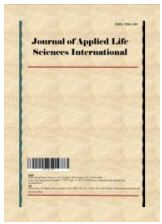

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Molecular Characterization and *in-vitro* Regeneration of Wild *Ganoderma lucidum* from Abuja, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Authors CEO, AAH and AIA designed the study. Authors KO and GAEU performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Authors AAH, AIA, AA and PCO managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To demonstrate the importance of molecular identification of *G. lucidum* basidiomata, used as nutraceutics, in Abuja - Nigeria.
Study Design: Molecular characterization via comparative genomics and vitro regeneration of selected specimens of local *G. lucidum* from Abuja, Nigeria.
Place and Duration: Department of Biological Sciences, Nigerian Defence Academy, Kaduna Nigeria and Biotechnology Advanced Research Center, Sheda Science and Technology Complex, Abuja between February 2018 to June 2019.
Methodology: Genomic DNAs of twelve (12) selected specimens were isolate in good quantities and qualities that were amenable to sharp and distinct PCR amplifications and Sanger's sequence analyses. The molecular identification was performed using the internal transcribed spacers (ITS) sequences amplified from the samples to run similarity search with reference database sequences

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in the gene bank. *In-vitro* regeneration of the samples using tissue culture techniques in the laboratory was carried out following optimization of the surface sterilization, regeneration of pure mycelia and pure spawn formation.
Results: Nucleotide sequence data mining of the national centre for biotechnology information (NCBI) with the query sequences using basic local alignment search tool (BLAST) showed that 25% of the samples are not *G. lucidum*. This implies a significant difference between the morphological and molecular identification at (n-1) degree of freedom with (p= .01).
Conclusion: The molecular identification and *in-vitro* regeneration of local *G. lucidum* is indeed a necessity for proper and effective utilization of the mushroom because there is a significant potential margin of error in the use of morphological characteristics for *G. lucidum* identification as observed through this molecular analysis.

Keywords: Ganoderma lucidum; tissue culture; characterization; phylogeny; DNA; BLAST; NCBI.

1. INTRODUCTION

The use of natural products with medicinal properties is as ancient as human civilization and for a long time till now, plants and microbes especially fungi are the main natural sources of drugs [1]. Their use as nutraceuticals and pharmaceuticals for prophylaxis, diagnosis and therapeutics has been an indispensable part of human life, in that they play critical roles in the general wellbeing of individuals [2]. The production of these agents is hugely dependent on the sources of the raw materials: their authenticity, availability, accessibility, possibility of preservation, reproduction/regeneration consistency in addition to the establishment of priorities in relation to their therapeutic indices [3]. The presence of very useful bioactive contents that are extractable from *G. lucidum* with highly beneficial pharmacological significances in the prevention and treatment of diseasesas documented by various scientific proofs [4] has led to production and marketing of its products as nutraceuticals. However, increasing use of *G. lucidum* as nutraceutical and/or pharmaceuticals is endangered by the wide morphological variation within its species and as such, a pertinent cause for its proper identification and micro-propagation using biotechnological methods [5].

There also seem to be uncertainties about the actual origin of the mushroom as there are reports of both European, Asian and other origins of this mushroom [6] probably because of its wide distribution across the globe owing to its high medicinal value. Similarly, some local Nigerian *G. lucidum* have been identified morphologically but not much has been done to ascertain their genetic authenticity in terms of clarity on their homology with reference strains in the database [7]. Identification has been based majorly on morphological species concept by decisions built on similarity and dissimilarity of characters, which are not very authentic as different biotic and abiotic factors that affect the morphology of mushrooms lead to faulty identification and classification [8].

The use of morphology to determine species boundaries in fungi especially for *Ganoderma* species has, however, proven inadequate because all characters are centred around one part of the life cycle and the fruit body which are relatively simple with limited available features for differentiating species [9]. Based on this, there is a high possibility of misuse of this medicinal mushroom for therapeutic purposes arising from problems of misidentification. This therefore, becomes counterproductive with detrimental consequences of *mycetism* and associated problems [10]. *Mycetism*' or mushroom poisoning refers to the harmful effects from ingestion of toxic substances present in mushrooms. This is always as a result of misidentification of toxic mushrooms as edible ones due to close morphological resemblance [11]. *Mycetism* ranges from slight gastro intestinal tract (GIT) upset, altered sympathomimetic and para-sympathomimetic effects to fatal consequences [12]. Regardless of the myths and legends associated with differentiating toxic mushrooms from edible ones, *mycetism* is still a very serious risk factor in mushroom utilization due to the prevailing environmental and climate change factors [13]. Researchers have also shown that some earlier termed edible mushrooms have turned out to contain toxins that exert cumulative negative effects in humans [13]. Hence, the best way to prevent *mycetism* is molecular authentication and *in-vitro* regeneration. The use of biotechnological tools especially tissue culture techniques as a portent tool in the development

of new drugs emerged in the 1990's and it's becoming an established process. Tissue culture techniques have been used not only to micro-propagate medicinal plants especially the endangered ones that are difficult to raise using traditional methods but also in mushroom cultivation technology for mass production and domestication [14]. Modern mushroom cultivation technology applies the principle of tissue culture to regenerate, domesticate and micro-propagate mushrooms of interest mostly medicinal ones for mushroom nutraceutical production [15].

1.1 Molecular Systematics of *G. lucidum*

G. lucidum is a polypore mushroom that is hard (even when fresh), corky, and flat, with a shiny red or greenish or yellow or light/dark brown-varnished, kidney-shaped cap and depending on specimen age, white to dull brown pores underneath [16]. Among edible and medicinal mushrooms, most studies have been done on *Ganoderma* species especially *G. lucidum* because of their unique medicinal value [17]. A variety of commercial *G. lucidum* products are available in various forms, such as powders, dietary supplements, and tea [18].

The use of molecular genetics to elucidate the phylogenetic relationships and evolutionary patterns within and among the *Ganoderma* species is believed to be resolving most of the challenges associated with *Ganoderma* systematics. This is however, not without some minor species concept uncertainties in the *G. lucidum* complex due to its many inconsistencies in phylogeny and morphology [19]. Nuclear-encoded ribosomal RNA genes (rDNA) have been the main focus of analysis in fungal molecular systematics. These genes are arranged in tandemly repeated units containing the genes for the small subunit, (18s), (5.8s) and large subunit (25-28s) which are highly conserved among fungi. Each unit is flanked by one or more internal transcribed spacers (ITS) regions which are highly variable and easy to amplify that makes them very useful in distinguishing between *Ganoderma* species [20]. This ITS sequence analysis method is a barcode-based species identification approach which relies on an overall sequence identity between the query sequence and those in the reference databases [21]. The query sequences are obtained via a polymerase chain reaction (PCR) mediated process that uses ITS primers to amplify the targeted sequences which are then subjected to sequencing to obtain the actual configuration of the nucleotides.

1.2 *Ganoderma lucidum in-vitro* Regeneration

In their natural habitats, availability of mushrooms is seasonal. Depending on the species, some may be available just at the onset of rains, others during the rains, yet others when the rains are winding up [22]. This leaves a vacuum in the usefulness of mushrooms during dry season or summer periods and hence emphasizes the need for mushroom cultivation.

In-vitro regeneration of *G. lucidum* can be possibly carried out on sterile substrate using a semi intensive or intensive cultivation process under aseptic condition *in-vitro*, which involves isolation of pure cultures from the mushroom's fruit bodies germinated from spawn [23]. The use of *in-vitro* aseptic culture of cells, tissues, organs of organisms under controlled nutritional and environmental conditions [24] to produce resultant clones that are proto type of the selected genotype [25] of that organism is known as tissue culture. This has become an important biotechnological approach for producing reliable and commercial quantities of desired species. *G. lucidum* tissue culture will no doubt go a long way in providing reliable sources for *G. lucidum* nutraceuticals and pharmaceuticals. The process of mushroom tissue culture involves: i) Parent sample collection using the various morphological features as guide, ii) surface sterilization, which involves the use of appropriate concentrations of specific sterilants and surfactants to kill surface contaminants with minimal effect on the sample cells. iii) Media preparation involving a combination of artificial compounds needed to provide a balanced nutrient for the feeding and nourishment of the cultured samples. iv). Maintenance, regeneration and multiplication of mycelia at appropriate optimal conditions. v) Spawn development; which involves impregnation of suitable substrates with pure mycelia, and vi) Fruit body production, which involves regeneration of the mushroom by inoculating pure spawn at optimal conditions into suitable sterile substrate preferably compost made of agricultural wastes (like rice straw, rice bran, wheat straw, pulp, corncobs, cocoa shell wastes, cotton seed bulb, maize husks and cassava peelings [26].

2. MATERIALS AND METHODS

2.1 Samples Collection/ Study Area

A total of twelve (12) selected samples of *G. lucidum* species, collected from the various area councils in Abuja, (2 from each area council) were morphologically identified at the Biodiversity unit of Biotechnology Advanced Research Centre of Sheda Science and Technology Complex (SHESTCO). Abuja is located in the centre of Nigeria and has a land area of about 8,000 square kilometres and falls within latitude 745' and 739'. The area councils that make up Abuja include: *Abaji, Bwari, Gwagwalada, Kuje, Kwali* and *Abuja municipal area council (AMAC)*.

2.2 Genomic DNA Extraction

Modified [27] method was used for the DNA extraction. The major modifications to the original protocols include synergistic combination of antioxidants (β-ME, PVP), three times CIA treatment, three times ethanol wash with 70% and absolute ethanol, extension of incubation periods in CTAB buffer at 65°C, in the freezer at -20°C and centrifugation period after freezing at 20°C.

2.3 PCR Amplification using ITS-Primers

A set of two (2) ITS primers were obtained from *Inqaba* Biotech and used for the PCR run. They are: ITS1- (TCC GTA GGT GAA CCT GCG G) ITS4- (TCC TCC GCT TAT TGA TAT GC). Polymerase chain reactions were carried out for ITS amplification in 25 µl reaction mixture containing 200 ng template DNA, 0.5 µM forward, 0.5 µM reverse primer, 12.5 µl Dream *Taq* PCR master mix and 2 µl of 50 µg/ml BSA. The amplification reaction was carried out in a Peltier-based thermal cycler using a program cycle of 94°C for 4minutes followed by 35 cycles of 94°C for 1 minute, 56°C for 2 minutes 72°C for 2 minutes, 72°C for 10minutes. The resulting PCR products were screened 1.2% agarose gel, stained with SYBR safe fluorescent dye (10,000X), then visualised and captured with *Alpha innotech* Gel visualisation and documentation system as shown below in Fig. 3. DNA step ladder (100bp) was used as gene ruler for bands molecular weight estimation [28].

2.4 Sequencing and Sequence Analyses

The PCR products were cleaned with Zymo research PCR clean up kits and subjected to Sanger's sequencing using Applied Biosystems International (ABI) automated sequencer model ABI 13130.

The sequence data obtained were used as query sequences to perform sequence data similarity search against curated ITS sequences in Gene Bank using Basic Local Alignment Search Tool (BLAST).

2.5 *G. lucidum* Tissue Culture

Two different types of media were used for culturing of the samples. They are Malt extract agar (MEA) and Potato Dextrose Agar (PDA) prepared according to the manufacturer's instructions, 50 g/L and 39 g/L respectively, sterilized and dispensed into petri- dishes (50ml/ plate) under aseptic condition [29].

2.5.1 Surface sterilization, culturing and maintenance

Cut pieces of *G. lucidum* samples were cultured in the two different media prepared following surface sterilisation optimisation process, incubated at optimal conditions in a growth chamber (*Bosschendijik 193 Hot-Cold*) and kept under surveillance for 2 weeks. The resultant mycelia were continually `sub-cultured, multiplied and maintained in different vessels to obtain pure mycelia that were used for spawn formation [30].

2.5.2 Spawn formation

Pure cultures of the mycelia were transferred aseptically to sterilized substrates in specialised bottles containing wheat and guinea corn substrates and incubated at 25°C and 55% temperature and humidity respectively in the dark with adequate provision for sterile aeration as they developed and colonised the substrate. The substrates were prepared by parboiling large quantities of clean dry wheat and guinea corn grains followed by air-drying after which they were mixed with 1% $CaSO_4$ and then transferred into the specialised heat resistant bottles with aeration outlet and sterilised by autoclaving at 15 lb/in^2 pressure and 121°C for 30 minutes. The substrates were later inoculated with the pure mycelia and incubated at 37°C in the growth chamber in the dark for 3 weeks [31].

2.5.3 Compost substrate preparation

This was carried out by chopping into pieces the corn straw and thorough mixing of the constituents in their specific proportions in the

following proportions: compost 1 -sawdust/ poultry dungs (2:1), compost 2 – saw dust / cassava peels (1:1) and compost 3 - corn straw / cassava peels substrates (1:1). This was followed by wetting and boiling in appropriate quantities of water for about 10 minutes after which they were drained and spread out to cool before $CaSO_4$ and carbamide were added in to the mixtures in the proportions of 1 and 2% respectively and mixed very well. The mixture was boiled and cooled again before packing into special plastic vessels lined with sterile nylon bags. The covers of these vessels were constructed to have holes meshed with sterile cotton wools to allow for adequate aeration devoid of contamination and temperature inconsistency [32].

2.5.4 Spawning of the mushroom compost substrate

The matured pure spawns were removed from the glass bottles where they were grown and teased out into pieces on two layers of the composts; the first layer at about 3-4 inches below the surface while the second layer at about another 3-4 inches below the first layer in the different compost beds. This was followed by incubation at temperature between 37-40^{0}C and humidity of 65-70% and kept under surveillance with addition of little quantities of sterile water per day [29].

2.5.5 Spawn-run fruit body regeneration

Following the emergence of some mini button-like structures suspected to be primordials of fruit-bodies that were observed on the composts three weeks after the inoculation, the composts were opened and exposed to bright light, high humidity (70%) and lower temperature (25°C) under a contained sterile condition in the growth chamber for further development.

2.6 Experimental Design

The experimental design used for the sampling and analysis was randomised Complete Block Design (RCBD) and simple percentage while the statistical package is Chi square as shown in Table 3.

3. RESULTS AND DISCUSSION

3.1 Results

The results of the genomic DNA extraction and PCR amplifications of the samples on agarose gel electrophoresis shown below in Figs. 2 and 3. respectively were used to analyse the banding patterns and the sizes of the bands in base pairs (bp). The concentrations and purity levels of the DNA samples are highlighted in Table 1. The sequence results obtained were edited and used as query to carry out data mining for sequence similarity search using BLAST. Analysis of the BLAST results showed that nine out of the twelve selected samples were confirmed to be *G. lucidum* samples while the other three were found to be *Ganoderma cupreum, Ganoderma fornicatum* and *Ganoderma applanatum.* The summary of the sequence alignment results of the specimens using BLAST is represented in Table 2. The phylogenetic tree constructed from these results shown in Fig. 1 is a neighbour joining tree without distance corrections using Clustal omega tool. It elucidates a simple evolutionary relationship among the specimens.

Table 1. Analysis Result of Genomic DNA Extractions of the *G. lucidum* samples

Sample no	Absorbance (OD_{260nm})	Absorbance (OD_{280nm})	Concentration (ng/µl)	Purity check ($A_{260nm}/_{280nm}$)
1	0.14	0.078	350.00	1.79
2	0.164	0.072	410.00	1.80
3	0.179	0.106	447.50	1.78
4	0.166	0.092	415.00	1.80
5	0.163	0.091	407.50	1.79
6	0.118	0.065	295.00	1.81
7	0.143	0.080	357.50	1.80
8	0.108	0.059	270.00	1.80
9	0.113	0.063	282.50	1.80
10	0.186	0.103	465.00	1.80
11	0.108	0.102	465.00	1.81
12	0.169	0.09	422.50	1.80

Table 2. Showing the summary of sequence alignment result with BLAST at a glance

Sample no	Origin	Closest hit	Voucher number	Accession number
1	Abaji	*G. cupreum*	BUTH1	AY569450.1
2	Abaji	*G. lucidum*	XXL22	MH553170.1
3	Bwarri	*G. fornicatum*	TNM-F00-10592	JX840347.1
4	Bwarri	*G. lucidum*	77002	G654160.1
5	Gwagwalada	*G. applanatum*	BL26	JX501311.1
6	Gwagwalada	*G. lucidum*	MS183CA	KF146177.1
7	Kuje	*G. lucidum*	BEOFB434	AY884175.1
8	Kuje	*G. lucidum*	XLLZ	JN588589.1
9	Kwali	*G. lucidum*	AP-16	MG706228.1
10	Kwali	*G. lucidum*	AI-R15	MF419231.1
11	AMAC	*G. lucidum*	CCBAS707	MH160085.1
12	AMAC	*G. lucidum*	RNK1	JQ781853.1

Table 3. Showing the statistical data at a glance

Sample	Mol. identity	Morph. identity	Percentage identity
G. lucidum	9	12	75
Non *G. lucidum*	3	0	25

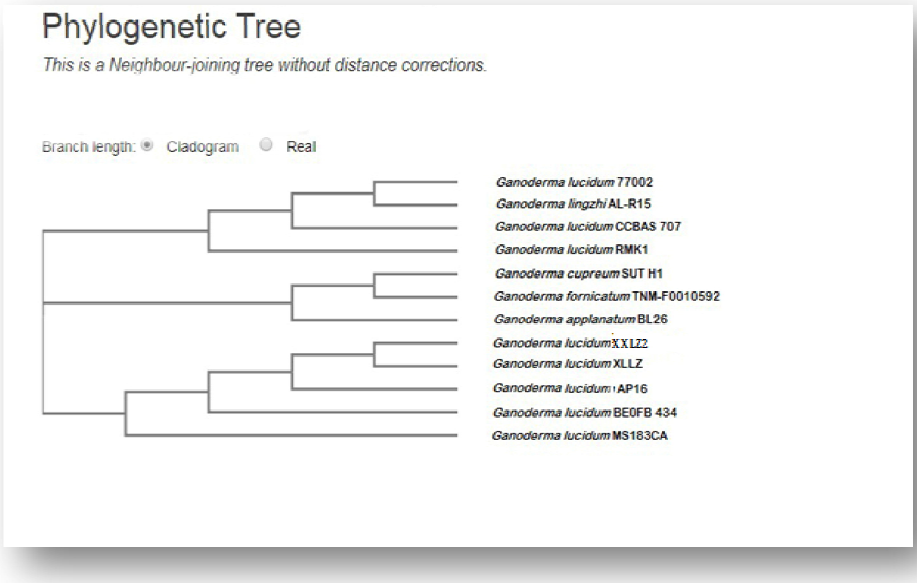

Fig. 1. Phylogenetic tree of the wild *G. lucidum* specimens from Abuja environs

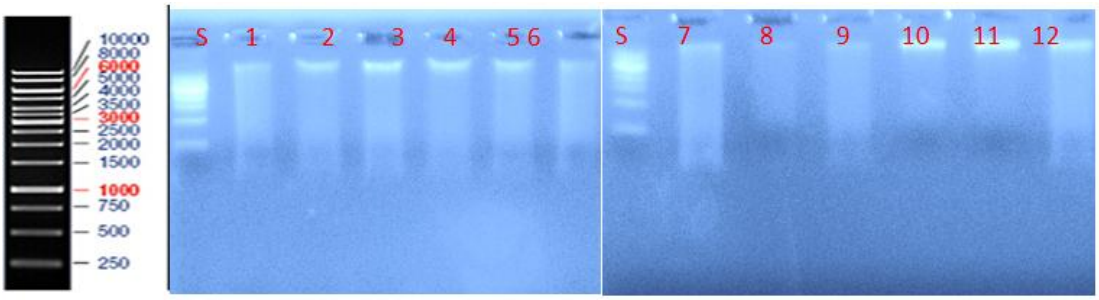

Fig. 2. Electrophoregram of genomic DNA extraction of the *G. lucidum* samples

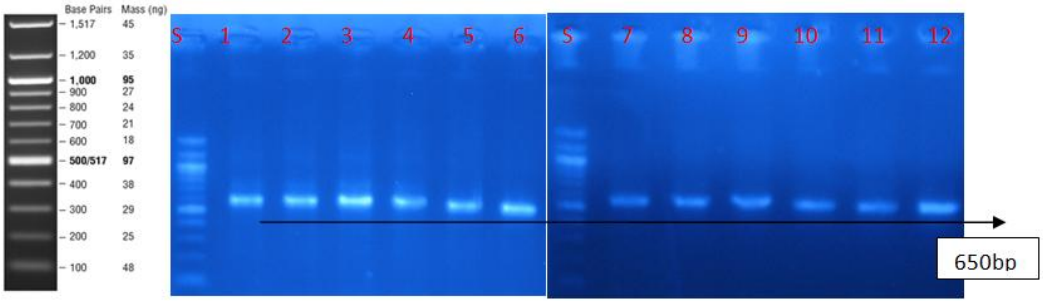

Fig. 3. Electrophoregram of PCR result of the *G. lucidum* samples

The in-vitro regeneration of *G. lucidum* via tissue culture technique was largely successful as mycelia regeneration, pure spawn production and fruit body regeneration were developed as shown in Fig. 4.

3.2 Statistical Analysis

Results are expressed as X^2_{cal} vs X_{tab} at (n -1) degree of freedom. Chi square (X^2) was used to analyze data and a difference of (P=.01) was considered significant.

Degree of freedom (DF) n-1= 1
Confidence level (P) = 0.01

$$X^2 = [(O - E) - 0.5^2 / E] + (O - E) - 0-5^2 / E.$$
$$0.42 + 6.25 = 6.67$$

X^2 at (n-1) d/f and p=.01; $X^2_{cal} > X^2_{tab}$; Null hypothesis is rejected;

There is significant difference between the observed quantity by molecular characterization and the expected quantity by morphological characterisation. Thus H_o is rejected.

3.2 Discussion

Genomic DNA with good quality and quantity from the selected raw samples of *G. lucidum* was needed for the various analyses and downstream applications like PCR and sequencing [33].

The optimized protocol which is a modification of CTAB extraction procedure of [27] with careful amendments that led to the isolation of good genomic DNA from the samples. The PCR result yielded strong and reliable amplification products of sizes around 600bp which is within the range of the required fragment line with [34]. The sequence alignment results which shows 75% *G. lucidum* and 25% non-*G. lucidum* of the selected samples, presents a significant difference between the morphological and molecular characterization at (n-1) degree of freedom and (p= .01).This on one hand is a confirmation of the intra species morphological variation within this mushroom and on the other hand emphasises the need for molecular authentication for proper utilisation of this mushroom in line with [5]. The phylogenetic tree constructed using Clustal omega tool is a simple distance matrix analysis showing neighbour- joining tree without distance corrections which primarily reveals the basic inter relationship` among the samples in relation to the homologues/ closest identical organisms from the BLAST results. From this simple tree, the *G. lucidum* samples are not a monophyletic species as pointed out by [35] and exist in two distinct lines in conformity to [6] and with closely related intermediate forms as G. *cupreum, G. fornicatum* and G. *applanatum.* This however is in tune with the reports of [36] and [35] which stated that laccate *Ganoderma* species (*lucidum, oregonens e*and *rasinecium*) are greatly synonymous as the collections named *G. lucidum* from different parts of the world are scattered in several separated lineages in phylogenetic analyses of the genus.

The result from *G. lucidum* tissue culture specifies that the samples required an optimal concentration of 2.0% sodium hypochlorite for about 30 minutes after 1 min of 70% ethanol wash which is in conformity with [32] probably due to the very hard texture and possible endophytic contamination of *G. lucidum* samples. The mycelia production which started from the 8th day after culturing was in conformity with [37]. The spawn formation was matured within 10 days of inoculation and also in line with [37] and [38]. These techniques were highly susceptible to contamination and as such were repeatedly carried out at optimal and aseptic conditions with absolute handling meticulousness in line with [39].

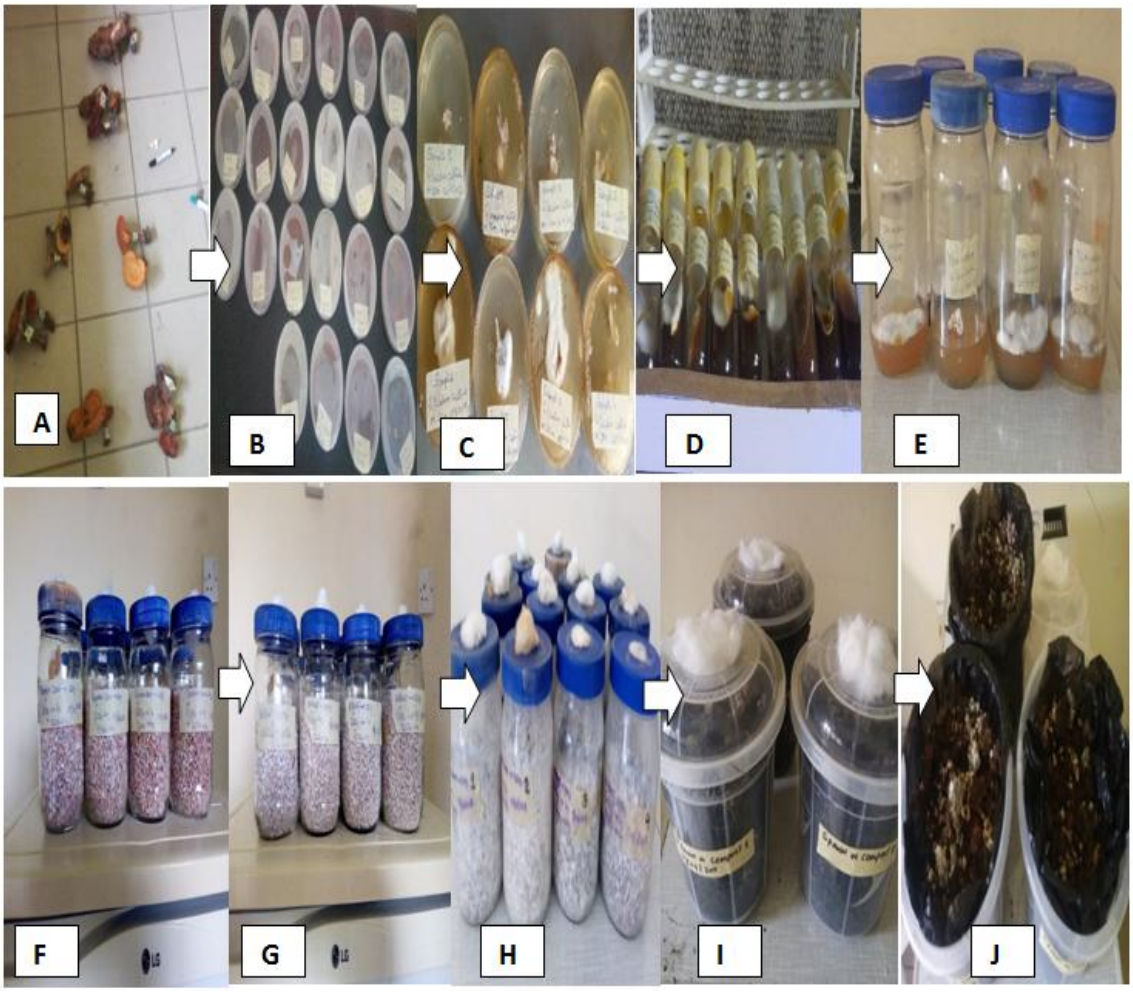

Fig. 4. Pictures of the in-vitro regeneration process (from sample selection to development of fruit bodies)

Key; A-Samples selection and labelling; B-Media and culturing of samples;
C-E Sub-culturing and maintenance of pure mycelia; F- H Spawn production;
I-J Fruit body regeneration

The Potato Dextrose Agar (PDA) media was preferred to the Malt extract agar (MEA) media in that, its production of mycelia was progressively moderate with less contamination. Production of pure spawns devoid of contamination was from resultant pure mycelia produced by means of multiple sub-culturing processes at aseptic conditions with optimal temperature and humidity (25^0C and 55%) respectively in culture growth chamber in line with [40] and [41]. The slow development of the fruit bodies could be attributed to some probable reasons which include moisture levels of the compost substrate and /or unfavourable compost condition. This is because the quantity and quality of raw materials used to make mushroom compost are highly variable with different mushroom strains as well as the moisture- temperature- microbial balance in the composts which are known influence performance in terms of spawn- run fruit bodies' development as highlighted by [42].

4. CONCLUSION

Molecular characterisation and in-vitro regeneration of local *G. lucidum* from Abuja, Nigeria, for proper identification and micro-propagation respectively, present the use of modern biotechnology tools for authentic specimen identification and domestication of medicinal mushrooms to prevent misapplication and *mycetism*. From the result of this work, the

potential margin of error in the use of morphological characteristics for *G. lucidum* identification was observed through its molecular analysis to be about 25%. The *in-vitro* regeneration was largely successful and promising as its tissue culture procedure under laboratory conditions was optimized. The use of molecular authentication as well as developing true-to- type species by means of tissue culture is therefore recommended for effective utilisation of this mushroom especially in large quantities as nutraceuticals and pharmaceuticals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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