International Research Journal of Advanced Engineering and Science
ISSN (Online): 2455-9024

Morphological Analysis and Molecular Polymorphism Detection of Wild Mushroom Strains by DNA Fingerprinting Using Molecular Marker

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Abstract— *The investigation was conducted for morphological analysis and molecular polymorphism detection of six edible wild mushroom strains such as K-5, K-30, B-36, B-60, M-4 and R-108. In the morphological study, the highest amount of biological yield (46.5g/pac.) was observed in B-60 where as it was the lowest (25.5g/pac.) in K-30. The highest number of fruiting body (8.7g/pac.) was found in R-108 and the lowest numbers of fruiting body (5.3 g/packet) were found in M-4. In case of the number of effective fruiting body, it was the highest (5.8 g/pac.) in B-36 and the lowest (3.5g/pac.) in K-30. On the other hand, the highest and the lowest length of stalk were observed in B-60 and K-30; the figures were 4.8 cm/packet and 2.25 cm/packet respectively. The lowest length of stalk was observed in K-30. The highest diameter of stalk (0.98 cm/packet) was shown in B-60. The lowest diameter of stalk (0.49 cm/packet) was shown in R-108. The highest diameter of pileus (7.78 cm/packet) was got in B-60. The lowest diameter of pileus (5.31 cm/packet) was got in K-5. Among the six strains the thickness of pileus was highest and lowest 0.6cm (B-60) and 0.3cm (R-108) respectively. Six RAPD markers were used to analysis on six strain and found 106 scorable bands. Among the 106 RAPD bands, 36 bands were monomorphic and 70 bands were polymorphic. Out of seven random primers, the maximum polymorphism was observed by primer OPA- 4 (92%). Dendrogram analysis revealed that, it is consist of main two classes C1 (B-60) alone and C2 (K-5, K-30, B-36, M-4 and R-108) are grouped cluster. C2 formed sub-cluster SC1 and SC2. Strain K-5 alone formed sub-cluster 1(SC1) and K-30, B-36, M-4 and R-108 strain formed sub-cluster 2 (SC2). Sub-cluster 2 (SC2) formed sub-cluster SC3 and SC4 where R-108 strain alone formed sub-cluster 3 (SC3) and K-30, B-36, M-4 strain formed SC4. Besides, sub-cluster 4 (SC4) formed SC5 and SC6, Strain K-30 alone formed SC5 and B-36 & M-4 formed sub-cluster 6 (SC6). Overall Results indicated that the strain B-60 was shown to be outliers in the dendrogram and distantly related with other strains based on their genetic distances.*

Keywords— *Mushroom strains, Molecular polymorphism, RAPD markers, Electrophoresis, Genetic distances.*

I. INTRODUCTION

Mushroom is a large reproductive structure of edible fungi. The vegetative part of mushrooms is mainly consisting of thread-like long thin mycelia which under suitable condition forms fruiting bodies. Mushroom is very popular in vegetarian and non-vegetarian for its nice flavor. It contains high amount of protein, vitamins, minerals and very low amount of fat. It is used as the development of immune system, increase insulin hormone release, anti-cancer agent and decrease cholesterol level of blood. Different species of mushrooms are cultivated in Bangladesh such as oyster, milky, button, straw, shitake, reshi etc. In many different cuisines, including Chinese, Korean, European, and Japanese, mushrooms are frequently utilized in cooking. The "meat" of the vegetable world, as a saying goes [1]. Food mushrooms are a good source of vital minerals like selenium, copper, and potassium as well as B vitamins like riboflavin, niacin, and pantothenic acid. minimal in sodium and vitamin C, with minimal levels of fat, carbohydrates, and calories. On a dry weight basis, it contains 19%-35% protein, compared to 7.3% in rice, 13.2% in wheat, and 25.2% in milk [2]. Unsaturated fatty acids are present in the 4% dry weight of fat [3]. Some therapeutic mushroom isolates have been discovered through research to have positive cardiovascular, anticancer, antiviral, antibacterial, antiparasitic, anti-inflammatory, and anti-diabetic effects. Many extracts, including polysaccharide-K and polysaccharide peptide lentinan, are currently used as adjuvants to chemotherapy and radiation treatments in China, Japan, and Korea [4]. Mushrooms are effective against cancer, diabetes, ulcers, and lung problems [5]. Due to their hematological, antibacterial, antiviral, and antioxidant activities, mushrooms will increasingly be used as food supplements, food additives, and in the pharmaceutical business [6]. Using PCR, the RAPD (Random Amplified Polymorphic DNA) approach can be used to detect genetic variation. RAPD technology afford a chance to examine variety and to provide genetic typing for differentiating mushroom spawns for strain protection from the genus Agaricus [7]; RAPD analysis was first developed to detect polymorphism between organisms, despite the absence of sequence information, to produce genetic markers, and to construct genetic maps [8]. In Bangladesh, mushroom is a unique non-traditional horticulture crop grown indoors, both as a seasonal crops and round-the-year under the controlled and natural environmental condition. Mushroom cultivation in this country was started very late in 1976s. But institutionally mushroom cultivation began in Bangladesh Horticulture Centre, Sobhanbag, Savar, Dhaka in 1979-1980 with the assistance of Japan Overseas Cooperative volunteers (JOCV). In the objectives of study to yield and yield contributing parameters of wild mushrooms, to determine molecular

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ISSN (Online): 2455-9024

polymorphism of wild mushroom strains by DNA fingerprinting using RAPD markers and to determine genetic diversity and relationship among six edible mushroom strains.

II. Materials and Methods

Mushroom cultivation

For mushroom cultivation, 500 g-sized sawdust packets containing pure culture, mother culture, and spawn were made, infected, and incubated according to a process established and described by Sarker [9]. After mycelium running was finished, spawn packets were sliced into "D" shapes and moved to the culture house for the commencement of fruiting bodies. 30 to 350 C was the ambient temperature, while 85 to 90% of the air was humid. To maintain the temperature and relative humidity, water was sprayed four to five times daily.

Fig. 1. Wild mushroom cultivation

Sample collection

Six different wild mushroom strain such as- strain (K-5), strain (K-30), strain (B-36), strain (B-60), strain (M-4), strain (R-108) was used as material which has been collected from Mushroom Development Institute, Sobhanbag, Saver, Dhaka, Bangladesh. They were collected from hilly area of Bangladesh showed in Table-1. These natural mushrooms are greatly popular for commercialization, which also used as materials for DNA isolation.

TABLE 1. List of wild mushroom strains along with their common name and morphological features of six natural wild mushroom strains in different area.

Strain	Local Name	Common Name	Location	Habitat	Remark
K-5	Gashole	Oyster	Khagrachori	Konok tree	Edible, explot easier
K-30	Gashole	Oyster	Khagrachori	Woodlog	Edible, popular
B-36	Gashole	Oyster	Khagrachori	From farm	Cream color, edible not so popular
B-60	Daramara	Oyster	Bandarban	Hardwood darman	Edible and very popular
M-4	Gashole	Oyster	Mymensingh	Woodlog	Edible, small, fruit body white
R-108	Gashole	Oyster	Rangamati	From farm	Edible, popular in market.

Isolation of DNA

A modified CTAB method was used to isolate total genomic DNA from wild mushrooms [10]. Quality was determined by 260/280 nm UV absorption ratios. If the ratio higher than 2.0, generally indicates RNA contamination and if the ratio lower than 1.8 normally indicates protein contamination during extraction process. Good quality DNA should give the ratio (A260/A280) in the range of 1.8 - 2.0 [11].

Quantification of DNA

The spectrophotometric approach and visual estimation were both employed to calculate the amount of separated DNA. By comparing the DNA in the electrophoresis agarose gel with a known molecular weight marker, the visual amount of DNA was calculated. By measuring absorbance at 260 nm, the amount of DNA was calculated using the spectrophotometric approach.

Electrophoresis

To place the tray such that the sample wells were close to the cathode, the gel was kept horizontal and placed into the bottom of the electrophoresis chamber. During electrophoresis, the DNA sample would move from the cathode (-ve) to the anode (+ve). For 1.5 hours, electrophoresis was performed at 100 Volts. The migration of the dye in the loading buffer was used to track the separation process. The gel length was approximately three-fourths (34) of the way filled with bromophenol-blue dye when the electrophoresis was finished and stopped.

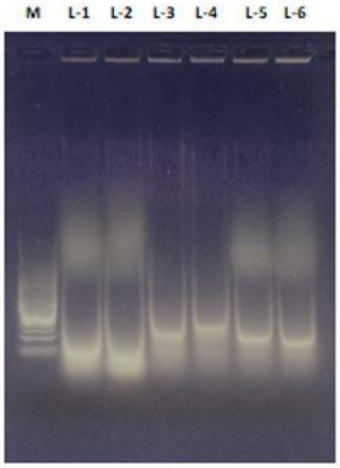

Fig. 2. L-1 contain K-5, L-2 contain K30, L-3 contain B-36, L-4 contain B-60, L-5 contain M-4 and L-6 contain R-108.

PCR amplification

The PCR protocols also proceeded with certain modifications [12],[13]. The amplifications were carried out in a thermal cycler (Genius, Techne, Cambridge Limited) adjusted for the program PCR-1 to 40 cycles for the complete DNA synthesis. PCR amplification was carried out in a 10 µl reaction mixtures containing 2 µl (50 ng/ µl) of genomic DNA, 2 µl (10 µM) primer, 1 µl (10X) PCR buffer, 0.6 µl (25 mM) MgCl2, 1 µl (2.5mM) dNTPS, 0.2µl (1 unit) Taq DNA polymerase (Promega, USA) and 3.2 µl deionized distilled water. The method consisted of 3 stages: denaturation of the DNA chain in simpler sequences of 94°C for 1 min, during regular cycles (the

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initial denaturation was longer: 1 min); hybridization of the primers to complemental sequence sites in the denatured DNA chain at 37°C for 1 min and primers extension for the synthesis of the DNA complemental sequences at 72°C for 7 min, followed by cooling at 40C for infinite period. Amplification products were electrophoresed in 1.5% agarose gels in TBE (1X/) buffer and detected after ethidium bromide staining [14]. RAPD bands were observed under Ultra Violet light on a transelluminator and captured by taking photos.

III. Results and Discussion

Morphological analysis of the collected six wild mushroom strains

The morphological analysis was presented in Table-3 and revealed the highest amount of biological yield (46.5g/packet) was observed in B-60 where as it was the lowest (25.5g/packet) in K-30. The highest number of fruiting body (8.7g/packet) was found in R-108 and the lowest numbers of fruiting body (5.3 g/packet) were found in M-4. In case of the number of effective fruiting body, it was the highest (5.8 g/packet) in B-36 and the lowest (3.5g/packet) in K-30. On the other hand, the highest and the lowest length of stalk were observed in B-60 and K-30; the figures were 4.8 cm/packet and 2.25 cm/packet respectively. The lowest length of stalk was observed in K-30. The highest diameter of stalk (0.98 cm/packet) was shown in B-60. The lowest diameter of stalk (0.49 cm/packet) was shown in R-108. The highest diameter of pileus (7.78 cm/packet) was got in B-60. The lowest diameter of pileus (5.31 cm/packet) was got in K-5. Among the six strains the thickness of pileus was highest and lowest 0.6cm (B-60) and 0.3cm (R-108) respectively (Table-2).

TABLE 2. Morphological traits of fruiting body of six wild mushroom strains

Strain	Biological Yield (g/packet)	No. of Fruiting Body	No. of Effective Fruiting Body	Length of Stalk	Width of Stalk	Width of Pileus	Thickness of Pileus
K-5	27.5	7.0	4.0	3.28	0.78	5.31	0.40
K-30	25.5	6.6	3.5	2.25	0.92	6.24	0.56
B-36	40.1	6.5	5.8	4.18	0.75	5.80	0.50
B-60	46.5	6.0	3.8	4.80	0.98	7.78	0.60
M-4	30.4	5.3	5.8	2.98	0.68	6.54	0.35
R-108	33.2	8.7	5.1	4.30	0.49	6.58	0.30

RAPD-PCR analysis

Using the computer program Alpha View 3.2, all RAPD bands were scored. Calculations were made for each primer's total number of bands, size ranges (bp), total number of bands per variety, number of polymorphic bands, and percentage of polymorphism. For each of the four date palm kinds tested, DNA fragments that were amplified by a specific primer were scored for presence ('1') and absence ('0'). By employing the un-weighted pair-group method with arithmetic averages (UPGMA) cluster analysis with the STATISTICA program, a dendrogram was created to identify the genetic relatedness among the genotypes based on the distance matrix [15]. According to Pradhan,[16] fingerprinting keys were created based on the analysis.

Primer used

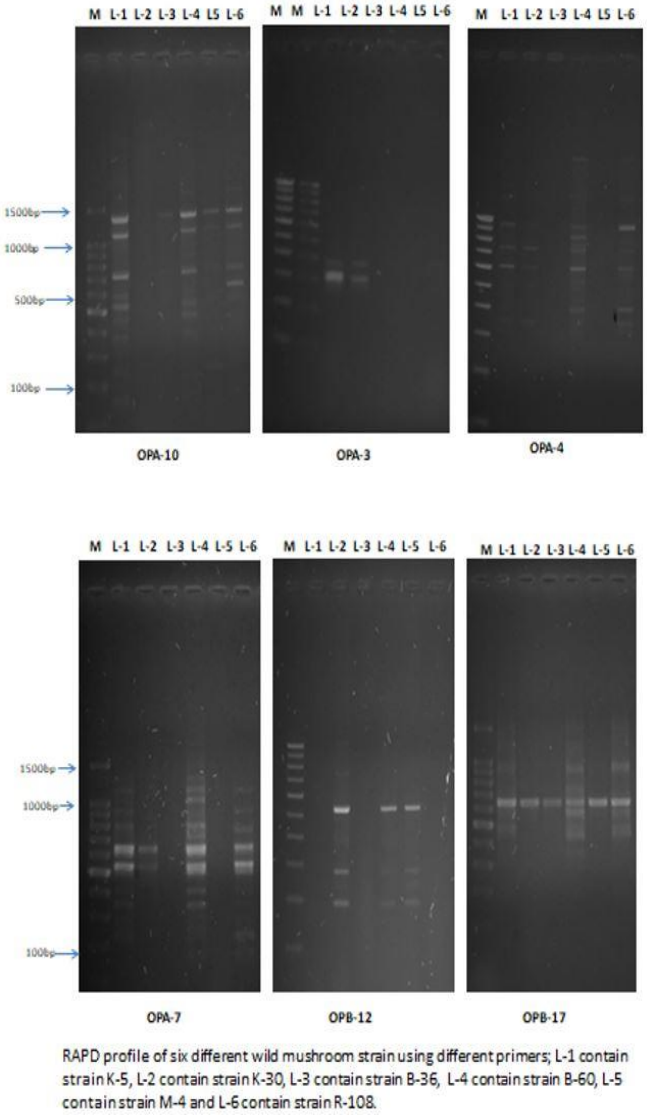
Six primers of random sequence (OPA 3, OPA 4, OPA 7, OPA 10, OPB 12 and OPB 17) were used on six wild type mushroom strains for PCR (Table-3).

TABLE 3. List of 6 decamer RAPD primers with their sequences.

Sl. No.	Primer Code	Sequence 5' → 3'	% G+C Content
1	OPA 3	AGTCAGCCAC	60
2	OPA 4	AATCGGGCTG	60
3	OPA 7	GAAACGGGTG	60
4	OPA 10	GTGATCGCAG	60
5	OPB 12	CCTTGACGCA	60
6	OPB 17	AGGGAACGAG	60

DNA fingerprinting using RAPD markers

PCR based RAPD analysis against six mushroom strains (Figure-3) revealed in the Table 5. The sizes of the amplified bands in the six mushroom strains ranged from 273 bp to 1765 bp among the six RAPD primers.



RAPD profile of six different wild mushroom strain using different primers; L-1 contain strain K-5, L-2 contain strain K-30, L-3 contain strain B-36, L-4 contain strain B-60, L-5 contain strain M-4 and L-6 contain strain R-108.

Fig. 3. DNA fingerprinting of six wild type mushroom strain based Six RAPD primer through 1.5% agarose gel electrophoresis.

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OPA-3 revealed band sizes that ranged from 435-534bp, OPA-4 ranged from 273-1407bp; primer OPA-7 ranged from 688-1442bp, primer OPA-10 ranged from 277-1765bp, primer OPB-12 ranged from 289-873bp, primer OPB-17 ranged from 621-1403bp. Six RAPD primers were used to amplify the segments of DNA in six wild mushroom strains, which were found to be efficient for amplifying the genomic DNA. These primers showed significant band profiles on the tested varieties (mentioned in photo). The primer OPA-10 amplified the highest number of bands (27) and primer OPA-3 amplified the lowest number of bands (6). Highest polymorphism showed in the amplification bands by the primer OPA-4 (Table 4).

TABLE 4. RAPD primers with corresponding bands scored and their size ranges in selected wild mushroom strain.

Primer Codes	Size ranges (bp)	Total No. of bands scored	No. of monomorphic bands	No. of polymorphic bands	Polymorphism %
OPA 3	435-534	6	2	4	66.66
OPA 4	273-1407	25	2	23	92.00
OPA 7	688-1442	20	12	8	40.00
OPA 10	277-1765	27	5	22	81.00
OPB 12	289-873	16	8	8	50.00
OPB 17	621-1403	12	7	5	41.60
Total		106	36	70	

Genetic distances

The values of pair-wise comparisons of linkage distances analyzed by using computer software "Statistica" between strain were computed from combined data for the six primers, ranged from 15 to 44.1 (Table 6). The highest linkage distance (44.1) was found in mushroom strain R-108 vs. B-60. Comparatively higher distance was observed between mushroom strain R-108 vs K-5 and mushroom strain B-36. Mushroom strain R-108 vs B-36 than the other strain combination. The lowest linkage distance (15) was found in mushroom strain K-5 vs. B-36 strain pair (Table-5).

TABLE 5. Summary of linkage distances values six different cultivar pairs of wild mushrooms.

Mushroom Strain	K-5	K-30	B-36	B-60	M-4	R-108
K-5	0	28.0	25.0	39.0	31.0	30.8
K-30	28.0	0	15.0	39.0	17.0	25.1
B-36	25.0	15.0	0	30.0	16.0	24.1
B-60	39.0	39.0	30.0	0	34.0	44.1
M-4	31.0	17.0	16.0	34.0	0	28.1
R-108	30.4	25.1	24.1	44.1	28.1	0

Cluster analysis

Genetic relationship of six mushroom strains based in RAPD data using Unweighted pair group method with arithmetic mean (UPGMA) [17]. Genetic relationships among the six strains at the average distance of 38.0 showed two major clusters (C1 and C2) presented in the Figure- 4.

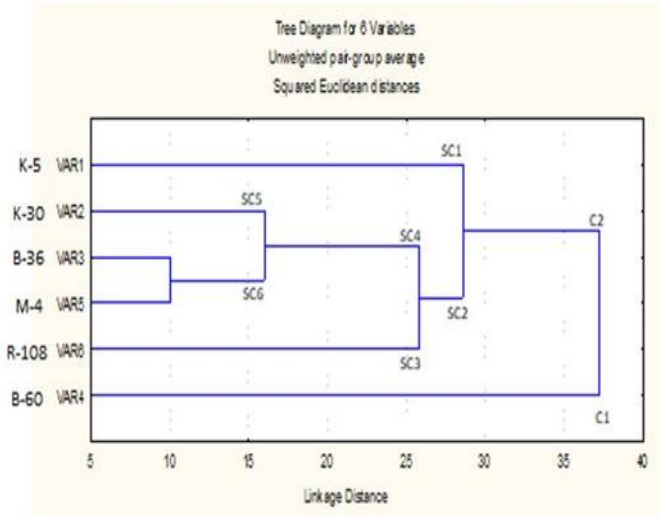

Fig. 4. Cluster analysis by Unweighted Pair Group Method of Arithmatic Means (UPGMA) of six wild mushroom strain based on six RAPD marker.

In the study of cluster analysis revealed that the mushroom strain B-60 formed separate cluster 1 (C1) in the dendrogram which indicates it different than all other mushroom strain. Dendrogram once again demonstrated other strain such as K-5, K-30, B-36, M-4 and R-108 are grouped cluster 2 (C2). At the linkage distance of 28.5 the major cluster C2 formed sub-cluster SC1 and SC2. Strain K-5 alone formed sub-cluster 1(SC1) and K-30, B-36, M-4 and R-108 strain formed sub-cluster 2 (SC2). At the linkage distance 26 sub-cluster 2 (SC2) formed sub-cluster SC3 and SC4 where R-108 strain alone formed sub-cluster 3 (SC3) and K-30, B-36, M-4 strain formed SC4. Besides, at the linkage distance 16 sub-cluster 4 (SC4) formed SC5 and SC6, Strain K-30 alone formed SC5 and B-36 and M-4 formed sub-cluster 6 (SC6). Results indicated that the strain B-60 was shown to be outliers in the dendrogram and distantly related with other strains based on their genetic distances.

The strain B-60 is a sustainable high yielding mushroom from other strain used in morphological investigation. It is well experienced that field performances of strain B-60 are superiorly different from other cultivated strain based on different criteria, such as biological yield, diameter of stalk, diameter of pileus, thickness of pileus etc.

The outcome is consistent with Yin's [7] findings, who looked into how 15 Chinese *P. pulmonarius* cultivars' genetic diversity was analyzed using molecular markers. A total of 21 RAPD primers were chosen for data generation based on the production of clear banding profiles. These RAPD primers allowed for the detection of 361 RAPD fragments in total, 287 (79.5%) of which were polymorphic. The 15 examined strains were divided into four clades by UPGMA trees, which showed structural similarity. The resolving ability of the combined RAPD markers in the separation between these strains was next assessed using visual DNA fingerprinting and cluster analysis.

IV. CONCLUSION

The Project work was conducted for morphological and molecular characterization of six wild mushrooms were used. From the morphological data shows strain B-60 wild mushroom

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is the best quality based on different criteria which approved by DNA fingerprinting result on the basis of RAPD markers and also revealed genetic relationship among the varieties.

ACKNOWLEDGEMENT

The author gratefully acknowledges the help received from the authority of Bangladesh University Grants Commission to provide fund allocation to finish the research work. We are also thankful to the Deputy Director of Mushroom Development Institute, Sobhanbag, Saver, Dhaka, Bangladesh due to providing samples to complete the project.

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