



ISOLATING THE MONOKARYON COLLECTION OF *Pleurotus* spp.

Tran Thi Ngoc My^{1,*}, Ho Bao Thuy Quyen², Pham Nguyen Duc Hoang³

¹Domesco Medical Import Export Joint Stock Corporation, 66 Highway 30, Cao Lanh City, Dong Thap Province

²Ho Chi Minh City Open University, 97 Vo Van Tan Street, District 3, Ho Chi Minh City

³Biotechnology Center of Ho Chi Minh City, 2374 Highway 1, District 12, Ho Chi Minh City

*Email: ngocmy0018116@gmail.com

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ABSTRACT

Pleurotus spp. is one of the most important cultivated mushrooms in the world by their nutrition and medicinal property. In Vietnam, although many *Pleurotus* species are popularly cultivated, but most of them are imported. These strains are easily degenerated when they are cultivated in large scale, because they are difficult to adapt to the environment of Vietnam. This research aims to construct the monokaryon collection of *Pleurotus* spp. with details of taxonomy, genetic diversity, cultivating traits, monokaryotic mating types focused on *Pleurotus* strains with the high yield, genetic stable and adaptability to Vietnam environment. 09 *Pleurotus* strains were collected in South Vietnam and signed PL1 to PL9. Identification by morphology and phylogenetic analysis based on ITS sequences showed that PL1 is *P. citrinopileatus*; PL2, PL6 and PL9 are *P. ostreatus*; PL3 is *P. cystidiosus*; PL4 and PL8 are *P. pulmonarius*; PL5 is *P. cornucopiae*; PL7 is *P. incarnatus*. AFLP analyses indicated a wide genetic diversity of the collected strains with the similar coefficient 52 – 90 %. Pair of PL2 and PL9 is the most closed genetic distance and pair of PL6 and PL7 is the furthest genetic distance. PL4, PL8 and PL1 adapted well to Vietnam environment. PL2, PL5, PL6 and PL9 are cold strains and formed fruiting bodies slowly. PL3 had the highest biological efficiency, but the longest harvesting period (2.5 – 3 months). Except PL7, the biological efficiencies of other strains are over 50 %. 120 monokaryotic isolates of PL1, PL2 and PL8 are collected and determined the mating type.

Keywords: *Pleurotus*, ITS, AFLP, monokaryon, compatible.

1. INTRODUCTION

In Viet Nam, although many *Pleurotus* species are popularly cultivated, but most of them are imported. These strains are easily degenerated when they are cultivated in large scale, because they are difficult to adapt to the environment of Viet Nam. For developing the cultivation of *Pleurotus* in Viet Nam, we construct the monokaryon collection of *Pleurotus* spp. with necessary information (taxonomy, genetic diversity, cultivating traits, monokaryotic mating

types) for the breeding programs to create *Pleurotus* strains with the high yield, genetic stable and adaptability to Viet Nam environment.

Pleurotus genus belongs to highly evolved class of Basidiomycota and most of these *Pleurotus* species have sexuality pattern of bifactorial heterothallism [1 – 4]. The heterothallism is regulated by two unlinked mating type factors (genes A and B). The segregation of mating type alleles in heterokaryon after meiosis follows the Mendelian pattern with two independent genes in the ratio of 1:1:1:1. The single spores germinates to give rise a homokaryotic mycelium. All of the combinations between homokaryons with heteroallelic conditions at both the loci give rise to compatible reactions and further of formation of fruit bodies. Compatible pairings of homokaryons are distinguished from incompatible pairings on presence or absent of clamp connection. Basing on this feature, homokaryons are collected and used for determining their mating type [3, 5].

Until now, evolutionary relationships of *Pleurotus* species are not well understood and many taxonomic problems exist in this group [4]. This condition is caused by their distributing all the world and excessive diversity in their morphology [4]. Besides, the development of *Pleurotus* cultivation also is a reason that makes classification complicated [6]. Many new *Pleurotus* species were cultivated and commercialized with the mistaken names [7]. The modern trend of mushroom classification is combination of morphology and molecular analyses. Morphology is still main proof and molecular information demonstrates result of classification again. ITS (Internal transcribed spacers) sequence is accepted as a fungi barcode and it also is the most plentiful data in GenBank [8]. So, in this research, *Pleurotus* species are classified by combination of morphology and ITS analyses.

Evolutionary history shows that the large diversity population survived and developed more than small diversity one. In breeding program, analysis diversity of strain collection is a necessary part. The further relationship genetic individuals are collected to breed together because of heterosis. In this research, AFLP (Amplified Fragment Length Polymorphism) technology is used to evaluate diversity of the *Pleurotus* collection. AFLP was developed by Vos et al. [9] and became one of the most popular methods evaluating genetic diversity. DNA fingerprint is made by cutting DNA genome by restricted enzymes and amplifying by PCR [9].

2. MATERIALS AND METHODS

2.1. Fungal samples

Total 9 *Pleurotus* strains were collected at 3 different areas in Southern Vietnam such as: PL1, PL2, PL5 strains from Cu Chi District, Ho Chi Minh City; PL6, PL9 strains from Phi Nom District, Lam Dong Province; PL3, PL4, PL8 strains from Dong Thap Province. All of them are commercial strains in mushroom farms.

2.2. Identification

For morphological identification, fresh basidiocarps (fruit-bodies) were used for identification based on Largent's guiding [10, 11].

The phylogenetic analysis was conducted based on ITS region. Firstly, total nuclear DNA was extracted from fresh tissue of basidiocarps by CTAB buffer (Hexadecyltrimethylammonium bromide) following Saghai-Marooof et al. [12]. ITS regions were amplified by DreamTaq DNA

Polymerase (ThermoFisher Scientific, USA) following manual of producer with pair primer ITS 4 [13] (GCATATCAATAAGCGGAGGA) and ITS5 [13] (GGAAGTAAAAGTCGTAACAAGG) (Biobasic, Canada). The amplified products were purified by MEGAquick Spin kit (iNtRon Biotechnology, Korea) and sequenced at MacroGen (Korea) with the same primers. DNA sequences were proofread to remove ambiguous signals by ATGC software (Genetyx, Japan). The sequences were then blasted on GenBank (NCBI, USA). Data set for phylogenetic analysis were established by combining above *Pleurotus* sequences and the other retrieved from GenBank that had the highest Max score. Two sequences KU355358.1 (*Hohenbuehelia tremula*), AY265835.1 (*Hohenbuehelia grisea*) from Genbank (NCBI, USA) were used as outgroup. The data set was aligned, made equal at end sides and constructed phylogenetic trees by MEGA 6.06 (Molecular Evolutionary Genetics Analysis, Free software, KurmaLab). The maximum likelihood (ML) tree was inferred based on quartet puzzling algorithm with the options of 1000 puzzling steps, model of substitution HKY [14].

2.3. Genetic diversity analysis by AFLP

The process is based on protocol of Pawlik et al. [15]. Firstly, total nuclear DNA was purified by treating with Ambion RNase A (ThermoFisher Scientific, USA) following producer's manual. The purified products were digested and reformed double strands by FastDigest *PstI* kit (ThermoFisher Scientific, USA). The digested-double strands DNA products were ligated with *PstI* adapter (Merging *PstIAF*- CTCGTAGACTGCGTACATGCA with *PstIAR*- TGTACGCAGTCTAC) [15] by Quick-Stick Ligase (Bioline, USA) following producer's manual. The final products were used as templates for both Non-selection PCR reaction and Selective PCR reaction.

Non-selection PCR reaction was performed to check digestion and ligation reactions by JumpStart REDTaq DNA polymerase (ThermoFisher Scientific, USA) and *PstIAF* as primer. The samples which had amplified products were chosen for selective PCR reactions. The samples which had not amplified products were procedured again until getting amplified products.

Selective PCR reactions were performed by JumpStart REDTaq DNA polymerase (ThermoFisher Scientific, USA) and 4 primers such as *PstIG* (GACTGCGTACATGCAGG), *PstIGC* (GACTGCGTACATGCAGGC), *PstIAAG* (GACTGCGTACATGCAGAAG) and *PstICAA* (GACTGCGTACATGCAGCAA). The amplified products were revealed by electrophoresis at 100 V in 3 hours. The procedure were repeated until having 3 continuously similar result replicates.

The bands that occurred in electrophoresis were manually scored as binary data for the presence or absence of fragments. This binary information was used to calculate Jaccard's pairwise similarity coefficient as implemented in the program NTSYSpc 2.1 (Exeter Software, USA). The unweighted pair group method with arithmetic averages (UPMA) generated from DNA band patterns using Nei and Li correlated coefficient [16].

2.4. Cultivating traits examination

Pleurotus strains were isolated and cultured on Modified Mushroom Complete Medium (MCM) Agar (Dextrose 20 g, peptone 2 g, yeast extract 2 g, thiamine HCl 12 µg, MgSO₄ 0.5 g, K₂HPO₄ 1 g, KH₂PO₄ 0.46 g, agar 15 g, distilled water 1000 ml and adjusted pH 6.5) [2]. Then, mycelia were transferred to spawn medium (Rice 100 g, rice bran 2 g, Gypsum 1 g and CaCO₃ 1

g and boiled together in 30 mins). Finally, inocula from spawn medium were inoculated into the fruiting media (Rubber sawdust 100 g, rice bran 10 g, Gypsum 1 g, CaCO₃ 1 g, MgSO₄ 0.2 g and control moisture at 60-65 %). Each 1.2 kg substrate was contained in the polyethylene bags, size 20 × 12 cm. Mushrooms were harvested when the mushroom cap surface was flat to slightly up-rolled at the cap margins. The harvested fruiting bodies in each bag were counted and weighed. The harvest stopped at the time each bag's weight remaining about 400 g.

The cultivating traits examination based on 3 parameters: the Mycelial Growth Rate (MGR), Fruit-body Forming Period (FFP) and Biological Efficiency (BE). Each *Pleurotus* strain was examination at scale 10 bags × 3 times. MGR (mm/day) = Height of bag (mm)/Total time for mycelia colonize all substrate (day) [17]. FFB (days) is total time from the fully mycelial colonized all bags until the fruit-body appearing. BE (%) = (Total weight of harvested fresh mushroom per bag (g)/Weight of dry substrate per bag (g)) × 100 [18]. Differences between the means of individual groups were assessed by one-way ANOVA, Tukey-Kramer Test.

2.5. Monokaryon collecting and determination

Basing on result of cultivating traits examination, the 3 best *Pleurotus* strains were chosen to collect and determine monokaryotic isolates.

Spores of each strains was collected from fresh basidiocarps and diluted by distilled water until concentration of 50-100 spores/ml. 0.5 ml diluted solution was spread out on plain agar and incubated at 26 – 28 °C. Each geminating spores were picked up by a needle and cultivated on Modified Minimum Medium (MM) Agar (Dextrose 20 g, L-asparagine 1 g, Thiamine HCl 12 µg, MgSO₄ 0.5 g, K₂HPO₄ 1 g, KH₂PO₄ 0.46 g, agar 15 g, distilled water 1000 ml and adjusted pH at 6.5).

40 single spore isolates were obtained for each *Pleurotus* strain. The single spore isolates of the same strain were hybridized together randomly to determine their mating types. Two φ 3 mm mycelial agar disks were cut out from seven days old monokaryotic isolates and placed at two opposite ends on MM Agar. The paired isolates were incubated at 26 – 28 °C in 10 days for mating. A small inoculum was taken from the meeting point of the paired monokaryons and observed the presence or absence of clamp connections on microscope (40X).

3. RESULTS AND DISCUSSIONS

3.1. Morphological identification

3.1.1. PL1 strain (Figure 1)

Pilei are rounded, 1 – 6.4 cm broad, plano-depressed, fleshy, bright yellow, dry, tomentous at center, glabrescent at least, margin inflexed and entire. Context is white and yellow near the epiderm, unchanging when bruised, thin, mild and inodorous to faint fragrant. Lamellae are white, crowded, decurrent. Stipe is eccentric, cylindrical, 1 – 5 cm length, 2 – 8 mm thick, white, solid, fleshy to fibrous, tomentous. Hyphal system is dimitic, with septa and clamp connection. Spore print is whitish. Spore (6.8 – 9.5 × 2.2 – 3.5 µm) are smooth, cylindrical, hyaline. Basidia are clavate, 25 – 32 × 4 – 8 µm, hyaline. Basidiole are ampule 22 – 32 × 4 – 5 µm. Gill trama are subparallel.

All macroscopic and microscopic features are similar with *P. citrinopileatus* [19]. There are also some different features of PL1 strain with strain in description of Bi et al. [19]. PL1

strain had dimittic hyphal system and many ampule basidioles, but Bi's strain had monomittic hyphal system and not mention about basidiole. However, description of Erast noted that this species with dimittic hyphal system and also not mention about basidiole [20]. So, according to morphology, PL1 strain belong to *P. citrinopileatus*.

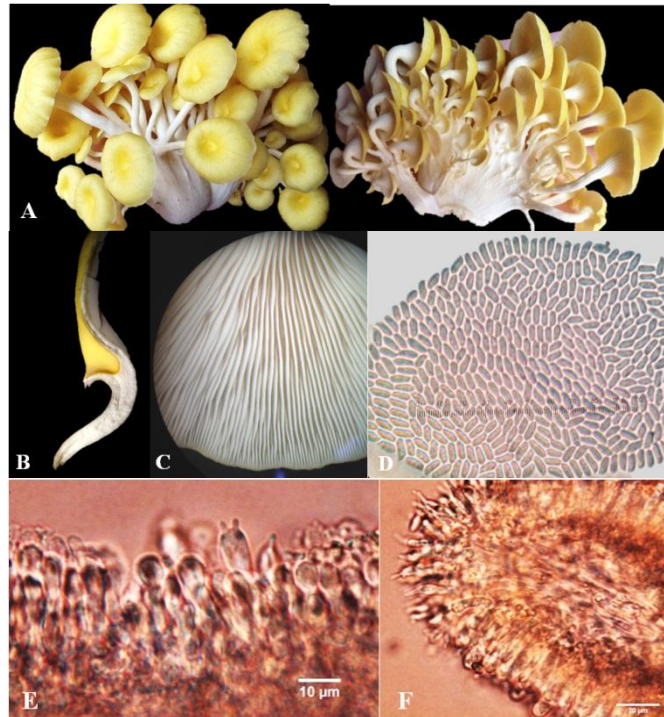


Figure 1. Morphology of PL1 strain.

(A)- Basidiocarp in cluster; (B) – Radial section of basidiocarp; (C) - Lamellae ; (D) – Spores;
(E) -Basidia ; (F) – Hymenium with the ampule basidioles.

3.1.2. PL2, PL6, PL9 strain (Figure 2)

Pileus PL2 (60 - 120 mm long × 100 -160 mm wide), PL6 (30 – 75 mm long × 24 – 70 mm wide), PL9 (40 -150 mm long × 5 - 170 mm wide), are fan-shaped or slightly rounded triangular, convex, undulate with age, with inflexed margin when young, thin- to moderately thick-fleshed, dark brownish grey to light brownish grey, frequently slightly pubescent at centre, sometimes with radial ridges or with appressed radial squamules towards margin. Lamellae are crowded, decurrent. Stipe PL2 (50 – 110 × 5 – 22 mm), PL6 (2 – 5 × 3 - 25 mm), PL9 (4 – 2 × 12 - 35 mm) is eccentric to lateral, connate, solid, whitish, in upper part longitudinally striate, frequently tomentose. Context is whitish. Spore print is whitish. Spores PL2 (8.3 – 12.2 × 3.7 – 4.3 µm, Q average = 2.4 – 2.7), PL6 (9 – 11.6 × 3.3 – 4.3 µm, Q average = 2.4 – 2.6), PL9 (9.5 – 13 × 3 – 4.5 (5) µm, Q average = 2.5 – 2.7) are oblong to cylindrical, smooth, hyaline. Basidia PL2 (28 – 34 × 4 - 6 µm), PL6 ((20) 25 – 35 × 4 – 6 (7) µm), PL9 (25 – 40 × 5.5 - 7 µm) are clavate, 2 – 4 spored. Cystidia are not observed, but lecythiform basidioles (or cystidium-like elements) present, PL2 basidioles (20 – 26,5 × 4,5 - 7 µm), PL6 basidioles (20 – 30 × 3 – 7 µm), PL9

basidioles ($20 - 3 \times 3,5 - 7 \mu\text{m}$). Hymenophoral trama is irregular, monomitic. Pileipellis is compact cutis, 40 to 200 μm thick. Pileitrama is monomitic, irregular.

Many these species's macroscopic and microscopic features are similar with *P. ostreatus* and *P. pulmonarius*'s descriptions [21]. But their pileipellis thick is more similar with *P. ostreatus* (40 – 180 μm) than *P. pulmonarius* (40 – 50 μm). So, according to morphology, these species belong to *P. ostreatus*.



Figure 2. Morphology of PL2, PL6, PL9 strain.

(A1) – Basidiocarp of PL2 strain; (A2) – Spores of PL2 strain; (B1) – Basidiocarp of PL6 strain; (B2) - Spores of PL6 strain; (C1) – Basidiocarp of PL9 strain ; (C2) - Spores of PL9 strain.

3.1.3. PL3 strain (Figure 3)

Basidiome are solitary to imbricate. Pileus is $12 - 17 \times 7 - 9 \text{ cm}$ ($W \times L$), pleurotoid, surface is brown to grayish brown with numerous punctiform squamules formed by surface cracking, more numerous toward the margin; margin entire, slightly to much festooned, hardly involute. Stipe is brown to grayish, almost lateral $3 - 12 \times 1.8 - 3.8 \text{ cm}$, tapered to the base. Context is white, fleshy when fresh, compact, corky when dry. Lamellae are white when fresh, yellow when dry, thinner toward the stipe, decurrent. Spores are hyaline, cylindrical-oblong, thin-walled, smooth, $10 - 16 \times 3.5 - 5 \text{ mm}$ $Q = 3.01$. Basidia are 2 to 4 spored, $30 - 42 \times 5.8 - 9.2 \mu\text{m}$. Cystidia are absent. Pileipellis is 80 – 100 μm broad, formed by clamped, brown, parallel, scanty branched, 3 – 5 μm diam hyphae; among them brown, thick-walled, cystidiform hyphae, 3.6 – 5.2 μm diam. Context is monomitic, formed by clamped, thin-walled, hyaline hyphae.

Culture characters. Mycelium hyaline, filamentous, dense, whitish near the inoculum, growth radial, well adhered to the substrate. At first, a large amount of hyaline, aerial mycelium was observed, more whitish later; coremia appearing on the inoculum. Micro-droplets are 1 – 2 mm in diameter. Following morphology, this species is most similar with *P. cystidiosus* [22].

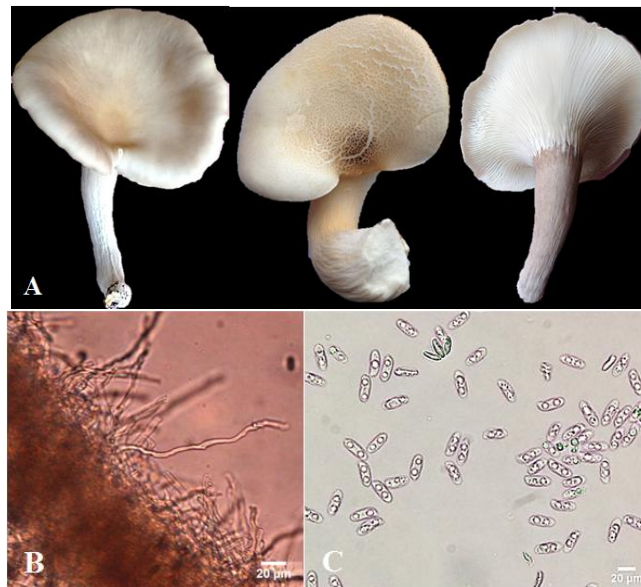


Figure 3. Morphology of PL3 strain.

(A) - Basidiocarp; (B) - Hyphae of the stipe trama; (C) - Spores.

3.1.4. PL4, PL8 strains (Figure 4)

Pileus PL4 (50 - 110 mm long × 43 - 160 mm wide), PL8 (40 - 100 mm long × 55 - 115 mm wide) are rounded flabelliform or semicircular, convex when young, later appanate to slightly infundibuliform, with inflexed margin when young, thin- to moderately thick-fleshed, whitish, pale yellowish to pale greyish brown, smooth, glabrous or slightly arachnoid, rarely somewhat squamose, frequently somewhat tomentose near the attachment point. Lamellae are crowded, decurrent, rather thin, up to 5 mm wide, whitish to pale cream, with entire or partly serrulate, concolorous or somewhat brownish edge. Stipes PL4 (5 - 9 × 15 - 45 mm), PL8 (5 - 14 × 20 - 55 mm) are eccentric to lateral, sometimes connate, solid, whitish, frequently tomentose. Context whitish. Spore print is whitish to cream, yellowish or pale ochre. Spores PL4 (8 - 11.3 (12.3) × 3 - 4.5 µm, Q average = 2.2 - 2.8), PL8 (8 - 11 (12) × 3.5 - 4.6 µm, Q average = 2.3 - 2.7) are cylindrical, thin-walled, smooth. Basidia PL4 (20 - 30 × 5 - 7 µm), PL8 ((18) 20 - 30 × 5 - 8 µm) are 2 - 4 spored. Cystidia are not observed. Pileipellis is compact cutis, 40 - 60 µm thick, made up of rather thin-walled hyphae, without distinct pigment. Pileitrama is monomitic, irregular.

Many these species's macroscopic and microscopic features are similar with *P. ostreatus* and *P. pulmonarius*'s descriptions [21]. But their pileipellis thick is more similar with *P. pulmonarius* (40 - 50 µm) than *P. ostreatus* (40 - 180 µm). So, according to morphology, these species belong to *P. pulmonarius*.

3.1.5. PL5 strain (Figure 5)

Pileus is 40 – 150 mm broad, convex when very young, soon with depressed to subumbilicate centre, with straight to inflected margin, thick flesh, pale grey-brown to pale creamy-brownish buff, more isabella at margin, smooth and glabrous, sometimes with outermost margin slightly tomentose. Lamellae are crowded, deeply decurrent, passing into conspicuous anastomosing ridges on stipe, rather thin, cream. Stipe is 30 – 50 × 3 -15 mm, subcentral to eccentric, fasciculate, cylindrical, occasionally somewhat tapering towards base, solid, whitish cream, occasionally with lilacinous tinge, entirely covered by anastomosing ridges, subpubescent to subgranulose, densely hairy at utmost base. Context is white in pileus and stipe. Spore print is whitish. Spores (9 – 11.6 × 3.3 – 3.7 μm , $Q = 2.6 - 3.2$, Q average = 2.8 – 3.1) are cylindrical to bacilliform, thin-walled, smooth. Basidia 25 - 35 × 5 - 7 μm , clavate, 2 – 4 spored. Cystidia not observed, but lecythiform basidioles (17) 20 - 30 × 4 – 6 μm , present. Hymenophoral trama irregular, monomitic. Pileipellis a 60 -120 μm thick cutis. Pileitrama monomitic.

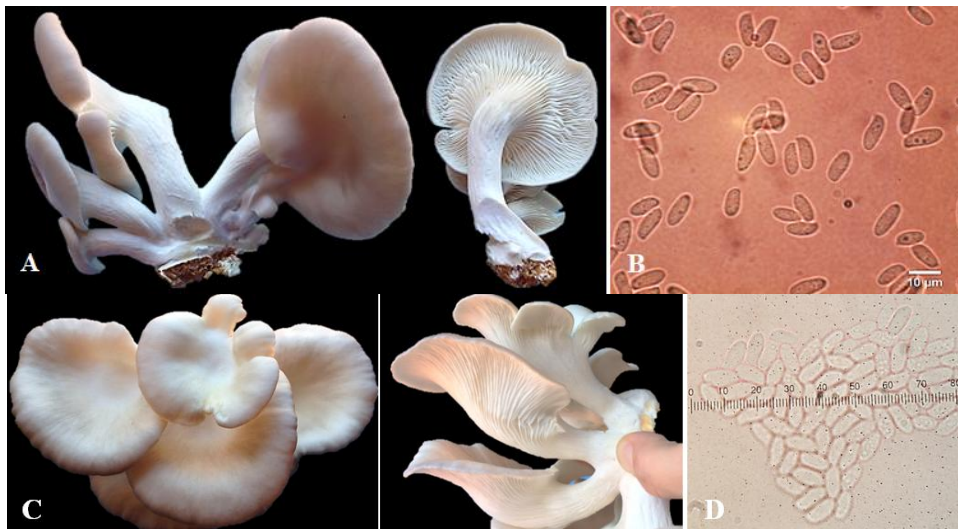


Figure 4. Morphology of PL4 & PL8 strain.

(A) – Basidiocarp of PL4 strain; (B) – Spores of PL4 strain; (C) – Basidiocarp of PL8 strain; (D) – Spores of PL8 strain.

P. cornucopiae is well characterized by the following combination of characters deeply decurrent lamellae passing into anastomosing ridges on the stipe, a pale pileus, and the sometimes dimitic trama. According to morphology, this strain belongs to *P. cornucopiae* [21].

3.1.6. PL7 strain (Figure 6)

Pileus is 65 - 134 × 58 – 11 mm (W × L), lobe, tomentose, pinkish, straight to inflexed margin, thick and tough flesh. Lamellae are crowded, deeply decurrent, pinkish. Stipe (3 - 15 × 15 - 20 mm) is eccentric, fasciculate, cylindrical, occasionally somewhat tapering towards base, solid, pink, tomentose. Context is pinkish in pileus and stipe. Spore print is pinkish. Spores (6 - 9 (-11.5) × 4.3 – 5 μm , $Q = 2 - 2.3$) are oblong to cylindrical, thin-walled, smooth. Basidia (15 - 20 × 3.5 - 5 μm) are clavate, 2 - 4-spored. Pleurocystidia are ventricose, 26 - 30 × 6 – 7 μm , present. Cheilocystidia are ventricose at center, sometime ampun, 23 – 36 × 7.5 - 12 μm .

Hymenophoral trama is irregular, monomitic. By morphology, this species is most similar with *P. incarnatus* [23].



Figure 5. Morphology of PL5 strain.

A – Different morphologies of basidiocarp from young to mature; (B) – Basidium; (C) – Radial section of basidiocarp; (D) - Hymenium; (E) – Top of hymenium with the lecithyform basidioles; (F) – Spores.

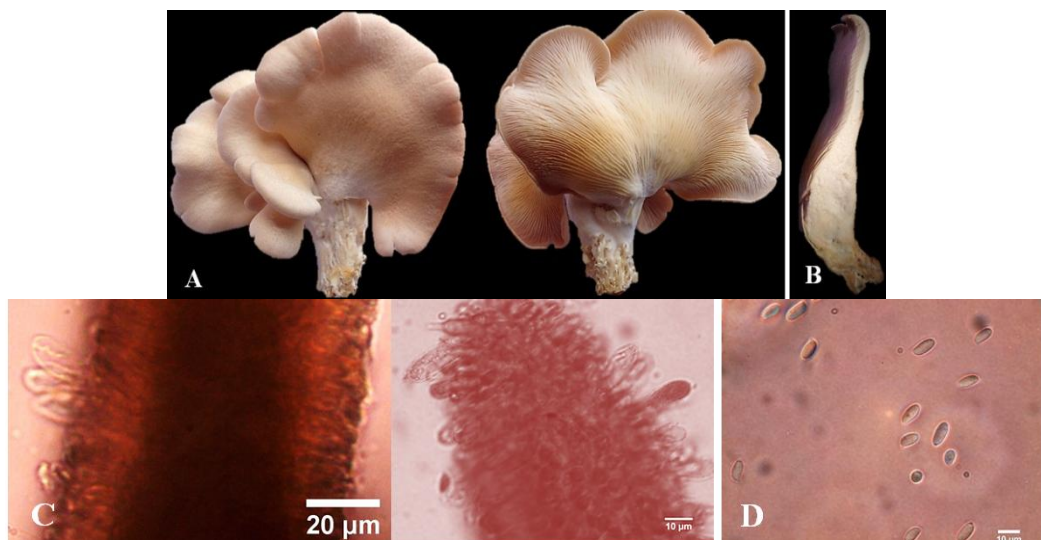


Figure 6. Morphology of PL7 strain.

(A) – Basidiocarp; (B) – Radial section of basidiocarp; (C) – Pleurocystidia; (D) – Spores.

3.2. Phylogenetic analysis

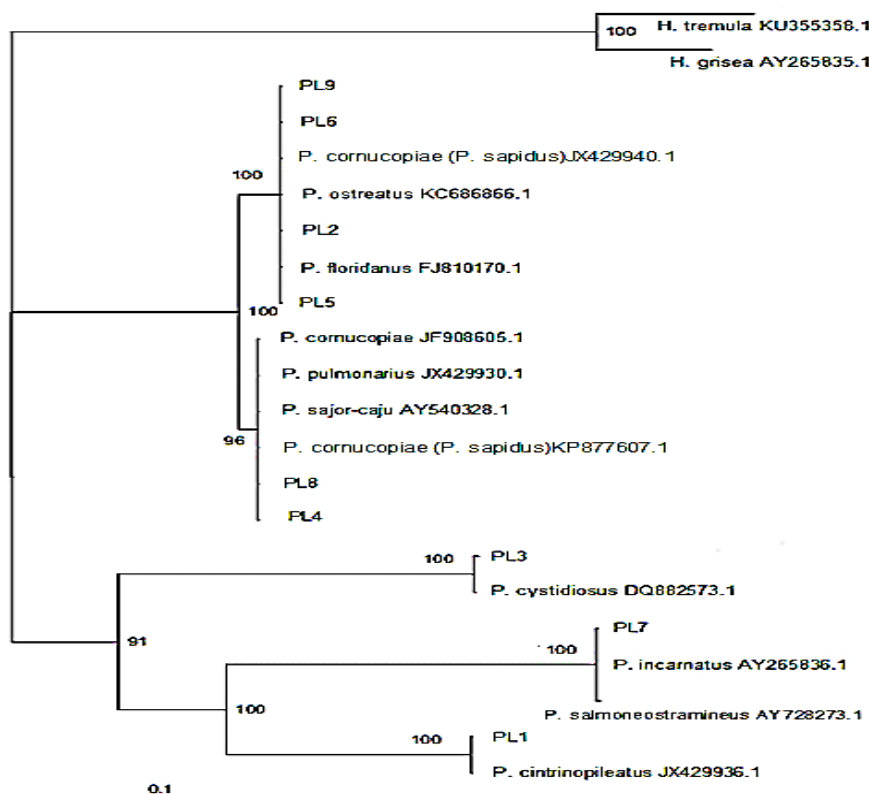


Figure 7. Maximum likelihood (ML) tree.

ML trees (Figure 7) showed that 9 *Pleurotus* strains and the most similar sequences in GenBank splitted into 4 groups, and these distributions were the same. PL7 strain with *P. incarnatus* AY265836.1 strain and *P. salmoneostramineus* AY72873.1 strain belonged to group 1. PL1 strain with *P. citrinopileatus* JX429936.1 belonged to group 2. PL3 strain with *P. cystidiosus* DQ882573.1 strain belonged to group 3. The other strains belonged to group 4. Among them, PL4, PL8 with *P. pulmonarius* JX429930.1, *P. cornucopiae* KP877607.1, *P. cornucopiae* JF908605.1 belonged to group 4A, and PL2, PL5, PL6, PL9 with *P. cornucopiae* JX429940.1 strain, *P. floridanus* FJ810170.1 strain, *P. ostreatus* KC686866.1 strain belonged to group 4B.

These results were compatible with the morphological identifications above and affirmed again the morphological identifications were right. The occurrence of *P. cornucopiae* in both 4A and 4B means *P. cornucopiae* is close with both *P. pulmonarius* and *P. ostreatus*. Basing on morphology, Hilber believed that *P. cornucopiae* was close with *P. ostreatus* species in Europe [24], Gonzales and Labarere also believed that *P. cornucopiae* is synonym of *P. ostreatus* by basing on SSU rRNA [25], while Zervarkis and partners claim that *P. cornucopiae* is close with *P. pulmonarius* species by basing on isozyme [26].

Collected *Pleurotus* strains were belonged to 6 species: PL1 belonged to *P. citrinopileatus*; PL2, PL6, PL9 belonged to *P. ostreatus*; PL3 belonged to *P. cystidiosus*; PL4, PL8 belonged to *P. pulmonarius*; PL5 was *P. cornucopiae*; PL7 belonged to *P. incarnatus*. Except PL7, the

others belonged to 4 incompatible group: group I - *P. ostreatus*, group II - *P. pulmonarius*, group IV - *P. cornucopiae*, group VII - *P. cystidiosus* [4]. This information is very useful for breeder and can help them for choosing the pair of strains that can breeding naturally.

3.3. Genetic diversity analysis by AFLP

This result showed the genetic diversity of 9 strain *Pleurotus* collected. It'll help the mushroom breeders can choose the strains that distant relation to make breeding dominances.

Table 1. Jaccard's pairwise similarities between *Pleurotus* strains based on AFLP polymorphic bands.

Strain	PL1	PL 2	PL3	PL4	PL5	PL6	PL7	PL8	PL9
PL1	1.0000000								
PL2	0.7083333	1.0000000							
PL3	0.6875000	0.6875000	1.0000000						
PL4	0.6666667	0.7500000	0.7708333	1.0000000					
PL5	0.6250000	0.7916667	0.6875000	0.7500000	1.0000000				
PL6	0.6041667	0.7708333	0.6250000	0.7291667	0.6875000	1.0000000			
PL7	0.5833333	0.6250000	0.7291667	0.6666667	0.6250000	0.5208333	1.0000000		
PL8	0.5625000	0.6875000	0.6666667	0.7708333	0.7291667	0.7083333	0.5625000	1.0000000	
PL9	0.6875000	0.8958333	0.7083333	0.7291667	0.7708333	0.7500000	0.6458333	0.7083333	1.0000000

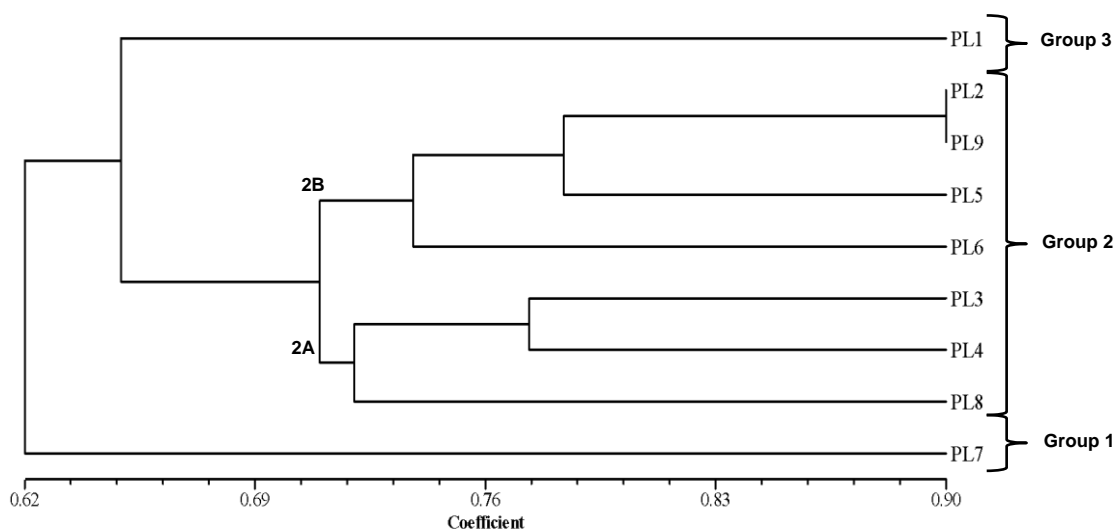


Figure 8. UPMA tree based on polymorphic AFLP marker shows genomic diversity of *Pleurotus* strains.

The result following Table 1 showed that diversity of 9 strains are high (similarity score from 0,52 – 0,9). Nine strains distributed into three main clusters: PL7 belonged to group 1, PL2, PL3, PL4, PL5, PL6, PL8, PL9 belonged to group 2 and PL1 belonged to group 3.

The genetic relation (Figure 8) of PL2 and PL9 is most distant (similarity score 0.90), while pair PL6 and PL7 is the most closed genetic relation (similarity score 0.52).

3.4. Cultivating traits examination

Table 2. Cultivating characteristics of tested *Pleurotus* strains

Strains	The mycelial growth rate (mm/day)	The fruiting forming period (days)	Biological efficiency (%)
PL1	9.09 ± 0.36 ^a	14.83 ± 0,91 ^d	50.88 ± 4.32 ^e
PL2	7.68 ± 0.32 ^d	18.60 ± 1.00 ^b	60.53 ± 2.78 ^d
PL3	5.66 ± 0.19 ^f	17.53 ± 1,81 ^c	75.04 ± 3.11 ^a
PL4	8.00 ± 0.17 ^c	10.14 ± 1,18 ^e	62.07 ± 3.59 ^d
PL5	9.18 ± 0.21 ^a	18.10 ± 0.67 ^{bc}	64.39 ± 2.11 ^{bc}
PL6	9.08 ± 0.21 ^a	21.07 ± 0,80 ^a	62.58 ± 2.83 ^{cd}
PL7	6.65 ± 0.32 ^e	18.76 ± 1,74 ^b	34.94 ± 4.43 ^f
PL8	8.67 ± 0.20 ^b	10.37 ± 1,16 ^e	66.72 ± 2.16 ^b
PL9	9.13 ± 0.23 ^a	20.30 ± 0,95 ^a	61.26 ± 2.04 ^d

Note: All data were illustrate as mean ± standard deviation.

The different lower case letters indicate the significant difference (Tukey-Kramer Test, $p < 0.05$) in each column categorizes. The alphabet lower case letters set were separated in each column.

Following Table 2, mycelial growth rate of PL5 strain was fastest (91.818 mm/day), PL3 strain's was slowest (56.663 mm/day). Fruiting forming period of PL4 and PL8 were fastest, about 10 days, the next were PL1, PL5, PL6 strains, their fruiting forming period were about 13 – 15 days, the last were PL2, PL3, PL7 and PL9, their one were about 17 – 20 days. Biological efficiency of PL3 was highest, 75.038 %, the next were PL5, PL6, PL8, PL9 about 50.876 – 66.716 % and that of PL7 strain was lowest 34.937 %.

Data collected in 3 times experiments and comparing with data of cultivation *Pleurotus* spp. on sawdust showed that except PL7 strain, the other strains had the good biological efficiency ; PL2, PL5, PL6, PL9 strains were cold strain, slow fruiting forming; PL4 and PL8 strains were the most compatible with Vietnam's climate (high biological efficiency, short fruiting forming period, high mycelial growth rate), the next is PL1 strain; PL3 strain had the highest BE, but be longest harvesting period, lasted 2.5 – 3 months [27 – 32].

3.5. Monokaryon collecting and determination

Basing on the results of cultivating experiments, three strain PL1, PL2, PL8 (Table 2) were chosen to collect and determine monokaryons. PL1 and PL8 was compatible with local's climate. PL2 had many morphological features that suitable with the consumers (fruit-body's shape, color, taste, etc.). These strain are suitable material sources for the breeding programs.

Table 3. PL1's monokaryon determination.

		Type A1B1				Type A2B2			Type A1B2			Type A2B1				
		04	06	07	10	03	09	13	08	12	14	01	02	05	11	
		18	20	21	25	15	17	22	19	27	28	16	23	24	29	
		31	35	38	40	26	32	33	34	36		30	37	39		
Type A1B1	04	21	-	-	-	-	+	+	+	-	-	-	(+)	(+)	(+)	(+)
	06	25	-	-	-	-	+	+	+	-	-	-	(+)	(+)	(+)	(+)
	07	31	-	-	-	-	+	+	+	-	-	-	(+)	(+)	(+)	(+)
	10	35	-	-	-	-	+	+	+	-	-	-	(+)	(+)	(+)	(+)
	18	38	-	-	-	-	+	+	+	-	-	-	(+)	(+)	(+)	(+)
	20	40	-	-	-	-	+	+	+	-	-	-	(+)	(+)	(+)	(+)
Type A2B2	03	22	+	+	+	+	-	-	-	(+)	(+)	(+)	-	-	-	-
	09	26	+	+	+	+	-	-	-	(+)	(+)	(+)	-	-	-	-
	13	32	+	+	+	+	-	-	-	(+)	(+)	(+)	-	-	-	-
	15	33	+	+	+	+	-	-	-	(+)	(+)	(+)	-	-	-	-
	17		+	+	+	+	-	-	-	(+)	(+)	(+)	-	-	-	-
Type A1B2	08	27	-	-	-	-	(+)	(+)	(+)	-	-	-	+	+	+	+
	12	28	-	-	-	-	(+)	(+)	(+)	-	-	-	+	+	+	+
	14	34	-	-	-	-	(+)	(+)	(+)	-	-	-	+	+	+	+
	19	36	-	-	-	-	(+)	(+)	(+)	-	-	-	+	+	+	+
Type A2B1	01	24	(+)	(+)	(+)	(+)	-	-	-	+	+	+	-	-	-	-
	02	29	(+)	(+)	(+)	(+)	-	-	-	+	+	+	-	-	-	-
	05	30	(+)	(+)	(+)	(+)	-	-	-	+	+	+	-	-	-	-
	11	37	(+)	(+)	(+)	(+)	-	-	-	+	+	+	-	-	-	-
	16	39	(+)	(+)	(+)	(+)	-	-	-	+	+	+	-	-	-	-
	23		(+)	(+)	(+)	(+)	-	-	-	+	+	+	-	-	-	-

Note : + : clamp connection forming ; (+) : pseudo- clamp forming ; - : not forming clamp connection.

So, there were 120 monokaryotic isolates collected and determined following the results of mating test (Tables 3, 4, 5). PL1 strain had 12 monokaryotic isolates type A1B1, 9 monokaryotic isolates type A2B2, 8 monokaryotic isolates type A1B2 and 11 monokaryotic isolates type A2B1. PL2 strain had 11 monokaryotic isolates type A1B1, 11 monokaryotic isolates type A2B2, 9 monokaryotic isolate type A1B2 and 9 monokaryotic isolate type A2B1. PL8 strain had

6 monokaryotic isolates type A1B1, 6 monokaryotic isolates types A2B2, 16 monokaryotic isolates type A1B2 and 12 monokaryotic isolates type A2B1. All of monokaryotic isolates were kept as living cultures collection for next researches about *Pleurotus* hybridization.

Table 4. PL2's monokaryon determination.

		Type A1B1				Type A2B2				Type A1B2			Type A2B1		
		01	02	06	11	05	07	09	16	10	13	14	03	04	08
		12	17	25	29	20	23	24	26	18	19	21	15	22	27
		36	38	39		30	37	40		28	34	35	31	32	33
Type A1B1	01 25	-	-	-	-	+	+	+	+	-	-	-	(+)	(+)	(+)
	02 29	-	-	-	-	+	+	+	+	-	-	-	(+)	(+)	(+)
	06 36	-	-	-	-	+	+	+	+	-	-	-	(+)	(+)	(+)
	11 38	-	-	-	-	+	+	+	+	-	-	-	(+)	(+)	(+)
	12 39	-	-	-	-	+	+	+	+	-	-	-	(+)	(+)	(+)
	17	-	-	-	-	+	+	+	+	-	-	-	(+)	(+)	(+)
Type A2B2	05 24	+	+	+	+	-	-	-	-	(+)	(+)	(+)	-	-	-
	07 26	+	+	+	+	-	-	-	-	(+)	(+)	(+)	-	-	-
	09 30	+	+	+	+	-	-	-	-	(+)	(+)	(+)	-	-	-
	16 37	+	+	+	+	-	-	-	-	(+)	(+)	(+)	-	-	-
	20 40	+	+	+	+	-	-	-	-	(+)	(+)	(+)	-	-	-
	23	+	+	+	+	-	-	-	-	(+)	(+)	(+)	-	-	-
Type A1B2	10 21	-	-	-	-	(+)	(+)	(+)	(+)	-	-	-	+	+	+
	13 28	-	-	-	-	(+)	(+)	(+)	(+)	-	-	-	+	+	+
	14 34	-	-	-	-	(+)	(+)	(+)	(+)	-	-	-	+	+	+
	18 35	-	-	-	-	(+)	(+)	(+)	(+)	-	-	-	+	+	+
	19	-	-	-	-	(+)	(+)	(+)	(+)	-	-	-	+	+	+
Type A2B1	03 27	(+)	(+)	(+)	(+)	-	-	-	-	+	+	+	-	-	-
	04 31	(+)	(+)	(+)	(+)	-	-	-	-	+	+	+	-	-	-
	08 32	(+)	(+)	(+)	(+)	-	-	-	-	+	+	+	-	-	-
	15 33	(+)	(+)	(+)	(+)	-	-	-	-	+	+	+	-	-	-
	22	(+)	(+)	(+)	(+)	-	-	-	-	+	+	+	-	-	-

Note : + : clamp connection forming ; (+) : pseudo- clamp forming ; - : not forming clamp connection.

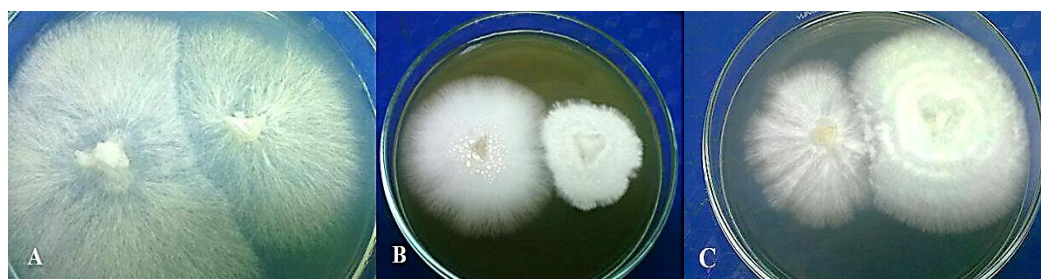


Figure 9. Morphological monokaryon and touching breeding types.

Table 5. PL8's monokaryon determination.

		Type A1B1		Type A2B2		Type A1B2						Type A2B1						
		02	03	06	12	01	04	10	11	13	14	05	08	09	18			
		07	15	16	21	17	19	22	24	27	30	20	23	25	26			
		37	40	29	31	34	35	36	38			28	32	33	39			
Type A1B1	02	15	-	-	+	+	-	-	-	-	-	-	-	-	(+)	(+)	(+)	(+)
	03	37	-	-	+	+	-	-	-	-	-	-	-	-	(+)	(+)	(+)	(+)
	07	40	-	-	+	+	-	-	-	-	-	-	-	-	(+)	(+)	(+)	(+)
Type A2B2	06	21	+	+	-	-	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	-	-	-	-
	12	29	+	+	-	-	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	-	-	-	-
	15	31	+	+	-	-	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	-	-	-	-
Type A1B2	01	24	-	-	(+)	(+)	-	-	-	-	-	-	-	-	+	+	+	+
	04	27	-	-	(+)	(+)	-	-	-	-	-	-	-	-	+	+	+	+
	10	22	-	-	(+)	(+)	-	-	-	-	-	-	-	-	+	+	+	+
	11	30	-	-	(+)	(+)	-	-	-	-	-	-	-	-	+	+	+	+
	13	34	-	-	(+)	(+)	-	-	-	-	-	-	-	-	+	+	+	+
	14	35	-	-	(+)	(+)	-	-	-	-	-	-	-	-	+	+	+	+
	17	36	-	-	(+)	(+)	-	-	-	-	-	-	-	-	+	+	+	+
19	38	-	-	(+)	(+)	-	-	-	-	-	-	-	-	+	+	+	+	
Type A2B1	05	25	(+)	(+)	-	-	+	+	+	+	+	+	+	+	-	-	-	-
	08	26	(+)	(+)	-	-	+	+	+	+	+	+	+	+	-	-	-	-
	09	28	(+)	(+)	-	-	+	+	+	+	+	+	+	+	-	-	-	-
	18	32	(+)	(+)	-	-	+	+	+	+	+	+	+	+	-	-	-	-
	20	33	(+)	(+)	-	-	+	+	+	+	+	+	+	+	-	-	-	-
	23	39	(+)	(+)	-	-	+	+	+	+	+	+	+	+	-	-	-	-

Note : + : clamp connection forming ; (+) : pseudo- clamp forming ; - : not forming clamp connection.

There were 4 monokaryon's colony types: rooting type (colonies in Figure 9A), cotton type (left colony in Figure 9B), dense mycelial type (right colony in Figure 9B), and concentric striate type (right colony in Figure 9C). Cotton mycelium's growth was fastest. Dense mycelium's growth was slowest. And monokaryons's mycelium always grow slower than their dikaryon's mycelium.

There were 3 touching breeding types such as boundary type in Figure 9A, covering type in Figure 9B and edge type in Figure 9C. All of breeding pairs made boundary or covering type were always not compatible, not forming clamp connections, similar to results of *P. ostreatus* monokaryons breeding each other [33 – 35].

4. CONCLUSION

Following morphology and phylogenetic analysis, 9 *Pleurotus* strains were identified. PL1 is *P. citrinopileatus*; PL2, PL6, PL9 are *P. ostreatus*; PL3 is *P. cystidiosus*; PL4, PL8 are *P. pulmonarius*; PL5 is *P. cornucopiae*; and PL7 is *P. incarnatus*. Genetic analysis by AFLP showed that diversity of those strains is high which similarity score from 0.62 – 0.9. AFLP analysis can reveal the genetic relationship not only among different species strains but also among same species strains.

All strains except PL7 had the good trait in cultivation. Among them, strains PL2, PL5, PL6, PL9 are temperate adaptation, slow fruiting forming; strains PL4 and PL8 are the most compatible with Vietnam's climate.

The living stock culture collection of monokaryotic *Pleurotus* isolates was set up from strain PL1 (*P. citrinopileatus*), strain PL2 (*P. ostreatus*) and strain PL8 (*P. pulmonarius*) with all data about the mating types, cultivation trait of parental strains and identification. There were 3 touching breeding types among them boundary type, covering type and edge type. All of breeding pairs made boundary or covering type were always not compatible, not forming clamp connection.

This is the first living culture collection of monokaryotic isolates of *Pleurotus* spp. with full characteristics of their parent in southern Vietnam. It contributes the *Pleurotus* cultivation industry by hybrid strains which native adaptation and stable genetic for large scale producing.

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