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ISSR-Assisted Breeding of Excellent New Strains of *Ganoderma lingzhi* through Single-Spore Selfing

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Abstract: To improve our understanding of the selfing of *G. lingzhi* basidiospore monokaryons and increase the efficiency of breeding excellent strains, 52 basidiospore monokaryons were isolated from a commercial *G. lingzhi* strain (laboratory number P). A severe partial segregation was observed using the chi-square test, the growth rate of the monokaryotic strains was normally distributed, and colonies exhibited 5 forms. The genetic diversity of the monokaryotic strains was further demonstrated by intersimple sequence repeat (ISSR) analysis, and the similarity coefficient was in the range of 0.49–1, which was consistent with the genotype classification results. In total, 14 AxBx monokaryotic strains were randomly selected for selfing with the 1 AyBy strain when the similarity coefficient was 0.76, and a total of 14 offspring were obtained via selfing, all of which were incompatible with their parents. The traits of the selfing progenies were diverse. The mycelial growth rate, fruiting body yield, and polysaccharide, triterpene, and sterol contents were the main indices. According to the membership function value, 71.43% of the selfing progeny were super parent, and the A88 strain with the best comprehensive traits was selected. These findings prove that ISSR molecular marker-assisted breeding reduces blindness, greatly reduces workload, and improves work efficiency.

Keywords: *Ganoderma lingzhi*; basidiospore monokaryons; selfing; ISSR; genetic diversity

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1. Introduction

Ganoderma lingzhi is a well-known medicinal fungus with important economic value that has a history of more than 2000 years in China [1]. *Ganoderma lingzhi* reportedly contains hundreds of bioactive secondary metabolites, consisting mainly of triterpenoids, polysaccharides, fatty acids, and sterols [2,3], and has a substantial effect on a variety of diseases. For example, pharmacological experiments showed that the *G. lingzhi* triterpenoids GL-4 and GL-8 had moderate cytotoxic effects on different cancer cell lines, inhibited nitric oxide production induced by lipopolysaccharide in RAW264.7 macrophage cells, and had dual anti-cancer and anti-inflammatory effects [4]. In addition, *G. lingzhi* also exhibits pharmacological effects such as immune regulation, antioxidant, germicidal, and hypoglycaemic properties [3,5–7]. Today, *G. lingzhi* products are greatly welcomed by the market with the increase in people's health awareness. At present, China's *G. lingzhi* industry is developing rapidly. The China Edible Fungi Association has reported that the output of *G. lingzhi* exceeded 250,000 tons in 2022, an increase of 21.63% from the output of 2021, and the output value of about 21.5 billion yuan in 2022.

Excellent strains are the basis of and the key to the healthy and rapid development of the *G. lingzhi* industry. It is highly important to select high-yield and quality strains, because continuous asexual reproduction in commercial production usually leads to strain quality degradation [8,9]. At present, the breeding methods commonly used for *G. lingzhi* include mutagenesis [10], crossbreeding [9], protoplast fusion [11], and molecular breeding [12]. Article 36 of the Standard for the Chinese Standard for the Production Quality Management

of Chinese Medicinal Materials requires that polyploid or haploid cultivars, interspecific hybrids, and transgenic varieties produced by artificial intervention are prohibited in the breeding of Chinese medicinal materials. From this point of view, crossbreeding is the best way to select *G. lingzhi* for medicinal fungi. Crossbreeding, which can be divided into sexual and asexual, is one of the most commonly used methods to obtain new high-yield quality cultivars of edible fungi. Mushrooms produce spores through sexual reproduction in nature. After spore germination, sexual monokaryotic strains are produced, and fertile mating occurs between compatible monokaryotic strains [13]. Selfing is a kind of crossbreeding. Because sexual spores undergo meiosis and are the product of genetic recombination, they have high genetic diversity, resulting in differing traits in offspring [14,15], simultaneous selfing aggregation, and homozygous genes [13]. The F1 selfing progeny of *Agrocybe salicacola* were comprehensively analysed based on their qualitative traits (colour, deformity, and growth characteristics) and quantitative traits (shape, yield, size, and the number of fruiting bodies). It was found that 18% of the selfing progenies had better agronomic traits than their parents, and the dominant lines could be selected from the selfing progenies groups according to their different breeding purposes [16]. Strains with excellent agronomic properties were also cultivated in *Lentinula edodes* through selfing [17]. However, the traditional method of selfing breeding randomly selected basidiospore monokaryons for selfing and selected excellent new strains in a large number of progenies, which was blind and had a large workload.

The evaluation of genetic diversity will increase the effective use of genetic variation in breeding [18]. The genetic diversity analysis of strains can be performed in a variety of ways in edible fungi [19], for example, simple sequence repeat (SSR) [20], intersimple sequence repeat (ISSR) [21], random amplified polymorphic DNA (RAPD) [22], and sequence-related amplified polymorphism (SRAP) marker methods [23]. Different molecular markers have different limitations. ISSR markers can overcome these limitations and are widely used because of their high polymorphism, strong repeatability, simplicity, speed, and economy [24]. For example, ISSR molecular marker technology was conditioned to analyse the genetic diversity of 22 wild strains and 2 cultivars of *Auricularia heimuer* in 13 ecological regions in China, and it was found that the genetic differences within the region or between neighbouring strains were minimal, and that the genetic differences between the wild strains and the cultivated strains were the greatest [25]. In addition, ISSR molecular markers have also been conditioned to determine the genetic diversity of macrofungal monokaryotic strains, which is conducive to the breeding of excellent strains [26].

The mating compatibility that allows for heterokaryosis and fruiting body formation exists only between the mycelium carrying genes of different mating types and is the basis of crossbreeding [27]. *Ganoderma lingzhi* exhibits a tetrapolar heterozygotic mating system that produces a dikaryon capable of developing into a fruiting body through the fusion of two monokaryotic strains with different mating alleles at the A and B mating type sites [28]. After fruiting body maturation, the four types of haploid basidiospores are formed in large quantities in basidia via meiosis [9]. The genetic diversity of *G. lingzhi* basidiospore monokaryons was analysed, and the strains were classified according to the genetic distance in this study. Then, according to the classification results, monokaryotic strains were selected for selfing. Finally, the mycelial growth speed, fruiting body yield, and polysaccharide, triterpene, and sterol contents were used as the main indices, and the strains with an excellent overall performance were selected from among the hybrid strains, according to their membership functions.

2. Materials and Methods

2.1. Fungal Strains

The strains used in this paper are shown in Table 1. The parent strain analysed in this study was a commercial *G. lingzhi* strain, laboratory number strain P, which was preserved by the Edible and Medicinal Fungus Team at the Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences. The strain P was maintained on

potato dextrose agar (PDA) slants containing 200 g potato (immersed), 20 g of glucose, 1.5 g of KH_2PO_4, 1.5 g of K_2HPO_4, 2 g of $MgSO_4$, 20 g of agar, and water up to 1 L at a natural pH of 4 °C.

Table 1. Sample data of *G. lingzhi* strains.

Code	Strain No.	Type	Code	Strain No.	Type	Code	Strain No.	Type
1	P	parental strain	24	43	AyBy	47	306	AxBx
2	20	AyBy	25	44	AxBx	48	337	AxBx
3	21	AxBx	26	45	AyBy	49	355	AxBx
4	22	AyBy	27	46	AyBy	50	382	AxBx
5	23	AyBy	28	48	AxBx	51	413	AxBx
6	24	AyBy	29	49	AyBy	52	418	AxBx
7	25	AyBy	30	50	AxBx	53	426	AxBx
8	26	AxBx	31	52	AxBx	54	A21	21 × 31
9	27	AyBy	32	53	AxBx	55	A28	28 × 31
10	28	AxBx	33	54	AxBx	56	A53	53 × 31
11	29	AyBy	34	56	AxBx	57	A54	54 × 31
12	31	AyBy	35	63	AxBx	58	A88	88 × 31
13	32	AxBx	36	78	AxBx	59	A97	97 × 31
14	33	AyBy	37	83	AxBx	60	A203	203 × 31
15	34	AyBy	38	88	AxBx	61	A217	217 × 31
16	35	AyBy	39	92	AxBx	62	A226	226 × 31
17	36	AyBy	40	97	AxBx	63	A337	337 × 31
18	37	AyBy	41	159	AxBx	64	A382	382 × 31
19	38	AyBy	42	203	AxBx	65	A413	413 × 31
20	39	AyBy	43	216	AxBx	66	A418	418 × 31
21	40	AyBy	44	217	AxBx	67	A426	426 × 31
22	41	AyBy	45	226	AxBx			
23	42	AyBy	46	300	AxBx			

× stands for selfing.

2.2. Collection of the Basidiospores and Isolation of the Monokaryotic Strains

To harvest the *G. lingzhi* spores, paper was wrapped around fruiting bodies during the maturation period, and sterile sulphate paper was placed on pileus. The spores collected on the sulphate paper were scraped into a centrifuge tube under sterile conditions, and sterile water was added to resuspend the spores on the second day. After the concentration was adjusted to approximately 1×10^6 spores/mL, 0.1 mL of the spore suspension was collected and uniformly coated on PDA (CM123, Land Bridge, Beijing, China) media and incubated at 25 °C.

After spore germination, a single colony was carefully selected on PDA (CM123A, Land bridge, Beijing, China) culture media with an inoculation needle, and a monokaryotic strain without clamp connections was observed under a 400× optical microscope (NLCD500, Jiangnan Yongxin Optical Co., Ltd., Nanjing, China).

2.3. Spore Mating Type Analysis and Chi-Square Test

For mating type determination, the methods of Ke et al. [29] were used. The chi-square test was used to detect 4 types of basidiospore monokaryons to verify whether the proportion of monokaryotic strains of the different mating types exhibited free separation.

2.4. ISSR Profiling

Genomic DNA was extracted using an Ezup Column Fungi Genomic DNA Purification Kit (B518259, Sangon Biotech, Shanghai, China). The ISSR primer sequences and amplification reaction conditions followed those described by Li et al. [11].

2.5. Mycelial Growth Rate and Fructification

The activated mycelia were inoculated in the centre of the PDA media and cultured in a constant temperature incubator at 25 °C. The colony radius was measured on the 5th day after inoculation, and the mycelial growth rates were calculated (mm/day).

The activated mycelia were inoculated in a test tube containing sawdust media (30 mm × 200 mm). The sawdust media contained 76% sawdust, 20% wheat bran, 2% gypsum, 2% glucose, and 60% water content. After inoculation, the mycelia were cultured vertically in a constant temperature incubator at 25 °C. The mycelia height was measured on the 12th and 17th days after inoculation, and the mycelial growth rate was calculated (mm/d).

The composition of the experimental mushroom cultivation material was the same as that described above. Each mushroom bag weighed 450 g and was incubated in a constant temperature incubator at 25 °C after inoculation. After complete colonization, the cultures were moved to the greenhouse for the fruiting experiment, and the white edge of the pileus disappeared as the fruiting bodies matured.

2.6. Determination of Total Polysaccharides, Triterpenes, and Sterols in Fruiting Bodies

The fruiting bodies were placed in an electric blast drying oven (GZX-9140, Boxun, Shanghai, China) at 60 °C and then crushed after drying, after maturation. The crushed fruiting bodies were used for the determination of total polysaccharides, triterpenes, and sterols. Specific methods referred to the method of the Pharmacopoeia of the People's Republic of China improved by Li et al. [11].

2.7. Statistical Analysis

Based on the ISSR amplification electrophoretic map, a 0/1 matrix was established, the genetic similarity coefficient was calculated by NTSYS-pc 2.10 software based on the DICE coefficient, and cluster analysis was performed by the unweighted pair group method with an arithmetic mean (UPGMA) [25]. All experiments included three or more biological and technical replicates. IBM SPSS Statistics 23 was used for the statistical analysis of the experimental data, and GraphPad Prism 8.0 was used to construct the diagrams. The letters in the diagrams indicate significant differences at the $p < 0.05$ level. The membership function method was used to evaluate the hybrid strains comprehensively, and the calculation formula was as follows:

$$\mu(Xi) = (Xi - Xmin)/(Xmax - Xmin) \quad (1)$$

where $\mu(Xi)$ is the membership function value, i is the indicator, Xi is the measured value of an indicator, and $Xmax$ and $Xmin$ are the maximum and minimum measured values of an indicator, respectively [30]. After calculating each index of fruiting bodies of the same strain, the comprehensive quality measurement value of the strain was obtained by arithmetic averaging, and the strains were ranked.

3. Results

3.1. Characteristics of the Monokaryotic Strains

A total of 52 basidiospore monokaryons were selected via microscopic examination (Figure S1), among which AxBx:AyBy:AxBy:AyBx = 15:11:0:0 (Figure 1A), $\chi^2 = 54.46$, and $\chi^2_{0.05} = 7.81 < 54.46$ when n = 3 degrees of freedom, which did not meet the 1:1:1:1 free separation ratio, exhibited partial separation. Figure 1B shows the mycelial growth rate frequency of the monokaryotic strains on PDA media. The growth rate of monokaryotic strains presented a normal distribution with a continuous change trend, exhibiting an average value of 3.7. According to the Kolmogorov–Smirnoff (K–S) test, $p = 0.2$, which was greater than 0.05 and conforms to a normal distribution, indicating that the mycelial growth rate was a quantitative trait controlled by polygenes. The lowest mycelial growth rate was 2.41 ± 0.19 mm/d, and the highest was 6.16 ± 0.17 mm/d, with a coefficient of variation

of 0.23, exhibiting a large difference and a significant character separation (Figure 1C). There were five types of mycelia overall, namely, compact, fluffy, central compact, grey, and fading, with most being of the central compact type and three being fading types (Figure 1D).

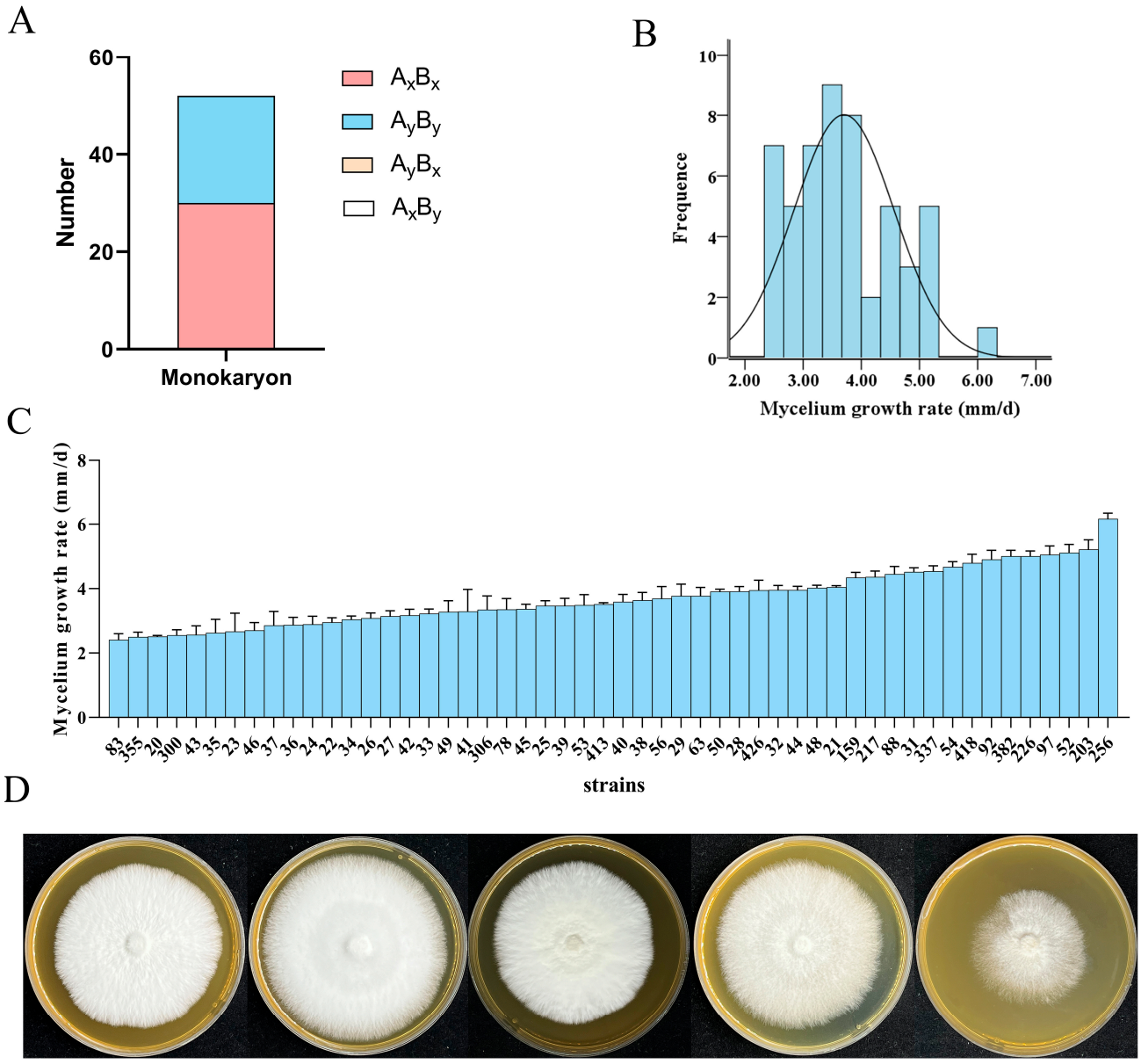

Figure 1. Characteristics of the monokaryotic strains. (**A**) The number of monokaryotic strains of the four genotypes. (**B**) Mycelial growth rate frequency distribution. (**C**) Mycelial growth rate. (**D**) Colony morphology of the monokaryotic strains. The morphology of the colony from left to right are named as compact, fluffy, central compact, grey, and fading.

3.2. Genetic Diversity Analysis of the Monokaryotic Strains

ISSR-PCR was performed on 52 monokaryotic strains and their parents using 12 primers (Table 2). The different primers were amplified between 1 and 10 bands, and a total of 57 bands were amplified, with an average of 4.75 bands per primer. The numbers of polymorphic bands amplified by each primer and the polymorphism ratio were statistically analysed. The average polymorphic band ratio was 98.32%, and the polymorphism ratio of the bands amplified by the 11 primers was 100%, while the polymorphism ratio of the bands amplified by ISSR4 was 0%. The ISSR4 primer may facilitate the identification

of the parent and its basidiospore monokaryons. In addition, the bands amplified with ISSR1, ISSR6, ISSR8, ISSR9, ISSR10, ISSR11, and ISSR16 exhibited one genotype, and the other genotype was absent, which could be used as the basis for genotype identification. In conclusion, the frequency of genetic polymorphisms among the monokaryotic strains was high.

Table 2. ISSR primer amplification results and polymorphism analysis.

Primer	Sequence (5′-3′)	Total Bands	Polymorphic Bands	Percentage of Polymorphic Bands (%)
ISSR-1	CACCACACACACACACA	5	5	100
ISSR-3	GAGAGAGAGAGAGAGACC	3	3	100
ISSR-4	AGCAGCAGCAGCAGCAGCG	1	0	0
ISSR-5	TGCACACACACACAC	8	8	100
ISSR-6	GAGAGAGAGAGAGAGAT	4	4	100
ISSR-7	AGAGAGAGAGAGAGAGC	2	2	100
ISSR-8	CACACACACACACACAT	4	4	100
ISSR-9	GAGAGAGAGAGAGAGACT	5	5	100
ISSR-10	TTCCCTTCCCTTCCC	4	4	100
ISSR-11	GTGACACACACACAC	10	10	100
ISSR-12	AGTGTGTGTGTGTGT	5	5	100
ISSR-16	GGATGCAACACACACACAC	6	6	100
	All number	57	56	
	Average value	4.75	4.67	98.32

The results of the cluster analysis are shown in Figure 2. In total, 53 strains were divided into 2 categories at a similarity coefficient of 0.49, which was consistent with the genotype classification results. The similarity of strains 48, 32, and 50, in terms of the AxBx genotype was 100%. The similarity of all monokaryotic strains of the AyBy genotype was 100%. The parental and AxBx genotype strains were grouped into class 1. The AxBx genotype strains exhibited a greater genetic diversity, and the AyBy genotype strains exhibited a greater similarity.

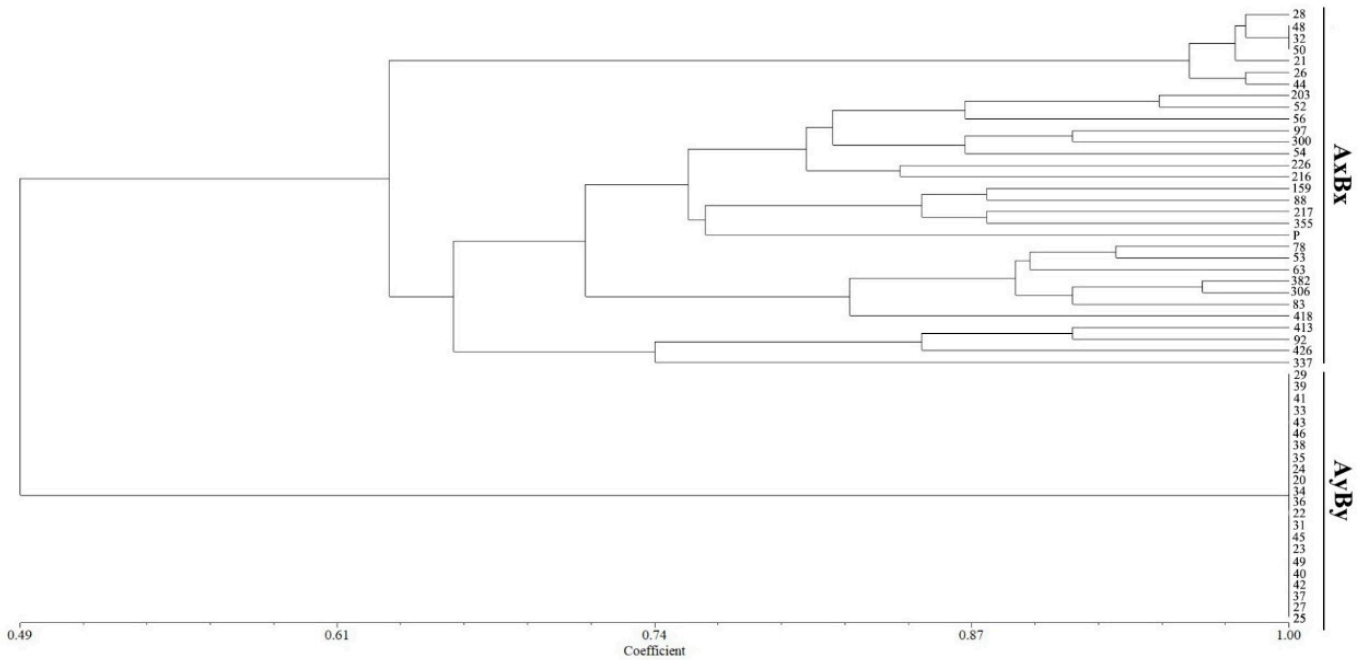

Figure 2. ISSR affinity cluster analysis of the basidiospore monokaryons.

The traditional breeding method randomly selects the mating basidiospore monokaryons for selfing, which is blind and usually forms dozens of selfing combinations. According to the results of the cluster map, 53 strains were divided into 8 categories at a similarity coefficient of 0.76, and 14 AxBx strains (28, 21, 203, 226, 97, 54, 88, 217, 53, 382, 418, 413, 426, and 337) were randomly selected for crossing with the AyBy genotype 31 in this study. A total of 14 selfing progenies were formed.

3.3. Identification of the Hybrid Strains

The 14 hybrids of the randomly selected AxBx strain and AyBy genotype 31 produced clamp connections (Figure S2), which indicated that the hybridisation was successful. As shown in Figure 3A, the mycelia of the monokaryotic strains were whiter and thicker, while the mycelia of the strain after hybridisation were sparser compared to those of the strain before hybridisation. Another obvious feature was that the AxBx monokaryotic strain had a greater effect on the morphology of the hybrid strains, such as strains 21 and A21 and 28 and A28, which exhibited a flocculent colony morphology. In addition, the colony morphology of the obtained hybrid strains was similar to that of the parent P. ISSR electrophoretic results. (Figure 3B) showed that the electrophoretic bands of hybrids were a combination of the monokaryotic strains bands. According to the antagonism test (Table S1) and the ISSR results (Figure 3C), 14 hybrids were obtained via hybridisation, and they were all different from parent P. The results for hybrids A28 and A21 showed the same strain. The calculated genetic similarity coefficient was 0.98, which indicates that A28 and A21 are very close to each other.

3.4. Agronomic Traits and Comprehensive Evaluation

To compare the correlation between the growth rate of the monokaryotic strains and the growth rate of the hybrid progeny mycelia, and between the growth rate of mycelia on different media, the growth rates of mycelia on the PDA media and sawdust media were measured. The growth rate of monokaryotic strain 413 on the PDA (5.40 ± 0.85 mm/d) was significantly greater than that of the other strains, and the growth rate of strain 337 was the lowest (3.64 ± 0.29 mm/d) (Figure 4A). The growth rate of the hybrid strains on the PDA media was greater than that of the monokaryotic strains, and the growth rate of the hybrid strains ranged from 6.95 ± 0.23 mm/d–8.68 ± 0.33 mm/d, with an average growth rate of 7.58 mm/d. The K–S test showed that $p = 0.102$, which was greater than 0.05 and was consistent with a normal distribution. Strain P may have degenerated, and its growth rate (3.69 ± 1.16 mm/d) was significantly lower than that of the hybrid strains (Figure 4B). The growth rate of the monokaryotic strains was relatively concentrated on the sawdust media, and the growth rates of these 10 strains were significantly greater than that of the remaining 5 strains. The growth rate of strain 97 was the lowest, at 3.15 ± 0.21 mm/d, and the growth rates of the monokaryotic strains were normally distributed in the sawdust media (Figure 4C). The growth rate of P was relatively fast (5.75 ± 0.37 mm/d) on the sawdust media, which was greater than the average growth rate of the offspring (5.52 mm/d); the growth rate of A337 was the greatest (6.89 ± 0.43 mm/d), and that of A21 was the lowest (4.77 ± 0.91 mm/d). The median growth rate of the hybrid strains was 5.46 mm/d, which was lower than the average (Figure 4D). By calculating the Pearson correlation coefficient, the growth rate correlation between the AxBx strain and the hybrid offspring on the PDA ($r^2 = -0.299$, $p = 0.299$) and the growth rate correlation on the sawdust media ($r^2 = 0.34$, $p = 0.235$) were poor. There was a negative, but not significant, correlation between the growth rate of the monokaryotic strains on the PDA and sawdust media ($r^2 = -0.367$, $p = 0.197$) and between the growth rate of the hybrid progeny on the PDA and sawdust media ($r^2 = -0.465$, $p = 0.094$).

The fresh weight and total contents of polysaccharides, triterpenes, and sterols in fruiting bodies of *G. lingzhi* were normally distributed (p values were 0.2, 0.17, and 0.2, respectively). A21 and A28 had weak anti-bacterial abilities and failed to produce fruiting bodies, the phenotype of the other strains was normal (Figure S3). The fruiting body

formation rate was 85.71%. The fresh weight of the parent strains was 20.94 ± 4.96 g, and the fresh weight of A413 in the offspring was the highest, reaching 33.64 ± 1.97 g, which was 1.6 times that of the parents. The consistency in the fresh weight of A54 was poor (mass range 18.45–38.84 g). There was no significant difference between A418 and its parents, and A426 was the only strain with a lower mean fresh weight than its parents (Figure 4E). The polysaccharide content of P was high (2.47 ± 0.02%), the polysaccharide content of A382 was not significantly different from that of P, and the polysaccharide content of the other progeny was significantly lower than that of P, but the polysaccharide content of all strains exceeded the pharmacopoeia standard (Figure 4F). The triterpene and sterol contents of A382 were the lowest (0.72 ± 0.01%), and the triterpene and sterol contents of P were 1.03 ± 0.02%, which were not significantly different from those of A203 and A426, while those of A54, A88, and A337 were greater than those of their parents. The highest triterpene and sterol contents of A88 was 1.10 ± 0.07, which was not significantly different from that of A337 (Figure 4G).

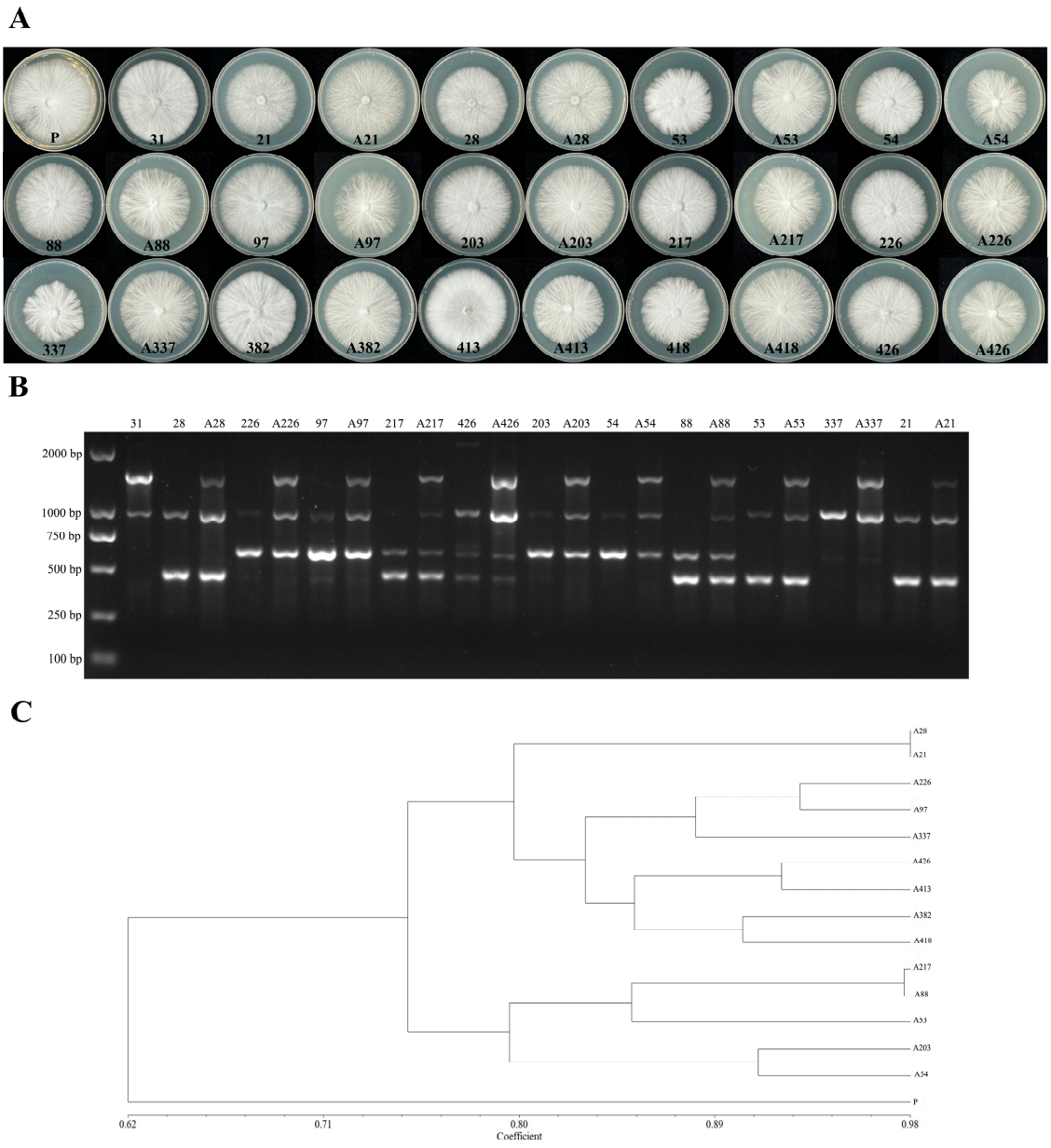

Figure 3. Identification of the hybrid strains. (**A**) Colony morphology. (**B**) ISSR electrophoretic map of the monokaryotic strains and their hybrids. (**C**) Phylogenetic cluster analysis of the hybrid strains.

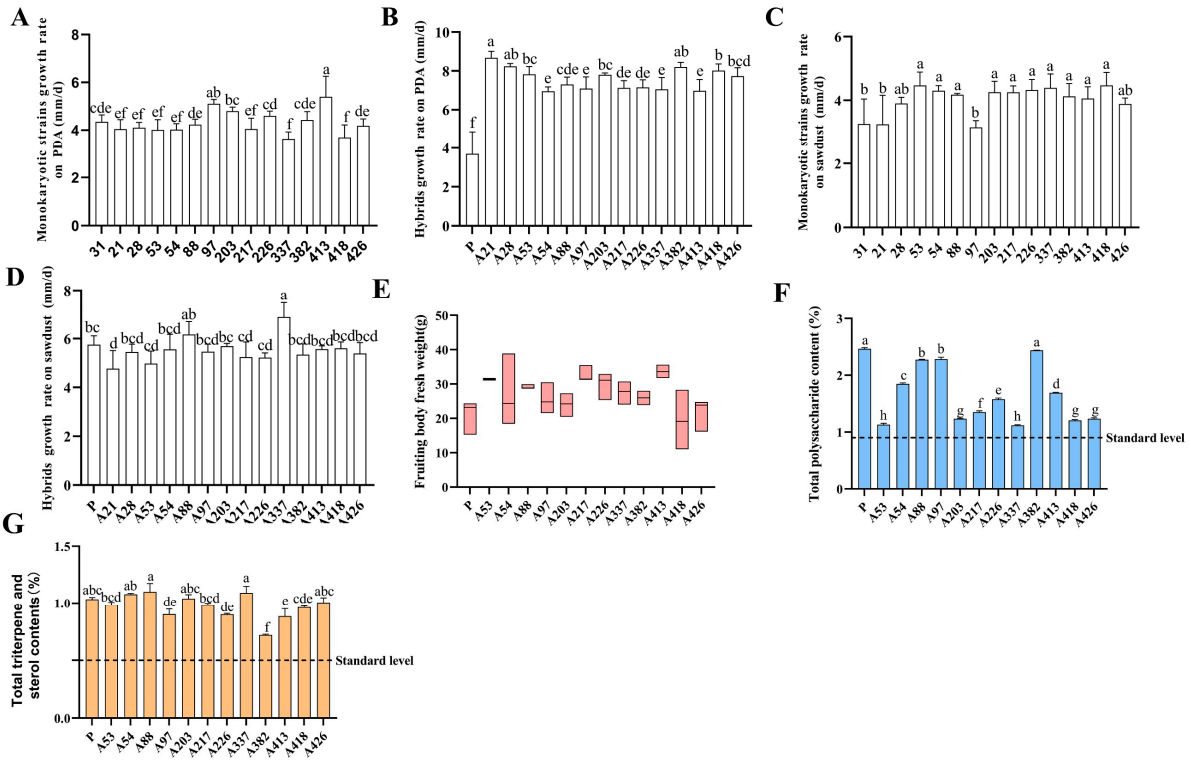

Figure 4. Agronomic traits. (**A**) Growth rate of the monokaryotic strains on the PDA media; (**B**) Growth rate of the hybrid strains on the PDA media; (**C**) Growth rate of the monokaryotic strains on the sawdust media; (**D**) Growth rate of the hybrid strains on the sawdust media; (**E**) Fruiting body yield; (**F**) Total polysaccharide content of the fruiting body; (**G**) Triterpenoid and sterol contents in the fruiting bodies. The values are the means ± standard deviations of three or more independent experiments. Different letters indicate significant differences in sample comparisons ($p < 0.05$ according to Duncan's test).

The mycelial growth rate on the PDA media and sawdust media, fruiting body yield, and total polysaccharide, triterpene, and sterol contents were compared, based on the membership functions (Table 3). A88 had the best comprehensive traits, and A418 had the worst. P ranked 11th due to its low growth rate on the PDA and low yield, and 10 strains had more comprehensive traits than P, with a super parent rate of 71.43%. Only a few selfing combinations are needed to obtain a new strain that is superior to the parent, compared to the traditional breeding method.

Table 3. Comprehensive evaluation of the hybrid strains.

Strain	μ (x1)	μ (x2)	μ (x3)	μ (x4)	μ (x5)	Average Value	Ranking
P	0.10	1.00	0.83	0.40	0.00	0.47	11
A53	0.84	0.01	0.69	0.00	0.92	0.49	9
A54	0.55	0.54	0.94	0.30	0.72	0.61	3
A88	0.21	0.86	1.00	0.63	0.80	0.70	1
A97	0.40	0.86	0.47	0.26	0.75	0.55	5
A203	0.32	0.08	0.84	0.37	0.91	0.51	8
A217	0.93	0.17	0.69	0.14	0.76	0.54	6
A226	0.75	0.34	0.47	0.13	0.77	0.49	10
A337	0.57	0.00	0.97	1.00	0.74	0.66	2
A382	0.46	0.98	0.00	0.19	1.00	0.53	7
A413	1.00	0.42	0.43	0.31	0.73	0.58	4
A418	0.00	0.06	0.64	0.33	0.96	0.40	13
A426	0.15	0.09	0.75	0.22	0.90	0.42	12

4. Discussion

Basidiospore monokaryons are the product of gene recombination during meiosis, and they exhibit high genetic diversity [31]. The screening of the monokaryotic strains with good traits is an important prerequisite for improving the efficiency of new mushroom variety development [17]. Theoretically, *G. lingzhi* can produce spores of four genotypes in the same proportion [32]. In this study, only two spore genotypes were isolated, $\chi^2 = 54.46$, and $\chi^2_{0.05} = 7.81 < 54.46$ when n = 3 degrees of freedom, which did not meet the 1:1:1:1 free separation ratio but exhibited partial separation, resulting in severe partial segregation, which has been reported in a variety of edible fungi [33]. It has been reported that there are differences in the germination ability or the survival ability of spores carrying different mating types, leading to the occurrence of partial segregation in sexual monokaryotic strains isolation [34]. There were differences in the growth rates of monokaryotic strains. In this study, the growth rates of the monokaryotic strains showed a normal distribution with a continuous change trend, indicating that the mycelial growth rate was a quantitative trait controlled by polygenes. Currently, it is believed that the mating type A site is related to the mycelial growth rate, and QTL mapping has revealed the main genomic areas responsible for growth rate regulation and control. The primary effect site that controls the mycelial growth rate is near the mating type A site [35,36]. For example, the overexpression of *LeHD1* gene can increase the mycelial growth rate of mushrooms by 8.6–18.5%, and the silencing of *LeHD1* can reduce the mycelial growth rate by 8.5–26.8% [37]. The diversity of basidiospore monokaryons is also reflected in colony morphology. In this study, the basidiospore monokaryons exhibited five colony morphologies, and one colony morphology showed obvious weakness. Molecular markers were important for analysing genetic diversity, and most previous studies have focused on the dikaryotic cultivars and wild strains [20,38]. Few studies have analysed basidiospore monokaryons. Using ISSR molecular markers, we found that at a similarity coefficient of 0.49, these 53 basidiospore monokaryons of *G. lingzhi* were divided into two categories, which was consistent with genotype classification results. The AxBx strains had high genetic diversity, while the AyBy genotype had a similarity coefficient of 100%. Based on these results, strains can be selected for hybridisation, which is a scientific and reasonable approach for reducing the workload. The genetic diversity of the basidiospore monokaryons of edible fungi, such as *Auricularia heimuer*, *Lentinula edodes*, and *Pleurotus ostreatus*, was also proven by the molecular markers, indicating that it is feasible to screen for Mono–mono mating via molecular markers in edible and medicinal fungi. However, the results showed that hybrid strains were not strictly classified according to the mating type, which may be related to the characteristics of the parental strains. This method can be used as an auxiliary means for identifying the mating type [26,39,40].

ISSR-assisted monokaryotic strains selfing is one of the most promising breeding methods for *G. lingzhi*. On the one hand, the breeding methods for Chinese medicinal materials are restricted by the Chinese Standard for the Production Quality Management of Chinese Medicinal Materials. On the other hand, according to the classification results of ISSR genetic diversity, the mycelial growth rate, yield, and polysaccharide, triterpene, and sterol contents of the 14 hybrids were diverse. The growth rate is an important index for breeding new strains, and the rapid growth of strains can reduce microbial contamination and shorten the production cycle [41]. We found that there was little correlation between the growth rate of monokaryotic strains and the growth rate of the mycelia of hybrids, and the growth rate of hybrids was greater than that of the monokaryotic strains, which was also reported in macrofungi [42], possibly due to the complementary coordination mechanism between the two cell nucleus in somatic cells [34,43]. Transcriptome analysis of the hybrid dikaryon and the two parental monokaryotic strains of *Pleurotus ostreatus* showed that 7953 and 7787 genes were upregulated more than 3 times, and that 8421 and 7425 genes were downregulated, respectively, compared to their parental monokaryotic strains. These genes are related to macromolecule utilization, cell material synthesis, stress resistance and signal transduction. This also proved that these two nuclei were co-ordinated [44]. There may be various reasons for the low growth correlation of mycelia in the PDA media and sawdust

media. The composition of the PDA media is simple, and mycelia can directly absorb and utilize substances such as glucose, while the sawdust media is composed mainly of wood chips, and the composition is complex and diverse. Extracellular enzymes, such as lignocellulosic degrading enzymes, need to be secreted to degrade the matrix before it can be absorbed and utilized [45]. It is also necessary to secrete organic acids to regulate environmental pH to create the optimal conditions for growth and development [46]. Polysaccharides and triterpenes are the main active substances of *G. lingzhi*., which reflects the strain quality [11]. Fruiting body yield is an important agronomic trait, and the traits of selfing offspring are diverse, with yields per packet ranging from 19.49–33.64 g and polysaccharide contents ranging from 1.12–2.44%. The contents of triterpenes and sterols ranged from 0.72 to 1.1%, and the highest polysaccharide, triterpene and sterol contents were observed in A413, A382, and A88, respectively, which could meet different market demands. The membership function value is a comprehensive value obtained by using the basic theory of fuzzy mathematics and the membership function method from the perspective of the membership degree [47]. In combination with the five important traits, the growth rate on PDA and sawdust media, the yield, the polysaccharide content, and the triterpene and sterol contents, the membership function value is sorted according to the membership function value. The results showed that the percentage of selfing progeny that performed better than the parental strains was 71.43%, and A88 was the best selfing progeny, which may be a good strain for commercial cultivation.

In this study, we confirmed the rich genetic diversity of the *G. lingzhi* basidiospore monokaryons by ISSR, which can also be used as an auxiliary means for identifying the mating type. According to the ISSR clustering results, monokaryotic strains can be screened for selfing, the best comprehensive characteristics of the strains can be obtained, and the workload can be greatly reduced.

5. Conclusions

In this study, 52 basidiospore monokaryons were isolated from the fruiting bodies of one commercially cultivated *G. lingzhi* (laboratory number strain P) in Jilin Province, China. The mating type was partial segregation. ISSR molecular markers proved that basidiospore monokaryons had rich genetic diversity. The clustering results can be used as the basis for the screening selfing of monokaryons. A similarity coefficient of 0.49 can distinguish monokaryons with different mating types. At the level of 0.76, the super parent rate of the selfing progenies of monokaryons randomly selected according to the grouping results reached 71.43%. This study proves that ISSR molecular marker-assisted self-breeding can effectively select new strains of *G. lingzhi* with high yield and high quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14050745/s1, Figure S1: Morphology of monokaryotic strains mycelium; Figure S2: Morphology of hybrid mycelium; Figure S3: Fruiting body morphology of hybrids; Table S1: Antagonism between parents and hybrids.

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