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Molecular characterization and cultivation of edible wild mushrooms, *Lentinus sajor-caju, L. squarrosulus* and *Pleurotus tuber-regium* from Sri Lanka

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Abstract

Tropical wild edible mushrooms are nutritious, delicious and economically important fungal bioresources. In this study, two edible *Lentinus* species and one *Pleurotus* species were collected from Central and Western provinces of Sri Lanka. After the initial morphological identification, growth rates and culture characteristics were observed in four different culture media. Genomic DNA was extracted and PCR amplifications of fungal barcode, nuclear ribosomal internal transcribed spacers 1, 2 and 5.8S (ITS) region were carried out. Molecular phylogenetic analyses confirmed the identity of the isolates as *Lentinus squarrosulus, L. tuber-regium* and *Pleurotus sajor-caju*. The possibilities of cultivation of the species collected were investigated using rice and corn as the spawn media, while rubber and mango saw dust were used as the basal media. A commercial *P. ostreatus* strain was used as a positive control strain alongside all experiments. All three newly collected species showed the highest growth rates on Potato Dextrose Agar (PDA) and the highest mycelial density on corn spawn. Mycelia of all the isolates showed the highest growth rate in mango saw dust medium. *Pleurotus tuber-regium, L. sajor-caju* and *L. squarrosulus* produced fruit bodies 69, 88 and 49 days after spawning respectively. Highest mean yield for *P. tuber-regium* was observed in rubber saw dust (110.617 g ± 15.828), for *L.* sajor-*caju* in mango saw dust (13.978 g ± 3.656) and for *L. squarrosulus* in rubber saw dust (51.350 g ± 0). Based on the available information, this is the first successful cultivation of *L. sajor-caju* and *P. tuber-regium* strains collected from Sri Lanka.

Key words – Basidiomycetes – DNA barcoding – spawn production

Introduction

Mushrooms, the earliest form of fungi known to mankind, are currently considered as functional food due to their ability to render many nutritional and medicinal benefits to humans (Muhammad & Suleiman 2015). Sri Lanka is rich in wild edible mushrooms similar to many tropical countries, but most of this knowledge can be stated as 'hidden' among local communities (Hewage 2015). Approximately, out of about 2000 mushrooms that were known to be edible, only about 130 species have been domesticated globally (Li et al. 2021, Thawthong et al. 2014). These

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domestication attempts are more often associated with the testing for cultivation in rather unconventional novel substrates in addition to common lignocellulosic agricultural wastes. In Sri Lanka, the status of edible wild mushroom cultivation is still at infancy with the knowledge restricted to a few species belonging in the genera *Agaricus*, *Pleurotus*, *Ganoderma* (Rajapakse et al. 2010), *Schizophyllu*m (Ediriweera et al. 2015, Dasanayaka & Wijeyaratne 2017), *Auricularia polytricha* and *Lentinus squarrosulus* (Ediriweera et al. 2015). However, some edible mushroom species closely resemble nonedible or poisonous mushroom species (Jose & Radhamany 2012). Thus, accurate identification of a mushroom species is essential before a proper cultivation attempt. On the other hand, in addition to the analysis of nutritional content and chemical analyses that include heavy metal concentrations of a mushroom strain, precise identification, is essential before commercializing it as a food source. In addition to the morphological identification based on taxonomic characteristics, molecular level studies such as DNA barcoding for precise taxonomic identifications and use of phylogenetic analyses in confirming taxonomic identities are paramount in providing back up for these cultivation trials.

Almost all the species in genus *Lentinus* are considered to be edible (Thawthong et al. 2014). Some common edible *Lentinus* species include *L. edodes*, *L. giganteus*, *L. polychrous* and *L. strigosus*. *Pleurotus djamor, P. eryngii, P. flabellatus* and *P. ostreatus* are examples for some common edible tropical *Pleurotus* species. *Pleurotus tuber-regium* is a tropical fast-growing white rot fungus that produces fruit bodies and prominent sclerotia which are both edible. It is commonly consumed in African countries. *Lentinus squarrosulus* is an edible mushroom with a significant medicinal and a nutritional value, and is underutilized in most parts of the world except in Asia and Africa (Lau & Abdullah 2017, Omar et al. 2011). It is rich in phenolic compounds, flavonoids, immunostimulatory glucans, lectins, carbohydrates, proteins and minerals (Lau & Abdullah 2017). *Lentinus sajor-caju* is another wild edible mushroom which was earlier classified under *Pleurotus* but was later placed in *Lentinus* (Pegler 1975). It is an excellent edible mushroom with a high protein content (Mortimer et al. 2014). Its protein content was found to be greater than *Agaricus bisporus, Lentinus edodes, Pleurotus ostreatus* and *Volvariella volvacea* (Chang et al. 1981).

With the impact of global warming and due to the growing demand for protein rich food sources for the increasing global population, wild mushroom cultivation and commercialization has become a priority in rural agriculture and food industry. Modern biotechnology has revolutionized many facets of mushroom science including cultivation technologies, conservation of germplasm, development of high yielding varieties and other improved varieties with high biological efficiency, high nutritional value, better taste, aroma and longer shelf life. Molecular biological techniques have many applications in genetic improvements and identification of cultivated mushrooms. Protoplast technologies, lignocellulose degradation through mushrooms and bioremediation through mushrooms are some other novel pathways where biotechnology aids in mushroom science (Singh et al. 2009).

The objectives of this study are to optimize growth, spawn media, culture and fruiting conditions of three edible wild mushroom species, *Lentinus sajor-caju*, *L. squarrosulus* and *Pleurotus tuber-regium* and to establish their molecular identification and phylogenetic relationships.

Materials & Methods

Sample collection, tissue isolation and morphological identification

The fruit bodies of *P. tuber-regium* were collected from Dombawela, Matale (7.5564^{o}N 80.6105^{o}E), *L. sajor-caju* from Gangodawila, Nugegoda (6.8549^{o}N 79.9042^{o}E) and *L. squarrosulus* from Ragama (7.0346^{o}N 79.9176^{o}E) in Sri Lanka. Morphological characteristics were studied and a tentative identification was established by comparing with the available original taxonomic literature. For fungal isolation, the caps of the fruit bodies were surface sterilized using 70% ethanol, a piece of sterile interior gill/stipe tissue was extracted and placed on petri plates containing Potato Dextrose Agar (PDA). Plates were incubated at room temperature (average =

30 °C) and through subsequent sub-culturing, pure cultures were obtained. Isolates were deposited in the Fungal Culture Collection maintained by the Department of Botany, Faculty of Technology, University of Sri Jayewardenepura (USJCC).

Morphological characterization of fungal isolates

Isolates were cultured on four different culture media; PDA, Malt Extract Agar (MEA), Corn Meal Agar (CMA) and Straw Extract Agar (SEA). SEA was prepared by adding 2% (w/v) agar to paddy straw extract prepared by boiling 50 g of paddy straw in 1 L of distilled water. Mycelial plugs of 0.75 cm diameter were inoculated at the center of the media plates, incubated at room temperature and the colony diameters were measured daily across six locations in three replicate plates. Colony morphologies of the isolates in the above media were also noted after 7-d of incubation.

DNA extraction, PCR and sequencing

Mycelia from 7 d old cultures were used for genomic DNA extraction which was carried out in accordance with a modified method described by Arnold & Lutzoni (2007). The forward (ITS 1) and reverse (ITS 4) PCR primers, ITS 1 and ITS 4 (White et al. 1990) were used for the amplification of ITS region in a BIORAD® T100™ Thermal cycler. The PCR conditions and the number of cycles were selected as described in Manamgoda et al. (2012). The PCR products were then visualized in 2% (w/v) agarose gel to confirm the amplification and were sequenced using Sanger DNA sequencing facility at Macrogen, Seoul, Rep. of Korea.

Molecular identification of cultures

The initial identifications of the ITS sequences generated in this study were obtained by the BLASTN in NCBI (http://www.ncbi.nlm.nih.gov/). To confirm their phylogenetic placement, molecular phylogenetic analyses were carried out for the three isolates along with ex-type sequences or reference sequences obtained from GenBank. Sequences were assembled with BioEdit v7.2.5 software. The assembled consensus sequences were aligned with ClustalX and optimized with MAFFT v.7. The PAUP* Version 4.0 b10 (Swofford 2003) was used to perform maximum parsimony (MP) analyses. The phylogenetic trees were visualized with FigTree version 1.4.0. All new sequences generated in this study were submitted to GenBank.

Spawn media preparation

Spawn media were prepared according to the method described by Singh et al. (2017) with modifications in chemical ratios. Intact, properly washed rice (with husk) and corn grains were boiled for 15 min after soaking for 12 h in water. Then, the grains were sieved, dried until they cool down to room temperature and amended with gypsum ($CaSO_4.2H_2O$) and lime ($CaCO_3$). Rice grains were amended with two ratios of gypsum i.e. with 0.5% (w/w) gypsum and with 2% (w/w) gypsum in two separate batches. Corn grains were amended with 1.2% (w/w) gypsum and 0.3% (w/w) lime.

Then, approximately 100 g of grains were put into 250 mL Erlenmeyer flasks in three replicates, sealed with cotton wool and sterilized at 121 ^oC and 100 kPa (15 lb/in^2) pressure for one hour in the autoclave. The sterile grains were inoculated with three triangular portions (approximately 8 cm^2) of pure fungal cultures and incubated at room temperature. Number of days (d) taken for full ramification of grains and the visual density of the mycelia were noted.

Substrate preparation

Mango (*Mangifera indica*) and rubber (*Hevea brasiliensis*) sawdust were separately used as the main carbon source to prepare substrates. The substrate mixture was prepared with 10% rice bran, 2% dolomite ($CaMg(CO_3)_2$), 0.2% $MgSO_4$ and 1% commercially available cereal mix (corn, rice, mung bean and soy) on weight basis. An adequate amount of water was added and homogenized. Polypropylene bags (5 × 11 inches) were filled with approximately 600 g of the

mixture, narrow outlets were made at the top of the bags with plastic pipe rings and the outlets were sealed with cotton wool plugs. The bags were sterilized at 121 ^{o}C and 100 kPa (15 lb/in^2) pressure for one hour in the autoclave. After cooling, they were separately inoculated with pre-prepared rice (0.5% (w/w) gypsum) and corn (1.2% (w/w) gypsum and 0.3% (w/w) $CaCO_3$) spawn. Three replicate bags were prepared for each sawdust-spawn combination. Bags were incubated at room temperature under dark conditions until the mycelia fully covered the bag and the time taken for the complete coverage of the bags was recorded.

Determination of yield and moisture content

The mushroom cultivation bags were opened and placed in the cropping room. They were maintained at room temperature (30 ^{o}C) and 70% relative humidity and were watered daily. Time taken for the emergence of primordia in each bag was recorded. Once a flush is harvested, the fresh weight (g) and the number of fruit bodies in the flush were noted. To obtain the dry weight, mushrooms were dried in a hot air oven maintained at 40 ^{o}C till a constant dry weight was obtained. The moisture content was calculated and recorded as a percentage.

A *P. ostreatus* fungal culture which was isolated from a commercially grown mushroom available in the market was used as a control for all the experiments related to mushroom cultivation.

Results

Morphological characteristics of mushrooms and cultures

Comparison of the macromorphological characteristics (Fig. 1, Table 1), which included the nature and the colour of the pileus, stipe and gills, with the original descriptions for the species aided in confirming their tentative identity.

Fig. 1 – Mushrooms collected in this study in their natural habitats. A Fruit bodies of *Pleurotus tuber-regium* on wood of *Mangifera indica.* B *Lentinus sajor-caju* on a dying tree stump of *Tabebuia rosea.* C *Lentinus squarrosulus* on wood of *Mangifera indica.*

Table 1 Major morphological characteristics of the mushroom specimens collected and their morpho-identification.

Specimen	Morphological characteristics	Morphology-based identification
LEN001	*Pileus*-white to cream, pale ochraceous, more or less fuliginous or brownish, convex pileus with an umbilicate center; *Margin*-smooth, incurved; *Stipe*-short, cylindrical, concolorous with the pileus, central, eccentric or lateral; *Base*-abrupt; *Annulus*-1-3 mm wide, firm with an entire edge; *Gills*-deeply decurrent, very crowded, narrow; *Flesh*-tough and pliant; *Smell*-mushroomy	*L. sajor-caju*

Table 1 Continued.

Specimen	Morphological characteristics	Morphology-based identification
LSK005	*Pileus*-deeply infundibuliform, brown with minutely scurfy squamules; *Margin*-incurved, puberulous, entire, contains small floccose fragments of the veil; *Stipe*-central, subcylindrical, concolorous with the pileus; *Base*-abrupt or attenuate into the ground; *Gills*-deeply decurrent, very crowded, thin, narrow, appear as fine grey lines; *Flesh*-thick at the center, cheesy and hard; *Smell*-mushroomy	*P. tuber-regium*
M013	*Pileus*-white, convex, gradually becomes plane, umbilicate, pale straw-color to pale ochraceous, more or less furfuraceous-squamulose with small and often subrevolute scales; *Margin*-lacerated; *Stipe*-subcylindric, fibrous, excentric and rarely lateral; *Base*-scurfy-squamulose, abrupt; *Gills*-deeply decurrent, crowded, thin; *Flesh*-thick at the center, dry, tough and flaccid; *Smell*-mild	*L. squarrosulus*

Some notable differences were observed in the colony morphologies (Fig. 2) of the three isolates when they were grown on the four selected media (PDA, CMA, MEA and SEA). Circular, white, aerial, raised colonies of *Lentinus sajor-caju* with filiform margins were observed in all media. Colonies on PDA were opaque, cottony and progressively becoming tufty while on CMA, a concentric growth ring pattern was seen. Mycelial density was observed to be sparse in CMA, MEA and SEA when compared with PDA. *Pleurotus tuber-regium* on PDA and MEA showed irregular, aerial, raised colonies that are opaque, cottony and white with comparatively dense mycelial mats and filiform/undulating margins. Colonies on both CMA and SEA were translucent and white hyaline with sparse mycelia. Colonies on SEA were slightly circular. Colonies on PDA and CMA showed a distinct irregularity and a rhizomorphic nature while PDA produced *in vitro* primordial initials. *Lentinus squarrosulus* produced circular, aerial and white colonies with filiform margins. On PDA they were dense, raised, opaque and cottony. On MEA the colonies were dense, opaque to translucent and felt textured. Both SEA and CMA produced translucent to transparent colonies with extremely sparse mycelial mats. Browning of mycelia was observed in PDA with time.

Variations among the mycelial growth rates of the three isolates were observed in the four media and the highest mycelial colonization rate was seen on PDA for all three isolates (Table 2).

Molecular characterization

In the identification of the two *Lentinus* species, MP analysis for the ITS alignment consisted of 32 taxa including the outgroup taxon *Pleurotus cystidiosus* var *formosensis*. Analysis revealed that out of the 582 total characters, 289 were constant, 241 were parsimony informative while 52 characters were parsimony uninformative. The analysis generated 4 parsimonious trees and the 1st tree (TL = 653, CI = 0.712, RI = 0.837, RC = 0.596 and HI = 0.288) with bootstrap 50% majority rule consensus is shown in Fig. 3. The isolate LEN001 (this study) was clustered to the three *L. sajor-caju* species used in the tree with a high bootstrap support value of 87 and thereby it can be classified under *L. sajor-caju*. Isolate M013 (this study) is clustered close to the other two *L. squarrosulus* species used in the tree, confirming its identity as *L. squarrosulus*.

In the identification of the *Pleurotus* species, maximum parsimony analysis for the ITS alignment consisted of 105 taxa including the outgroup taxa *Hohenbueheli auriscalpium* and *Hohenbueheli mastrucata.* Analysis revealed that out of the 712 total characters, 354 were constant, 334 were parsimony informative while 24 characters were parsimony uninformative. The analysis generated 30 compatible parsimonious trees and the first tree (TL = 1185, CI = 0.551, RI = 0.920,

RC = 0.507 and HI = 0.449) with bootstrap 50% majority rule consensus is shown in Fig. 4. Isolate LSK005 (this study) grouped with the sequences of *P. tuber- regium* sequences available in GenBank, which further confirmed its identity.

The newly obtained sequences in this study were submitted to GenBank. (GenBank accession numbers for ITS: *P. tuber-regium* – MK346338, *L. sajor-caju* – MK346337 and *L. squarrosulus* - MK346335).

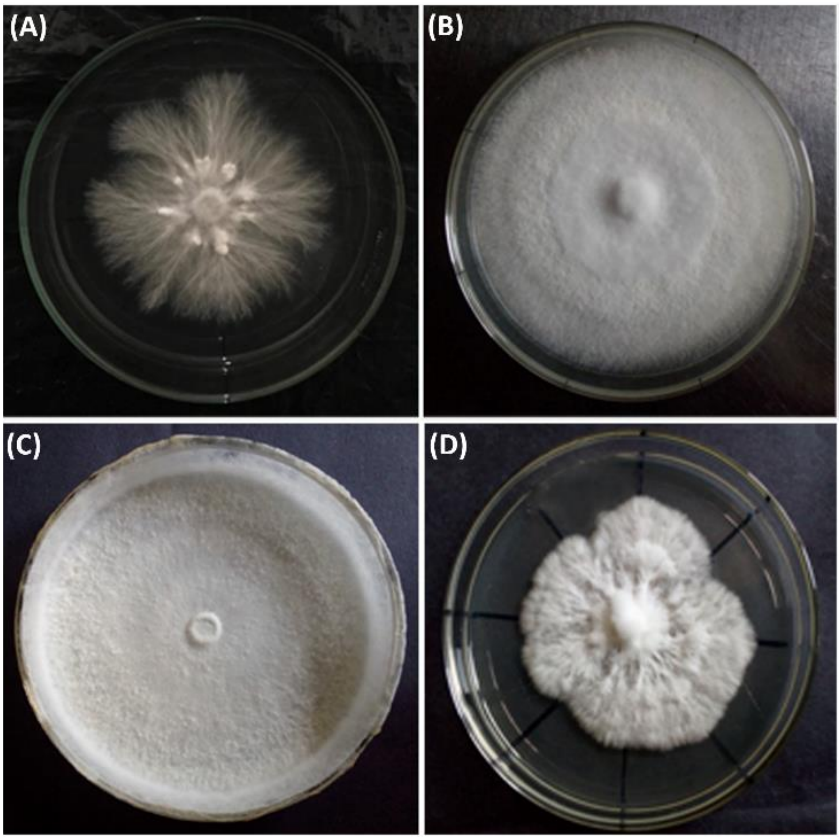

Fig. 2 – Differences in colony morphologies of the isolates and the positive control cultured on PDA. A *Pleurotus tuber-regium.* B *Lentinus squarrosulus.* C *Lentinus sajor-caju.* D *Pleurotus ostreatus.*

Table 2 Mean growth rates of the three isolates on selected culture media.

Mushroom species	**Mean growth rates in culture media (cm/day)**			
	PDA	**MEA**	**CMA**	**SEA**
P. tuber-regium	0.86 ± 0.044	0.25 ± 0.05	0.36 ± 0.04	0.62 ± 0.02
L. sajor-caju	2.06 ± 0	1.19 ± 0	1.65 ± 0.01	1.17 ± 0.01
L. squarrosulus	1.66 ± 0	0.96 ± 0	1.19 ± 0	1.05 ± 0.02
P. ostreatus	0.67 ± 0.09	0.96 ± 0.07	0.70 ± 0.10	0.41 ± 0.01

PDA = Potato Dextrose Agar, CMA = Corn Meal Agar, MEA = Malt Extract Agar, SEA = Straw Extract Agar

Growth rates in spawn media

The isolate of *L. sajor-caju* colonized well in all three spawn media types tested with an abundant mycelial density (Table 3). *Pleurotus tuber-regium* took the longest duration out of all the isolates to completely colonize all of the media tested and it showed a characteristic low mycelial density in all media. All isolates showed the highest rate of mycelial colonization in corn grain medium with *L. sajor-caju* taking 5 d, *P. tuber-regium* taking 6 d and *L. squarrosulus* taking 6 d. The control strain, *P. ostreatus* showed a similar trend with the test strains, also fully colonizing the corn medium (6 d) with a greater efficiency than the rice medium.

Table 3 The differences in mycelial density and rates of colonization, in terms of number of days taken for complete colonization of the grain medium, of the three isolates and the control on different grain spawn media at room temperature. The differences are represented semi quantitatively relative to each other based on visual comparison where "+++" is highly dense and "+" is least dense.

Mushroom species	Rice spawn 1 (0.5% gypsum)		Rice spawn 2 (2% gypsum)		Corn spawn (1.2% gypsum, 0.3% $CaCO_3$)	
	Time (d)	Mycelial density	Time (d)	Mycelial density	Time (d)	Mycelial density
P. tuber-regium	15	++	10	+	6	+
L. sajor-caju	5	+++	7	+++	5	+++
L. squarrosulus	7	+++	8	+++	6	+++
P. ostreatus	12	++	7	++	6	+++

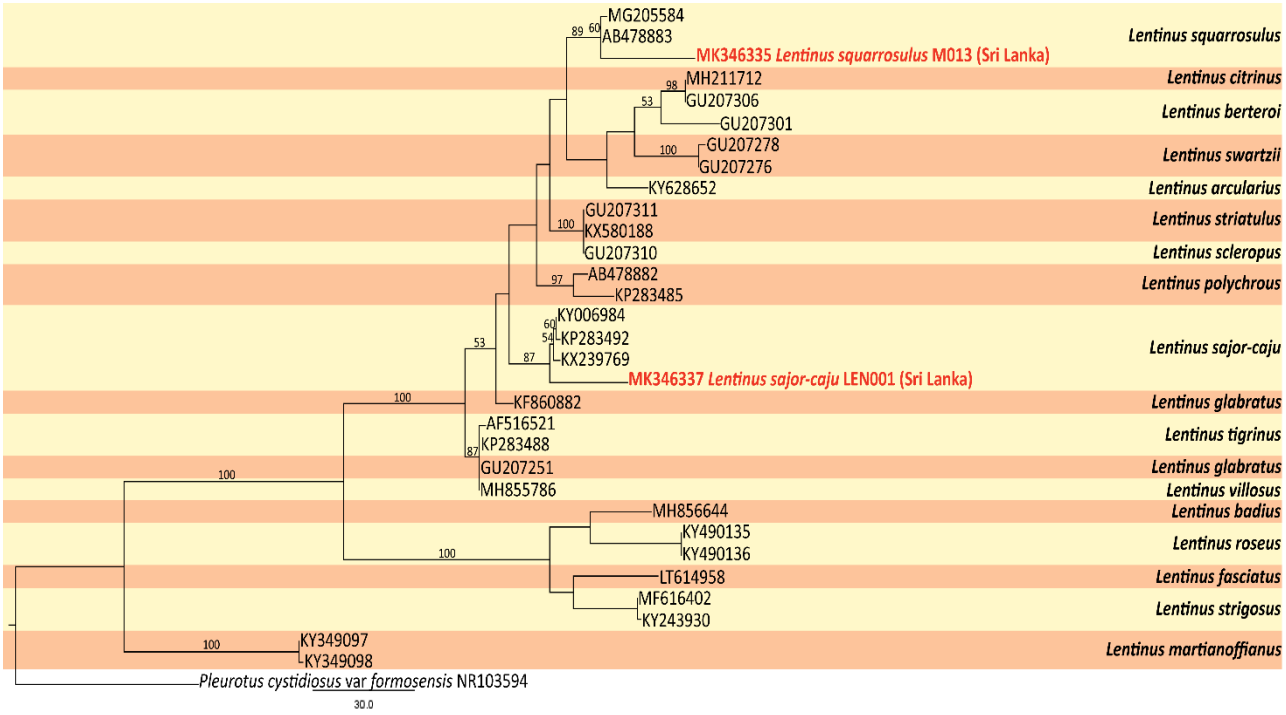

Fig. 3 – Phylogram generated for the isolates LEN001 and M013 (indicated in red) from maximum parsimony analysis based on ITS sequences. Bootstrap support values (1000 replicates) which are ≥50% are displayed above each branch. The tree is rooted with *Pleurotus cystidiosus* var *formosensis*.

Spawn run and fructification

Results obtained for the fungal colonization in rubber and mango saw dust media are summarized in Table 4. In *L. sajor-caju*, fruiting occurred only in the MSD-CS combination (Table 4) with a maximum of two flushes. Exactly three primordia appeared on each bag (total of nine) out of which only seven developed into mature fruit bodies. A mean fresh weight of 13.978 g ± 3.656 was observed for the harvest (Fig. 5) in MSD-CS. Eventhough primordia were initiated in RSD-RS and MSD-RS combinations, they disintegrated with no development into fruit bodies.

Pleurotus tuber-regium fructified in all of the substrate-spawn combinations tested. RSD-RS took the shortest time to produce primordia i.e. 69 d. Highest mean number of fruit bodies was also seen for RSD-RS but most of the initiated mushrooms went on to produce misshapen caps. The highest mean yield for the 1st flush (10.617 g ± 15.828) was recorded for RSD-CS but misshapen

caps were formed here as well with abnormally extended stalks which significantly added up to the weight (Fig. 6A, E). Bags of RSD-RS and MSD-CS combinations produced three flushes. Mean yield of the subsequent flushes gradually decreased.

Lentinus squarrosulus fructified in all of the substrate-spawn combinations tested. Both the highest number of fruit bodies and the highest mean yield for the 1st flush was observed in the RSD-RS combination (Fig. 7). Even if the mean yield of the 1st flush is the lowest, the MSD-CS combination produced the highest number of flushes i.e. four. This combination also took the least duration (49 d) for the appearance of primordia.

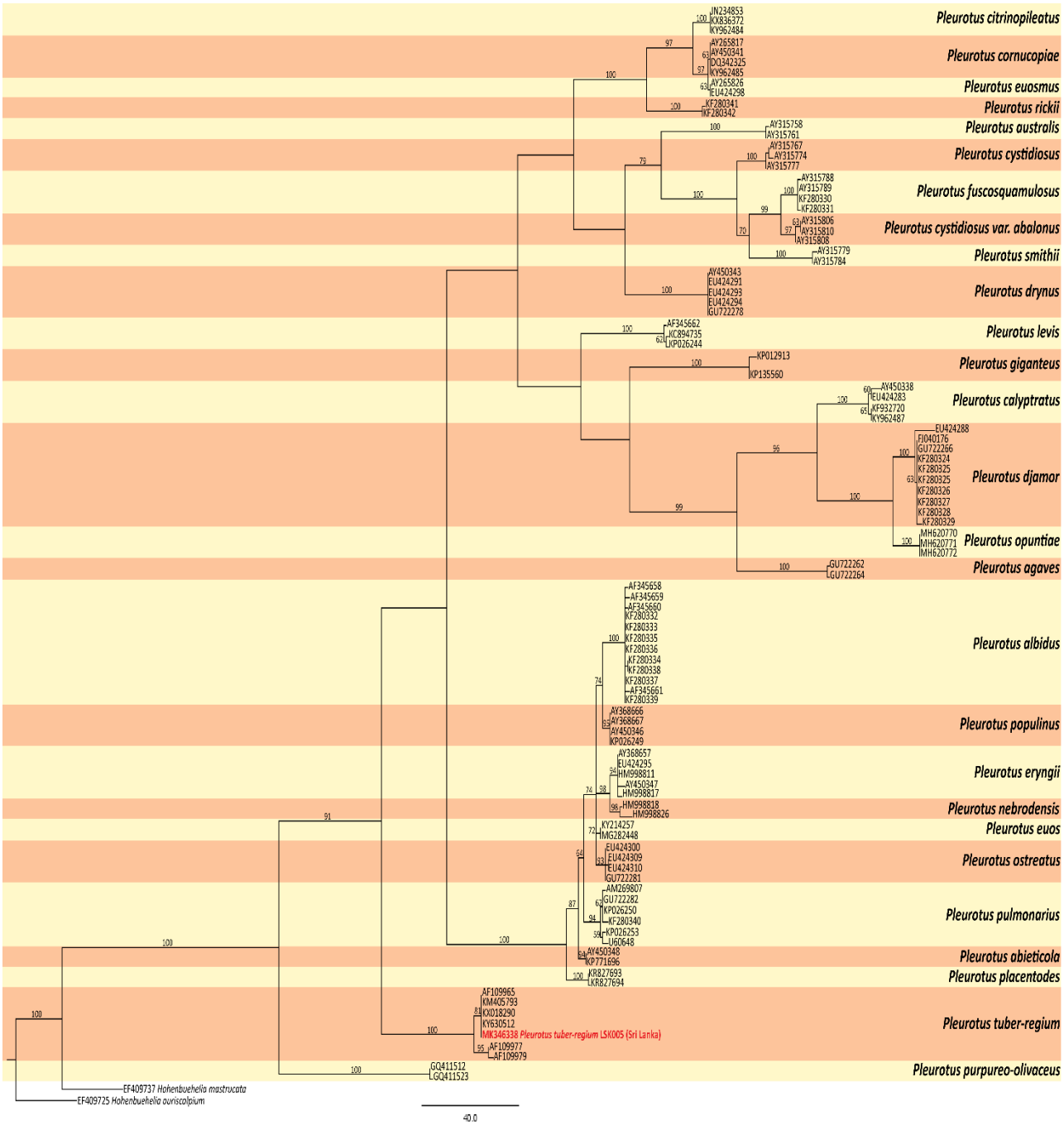

Fig. 4 – Phylogram generated for the isolate LSK005 (indicated in red) from maximum parsimony analysis based on ITS sequences. Bootstrap support values (1000 replicates) which are ≥50% are displayed above each branch. The tree is rooted with *Hohenbueheli auriscalpium* and *H. Mastrucata* (Pleurotaceae).

Fig. 5 – Phenotypic characteristics of *Lentinus sajor-caju* isolate LEN001 harvest. A Fruiting in substrate bags with three mature fruit bodies. B Surface view of the basidiocarp displaying the white-cream, dry pileus. C Young fruit body developing from a primordia (Annulus developed from the stipe around the primordial pileus is clearly visible). D Developing young fruit body. E Bottom view of a fruit body showing the decurrent formation of gills.

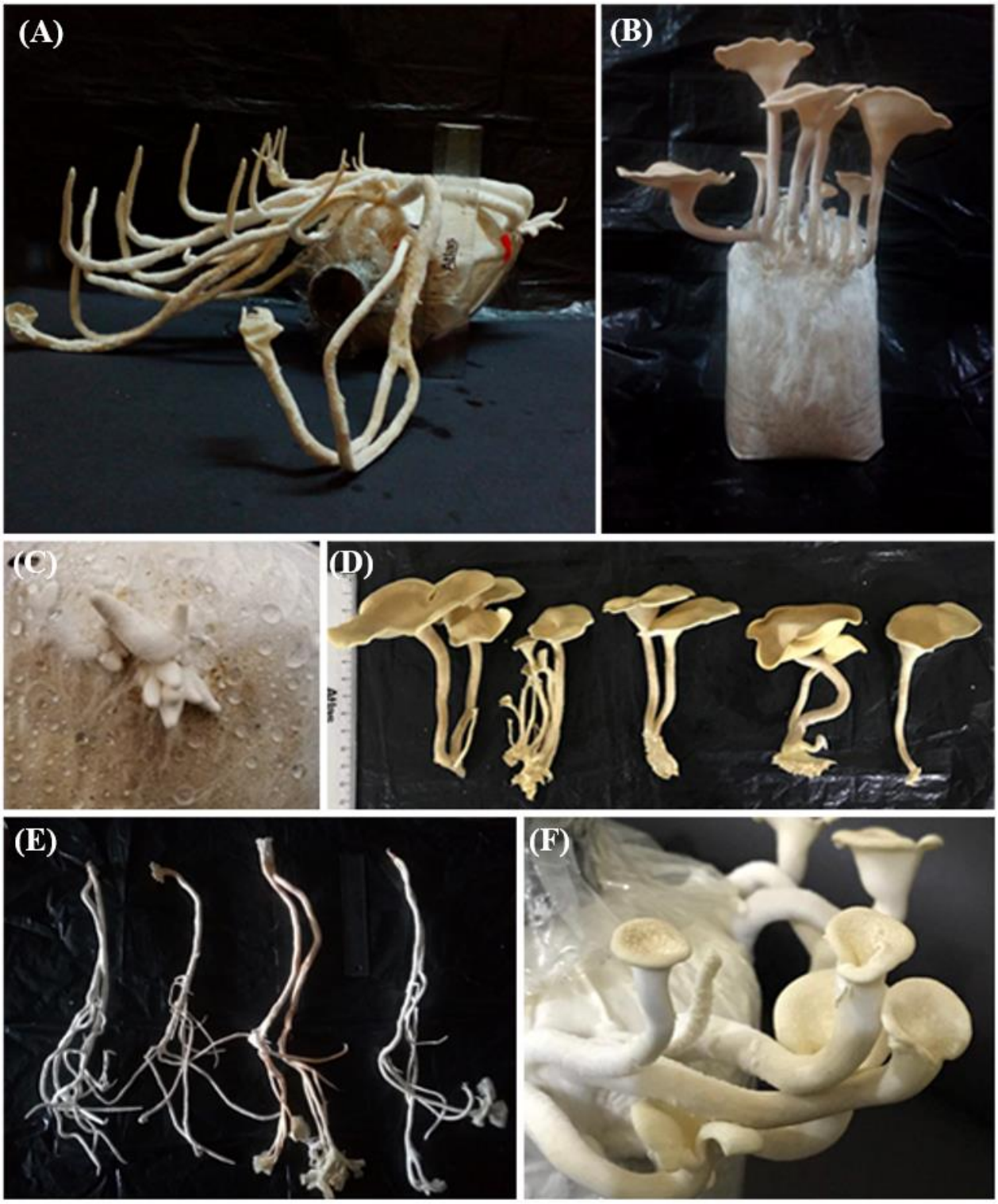

Fig. 6 – Phenotypic characteristics of *Pleurotus tuber-regium* isolate LSK005 harvest. A Fruiting on RSD-CS substrate with long stalk lengths. B Fruiting on MSD-CS substrate. C Young fruit bodies emerging from the sclerotia initials in substrate bags. D 1st flush of one replicate bag of MSD-CS combination. E 1st flush of one replicate bag of RSD-CS combination. F Young and developing fruit bodies of MSD-CS combination show the surface features of the developing caps.

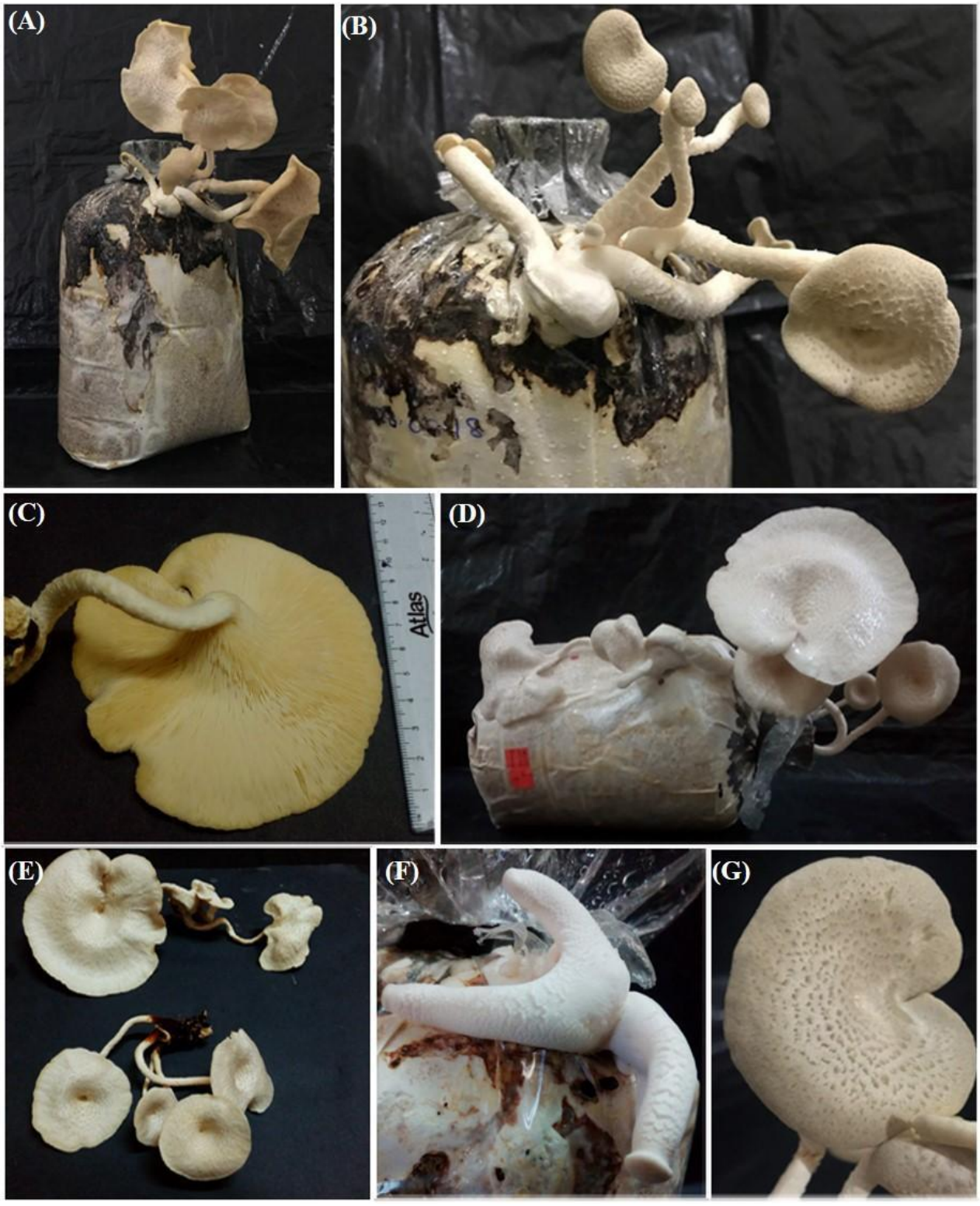

Fig. 7 – Phenotypic characteristics of *Lentinus squarrosulus* isolate M013 harvest. A, B 1st flush on MSD-CS substrate-spawn combination. C Gill arrangement on the underside of the fruit body. D, E 1st flush on MSD-CS combination. F Young fruit bodies emerging from primordia. G Surface view of the fruit body clearly depicting the squamules on the pileus.

Table 4 Yield data and mycelial colonization rates of the isolates in the four substrate-spawn combinations.

	Substrate-spawn combination			
P. tuber-regium	**RSD-RS**	**RSD-CS**	**MSD-RS**	**MSD-CS**
Time for full colonization of bag (d)	37 ± 3	39 ± 0	35 ± 2	38 ± 1
Time until 1st primordia appear (d) (1st flush)	69 ± 0	74 ± 3	95 ± 0	84 ± 5
No. of fruit bodies (1st flush)	36 ± 0	30 ± 6	11 ± 0	10± 2
Mean yield (g/bag) (1st flush)	32.560 ± 0	110.617 ± 15.828	23.250 ± 0	24.400 ± 8.877
Moisture content (%)	68.0	87.3	63.9	64.0
L. sajor caju	**RSD-RS**	**RSD-CS**	**MSD-RS**	**MSD-CS**
Time for full colonization of bag (d)	22 ± 5	27 ± 1	22 ± 1	19 ± 1
Time until 1st primordia appear (d) (1st flush)	-	-	-	88 ± 7
No. of fruit bodies (1st flush)	-	-	-	2 ± 1
Mean yield (g/bag) (1st flush)	-	-	-	13.978 ± 3.656
Moisture content (%)	-	-	-	83.8
L. squarrosulus	**RSD-RS**	**RSD-CS**	**MSD-RS**	**MSD-CS**
Time for full colonization of bag (d)	27 ± 2	31 ± 3	23 ± 0	19 ± 0
Time until 1st primordia appear (d) (1st flush)	85 ± 0	67 ± 0	84 ± 5	49 ± 1
No. of fruit bodies (1st flush)	9 ± 0	6 ± 0	5 ± 3	4 ± 2
Mean yield (g/bag) (1st flush)	51.350 ± 0	44.134 ± 0	24.847 ± 21.37	18.226 ± 6.621
Moisture content (%)	85.2	80.9	89.1	83.7
P. ostreatus	**RSD-RS**	**RSD-CS**	**MSD-RS**	**MSD-CS**
Time for full colonization of bag (d)	28 ± 1	33 ± 1	33 ± 0	41 ± 0
Time until 1st primordia appear (d) (1st flush)	68 ± 4	76 ± 3	65 ± 16	76 ± 0
No. of fruit bodies (1st flush)	8 ± 2	4 ± 4	6 ± 5	7 ± 4
Mean yield (g/bag) (1st flush)	48.583 ± 41.787	25.920 ± 11.116	56.098 ± 37.722	63.784 ± 30.572
Moisture content (%)	88.5	85.7	91.1	90.9

RSD-RS = rubber sawdust-rice spawn, RSD-CS = rubber sawdust-corn spawn, MSD-RS = mango sawdust-rice spawn, MSD-CS = mango sawdust-corn spawn

Discussion

All three mushroom species studied were wood decaying saprobic fungi commonly encountered in Sri Lanka. Specimen of *L. sajor-caju* was collected from a dying tree stump of *Tabebuia rosea* tree while the specimens of both *P. tuber-regium* and *L. squarrosulus* were collected from decaying mango wood substrates in home gardens. All three mushroom species were known as edible wild species according to available literature as well as the traditional knowledge of local communities (Braun et al. 2000, Lau & Abdullah 2017, Mortimer et al. 2014).

The results of the molecular phylogenetic analyses reveal the placement of LEN001 and M013 isolates within the genus *Lentinus* and LSK005 under *Pleurotus.* Eventhough, the two isolates LEN001 and M013 are clustered along with their respective reference sequences, both of them can be considered as genetically diverse cryptic strains compared to the rest in each clade. However, both M013 and LEN001 showed highly similar morphological characteristics to *L. squarrosulus* and *L. sajor-caju* respectively.

Based on the growth rate studies of the three mushrooms, *L. sajor-caju* took 4-d to completely cover the PDA plate, while both MEA and SEA showed comparatively slower rates. *Pleurotus tuber-regium* showed comparatively low growth rates in all media tested in this study. Appearance of *P. tuber-regium* primordia was observed only in PDA. The primordial formation on media plates can be determined by several factors including the chemical composition of the agar medium as well as by the quantity and the composition of the vegetative mycelium (Wood 1976). A rich mycelial growth on a complex nutrient medium like PDA might have favoured this primordia formation. Isikhuemhen et al. (1999) describe the appearance of small, white clumps of mycelia/primordia/sporophore initials that are associated with a characteristic oozing of a colourless liquid in wheat straw substrate bags. This observation is similar to the ooze observed along with the *in vitro* primordia in this study. This ooze is found to be potentially linked with a nematicidal activity characteristic to the fungus (Anyalewechi et al. 2009). Observations of the growth of *L. squarrosulus* in SEA agree with the results of Mensah & Obodai (2014) in terms of concentric growth rings in rice straw agar and development of a brown color pigmentation in older cultures due to possible enzymatic browning or polyphenolic compounds (Mensah & Obodai 2014).

Since all three isolates showed the highest mycelial colonization rate and highest mycelial density in corn grain, it can be suggested as an optimum medium for the grain spawn preparation of wild mushroom varieties intended for cultivation. Corn has been used as a spawn medium around the world but its use has not been recorded in Sri Lanka as a spawn medium previously. The results showed that the corn spawn media can be used in the successful cultivation of the Sri Lankan *Lentinus* and *Pleurotus* mushroom species. Gypsum amendment intends to supply a nonstick effect to the grains to prevent clumping and to improve aeration. $CaCO_3$ helps to maintain the pH at a desired level (usually around pH 7). Fast-growing, rapidly decomposing hardwoods are generally better for the spawn run due to high ratios of starch-enriched sapwood to heartwood which encourage rapid initial growth, resulting in full colonization in a short time (Stamets 2000). Both rubber and mango sawdust fall under these criteria and are also easily available at low costs in Sri Lanka as a waste material of the wood processing industry. According to the results, the best substrate-spawn combination for *L. sajor-caju* and *L. squarrosulus* is MSD-CS (Table 4), taking approximately 20 d only for complete coverage of the bags while for *P. tuber-regium*, it was MSD-RS taking around 35 d. It took *P. ostreatus* a minimum duration of approximately 28 d to completely colonize the bags in RSD-RS combination. Comparatively, out of the three strains, only *P. tuber-regium* has taken more time than *P. ostreatus* for the spawn run.

Lentinus sajor-caju fruit bodies took 3–4 d to develop into maturity which is in agreement with Hussein et al. (2016). In the Hussein et al. (2016) study, primordia have appeared after 42–59 d and in Ragunathan & Swaminathan (2003) study, after 21–30 d of spawning, whereas it took 88 d to produce primordia in this cultivation trial. The possible reasons for the degradation of the primordia that were formed on RSD-RS and MSD-RS can be stated as the uncontrolled environmental parameters and especially the drying up of the structures. Substrate bags of most of the combinations got severely contaminated creating no possibility to fruit at all and were discarded as a result.

The reasons for the deformities in *P. tuber-regium* fruit bodies, as also stated by Stamets (2000), can be given as lack of light, excessive relative humidity and excessive temperature, all of which couldn't be effectively regulated within the cropping chamber. *P. tuber-regium* is a common sclerotia producing fungus and studies like Fasidi & Ekuere (1993) were focused mainly on sclerotia yield. But all the strains are not essentially positive for the production of sclerotia because

as Isikhuemhen et al. (1999) describe, some Australian and Indonesian strains have directly formed fruit bodies without sclerotia which is also true for the strain used in this study. In another study by Jonathan et al. (2008), an average of 15 fruit bodies in 1st flush were obtained in composted *Khaya ivorensis* (African mahogany) sawdust. Comparing with the *Pleurotus ostreatus* positive culture, the RSD-CS combination produced a higher mean yield (1st flush) than that of the positive control.

Within the observed cropping duration, MSD-CS combination producing the highest number of flushes for *L. squarrosulus* within a short time is important when it comes to commercial level cultivation of this strain as this will help to increase the return to the farmers. Oghenekaro et al. (2009) have cultivated *L. squarrosulus* in five sawdust types of different tropical tree species and have obtained a maximum mean yield for the sawdust of *Brachystegia nigerica* (16.17 g ± 1.25) which is lower than the mean yields seen in our study (18.226 g ± 6.621 to 51.350 g ± 0). These variations may be due to strain level variations, differences in environmental parameters or the differences in the amounts of substrate chosen. The results observed in our study closely agree with the study by Ediriweera et al. (2015) who reported that mean yields as high as 54.079 g ± 3.61 were observed for rubber sawdust and 50-60 d were taken for primordia development. In the present study, primordia emerged between 50 to 84 d of spawning. Dimensions of the polypropylene bags (22 cm × 12.5 cm), ratios and the types of chemicals used for media supplementation, relative humidity during incubation and mode of inoculation of substrate bags used in the Ediriweera et al. (2015) study are different from the conditions used in this study. These variations of conditions can be held accountable for the differences in yield values of *L. squarrosulus* obtained in the two studies.

Generally, in *Pleurotus ostreatus*, the moisture content was found to be around 80–95 g/100 g (Valverde et al. 2015). The calculated moisture content values for the mushrooms considered here ranged between 69.1%–89.1%, which cannot be stated as very high. An inherent dryness/toughness with rapid loss of moisture could be observed for *P. tuber-regium* in RSD-RS, MSD-RS and MSD-CS which accounts for the low moisture percentages in those substrates. Comparatively lower moisture contents indicate a higher dry matter content in these mushrooms. To more accurately decide on a substrate-spawn combination that would produce the optimum yields for each of these mushroom strains, the incubation needs to be carried out in controlled environment conditions and the bags need to be monitored for longer periods of time.

According to the available published data, this is the first successful cultivation record for *L. sajor-caju* and *P. tuber-regium* strains from Sri Lanka and it shows the potential of cultivating wild *Lentinus* and *Pleurotus* mushrooms in Sri Lanka. The DNA barcodes, molecular phylogeny, along with the taxonomic diagnosis are provided in this study, with complete morphological descriptions linked with authentic strains. Therefore, the strains developed in this study are available as the first well-characterized strains of *L. sajor-caju*, *L. squarrosulus* and *P. tuber-regium* in Sri Lanka. These three strains can be successfully cultivated in an average temperature of 30 °C by providing with an optimized growth medium for culturing and an optimum growth substrate for colonization.

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