

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.708.361>

Molecular Characterization and Phenotypic Study of New *Pleurotus djamor* Isolate KKM 1

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ABSTRACT

Recently we found new *Pleurotus* sp. isolate KKM 1 that grew on wood logs. The experiments were conducted to study its morphological and phenotypic characters of mycelium and basidiocarps of *Pleurotus* isolate KKM 1 compared with other cultivated mushroom varieties such as *P. eous* var. APK1, and *P. djamor* var. MDU1 and *P. florida*. *Pleurotus* isolate KKM 1 was confirmed as *Pleurotus djamor* by ITS sequencing of rDNA region. Thus, the new *Pleurotus* sp. isolate KKM1 was named as *P. djamor* isolate KKM1. The mycelial growth of *P. djamor* isolate KKM 1 appeared loose, non-rhizomorphic type and thin mycelium whereas other cultivated mushroom varieties such as *P. djamor* var. MDU1 and *P. florida* produced compact and rhizomorphic type of mycelium. Basidiocarps of *P. djamor* isolate KKM 1 was distinct from other *Pleurotus* spp. *P. djamor* isolate KKM 1 produced fruiting bodies with rudimentary stipe or no stipe at all, whereas other tested *Pleurotus* spp. have visible stipe. Pileus diameter was the maximum in *P. djamor* isolate KKM1. *P. djamor* isolate KKM1 produced leathery type of pileus having less plectenchymatous tissue and its thickness is the minimum among the tested *Pleurotus* spp. *P. eous* var. APK1 produced pink coloured fruiting bodies whereas other species produced white colored basidiocarp. *P. djamor* isolate KKM 1 produced pileus with wavy margins/edges whereas in other *Pleurotus* spp. the margin/edges are smooth. Thus the present study shows the new *P. djamor* isolate KKM would be a potential isolate for mushroom cultivation and mushroom germplasm collection.

Keywords

Pleurotus,
Molecular
characterization,
ITS, Phenotype
analysis

Article Info

Accepted:
20 July 2018
Available Online:
10 August 2018

Introduction

Mushrooms are saprophytic fungi producing conspicuous sporocarps (fruiting bodies) and are collectively called as macrofungi. Mushroom has a large sporocarp, enough to be seen with the naked eye and can be picked

up by hand (Chang, 2012). Oyster mushrooms are a diverse group of saprotrophic fungi belonging to the genus *Pleurotus* (Kong, 2004). Oyster mushroom can grow at moderate temperatures, ranging from 20 to 30°C, and produces sporocarp at a humidity of 80–100%, on various agricultural waste

materials used as substrate. The climatic conditions and seasonal diversity is the most important factors for the cultivation of the oyster mushroom (Amin *et al.*, 2007). *Pleurotus* cultivation has recently increased due to desired taste as well as numerous nutritional and medicinal benefits. Development of new mushroom strains that can be adapted to the hot climatic conditions, higher yield potential and prolonged shelf life are the present day needs of commercial cultivation.

Giving more emphasis on development of mushroom variety having short crop duration and high biological efficiency, the present study was carried out to characterize new *Pleurotus* sp. isolate KKM1.

Materials and Methods

Isolation and culturing of the new *Pleurotus* sp. isolate KKM1

Well grown disease free sporocarps were collected and kept on a sterile tissue paper for 2-3 hours to evaporate the moisture present in the mushroom. The mushroom was surface sterilized with 70% ethyl alcohol using absorbent cotton and it was split opened longitudinally into two halves. Using a new sterilized blade, a small piece of tissue was cut from the centre of the split mushroom at the junction point of the pileus and stipe. The sterilized PDA medium was amended with 100 ppm of streptomycin sulphate to avoid bacterial contamination. When the temperature of the medium was cooled to bearable temperature (60 - 70°C), 20 ml was poured into sterile Petri plates (90mm) and allowed to solidify. Three tissue pieces, taken from the junction point of pileus and stipe, were then inoculated on the PDA medium at equidistance in triangular position and incubated at 28°C. The plates were observed daily for the growth of the fungus. All these

works were carried out under aseptic conditions. The pure culture of the *Pleurotus* sp. isolate KKM1 was maintained on PDA slants for further use in this study. The stock cultures were maintained in PDA slants for long term storage under refrigerated conditions at 4°C.

Commercially produced mushrooms cultivars viz., *P. florida*, *P. djamor* var. MDU1 and *P. eous* var. APK1 were used in this study as standard isolates for comparison. For isolation of mycelium from these three isolates, the same procedure as described above was followed. The culture of all strains was maintained in PDA slant for further studies.

Molecular characterization of *Pleurotus* sp. Isolation of total genomic DNA from *Pleurotus* sp.

Total genomic DNA was isolated from *Pleurotus* sp. as described by (Lee *et al.*, 1988) with slight modifications. About 100 mg of mycelial mat was used for the isolation of the DNA. The mycelium was dried well using the blotter paper and immersed in 95-100 per cent ethanol for 10-15 mins and then ethanol was blot-dried (Avin *et al.*, 2013). Then the mycelium was ground with equal amount of acid-washed sand (for breaking the cell wall) using sterile pestle and mortar until mycelia tissue become paste. To ground mycelium, 1 ml of 2X CTAB buffer (hexadecyl trimethyl ammonium bromide) was added and mixed well. 750 µl of the mixture was taken in 1.5 ml Eppendorf tubes and incubated at 65°C for 25-30 mins. To that, 750 µl of chloroform was added and vortexed for 5 sec, incubated for 10 mins and centrifuged at 14,000 rpm for 15 minutes. The supernatant was collected and equal amount of isopropanol was added and incubated at -20°C for 30 - 45 minutes or overnight for DNA precipitation. The samples were centrifuged at 14,000 rpm for 10 minutes to

pellet the nucleic acids and washed with 70% ice cold ethanol. Finally the pelleted DNA was dried at 37°C. Finally, the isolated DNA was re-suspended in 50 µl of sterile water and quality and quantity of the isolated DNA was confirmed by resolving the DNA in the 1% agarose gel.

ITS sequencing of *Pleurotus* spp.

A PCR reaction was carried out using Emerald Amp® GT PCR master mix using genomic DNA of *Pleurotus* spp. as a template. ITS1-5.8S-ITS2 region of the rDNA was amplified using the primers ITS1 and ITS4. The following PCR conditions were followed. Initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 sec, annealing temperature at 50°C for 30 sec and extension at 72°C for 60 sec, and a final extension at 72°C for 10 min. The PCR products were verified by electrophoresis in 1% agarose gel.

The Primers used for amplification of ITS region were

ITS1 - 5' TCCGTAGGTGAACCTGCGG 3' (forward primer)

ITS4 - 5' TCCTCCGCTTATTGATATGC3' (reverse primer)

Sequencing of ITS and identification of species of *Pleurotus*

The PCR products were purified using FavorPrep GEL/ PCR purification kit. The purified ITS product was sequenced at Eurofins genomics India Pvt. Ltd. Bangalore. Then DNA sequences, in which clear chromatogram obtained, were made in Fasta format. This was used as input sequence (Query sequence) in nucleotide blast analysis program at NCBI database. The output was retrieved from the bioinformatics analysis tool and then, the organism showing major score

from the output is considered as the closely related species to the test fungus used in the study.

Mycelial growth pattern of *Pleurotus* spp.

To study the cultural and phenotypic characters of mycelial growth of *Pleurotus* sp. isolate KKM 1 along with the standard cultures, five millimetre culture discs were cut with sterilized cork borer from advancing margins of the colonies of fungus and inoculated on PDA plates supplemented with streptomycin sulphate (100ppm). The plates were incubated at 28°C. Three replications were maintained for each temperature. Radial growth of the mycelium was recorded when anyone of the Petri plates of the treatments was completely covered by mycelium.

Morphological characterization of basidiocarp of the *Pleurotus* spp.

The following phenotypic characters were gathered on *Pleurotus* sp. isolate KKM 1 with existing cultivars viz., *P.florida*, *P. djamor* var. MDU-1 and *P. eous* var. APK 1. Information on morphological characters viz., diameter of the pileus, pileus phenotype especially its margin/edge shape, stipe length, colour of the basidiocarp, thickness of the pileus, number of gills, and number of stipe present per bunch was recorded.

Results and Discussion

Molecular characterization of *Pleurotus* sp. isolate KKM1

Identification and confirmation of *Pleurotus* sp. isolate KKM1 by molecular technique

Based on the morphological character of basidiocarp, most of the mushroom fungi are identified at genus level. Likewise, the new

mushroom used in the study was identified at genus level as *Pleurotus* sp. and named as *Pleurotus* sp. isolate KKM1. Identification of unknown species of *Pleurotus* can be done by molecular technique such as ITS1-5.8S-ITS4 sequencing analysis which is one of the commonly used molecular methods for the identification of fungus at species and subspecies level (Bruns *et al.*, 1991; Hibbett, 1992).

Genomic DNA from the *Pleurotus* isolate KKM1, was isolated by CTAB method of DNA isolation. High molecular weight band was visualized on the agarose gel (Fig. 1A). Genomic DNA was used for amplification of ITS 1-5.8S-ITS 2 rDNA sequence region using primer pair ITS 1 and ITS 4. The PCR fragments of 700 bp size fragment was visualized as single band in agarose gel stained with ethidium bromide (Fig. 1B).

ITS 1-5.8S-ITS2 PCR products were cleaned up with PCR cleaned up kit to remove the residual primers, polymerase and salts in the PCR product, the cleaned up products were sequenced at Eurofin genomic private Ltd, India. The sequence was used for DNA database search using BLAST program. The BLAST search analysis of ITS 1-5.8S-ITS2 region of *Pleurotus* isolate KKM 1 matched with that of *Pleurotus djamor* at 99 % identified in the database.

Identification using the sequences of ITS region is typically the most useful method and also this method is applicable for molecular systematics at the species levels (De Beeck *et al.*, 2014). For identification of specific genera and species, the rDNA repeat unit, consisting of the subunits 18S, 5.8S and 28S rDNA interrupted by the internal transcribed spacer (ITS) and the intergenic spacer (IGS) is employed due to their specific sequences as a target region. The advantage of ITS sequencing is the identification of any

unknown fungal isolate using the database containing the corresponding sequence of previously identified fungal species or closely related species (Schmidt *et al.*, 2012). From the new *Pleurotus* sp. isolate KKM1, ITS sequence was PCR amplified and DNA fragments of 700 bp was eluted and sequenced. The results of ITS analysis indicated that the sequence was similar to that of ITS1-5.8S-ITS2 region of *P. djamor*. Thus, this new *Pleurotus* isolate KKM1 was named as *Pleurotus djamor* isolate KKM1.

Mycelial growth phenotype of *Pleurotus* spp.

On PDA medium, mycelial growth of *P. djamor* isolate KKM1 appeared loose, non-rhizomorphic type and thin mycelium whereas, *P. djamor* var. MDU1 and *P. florida* produced compact and rhizomorphic type of mycelium because they produce more mycelial branches from one place. *P. eous* var. APK1 showed polar growth defects and that formed constricted growth with cottony fluffy mycelium (Fig. 2). Similarly, the mycelium of *P. djamor* isolate KKM1 appeared loose, non-rhizomorphic type on the spawn substrate whereas all other *Pleurotus* spp. tested including *P. eous* produced compact and rhizomorphic type of mycelium.

Among the *Pleurotus* spp. tested, *P. djamor* var MDU1 grew well and attained the maximum growth of 89.00mm on PDA medium followed by *P. florida* and *P. djamor* isolate KKM 1 with the mycelial growth of 78 and 76 mm respectively. *P. eous* var. APK1 grew slowly on PDA medium (Table 1). Mishra *et al.*, (2015) reported similar type of mycelial growth pattern in several *Pleurotus* spp. The pattern of mycelial growth in *P. citrinopileatus* was thick and fluffy, whereas, that in *P. fossulatus*, *P. flabellatus* and *P. sapidus* were slightly fluffy. *P. djamor* showed the cottony growth. In the present

also, all the tested *Pleurotus* spp. except *P. djamor* isolate KKM1 produced thick cottony mycelial growth.

Phenotypic characterization of basidiocarp of *Pleurotus* spp.

The characterization of various *Pleurotus* isolate had been attempted by many scientists from time to time. In the present study, phenotypic characters of basidiocarps of four *Pleurotus* spp. were studied. Each *Pleurotus* sp. has typical distinguishing characters for easy identification. They are described below

Stipe length

Among the four *Pleurotus* spp. tested, *P. djamor* var. MDU1 produced fruiting bodies with long stipe (54.50 mm), which was on par with *P. florida* (52.25 mm). *P. djamor* isolate KKM 1 produced fruiting bodies with rudimentary stipe or no stipe at all. *P. eous* var. APK1 produced fruiting bodies having small sized stipe (26.50mm) (Fig. 3; Table 2).

Diameter of the pileus

Pileus diameter was maximum in *P. Djamor* isolate KKM1(119.25 mm) followed by *P.eous* var. APK-1 (97.75mm) and *P djamor* var. MDU1 (91.00mm). *P. florida* had small sized pileus with the diameter 88.25 mm (Fig.

3; Table 2).

Thickness of fruiting body

Thickness of the pileus depends on the amount of plectenchymatous tissue present in the pileus. The thickness of the pileus was measured near the junction point of pileus and stipe. *P. florida* produced fruiting bodies with maximum pileus (12.62mm). This was followed by *P. djamor* var. MDU1 and *P.eous* var. APK1 having the thickness of 8.62mm and 7.87mm respectively. *P. djamor* isolate KKM1 produced leathery type of pileus having less plectenchymatous tissue and the thickness of 5.62mm (Table 2).

Margin of fruiting body

P. djamor isolate KKM1 has the pileus with wavy margin whereas *P. florida*, *P. djamor* var. MDU1. And *P.eous* var. APK1 have pileus with smooth margins (Fig. 3; Table 2).

Colour of fruiting body

P. eous var. APK1 produced pink coloured fruiting bodies. *P. florida* produced creamy white coloured fruiting bodies. Whereas *P. djamor* isolate KKM1 and *P. djamor* var. MDU1 produced white coloured fruiting bodies (Fig. 3; Table 2).

Table.1 Effect of temperature on the mycelial growth of *Pleurotus* spp. on PDA medium

<i>Pleurotus</i> spp.	Mycelial growth (mm)*	
	4 th day	7 th day
<i>P. eous</i> var. APK-1	23.00 ^b	34.75 ^c
<i>P. djamor</i> var. MDU-1	62.50 ^a	89.00 ^a
<i>P. florida</i>	60.50 ^a	78.25 ^b
<i>P. djamor</i> isolate KKM 1	59.25 ^a	76.25 ^b

* Mean of four replications

The treatment means are compared using Duncan Multiple Range Test (DMRT).

In a column, mean values followed by a common letter (s) are not significantly different (P = 0.05).

Table.2 Phenotypic characterization of basidiocarp of *P.djamor* isolate KKM 1 and other *Pleurotus* spp

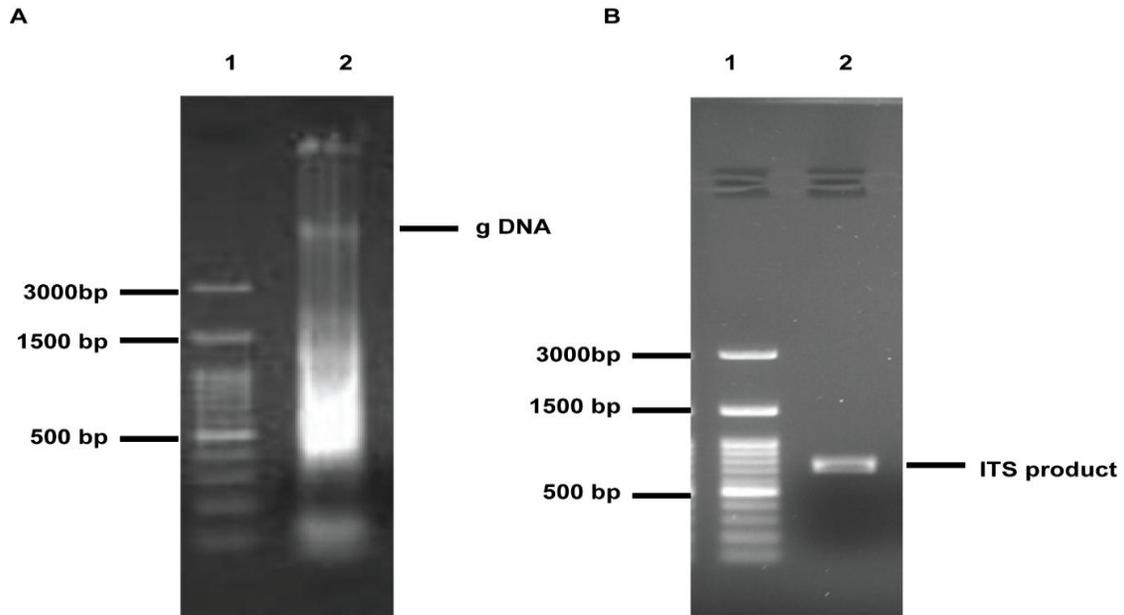
<i>Pleurotus</i> spp.	Diameter of the pileus(mm)*			Length of stripe(mm)*			appearance of pileus margin	Thickness of pileus (mm)*	Number of gills/cm ²	Colour of fruiting body
	Primordial stage	Mature stage	Harvesting stage	Primordial stage	Mature stage	Harvesting stage				
<i>P.eous</i> var.APK-1	11.25 ^b	57.75 ^c	97.75 ^b	12.35 ^a	21.75 ^c	26.50 ^c	Smooth	7.87 ^c	21.00 ^b	Pinkish
<i>P.florida</i>	5.25 ^c	86.50 ^a	88.25 ^c	10.25 ^{ab}	49.75 ^a	52.25 ^a	Smooth	12.62^a	19.50 ^c	Creamy white
<i>P.djamor</i> var. MDU-1	16.50 ^a	55.25 ^b	91.00 ^b	14.50 ^a	43.00 ^b	54.50^a	Smooth	8.62 ^b	11.50 ^d	White
<i>P. djamor</i> isolate KKM 1	15.25 ^a	80.75 ^b	119.25^a	5.55 ^b	11.50 ^d	15.12 ^b	Wavy and broken	5.62 ^d	23.50^a	White

*Mean of four observations

Treatment means are compared using Duncan multiple range test(DMRT).

In a column, mean values followed by a common letter(s) are not significantly different(P=0.05)

Figure.1 Molecular characterization new *Pleurotus* sp. isolate KKM1



A). Isolation of genomic DNA from *Pleurotus* sp. isolate KKM1. Lane 1: Lambda ladder
Lane 2: gDNA of *Pleurotus* isolate KKM
B) Amplification of ITS1-5.8S-ITS2 region from the genomic DNA of *Pleurotus* sp. isolate KKM1. Lane 1: 100 bp ladder and 1 and Lane 2:ITS of *Pleurotus* isolate KKM 1

Figure.2 Mycelial growth pattern of *Pleurotus* spp. on PDA medium

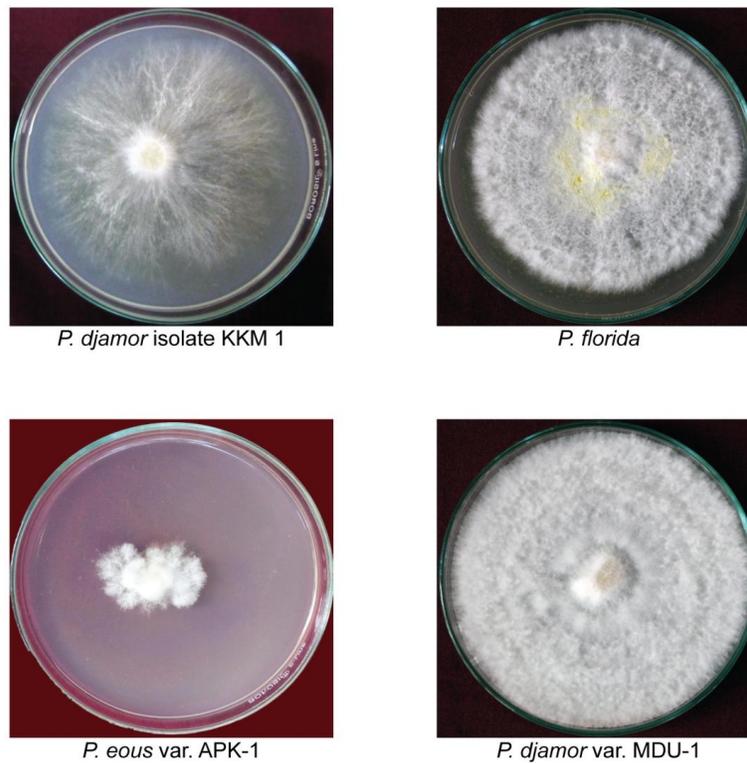
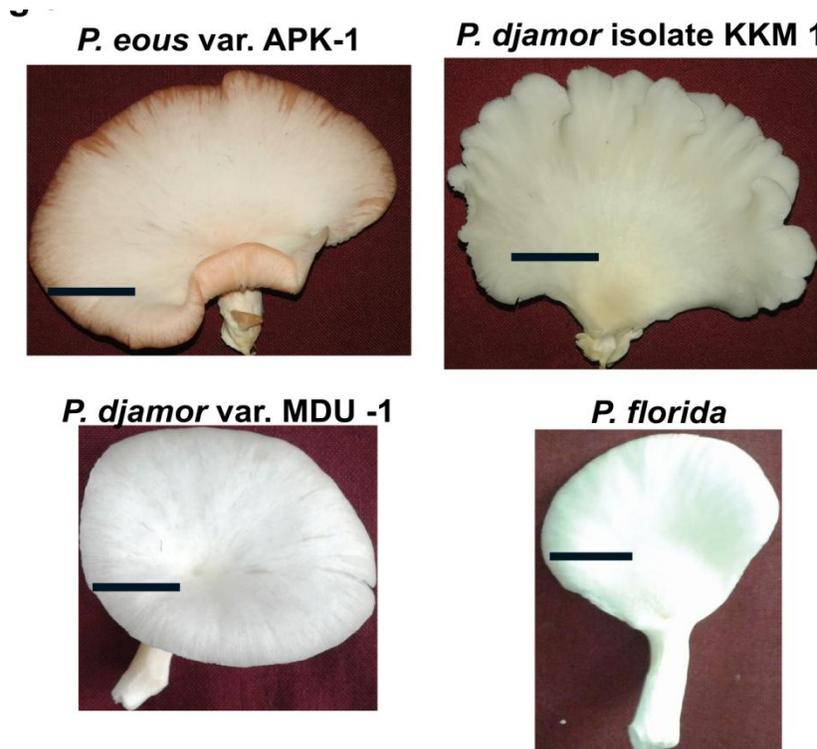


Figure.3 Phenotypic characterization of basidiocarps of *Pleurotus* spp



Number of gills

Generally *P. djamor* isolate KKM1 had thick cluster of gills at the under surface of pileus by recording 21 gills per cm² area. This was followed by *P. eous* var. APK1 which had 21gills /cm². *P. djamor* var. MDU-1 had the least number of gills (11.50 gills/cm²) (Table 2).

Mostly, the pileus of several *Pleurotus* spp. is white in color. The pileus of *P. djamor* isolate KKM1, *P. djamor* var. MDU1, and *P. florida* is white in color. The other commercial cultivar, *P. eous* var. APK1 has large pink colored pileus and small stipe. However, some studies reported that *P. djamor* species are pink in color Mishra *et al.*, (2015). Diameter of the pileus usually ranges between 70 to 130 mm. In the present study also the maximum pileus diameter was recorded in *P. djamor* isolate KKM1 with 119 mm and minimum in *P. florida* with the diameter of

88.25 mm. Among the various *Pleurotus* spp., tested, *P. flabellatus* showed the maximum pileus length of 139 mm followed by *P. ostreatus* (132mm) and minimum pileus diameter was observed in *P. sajorcaju* with 54 mm (Mishra *et al.*, 2015). Shukla and Jaitly (2011) characterized the seven different *Pleurotus* species based on the five different morphological traits such as stipe length (cm), cap diameter (cm), margin of fruit body, peripheral architecture of the pileus, colour of fruit body, total yield (kg), carbohydrate content (%) and protein content (%). Out of seven *Pleurotus* spp., five species were named as follows *P. citrinopileatus*, *P. djamor*, *P. florida*, *H. ulmarius* and *P. sajorcaju*. They also observed great diversity on morphological characters among all the five species of *Pleurotus*.

Usually, many *Pleurotus* spp. produce smooth pileus with long stipe. But *P. djamor* isolate KKM1 has large white colour pileus having

wavy margin and has small or rudimentary stipe. Thus, it is concluded that this new *P. djamor* isolate KKM1 has typical phenotypic features and characteristic mycelial pattern of loose non-rhizomorphic mycelial characters that distinguishes it from other *Pleurotus* spp. and this *P. djamor* isolate KKM1 could be used as new culture in mushroom germplasm collection and mushroom cultivation.

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How to cite this article:

Praveen, T., R. Reihana, V.K. Parthiban and Ramamoorthy, V. 2018. Molecular Characterization and Phenotypic Study of New *Pleurotus djamor* Isolate KKM 1. *Int.J.Curr.Microbiol.App.Sci*. 7(08): 3574-3582. doi: <https://doi.org/10.20546/ijcmas.2018.708.361>