbour-Joining method and distances computed using the Maximum Composite Likelihood (Tamura & Kumar, 2004). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 1000 replicates were shown next to the branches if the values were greater than 50%. The outgroup used for the ITS and nLSU sequences phylogenetic analysis included *Exidia recisa* (Ditmar) Fr. and *Ramaria ruballa* (Schaeff.) R. H. Petersen, which are closely related species but outside the in-group of the *Auriculariales* (Weiß & Oberwinkler 2001).

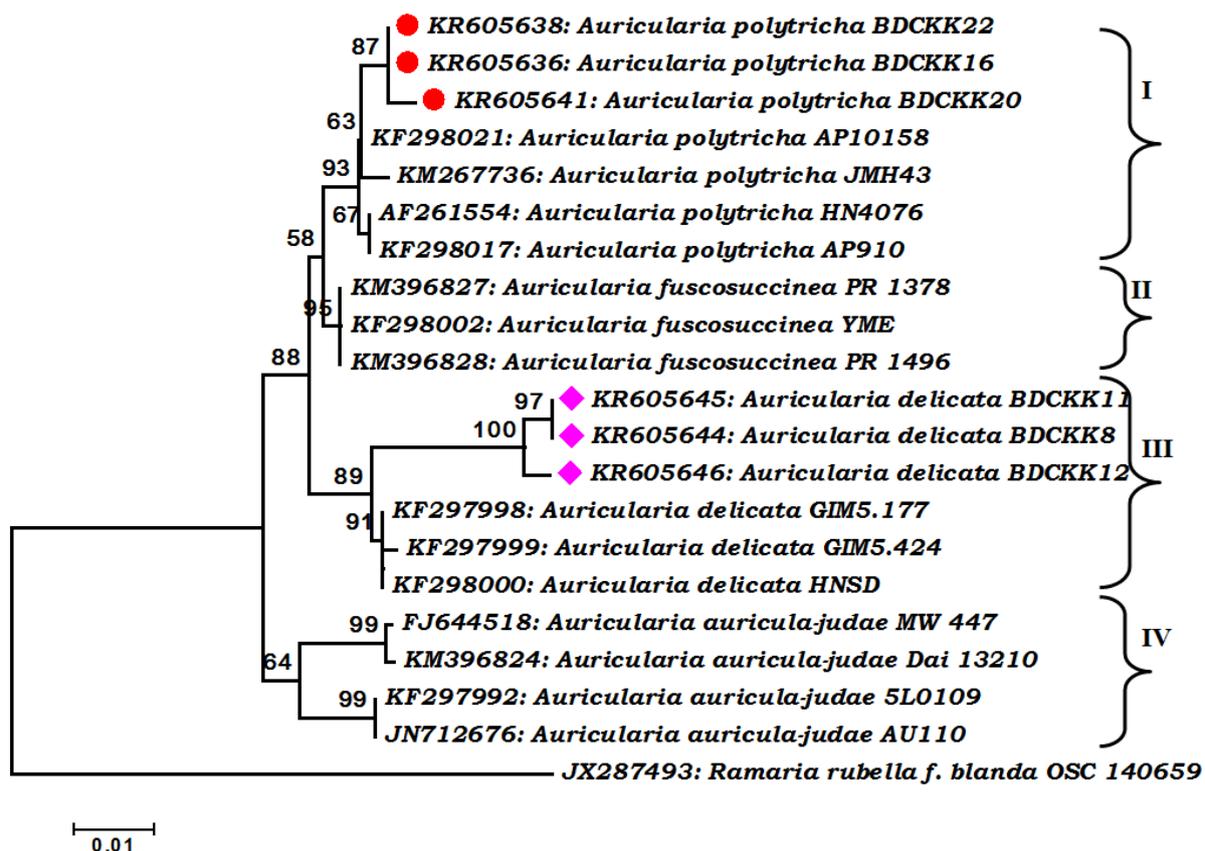


Fig. 3 – A neighbour-joining phylogenetic tree showing evolutionary relationship of the nLSU gene. The strains from this study are shown in different colours. The tree was constructed based on evolutionary distances computed using the Maximum-Composite Likelihood Method. The optimal tree with the sum of branch length = 0.18969980 is shown. The tree is drawn to scale and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (if >50%).

Results

Morphological identification

Morphological features of the wood ear mushrooms and the distinction of their internal basidiome structures are shown on Plate 1. Two groups I and II were identified with strains BDCKK16, BDCKK22 and BDCKK20 occurring in Group I. All Group I strains had long visible stipes and were black in colour. Strains BDCKK8, BDCKK11 and BDCKK12 were classified into Group II and were brown in colour with much shorter stipes. Variation within the internal basidiome structure was such that Group I strains had an average internal thickness of 0.8-1.2 mm. Other features of Group I strains were; abundant and gregarious abhymenial hairs – bearing separately visible individual hairs (5-10 μm), a thick *zona compacta* layer (21-40 μm) with an identifiable *zona subcompacta superioris/inferioris*, a large oblong *zona laxa superioris* (60-192 μm), a tiny medulla (18-52 μm), a narrower *zona laxa inferioris* (42-120 μm) and a hymenium. Internal features of Group II strains were similar to those of Group I except in the nature of the abhymenial hairs and the size of the medulla. The medulla (32-84 μm) was clearly visible and was surrounded by a larger *zona laxa superioris* (72-201 μm) and a much narrower *zona laxa inferioris* (15-35 μm). The abhymenial hairs were dense indistinguishable tufts of about 0.8 – 2.5 μm in length. The *zona compacta* was quite thin (15-27 μm) and it was not differentiated into a *zona subcompacta superioris* and a *zona subcompacta inferioris*.

Molecular analysis

The strain identities, accession numbers and country of origin of the GenBank ITS and nLSU gene sequences of *Auricularia* mushroom species which were closely related to the six strains from Kakamega Forest are listed on Table 1. Previous reports on morphological characterization indicated that the wood ear mushroom species *Auricularia auricula* were abundant in Kakamega Forest. Based on this report, three sequences of the ITS and the nLSU genes of *Auricularia auricula* obtained from the GenBank were included in the analysis and are displayed on Table 1.

Phylogenetic analysis of the ITS gene region

The ITS gene amplification resulted in 670 base pairs. The Maximum Composite Likelihood phylogenetic analysis was inferred by the Neighbour Joining tree shown on Fig. 2. Four distinct Clades I, II, III and IV were observed. The six samples from this study clustered within Clades I and II with bootstrap support values of 65% for *Auricularia polytricha* and 70% for *Auricularia delicata*. None of the Kakamega Forest wood ears clustered in Clades III and IV with GenBank sequences identified as *Auricularia auricula*, *Auricularia nigricans* and *Auricularia fuscusuccinea* indicating absence of these species amongst the samples analysed. Strains BDCKK16, BDCKK22 and BDCKK20 were monophyletic within Clade I clustering with *Auricularia polytricha* strains AP3, AP10158 and CSIRO(M) E7069 from the GenBank at bootstrap support values of 58%. Clade II consisted of strains BDCKK8, BDCKK11 and BDCKK12 which clustered with GenBank sequences identified as *Auricularia delicata* strains GIM5.424, GIM5.177a and HNSD, although the two groups were polyphyletic.

Phylogenetic analysis of the nLSU gene region

The Neighbour Joining nLSU gene phylogenetic tree based on Maximum Composite Likelihood analysis is shown in Fig. 3. A topology similar to the ITS gene phylogenetic tree was observed with four distinct clades I, II, III, IV. Strains BDCKK22, BDCKK16 and BDCKK20 were monophyletic within Clade I clustering with *Auricularia polytricha* strains AP10158, JMH43 and HN4076 at bootstrap support values of 93%. Clade II had GenBank sequences of species of *Auricularia fuscusuccinea* which had no representation within the samples from this study. Strains BDCKK8, BDCKK11 and BDCKK12 were identified as *Auricularia delicata* strains within Clade III clustering with GenBank sequences strains GIM5.177, GIM5.424 and HNSD. However, strains BDCKK8, BDCKK11 within Clade III appeared to be distantly related suggesting a separate delineation. Clade IV consisted of *Auricularia auricula-judae* strains which were not represented within the samples obtained from Kakamega Forest.

Discussion

Domestication of edible mushrooms collected from the wild requires adequate taxonomic description. Proper taxonomic identification will enhance the management and commercialization of indigenous mushroom species and reduce the impact of excessive harvesting from their wild habitats. Accurate identification of wild edible mushrooms has the extension advantage of labelling of strains with correct designations, as they are introduced into new regions (Tang et al. 2010). Incorrect designation of strains may negatively impact on mushroom breeding programmes and interfere with intellectual property rights protection. Taxonomic identification is therefore an important step towards efficient utilization of mushroom germplasm collected from the wild.

Taxonomic studies of the genus *Auricularia* has been challenged by the absence of reliable morphological characters that represent a clear genetic diversity amongst its species (Looney et al. 2013). In this study, Groups I and II strains were initially categorized based on external basidiome colours, presence or absence of a stalk and the shape (Fig. 1). However this variation was not sufficient in distinguishing between the two groups as was observed by Bandara et al. (2015) in a study of a novel *Auricularia* sp. from Thailand that these traits overlap among different species. Similarly, the wood ear mushrooms of Kakamega Forest could not be adequately distinguished using internal basidiome structures. Both groups had a medulla region surrounded by two zones of loosely arranged

hyphae known as *Zona laxa superioris* and *Zona laxa inferioris*. The medulla was however more prominent on Group II strains and smaller on Group I strains. The medulla is an important taxonomic variant in the *Auricularia* mushrooms and was used by Lowy (1952) to delimit the first ten species worldwide. Confounding observations in the presence/absence/size of the medulla in the *Auricularia* sp. have since been reported by Kirk et al. (2008) and Looney et al. (2013) which concur with the findings of this study. It was also evident that the nature of abhymenial hairs differed between Groups I and II strains, although Lowy et al. (1951) indicated that the hairs are not a sufficient criterion to allow a diagnosis of a species. These reports highlight the need for more morphological markers for identification of the genus *Auricularia*, or its combination with molecular markers. The DNA based molecular markers provide more accurate results in the speciation and cryptic diversity within the Basidiomycota (Miller & Buyck 2002).

Molecular taxonomy and phylogeny potentially resolves the ambiguity generated from morphological plasticity. In this study, the overall topologies of the phylogenetic tree inferred by both the ITS (Fig. 2) and nLSU genes (Fig. 3) using Neighbour-Joining Maximum Composite Likelihood were similar in resolving the six strains from Kakamega Forest. Strains BDCKK8, BDCKK11 and BDCKK12 were identified as *Auricularia polytricha* while strains BDCKK22, BDCKK16 and BDCKK20 were identified as *Auricularia delicata* at above 97% similarity values by BLAST analysis. Both the ITS and nLSU gene sequence analysis revealed very similar results in barcoding the Kakamega Forest strains. The minor differences observed in clade delineation of some strains correlate well with the current species concepts (Schindell & Miller 2005).

Analysis of the ITS and nLSU gene sequences revealed a wide evolutionary distance between *Auricularia delicata* strains from Kakamega Forest and those already deposited in the GenBank, signalling increased speciation of this group (Miller & Buyck 2002). The GenBank samples closely related to the Kakamega Forest strains had solely been described in China, and had a long evolutionary distance between them (Fig. 2 and Fig. 3). Although the very first described *Auricularia delicata* species were from Africa (Bresadola et al. 1887), it was surprising that none of the strains from this study clustered with any of the African strains in the GenBank. These observations on the phylogeny of *Auricularia delicata* support two major conclusions. Firstly, the wide variation could be attributed to the possible occurrence of a novel group of *Auricularia delicata* amongst the Kakamega Forest strains that is yet to be described. Alternately, it may be due to the large number of *Auricularia delicata* sequences from China already deposited in the GenBank, which resulted in higher similarity indices with the Kakamega Forest strains than with sequences from other parts of the world.

Strains identified as *Auricularia polytricha* showed a broad geographic variation with GenBank sequences based on both the ITS and nLSU genes (Chen et al. 2001). Although Looney et al. (2013) reported that *Auricularia polytricha* is a synonym of *Auricularia nigricans*, the strains from this study appeared to form a separate dichotomy based on the ITS tree. The Kakamega Forest strains had maximum similarity indices with representative samples from China, Germany, United States and Tanzania (Table 1). This wide distribution of the *Auricularia polytricha* clade indicates its better adaptability to diverse ecological regions compared to the *Auricularia delicata* strains which had a lower distribution. For instance, the Tanzanian strain JMH43 obtained from Kizimzumwi Forest near Kisarawe (Hussein et al. 2014) had 100% similarity index with strain BDCKK11 from Kakamega Forest. Kizimzumwi Forest is within the same geographic latitude as Kakamega Forest and has identical climatic conditions. At the same time, the indigenous tree species within the two regions are similar, yet *Auricularia* species are known to be highly selective to the host trees, which possibly explain the close identity of Kenyan and Tanzanian species. It is however necessary to further elucidate the evolutionary distribution of *Auricularia polytricha* strains closely related to the Kakamega Forest strains.

Conclusions

The first detailed study of the taxonomy of *Auricularia* mushrooms from Kakamega Forest used morphological markers and resulted in a dendrogram of three clusters, suggesting three distinct species. Molecular analysis using the ITS and nLSU genes has however shown that there are two

species; *Auricularia delicata* and *Auricularia polytricha* mushrooms in the forest. The ability of molecular markers to clearly delineate these species may also be extended to evaluate genetic variability within species with the possibility of identification of novel strains. An intra-genomic dichotomy appeared to occur in the *Auricularia delicata* strains based on the genetic distance of the nLSU gene sequences. Further exploration of the biodiversity of Kenyan wood ear mushrooms is necessary to examine their distribution and to identify type collections for herbarium preservation at the Kenya National Museums. This will increase scientific information on the existing wood ear species and also widen the nutritional and medicinal base of the rural populace who depend on the mushrooms, through conservation, cultivation and commercialization activities.

Acknowledgement

The authors are grateful to the Australian Government (AusAID) through a BecA-ILRI Hub-CSIRO initiative on Food and Nutrition Security for funding the wet lab processing of molecular data. The funding was awarded to Daniel Ojwang and Crispus Mbaluto through the African Biosciences Challenge Fund (ABCF) scholarship. Prof. Looney BP. from the Ecology and Evolutionary Biology Department in the University of Tennessee provided invaluable expertise in determining the authenticity of the *Auricularia delicata* strains from this study.

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