

Glycosyl linkage characteristics and classifications of exo-polysaccharides of some regionally different strains of *Lentinula edodes* by amplified fragment length polymorphism assay and cluster analysis

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Abstract

We report here the first combined amplified fragment length polymorphism (AFLP) analysis of genomic DNA fingerprinting data and cluster analysis of the exo-polysaccharide glycosyl linkage data of 10 regionally different strains of *Lentinula edodes* to compare their genetic and structural similarities and differences. In addition, the monosaccharide compositions, molecular weights, glycosyl structural linkages were investigated for the exo-polysaccharides extracted from these different phylogenetic groups of regionally different *L. edodes*. All exo-polysaccharides had similar molecular weight distribution between 1×10^4 and 3×10^6 Da and the monosaccharide composition analysis revealed the presence of heterogeneous materials containing glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose in different ratios. Among these monosaccharides, the glucose contents are the highest for all but one strain, indicating that glucose probably is the building block of the backbones of these exo-polysaccharides. The AFLP assay data helped to classify the 10 *L. edodes* strains into three distinct genetic groups. Gas chromatographic and mass spectrometric (GC–MS) data revealed five different glycosyl linkage types for these exo-polysaccharides. Most of the exo-polysaccharide backbone structures contain (1 → 4)-linked- β -glucopyranosyl and (1 → 6)-linked- β -glucopyranosyl moieties. Arabinose 1 → 4 linkages and mannose 1 → 2 linkages also exist in all strains. The only differences among these linkages are their monosaccharide compositions leading to different degree of backbone and branch formations. Cluster analyses of the GC–MS data of the exo-polysaccharides of the 10 strains resulted in 10 dendrograms. However, four of the 10 dendrograms were identical and were obtained using the average, Ward and weighted linkage type method of Manhattan distance and using the Ward method of Euclidean distance. The results of cluster analyses were not very much different from that of the AFLP assay and allowed the comparison of genetic and structural similarities and differences.

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1. Introduction

Mushrooms, such as *rieshi* (*Ganoderma lucidum*) and *shiitake* (*Lentinula edodes*) have been cultivated and used for many years as flavorful and nutritious foods as well as precious medicines, e.g. immune-modulators, in the Far East [1,2]. Among them, the *shiitake* mushroom, *L. edodes*, is the mostly cultivated and is easily available from Korea, Russia, Taiwan,

China and Japan [3,4]. Of all the mushroom immune-modulators investigated, bioactive polymers, such as polysaccharides from *L. edodes* have been studied most extensively [5].

More than 100 cultivated *shiitake* strains have been developed. Traditional methods to differentiate various strain types in *shiitake* cultivars were usually carried out based on morphological, physiological and somatic compatibility characteristics [6]. Recently, additional molecular biological techniques, such as amplified fragment length polymorphism (AFLP) fingerprinting analyses were developed based on selective polymerase chain reaction (PCR) amplification of genomic DNA restriction fragments. It was demonstrated that AFLP analyses could reproducibly help identify a large number of polymorphic loci to differentiate various strains [7–9]. The AFLP technique could

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also successfully differentiate not only the diversity in flowering plants populations but also a wide variety of organisms [10–14].

Many carbohydrates are found in mushrooms including polysaccharides, such as glucans, mono- and di-saccharides, sugar alcohols, glycogen and chitin [15]. Of all, β -glucan has been identified as the major structural component among many polysaccharides with anti-cancer activities [16]. For example, Lentinan (extracted from cell wall of fruiting body) and KS-2 (a α -mannan peptide extracted from culture mycelia) of *L. edodes* are demonstrated to be immuno-potentiators and exhibit anti-cancer activities [17–20]. Structural elucidations for Lentinan show the presence of only glucose molecules with mostly β -(1 \rightarrow 3)-glucan linkages in the regular backbone chains and β -(1 \rightarrow 6)-glucan in side chains [21,22]. The major active constituent of the KS-2 mycelium extract is reported to be a hetero-glycan protein conjugate containing *ca.* 24.6% proteins and 44% monosaccharides in addition to nucleic acid derivatives and vitamins [23]. Shida et al. also reported the extraction of α -(1 \rightarrow 3)-D-glucan from fruiting bodies of *L. edodes* [24,25].

Glucans and glycans are believed to play key roles in cell communication, protein interaction and immunity. Thus, structural features and physical properties, such as the monosaccharide composition, molecular weight, types of backbone linkage and degree of (1 \rightarrow 6)-linked side-branching, helical conformation and solubility are all potentially important for biological functions [4,20,26–29]. In particular, the glycosyl linkages, formed by various monosaccharides at the nodes of the polysaccharide tree formations, would reveal essential structural information and have been mostly elucidated by gas chromatographic and mass spectrometric (GC–MS) techniques. However, the analyses of these linkage data are sophisticated, usually incomplete and difficult, at least because of the following reasons: (1) most polysaccharides are heterogeneous with high molecular weights, (2) there are many combinations of linkage types between any two monosaccharides, and (3) there is a lack of a complete set of standard materials of all linkage types that could be used as reference standards.

Recently, mathematical and statistical methods have been used to help understand the monomer composition of polysaccharide characteristics of woody plants and to establish polysaccharide classifications of Vochysiaceae [30,31]. Multiple linear regression analysis, principal component analysis and factor analysis methods have also been used to find the polysaccharide structural characteristics and their relationships with macrophage stimulatory activity of regionally different strains of *Lentinula edodes* [32]. Among these statistical methods, cluster analysis could be used to measure strain similarities if some experimental variables (i.e. glycosyl linkages) are potentially highly correlated [31]. Cluster analysis is a generic name for multivariate analysis techniques to create groups of objects based on their degree of association [33]. Clustering is useful in several exploratory pattern-analysis, grouping, decision-making and machine-learning situations, including data mining, document retrieval, image segmentation, and pattern classification [34].

In this paper, we wish to report the monosaccharide compositions, molecular weight distributions, glycosyl structural

linkages and glycosyl linkages classification of the exopolysaccharide extracted from 10 regionally different *L. edodes*. In addition, investigations by genomic AFLP assay and by statistical cluster analyses of GC–MS linkage data are compared to understand the relationships of genotypes and structural features of these species.

2. Experiment

2.1. *L. edodes* strains, growth conditions and polysaccharide isolation

The 10 strains of *L. edodes* are: No. 135 (L24) and No. 939 (L25) from China; Tainung No. 1 “white cap” (L1) and “red cap” (L4) from Taiwan; Japanese 271 (L11, L15), Jongxing 5 (L6), Jongxing 8 (L10), Hey-King-Gang (L21) and Jong-Wen 600 (L23) from Japan. The culture broth of the mycelia of the *L. edodes* was submerged fermented in the medium (pH 4.5) containing 2% oat, 0.5% yeast extract, 0.1% KH_2PO_4 , 0.05% MgSO_4 and 0.15% CaCO_3 with reciprocating shaking (150 rpm min^{-1}) for 14 days at 26 °C. The cultivation developed in the flasks was then transferred to static incubation and the liquid was filtered (membrane filter pore size > 0.45 μm) to collect the culture broth filtrate containing the secondary metabolites and then sterilized in an autoclave at 120 °C for 20 min. Three volumes of 95% ethanol were then added and the culture broth filtrate was stored at 4 °C overnight. The exo-polysaccharide precipitates were collected by centrifugation, followed by washing with 75% ethanol, and then freeze-dried. The solid mycelia pellets were also collected and washed with sterilized distilled water, and weighed after drying in an oven for 7 days at 60 °C.

2.2. Amplified fragment length polymorphism analysis

Mycelia were harvested onto filter paper, rinsed with distilled water, and freeze-dried. Genomic DNA for each progeny was extracted by using the DNeasyTH Plant Mini Kit (Qiagen, Hilden) and was eluted twice from the DNA binding column with 100 μL AE solution (50 mM sodium acetate pH 5.2, 10 mM ethylenediaminetetraacetate). AFLP analysis was carried out by using a modified procedure described by Vos et al. [7] and Terashima et al. [13], the instruction manuals of the AFLP core Reagent Kit (Invitrogen) and the AFLP Microbial Fingerprinting Kit (Applied Biosystems). After the DNA materials were digested with two restriction enzymes, *Mse* I and *Eco* RI, the adaptors were then added to prepare the AFLP template for sequence analysis. Two primer sets, *Eco* RI-AC-FAM/*Mse* I-CAA and *Eco* RI-AA-FAM/*Mse* I-CAC, were employed for selective amplification. Electrophoresis and detection of amplified fragments were performed using the 373 DNA Genetic Analyzer (Perkin-Elmer). Each gel track ranged from the size-marker 50 bp band to the 500 bp band. The band positions of selected primers were used to construct a similarity index. Patterns were normalized with reference to molecular mass of the internal ROX-labeled size standard that was added to each sample. After normalization, the levels of genetic similarity between the AFLP patterns were calculated with Pearson correlation. For

cluster analysis of AFLP banding patterns, the un-weighted pair group method using arithmetic averages (UPGMA) was used [35]. Internal standards of *L. edodes* strains used were BCRC 36024 (CBS 454.69, IFO 8340) and 36482 (MUCL 28773). Phylogenetic grouping was performed using Bio Numerics software.

2.3. Molecular weight and monosaccharide composition determination

The molecular weights for fractions of the exo-polysaccharide extracts were determined using a gel-chromatographic technique with distilled water (0.3 mL min^{-1}) as the mobile phase (Waters Hsp-Gel AQ5.0, 1515 isocratic pump, 717 plus injector and 2410 RI detector). The calibration curve was constructed with standard dextrans (Polymer Standards Service) with a molecular weight range from 1.8×10^2 Da to 2.1×10^3 kDa. Four distinct polysaccharide fractions were identified and labeled as A, B, C, and D, respectively.

Monosaccharide analysis was performed using the method of Blakeney et al. [36] and Hoebler et al. [37]. The exo-polysaccharide from culture broth filtrate (2.0 mg) was completely hydrolyzed in a sealed tube containing 0.3 mL of 2 M trifluoroacetic acid (TFA) at 120°C for 90 min. The converted monosaccharide alditol acetates were determined by gas chromatography (Varian 3800). Myo-inositol (1.0 mg) was added as an internal standard. The total amounts of monosaccharides of these exo-polysaccharides were determined using the phenol–sulfuric acid method [14,38,39].

2.4. Glycosyl linkage analysis

A sample containing 1.0 mg of each crude polysaccharide was methylated using the modified NaOH–DMSO (dimethyl sulfoxide) method at 25°C [40–42]. The per-methylated product was then hydrolyzed, reduced, acetylated and analyzed by GC–MS (*HP 6890/MSD 5973*). Myo-inositol (1.0 mg) was added as an internal standard. The relative abundances of all glycosyl linkages in the 10 samples were normalized to the myo-inositol internal standard of the L1 sample and were used for later cluster analysis [43]. The resulting data of all the above experiments are presented as a mean \pm S.D. from triplicates determinations.

2.5. Cluster analysis

Cluster analysis methods were applied to investigate the relationships between types of glycosyl linkages (using the relative GC–MS abundance data) and *L. edodes* strains. Two key steps are involved in cluster analysis. The first is the measurement of the object similarity based on the Euclidean distance or Manhattan distance between objects [34]. The second is to group the objects based upon the resulted distances (linkages). Linkage methods are based upon how the association between groups is measured.

The Euclidean distance ($D_{\text{Eucl.}}$) between any two objects x_i and y_j was probably the most commonly chosen type of distance

and in the multidimensional space was computed as below:

$$D_{\text{Eucl.}}(x, y) = \{\Sigma(x_i - y_i)^2\}^{1/2} \quad (1)$$

This method has certain advantages, e.g. the distance between any two objects is not affected by the addition of new objects to the analysis, which may be outliers.

The Manhattan distance ($D_{\text{Mann.}}$) is simply the average differences across dimensions. In most cases, the results of these distance measurements are similar to those of simple Euclidean distances [43,44]. However, the effect of single large differences (outliers) is dampened (since they are not squared) in this measure. The Manhattan distance is computed as below:

$$D_{\text{Mann.}}(x, y) = \Sigma|x_i - y_i| \quad (2)$$

In this study, both Euclidean and Manhattan distances with five clustering methods were used: average, complete, single, Ward's and weighted linkage type [44]. Further standardization (i.e. Z-score normalization) of variables has been undertaken to enable the comparison of variables to minimize the bias in weighting which may result from differing measurement scales and ranges [43]. Then, the linkage type similarity within each cluster was calculated and plotted. The analysis was performed using S-PLUS.

3. Results and discussion

3.1. *Lentinula edodes* culture

Recently, the submerged cultivation of mushroom has received much attention in Asian regions as a promising alternative for efficient production of its valuable metabolites, especially polysaccharides and ganoderic acids [45,46]. Traditionally, it usually takes several months to cultivate the fruiting body of the mushroom, and it is also difficult to control product quality during soil cultivation. There is a great need to supply the market with a large amount of lower cost and high-quality mushroom products. Therefore, submerged cultivation of mushroom could eventually supplement the need and prove useful over fruiting body cultivation.

The optimum environmental conditions for our current submerged culture growth and exo-polysaccharide production of mushrooms in liquid cultures are dependent on strains. Initially, the growth conditions of the culture were optimized that showed a stationary phase after 14 days. The biomass of the culture broth filtrate extracts of all strains was weighed to be in the range of $0.35\text{--}0.40 \text{ g (50 mL)}^{-1}$ and exo-polysaccharide contents between 0.15 and 0.59 mg mL^{-1} (Table 1). These values would be useful as references for optimization and pilot plant production studies in the future [47].

3.2. Strain typing/phylogenetic mapping

Fig. 1 shows the AFLP fingerprints of genomic DNAs of different *Lentinula edodes* strains. A dendrogram of the similarity index based on the bands obtained for selected two primer sets was plotted to distinguish the closely related strains as shown

Table 1
Mycelia dry weight and exo-polysaccharide contents of strains

Strains	Exo-polysaccharide content (mg mL ⁻¹)	Mycelia dry weight (g (50 mL) ⁻¹)
L1	0.58	0.37
L4	0.61	0.4
L6	0.53	0.38
L10	0.31	0.34
L11	0.48	0.4
L15	0.44	0.37
L21	0.2	0.36
L23	0.15	0.33
L24	0.59	0.38
L25	0.59	0.39

in Fig. 2. The 10 isolates of *L. edodes* were grouped into three distinct clusters by AFLP: (1) L24 and L25 isolates from China; (2) L1 and L4 isolates from Taiwan; (3) L6, L10, L11, L15, L21 and L23 isolates from Japan. A very close genetic homogeneity among cultivated strains of Japanese mushrooms, L6, L21, L10, L11, L15, and L23 was observed. Similarly, for the Taiwanese (L1 and L4) and Chinese (L24 and L25) mushrooms, the obtained AFLP fingerprints point out close resemblance to the genetic homogeneity. However, L24 and L25 mushrooms from China were quite different from the Japanese and Taiwanese mushrooms. The mushrooms, L1 and L4 were cultivated as hybrid strains comprising of Japanese strains SL-19 and 271.

The dendrogram obtained using AFLP (fingerprinting) analysis of *L. edodes* provides new insight into the population structure of this mushroom species and proves useful for phylogenetic type studies. The results appear promising and are well supported by results obtained using random amplified polymorphic DNA (RAPD) assay (fingerprinting) for the same species [48]. However, the time and cost efficiency, reproducibility

and resolution of AFLP are superior or at least equal to those of other markers, e.g. RAPD, allozymes, restriction fragment length polymorphism (RFLP) and microsatellites [49]. Therefore, application of AFLP fingerprint assay for phylogenetic studies of mushrooms can now be included among other reported species in population studies.

3.3. Molecular weight and monosaccharide composition

The results of molecular weight analysis indicated that all extracted polysaccharide materials fell into four mw fractions: A (mw > 2750 kDa), B (mw ~ 2700 kDa), C (mw ~ 534 kDa) and D (mw ~ 11.7 kDa) (Table 2 and Supplementary data, Fig. A). All exo-polysaccharides had similar molecular weight distribution between 1×10^4 and 3×10^6 Da.

The results showed that great differences existed in the distribution ratio of the high molecular weight fractions A. The contents of each fraction in decreasing order are listed below: A (mw > 2750 kDa): L15 ~ L10 > L25 > L23 ~ L4 > L6 ~ L21 > L24 > L11 > L1; B (mw ~ 2700 kDa): L10 > L25 > L24 > L15 ~ L4; C (mw ~ 534 kDa): L11 > L1 > L23; and D (mw ~ 11.7 kDa): L24 > L4 > L25 > L10 > L6 > L1 > L15 > L21 ~ L23 > L11. The contents of exo-polysaccharide materials are complex in nature and poses difficulties for better understanding of their importance and structure-function relationships (*vide infra*).

The monosaccharide composition data are listed in Table 3. These data revealed great differences in the distribution of glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose of the crude polysaccharide of all mushrooms, strongly indicating that these materials are heterogeneous [1]. Except for L24, the glucose contents of all other strains are the greatest. This presumably indicates that the polysaccharides backbone and branched side chains are formed by mainly glucose 1 → 4 and 1 → 6 linkages, respectively. For L4 polysac-

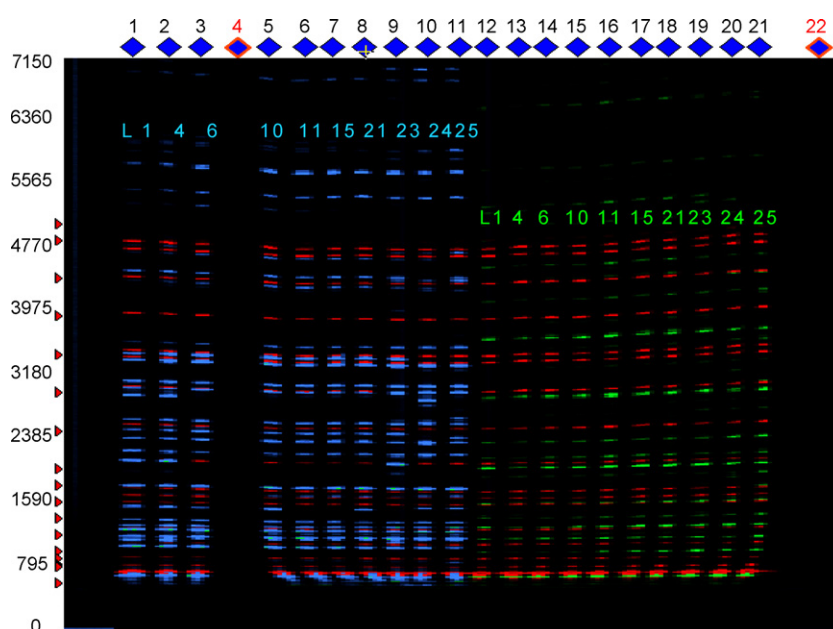


Fig. 1. AFLP fingerprints of genomic DNAs of different *Lentinula edodes* strains. Two primer sets were used: (left) *Eco* RI-AC-FAM/*Mse* I-CAA and (right) *Eco* RI-AA-FAM/*Mse* I-CAC.

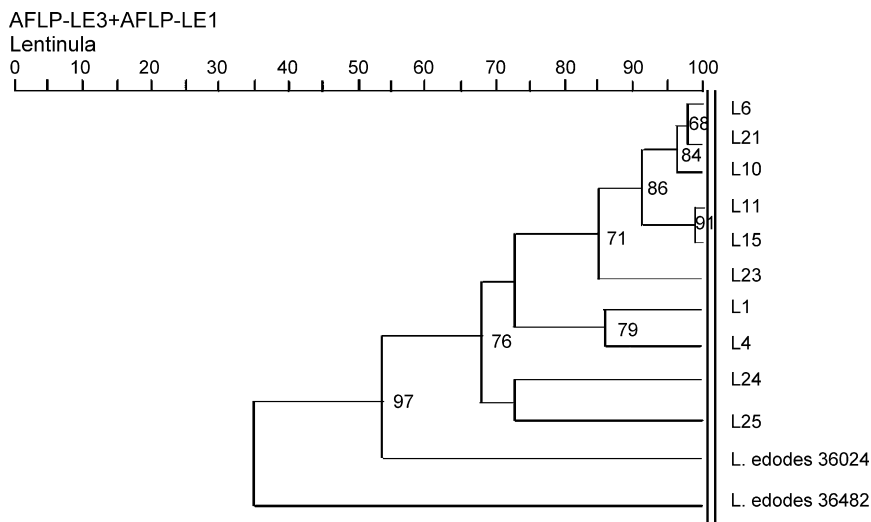


Fig. 2. Dendrogram of *L. edodes* constructed using AFLP assay.

Table 2
Molecular weight ratio of exo-polysaccharide from different strains

Strain/mw	L1	L4	L6	L10	L11	L15	L21	L23	L24	L25
Fraction A > 2,754,000	0.5	2.5	2.1	3.4	1.7	3.4	2.1	2.5	1.8	3.2
Fraction B 2,754,000	–	0.4	0.4	0.8	–	0.4	0.4	–	0.6	0.7
Fraction C 534,000	1.3	–	–	–	1.8	–	–	0.6	–	–
Fraction D 11,700	1.7	5	1.9	2.2	0.3	0.9	0.7	0.7	5.5	2.8

charide, the mannose composition is the greatest among all found monosaccharides, probably indicating that the backbone and/or branch contain more mannose 1 → 2 linkages (*vide infra*).

3.4. GC–MS analysis of glycosyl linkage

Fig. 3 shows the GC–MS result of the exo-polysaccharide extracted from L24, as an example of all GC–MS studies. It shows the presence of (1 → 4)-linked- β -glucopyranosyl and (1 → 6)-linked- β -glucopyranosyl moieties in the glucan, i.e. the peak of 1,4,5-tri-*O*-acetyl-1-deuterio-2,3,6-tri-*O*-methyl- β -glucitol with *m/z* values of 43, 59, 71, 87, 102, 118, 129, 142, 162, 173, and 233 represents glucose 1 → 4 linkage and the peak of 1,5,6-tri-*O*-acetyl-1-deuterio-2,3,4-tri-*O*-methyl- β -glucitol with *m/z* values of 43, 59, 71, 87, 102, 118, 129, 143, 162, 173, 189, and 233 represents glucose 1 → 6 linkage. Other peaks including 1,4,5,6-tetra-*O*-

acetyl-1-deuterio-2,3-di-*O*-methyl-glucitol, 1,4,5-tri-*O*-acetyl-1-deuterio-2,3-di-*O*-methyl-arabinitol, and 1,2,5-tri-*O*-acetyl-1-deuterio-3,4,6-tri-*O*-methyl-mannitol indicate glucose 1 → 4 and 1 → 6 linkage, arabinose 1 → 4 linkage, and mannose 1 → 2 linkage, respectively [36,38,41]. The completed GC–MS data are summarized in Table 4.

Based on the above analysis, the raw abundances (i.e. before data normalization) of the five linkage types of all 10 *L. edodes* strains are listed in Table 5. From the data in Table 5, it could be concluded that, for example, the exo-polysaccharide backbone of strain L24 is the longest among all strains because the 2,3,6-Me3-Glcp (representing glucose 1 → 4 linkage) abundance of L24 is the greatest [38]. The exo-polysaccharide of L24 has also more glucose 1 → 4 and 1 → 6 linkages (represented by 2,3-Me2-Glcp abundance) indicating more branches with 1 → 6 linkage [38]. On the other hand, the L24 branches have the least amount of glucose 1 → 6 linkages because the 2,3,4-Me3-Glcp (representing glucose 1 → 6 linkage) abundance is the least

Table 3
Monosaccharide composition of exo-polysaccharide from different strains

<i>Lentinula edodes</i>	L1	L4	L6	L10	L11	L15	L21	L23	L24	L25
Glucose %	55.44	23.1	50.95	56.2	47.12	68.93	47.36	56.36	88.82	55.58
Mannose %	32.28	49.22	31.83	26.89	36.24	20.02	33.5	26.72	7.23	30.91
Arabinose %	6.03	11.19	8.25	7.77	6.76	5.41	8.42	7.73	2.22	6.45
Xylose %	4.82	10.65	5.89	5.58	5.21	3.83	5.88	5.49	1.13	4.55
Galactose %	1.44	5.09	2.59	3.56	4.3	1.42	3.73	3.19	0.03	1.87
Fucose %	0	0.52	0.24	0	0.24	0.11	0.56	0.19	0.12	0.24
Rhamnose %	0	0.22	0.24	0	0.14	0.28	0.56	0.32	0.45	0.4

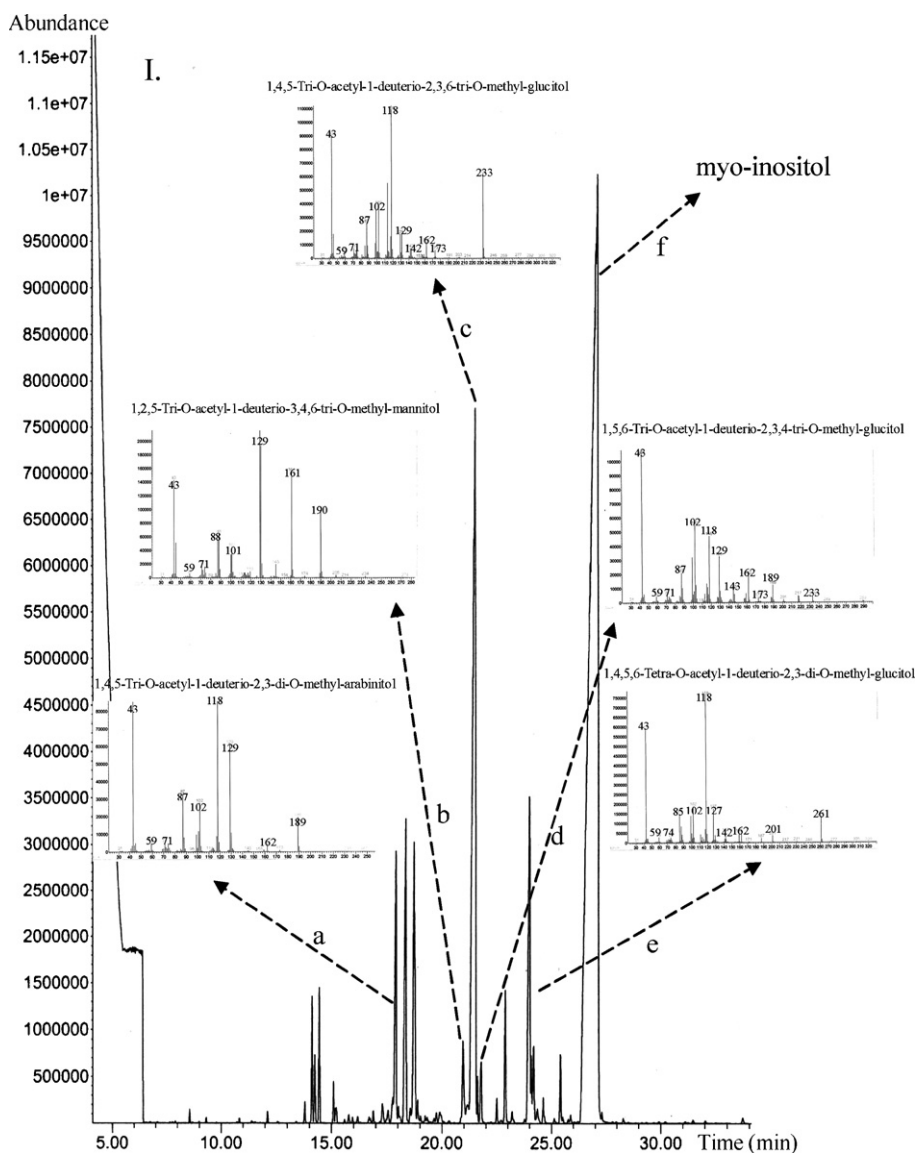


Fig. 3. Gas chromatography–mass spectrometry of L24 exo-polysaccharide.

among all. As an other example, the L15 exo-polysaccharide is most likely composed of glucose 1 → 4 backbone linkage, glucose 1 → 6 branch linkage, mannose 1 → 2 branch linkage and arabinose 1 → 4 branch linkage [41].

The sum of monosaccharide contents of glucose, mannose and arabinose constitutes ~98% of the total monosaccharides for L24. This is consistent with that found from GC–MS linkage data. For other strains, except for L4 with ~73% as the sum, the sums of the glucose, mannose, and arabinose contents are all

greater than 90%, and consistent GC–MS results were obtained. These indicate that the glucose 1 → 4 backbone linkage, glucose 1 → 6 branch linkage, mannose 1 → 2 branch linkage and arabinose 1 → 4 branch linkage are probably dominant in these exo-polysaccharides. However, the significance of these linkage data as related to strain similarities or differences is not easily decoded by just simple observation and therefore, statistical treatment, such as cluster analysis is employed and discussed next.

Table 4
GC–MS data from the methylated polysaccharides

Methylated sugars	Retention time (min)	Mass fragmentation (<i>m/z</i>)	Mode of linkage
2,3,6-Me3-Glcp	21.5	43, 59, 71, 87, 102, 118, 129, 142, 162, 173, 233	¹ Glcp ⁴
2,3,4-Me3-Glcp	21.8	43, 59, 71, 87, 102, 118, 129, 143, 162, 173, 189, 233	¹ Glcp ⁶
2,3-Me2-Glcp	23.9	43, 59, 74, 85, 102, 118, 127, 142, 162, 201, 261	¹ Glcp ^{4,6}
3,4,6-Me3-Manp	21	43, 59, 71, 88, 101, 129, 161, 190	¹ Manp ²
2,3-Me2-Arap	17.8	43, 59, 71, 87, 102, 118, 129, 162, 189	¹ Arap ⁴

Table 5
Methylation analysis of the raw polysaccharide glycosyl linkage abundances from different *Lentinula edodes* strains

Methylated monosaccharides/strains	Abundance $\times 10^{-6}$					
	Myo-inositol	2,3-Me3-Arap	3,4,6-Me3-Manp	2,3,6-Me3-Glcp	2,3,4-Me3-Glcp	2,3-Me3-Glcp
L1	9.2	2.6	1.4	3.35	0.9	0.9
L4	9.4	0.5	3.5	3.1	1.7	0.9
L6	10.3	0.9	1.7	3.9	1.4	1.7
L10	9.4	0.5	2.2	5.7	1.7	1.9
L11	9.6	1.2	2.5	4.9	1.7	1.7
L15	9.1	2.9	1.7	5.8	1.5	2.4
L21	8.8	1.1	1.7	4.4	1.4	1.4
L23	8.4	0.7	1.3	4.6	2.2	1.4
L24	10.2	0.3	0.1	7.7	0.7	3.6
L25	9.3	0.7	0.1	3.2	1.05	1.1

3.5. Cluster analysis

The results of cluster analysis of the five exo-polysaccharide linkage types using the normalized abundances data derived from Table 5 of the 10 *L. edodes* strains are shown in Fig. 4A, B and C and Supplementary data, Figs. B and C (the relative abundances of all glycosyl linkages in the 10 samples were normalized to the myo-inositol internal standard of the L1 sample and were used for cluster analysis [43]). The combination of Euclidean and Manhattan distance with five clustering methods resulted in 10 dendrograms. Interestingly, the 10 dendrograms form several groupings: one with four identical dendrograms obtained by using the average, Ward and weighted linkage type methods of Manhattan Distance and using the Ward method of Euclidean Distance (Fig. 4A), and another two with two identical dendrograms by using the average and weighted methods of Euclidean Distance (Fig. 4B) and by using the complete linkage type method of Euclidean and Manhattan Distance (Fig. 4C), respectively. From Fig. 4A, B and C, it is observed that L24 either forms an isolated cluster by itself or is close to L15. The Japanese L21, L23, L10, and L11 strains always form a second cluster with minor differences. The L1, L6, L25, and L4 strains form the remaining clusters with L1 and L25 strains being more similar and L4, and L6 strains being dissimilar. The cluster analysis results using the less common single linkage method of both Manhattan and Euclidean Distances are shown in Supplementary data, Figs. B and C which are similar to each other and are somewhat different from those in Fig. 4.

The different hierarchical clustering linkage type methods differ in how the distance between two clusters is computed [44] and resulted in different dendrograms. For example, the average linkage Distance between two clusters is defined as the average distance between pairs of objects, one in each cluster [43]. Average linkage tends to join clusters with small variances and is biased toward producing clusters with roughly the same variance. In Ward's method, the distance between two clusters is the sum of squares between the two clusters added up over all of the variables [43]. Roberts reported Ward's method as "a very efficient clustering method, but favors the grouping of small clusters" [44]. Also, Xu suggested that Average linkage method and Ward's method are among the better hierarchical clustering algorithms [43]. For our present studies, the cluster analysis

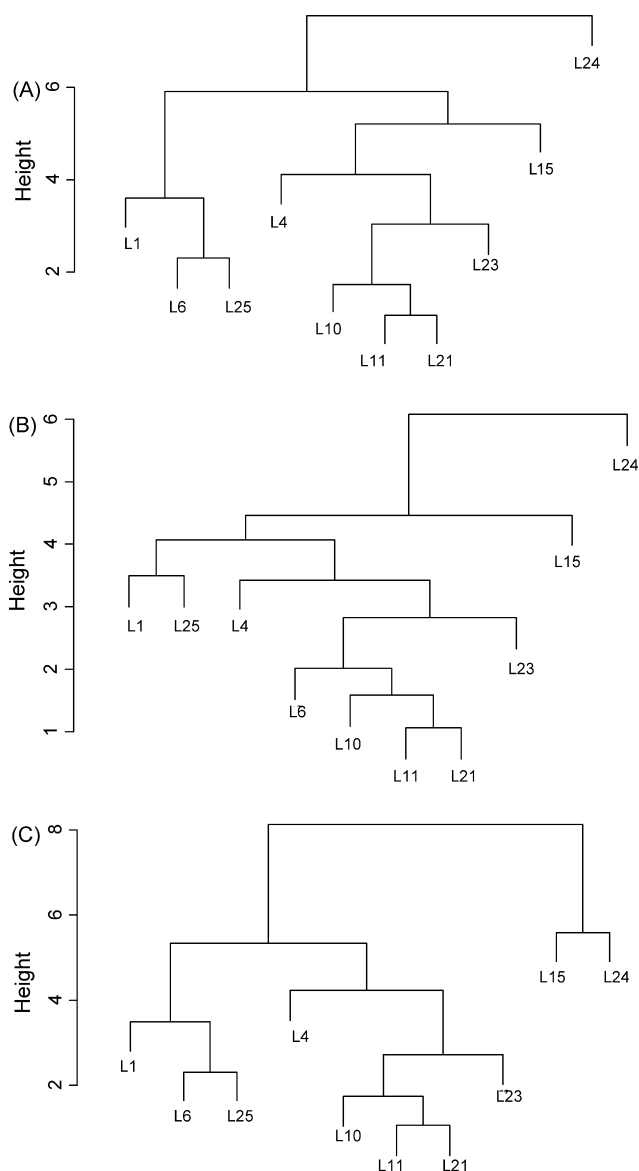


Fig. 4. (A) Cluster analysis using the average, Ward and weighted linkage type method of Manhattan Distance and using the Ward linkage type method of Euclidean distance. (B) Cluster analysis using average and weighted linkage type method of Euclidean distance. (C) Cluster analysis using the Complete linkage type method of Euclidean and Manhattan distance.

using the Ward's method and average linkage method with both Euclidean and Manhattan Distances resulted in two dendrograms (Fig. 4A and B). However, these dendrograms are all quite similar, with only the relative positions of L6 being different. If the complete linkage type method is considered (Fig. 4C), it is observed that, in addition to L6, the relative position of L15 is different from those of the average and Ward methods (Fig. 4A and B).

The results of the above cluster analysis are not very much different from that of the AFLP assay. The Japanese L10, L11, L21, and 23 strains are both genetically and structurally similar. Other strains show some minor genetic and structural differences. These differences could be attributed to the results of hybridization and other genetic differences.

4. Conclusion

In addition to the common monosaccharide compositions, molecular weight distributions, and glycosyl linkage information to classify different strains of *L. edodes*, we have carried out AFLP and cluster analyses using genomic and GC–MS glycosyl linkage data, respectively. The results of both AFLP and cluster analyses provide greater dimensions for mushroom classifications to account for the differences and similarities in their genetic and structural features. Specifically, for cluster analysis, both the average and Ward methods of Manhattan distance and Euclidean distance seem to be readily useful for this purpose. This approach should be useful for more strains or other species classification using valid structural data. More studies should definitely be carried out to further examine this point.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.aca.2007.04.021](https://doi.org/10.1016/j.aca.2007.04.021).

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