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MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF WILD MUSHROOMS IN KHARTOUM NORTH, SUDAN

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ABSTRACT

In a study of the diversity of wild mushrooms in Sudan, fifty-six samples were collected from various locations in Sharq Elneel and Shambat areas of Khartoum North. Based on ten morphological characteristics, the samples were assigned to fifteen groups, each representing a distinct species. Eleven groups were identified to species level, while the remaining four could not, and it is suggested that they are Agaricales *sensu* Lato. The most predominant species was *Chlorophylum molybdites* (15 samples). The identified species belonged to three orders: Agaricales, Phallales and Polyporales. Agaricales was represented by four families (Psathyrellaceae, Lepiotaceae, Podaxaceae and Amanitaceae), but Phallales and Polyporales were represented by only one family each (Phallaceae and Hymenochaetaceae, respectively), each of which included a single species. The genetic diversity of the samples was studied by the RAPD-PCR technique, using six random 10-nucleotide primers. Three of the primers (OPL3, OPL8 and OPQ1) worked on fifty-two of the fifty-six samples and gave a total of 140 bands. The three primers showed 100% polymorphism among samples, regardless of their groups, while the groups showed polymorphisms ranging from 0% to 100% among their samples with the three primers. A dendrogram was constructed using the Jaccard's similarity coefficient. The genetic similarities between samples ranged from 10% to 100%. Cluster analysis of the samples revealed distinct groupings. Both morphological characteristics and RAPD were useful in studying mushroom diversity, with some agreement between them. Accordingly, we believe that a hybrid system embodying both approaches can yield a more satisfactory identification of mushroom samples.

Keywords: wild mushrooms, RAPD, mushroom identification, Khartoum North, Sudan

INTRODUCTION

Fungi play an important role in the changes that take place around us because of their ubiquity and their astonishingly large numbers[1]. They fulfil many roles beneficial to humans[2]. However, fungi have been relatively poorly collected and studied from most countries, regions and habitats. This is at least in comparison to plants and larger animals that are considerably easier to collect and identify[3]. One of the large groups of fungi are the Basidiomycota with over 30 000 species. They include many familiar mushrooms and toadstools, bracket fungi, puffballs, earth balls, earth stars, stinkhorns, false truffles, jelly fungi, smuts, rusts and some less familiar forms[4]. In modern times, the cultivation and annual production of mushrooms has steadily increased[5]. The world has witnessed dramatic acceleration in total worldwide cultivated mushroom production and mushroom scientists are making a great effort to bring wild species under domestication to increase production[6], because growing awareness in the past two decades of the nutritional merits of mushrooms has increased consumption in an era in which people have become more concerned about human nutrition[5].

Sudan's ecological diversity is reflected in the richness of biodiversity, as seen in mushrooms. Nevertheless, very few studies were conducted on mushroom biology, with no studies targeting their diversity. Therefore, more studies to investigate the mushroom flora of the Sudan, particularly from the genetic perspectives, should be welcomed.

Molecular approaches have proven to be potent tools in the classification of complex fungal taxonomic groups, including mushrooms[7], as DNA techniques are quick and reliable to establish the identities of wild collections and are helpful in mushroom taxonomy[8]. However, macroscopic features are still important today for recognizing fungi and making an initial identification. Indeed, the comparison of DNA sequences obtained from fungi is meaningful only if these fungi have previously been characterized and named by conventional methods[4]. Therefore, the objective of this study was to investigate the diversity of wild mushrooms in Khartoum North (Sudan) using both traditional (morphological characteristics) and molecular techniques.

MATERIALS & METHODS

Collection of samples

Mushroom samples were collected from different areas in Khartoum North (Sudan), from February to September 2013. Intact button, medium-sized and mature specimens from each mushroom species were collected to represent different stages of mushroom's development; to ease identification. All field notes and macroscopic characteristics were recorded. Photos of samples to illustrate different characteristics and the growth environment were also taken, and each specimen was given a serial number. The samples were transported to the laboratory and were kept aerated throughout the period of examination.

Samples were cleaned with water and surface-sterilized with 70% ethanol. Small pieces of each fresh specimen were preserved in about 700 µl of CTAB (Cetyl trimethylammonium

bromide) buffer (5% CTAB, 1.2 M NaCl, 100 mM Tris-HCl, 20 mM) in a 15 mL capacity Falcon tube for DNA extraction.

Morphological identification of samples

The following morphological features and characteristics of each sample were used in identification: the overall size of the fruiting body, all characters of the cap (color, shape, size, surface and texture), the stalk (size, color, the presence of the ring, shape of the base, how it is attached to the cap, texture and whether it is hollow or solid) and the change in color when the cap or stalk are pressed. Also the type of spore-producing structure (gills, pores or spines) and its characters (spacing, size both in thickness and breadth, shape and color both before and after maturing of spores), and the presence of remnants of universal and partial veils were considered.

The characters of each collected sample were recorded during and immediately after collection. They were used to identify the samples with the aid of "The Morphing Mushroom Identifier (MMI) of MycoKey Fungus Identifier" (http://www.mycokey.com/newMycoKeySite/MycoKeyIdentQuick.html).
The species suggested by (MMI) were confirmed by referring to either the Mykoweb site (http://www.mykoweb.com/), The field guide to mushrooms of North America (McKnight and McKnight 1987) or to Mushrooms keys (http://www.mushroomexpert.com/).

DNA extraction

DNA extraction from fresh fruiting bodies was carried out using a CTAB-based protocol[9], with slight modifications. A small piece of fruiting body was crushed with about 700 µL of the extraction buffer (5% CTAB, 1.2 M NaCl, 100 mM Tris-HCl, 20 mM EDTA and 0.2 % β-Mercaptoethanol added just before use) using mortar and pestle, and then transferred to a 15 mL capacity Falcon tube. About 1 mL of extraction buffer was added and the mixture was incubated for about 70-90 min at 60-65 °C. Samples were centrifuged for 5 min at 6000 rpm. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant. Each sample was vortexed for a few seconds and was then centrifuged for 10 min at the same speed. This step was repeated two times to obtain a good quality DNA. The upper layer was transferred to a new tube and an equal volume of ice-cold isopropanol was added. Samples were incubated at -20 °C for one to several days. Then samples were centrifuged at 6000 rpm for 20 min, and the supernatant was discarded. The DNA pellet was washed twice with 70% ice-cold ethanol. The DNA pellet was air dried, and was then dissolved in 100 µL TE buffer {10 mM Tris-HCl (pH 8) and 1mM EDTA (pH 8)} and stored at 4 °C.

RAPD-PCR

The Random Amplified Polymorphic DNA (RAPD) technique[10] was followed to perform the molecular diversity of mushroom samples. The iNtRON's Maxime PCR Premix Kit (*i*-Taq, for 20 µL rxn) was used. Six 10-nucleotide random primers synthesized by Vivantis Technologies Sdn. Bhd were used; OPL3 (5'-CCAGCAGCTT-3), OPL8 (5'-AGCAGGTGGA-3), OPQ1 (5'-GGGACGATGG-3), OPL11 (5'-ACGATGAGCC-3), OPI2 (5'-GGAGGAGAGG-3) and OPI5 (5'-TGTTCCACGG-3). The template DNA was used in a wide range of concentration.

The thermal cycler used to conduct the PCR amplification was a Peqlab (Primus 96 advanced) machine, and the program suggested by Gallego and Martínez (1997)[11] to increase the reliability of the RAPD technique was followed.

Gel electrophoresis

PCR products were analyzed by running in a 1.2 % (W/V) agarose gel using 0.5X TBE (Tris-Borate-EDTA) as a running buffer for 1 hour and 15 min, 60 volts and 40.5 amp current. A DNA molecular weight standard (Vivantis VC 100bp Plus DNA ladder) was used. Then gels were visualized and photographed under UV light using a UV transilluminator.

RAPD data analysis

The bands of each primer were recorded. The presence of a band was counted as (1) and the absence as (0). The obtained binary data was used to construct a dendrogram (phylogenetic tree) according to the Jaccard's similarity coefficient[12] using the PAST software[13].

RESULTS & DISCUSSION
Morphological characterization of mushrooms

The morphological identification was done relying on many macroscopic characteristics. Based on ten morphological features and the morphological similarity, the 56 collected mushroom samples were classified into fifteen groups. Each group was found to represent a species (Table 1). Some of the samples are shown in Figure 2. The 56 mushroom samples belonged to three orders: Agaricales, Phallales and Polyporales. The order Agaricales was represented by four families: Psathyrellaceae, Lepiotaceae, Agaricaceae and Amanitaceae, while Phallales and Polyporales were represented by one family each. They were Phallaceae and Hymenochaetaceae, respectively and each of the two families included only one species.

Only eleven out of fifteen morphological groups were identified to species level. The other four groups (2, 3, 4, and 9) could not be identified and all of them appeared to be Agaricales *sensu lato*.

Molecular characterization (RAPD-PCR)

The DNA was analysed using six random primers. Three of the primers (OPI2, OPI5 and OPL11) did not show scoreable bands, while the other three amplified all samples except four (S21, S22, S23 and S24). They gave scoreable and reproducible bands and showed different banding patterns depending on the samples and primer used (Table 2).

A total of 140 DNA fragments were scored, all of which revealed variation among all samples, and the three primers showed 100% polymorphism between samples regardless of their groups. However, the groups showed various polymorphisms among their samples with the three primers. These polymorphisms ranged from 100% to 0% (Table 3).

Table 1: Assignment of the collected samples to groups (most likely spp.) and most likely classification according to morphological features and collection site

Groups	Samples/ group	Sample codes	Site of collection	Most likely species	Family
1	5	(S1,S2,S3,S17,S18)	SN, NG	*Podaxis pistillaris*	*Podaxacease, Agaricaceae*
2	3	(S4, S5, S6)	SN, NG	N.D.	
3	3	(S7, S8, S9)	SN	N.D.	
4	1	(S10)	SN	N.D.	
5	2	(S11, S12)	FA	*Coprinopsis cinerea*	*Psathyrellaceae*
6	4	(S13,S14, S15,S19)	SN, NG	*Lepiota castneidisca*	*Lepiotaceae*
7	1	(S16)	SN, NG	*Parasola auricoma*	*Psathyrellaceae*
8	3	(S21,S22,S23)	SN, NG	*Coprinopsis lagopus*	*Psathyrellaceae*
9	3	(S24,S25,S26)	SN, NG	N.D.	
10	4	(S27,S28,S29,S30)	SN, NG	*Psathyrella corrugis*	*Psathyrellacea*
11	15	(S39, S40, S41, S42, S43, S44, S45, S46, S47, S48, S53, S54, S55,S56, S57)	FA	*Chlorophyllum molybdites*	*Lepiotaceae*
12	4	(S49, S50, S51, S52)	FA	*Itajahya galericulata*	Phallaceae
13	2	(S58, S59)	FA	*Amanita vittadinii*	Amanitacea
14	5	(S60, S61, S62, S 63, S64)	UKF	*Psathyrella candolleana*	*Psathyrellaceae*
15	1	(S65)	SR	*Pseudoinonotus dryadeus*	Hymenochaetaceae (Polyporales)

SN= Sharq Elneel; NG= Noblez group greenhouses; FA= Faculty of Agric., University of Khartoum; UKF= University of Khartoum farm; SR= Staff residents, Faculty of Agric., university of Khartoum. ND= Not determined.

Table 2: Number of total bands and polymorphic bands given by primers OPL3, OPL8 and OPQ1 and polymorphism percentage

Primer	Total number of bands	Number of polymorphic bands	Polymorphism Percentage
OPL3	55	55	100%
OPL8	49	49	100%
OPQ1	36	36	100%

Table 3: Number of bands produced by each primer and polymorphism percentage among species

Groups	**No of samples**	**Primer OPL8**					**Primer OPQ1**					**Groups**				
		T. N	**N.P**	**U**	**P**	**P %**	**T. N**	**N.P**	**U**	**P**	**P %**	**T. N**	**N.P**	**U**	**P**	**P %**
1	5	19	0	10	19	100	22	1	12	21	95.45	20	1	11	19	95
2	3	18	1	11	17	94.44	10	3	4	7	70	10	0	7	10	100
3	3	19	0	12	19	100	15	7	2	8	53.33	16	1	8	15	93.75
4	1	8	-		-	-	5	-	-	-	-	10	-	-	-	-
5	2	9	6	3	3	33.33	10	6	4	4	40	14	4	10	10	71.43
6	4	14	6	0	8	57.14	16	1	7	9	56.25	17	3	7	14	82.35
7	1	3	-	-	-	-	7	-	-	-	-	7	-	-	-	-
8	3	0	0	0	0	-	0	0	0	0	-	0	0	0	0	-
9	2	1	1	-	0	0	6	5	1	1	16.67	7	6	1	1	14.29
10	4	18	2	8	16	88.89	16	1	5	15	93.75	12	2	2	10	83.33
11	15	33	0	8	33	100	12	5	1	7	58.33	10	5	1	5	50
12	4	14	2	7	12	85.71	9	9	0	0	0	5	5	0	0	0
13	2	15	4	11	11	73.33	8	5	3	3	37.5	9	9	0	0	0
14	5	9	6	1	3	33.33	8	8	0	0	0	6	6	0	0	0
15	1	8	-	-	-	-	5	-	-	-	-	6	-	-	-	-

TN= total number of bands; NP= non-polymorphic bands; U= unique bands; P= polymorphic bands; P%= percentage polymorphism

The highest polymorphism was observed with primer OPL3, where three different groups revealed 100% polymorphism (groups 1, 3 and 11) and only one group (group 9) gave 0% polymorphism. The other groups showed polymorphisms with this primer between 94.4 % and 33.3%. Primer OPQ1 also showed 100% polymorphism (group 2). Three groups showed 0% polymorphism (groups 12, 13 and 14). The polymorphism in other groups ranged from 95 % to 14.3 %. The least polymorphism among groups was observed with the primer OPL8. The highest polymorphism observed using this primer was 95.5% among group one samples. Two groups (12 and 14) showed 0% polymorphism. The other groups showed polymorphism ranging from 93.8% to 16.7% (Table 3).

A dendrogram was constructed (Figure 1) and used to compare and study the similarities and genetic relationships between the samples. The genetic similarities between samples ranged from 10% to 100%.

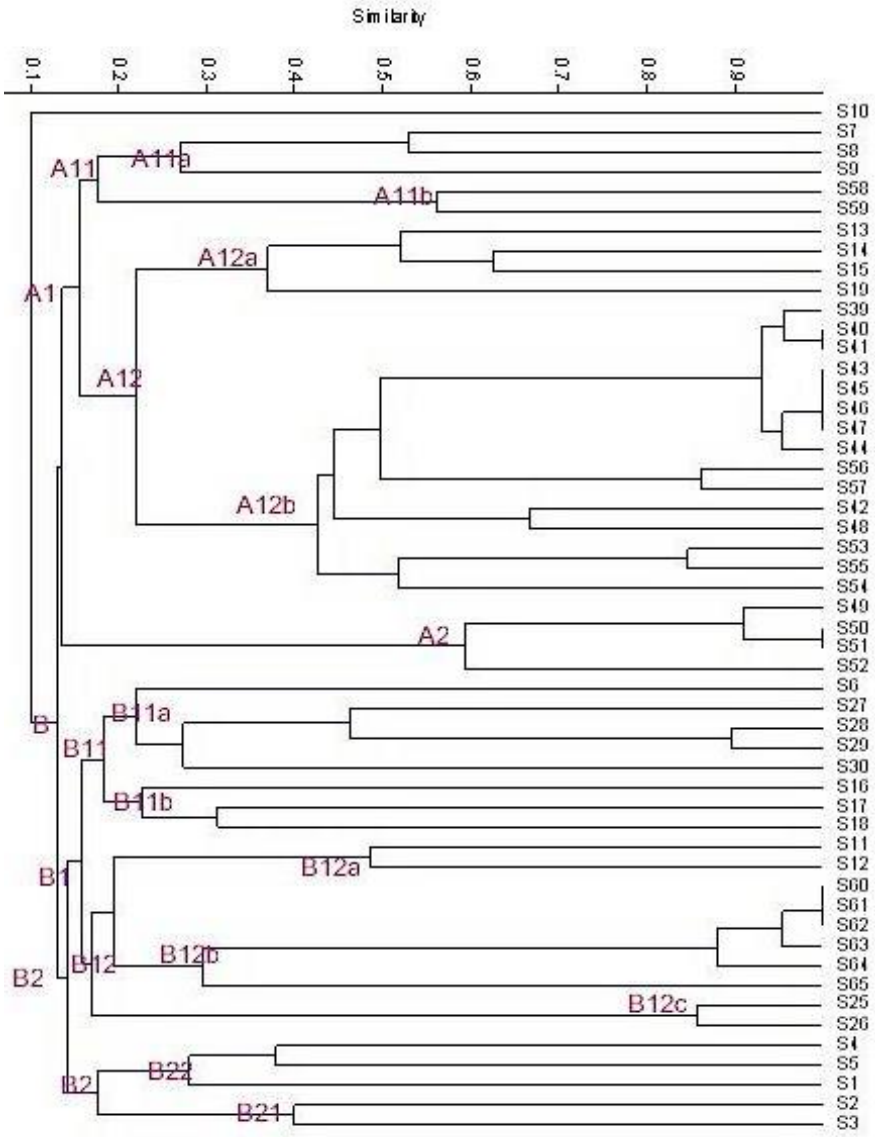

Figure 1: Dendrogram generated according to Jaccard's coefficient depicting genetic relationships among 52 mushroom samples based on three random primers

Cluster analysis of the samples revealed distinct groupings. The dendrogram categorized the fifty-two mushroom samples into two distinct clusters. The first cluster contained only one sample (S10), while all the other fifty-one samples came in the second cluster. The two clusters showed only 10 % similarity to each other.

Figure 2: [1]= group one (samples S1 and S17), A: S1, the whole fruiting body, B: S17, the whole fruiting body;
[2]= group two (samples S4 and S5), the whole fruiting bodies;
[3]= group six (samples S13, S14 and S15). A: S15, the lamella, stalk and the ring, B: S13 and S14, the upper part of the cap, C: S13 and S14, the lamella, stalk and the ring;
[4]= group eight (sample S21), A: the cap (upper part), B: the lamella and the stalk of the mature specimen;
[5]= Group ten (Sample S27 (A and B) and sample S28 (C and D), A: the lamella and the stalk, B: the whole fruiting body, C: the cap, stalk and the lamella, D: the cap with the striate margin;
[6]= Group eleven, samples S46, S47 and S48 growing gregariously in soil;
[7]= Group twelve (samples S49-S52). A: the egg stage, B: the fruiting body after bursting, C and D: The calyptras and gelpa in two different stages;
[8]= Group eleven (sample S55), A: the cap with the brown scales, B: the lamella and the stalk with the ring;
[9]= Group fifteen (sample S65) A: the upper part of the cap, B: the whole fruiting body, C: the pores, D: the lower part of the cap and the base

DISCUSSION

Identification of fungi to species level is a fundamental component of many research efforts in life sciences. This is true in applied as well as in basic research fields[14]. The species of fungi belonging to ecological proximity or different geographical origins can be classified through morphological and molecular markers[15]. However, the morphological traits do not provide a meaningful framework for evolutionary classifications[15]. Sometimes morphologically based groupings did not match molecular/genomic relationships among the species[16].

In the present study, the morphological identification was done relying on many macroscopic characteristics. Since there are no mushroom identification guides available for Sudan or even Africa, the identification was based on general guides and some of the electronic resources. Therefore, it was a bit difficult to identify all samples to species level. Only eleven out of fifteen morphological groups were identified to species level.

Group one (Five samples) was identified as *Podaxis pistillaris. Podaxis* is a gastroid mushroom within the family Agaricaceae and it is commonly known as "False Shaggy Mane" due to its resemblance with *Coprinus. comatus*, and it was initially identified as *Coprinus comatus.* Later it was identified as *Podaxis pistillaris* (L. Pers.) Morse[17]. The five samples have been collected from two different sites and although they have all been identified as *Podaxis pistillaris*, their fruiting bodies were not identical. This may be due to the effect of the environment on the morphological characteristics.

Group six consisted of four samples and it was classified as *Lepiota castneidisca*. *Lepiota* is small to large gill fungi with free (unattached) gills and a thin often fragile cap that separates readily from stalk. Stalk is slender, with a fixed or movable ring. Spore print was white to greyish yellow[18].

Group eleven was the largest group (fifteen samples) and it was classified as *Chlorophyllum molybdites.* The distinctive characteristic was the green spore print and greenish gills. It was growing gregariously in the lawns at the Faculty of Agriculture, University of Khartoum. Growing gregariously is also one of the distinguishing features of *Chlorophyllum molybdites*.

Group twelve (four samples) was classified as stinkhorns and they belonged to the family Phallaceae. The stinkhorns are easily identified due to their fetid smelling, sticky spore masses, or gleba, borne on the end of a stalk called the receptaculum or cap. The characteristic fruiting-body structure, a single, unbranched receptaculum with an externally attached gleba on the upper part, distinguishes the Phallaceae from other families in the Phallales. Morphological characters widely used to separate genera of the family Phallaceae include the presence of a campanula (head) at the apex of the receptacle in *Phallus*, indusium in *Dictyophora*, and calyptra in *Itajahya*[19]. This group of stinkhorns was identified as *Itajahya galericulata*.

Group thirteen was identified as *Amanita vittadinii*. *Amanita* is a large and important genus, and the characteristic features of *Amanita* include a white spore print and the presence of a volva, i.e. the torn remnants of a universal veil. The volva persists as a cup at the base of the stipe and broken volva fragments may also adhere to the cap. Most species also have a ring (annulus) on the stem, the remnant of the partial veil which protects the gills during fruit body development, but there is no ring in some species formerly classified as *Amanitopsis* (eg *A. veginata* and *A. fulva*)[4]. It has been reported[20] that amanitas in general are not as hard to identify as many gilled mushrooms, and plenty of good literature is available for the genus *Amanita*. Also, identification for many species is based on a set of fairly easily ascertained, reasonable characters.

Basically, a good matching was observed between morphological and molecular grouping of samples. However, several inconsistencies were noticed.

The modified protocol used for DNA extraction was very successful in obtaining DNA with very good quality. All samples that were morphologically identified as *Coprinopsis lagopus* did not give good DNA and did not give amplification results with all primers. The failure of the amplification of *Coprinopsis lagopus* DNA may have resulted from the low amount of DNA obtained. Since a small amount of the template DNA gives amplification results in the PCR, it is most probably that there was another reason for the failure, e.g. inhibition of the amplification due to the black pigment that colored the gills after maturation.

RAPD technique is known as good technique to indicate overall genetic relatedness/dissimilarity than morphological analysis[21]. A study carried out on Oyster mushroom (*Pleurotus* spp.) demonstrated that RAPD analysis and morphological evaluation were both useful for characterization, genetic diversity and identifying relationships among *Pleurotus* species of mushrooms[15]. It also revealed that RAPD analysis can be a very useful tool for mushroom growers for classification and maintenance of good quality spawns. The RAPD PCR program achieved good results and reproducible bands.

The 100 % polymorphisms obtained in the present study indicate the great genetic diversity among the mushroom samples and the suitability of the selected primers for studying the diversity as well. The similarity among groups was low. Even within each group similarity was found to be low in some groups, but, it was very high (100% in some cases) in the other groups. This result agrees to some extent with the morphological grouping and identification, since morphological identification placed the samples in different orders, families and genera. That means they were also morphologically diversified. Also, members of each group were taken from much close geographical sites, and the 100 % similarity was obtained with samples belonging to the same species (group) and collected from the same area/site. This 100% similarity is probably due to that they came from spores that were released by one fruiting body.

Members of the groups of samples identified as *Chlorophyllum molybdites* have shown considerable variation in both molecular and morphological characteristics. *Chlorophyllum molybdites* was the closest species to *Lepiota* (22% similar). This is supported by the fact that *Lepiota* and *Chlorophylum* are located in one family (Lebiotaceae).

Group thirteen (*Amanita vittadinii (*Moretti*)* Sacc, 1887) appeared with group three (not determined) in the same cluster. The closest cluster to them was the Lepiotaceae group which included *Lepiota* and *Chlorophyllum*. This result is plausible because *Amanita vittadinii* (Moretti) Sacc. previously had the name *Lepiota vittadinii* (Moretti*)* Quél, and had the name *Agaricus vittadinii* Moretti very early in1826, due to the presence of common characteristics between the two genera and *Amanita*. Also, it has been reported[22] that in many areas, *Chlorophyllum molybdites* is easily confused with some species

of *Amanita* (*Amanita thiersii*). This confusion is most probably due to some similar morphological characteristics.

Among all mushroom samples, those belonging to *Psathyrella* are the most related to *Coprinopsis*. They are grouped together. This genetic relatedness agrees with their morphological resemblance to some extent, and with their classical taxonomy. The family that contained *Coprinopsis* (Coprinaceae) was renamed Psathyrellaceae because *Coprinopsis* members are closely related to *Psathyrella*. The two genera produce fruiting bodies with hollow stipes[4]. A study that included six *Psathyrella* species suggested that certain coprinoids (*Coprinus*-like species with deliquescent gills now in the genus *Coprinellus* P. Karst) arose from a clade of psathyrelloid (fragile pileus, dark spores, non-deliquescent gills) taxa[23,24]. Also, it has been revealed that most of coprini, other than *Coprinus comatus* (the type species for *Coprinus*), are allied with members of the genus *Psathyrella*[25]. Consequently, most coprini have been reassigned to three newly defined genera included in the family Psathyrellaceae: viz., *Coprinopsis*, *Coprinellus*, and *Parasola*[25].

Members of group one that was identified as *Podaxis pistillaris* diverged from each other. S1, S2 and S3 came close to two samples from group two (S4 and S5) while S17 and S18 grouped with samples that belonged to Psathyrellaceae family with much relatedness to S16 that was identified as *Parasola auricoma.* One sample from group two (S6) was related to S17 and S18. This divergence may be due to the effect of the environment on the genetic material, and also it is probably that the diverged samples among the species represent different strains.

Since group two failed to be identified, it needs more work to determine the belonging of its samples, and to understand the relationships among them. However, this genetic relatedness agrees with the classical taxonomy. Hence, the two groups were suggested to be Agaricales *sensu lato*. Also, has been reported[25] that it is unclear which taxa are the closest relatives of *Podaxis*, but *Podaxis* seems to belong to the same general *Agaricus/Lepiota* clade with *C. comatus* and it appears on a lone branch in the *Agaricus*/*Lepiota* clade as has been reported[25]. Although *Podaxis* and *Coprinus comatus* are superficially similar, they are not closely allied, as was revealed by molecular studies[22, 26].
The puffballs group was the closest to Lepiotaceae (13 % similarity). Since the two were classified in two different orders, 13% molecular similarity agrees with the morphological groupings.

It could be concluded from the present results that identification of mushrooms, relying on morphological characteristics alone is not completely satisfactory, and as there are some poisonous species, it will be difficult and risky to depend solely on the morphological identification. Moreover, the morphological characteristics are highly affected by the environment. In this study, both morphological and molecular approaches revealed the great diversity among the studied mushrooms in Khartoum North, and good agreement between the two approaches was observed. The 100% polymorphism between samples shown by the RAPD technique indicates the great genetic diversity of Khartoum North mushrooms.

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