

Full Length Research Paper

Development of species-specific primers for identifying *Auricularia auricula-judae* using intergenic spacer 1 (IGS1) sequences

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Auricularia auricula-judae, an important edible and medicinal mushroom was first cultivated in China more than one thousand years ago. In this study, we developed a simple and rapid polymerase chain reaction (PCR)-based diagnostic tool for identifying *A. auricula-judae* from other morphologically similar *Auricularia* species. Based on the sequence analysis of intergenic spacer 1 (IGS1) in genus *Auricularia*, two species-specific primer combinations were designed from IGS1 sequence of *A. auricula-judae*, both of them can generate DNA fragments of different size unique to *A. auricula-judae*. The validity of this two primer combinations were confirmed by correctly amplifying species-specific fragments from genomic DNA of another 32 *A. auricula-judae* strains. To our knowledge, this was the first study on development of species-specific primers via IGS1 sequence in jelly fungi and also the first application of IGS1 sequence in genus *Auricularia*.

Key words: *Auricularia*, *Auricularia auricula-judae*, intergenic spacer, species-specific primer.

INTRODUCTION

Auricularia belonging to Basidiomycota, Agaricomycetes, Incertae sedis, Auriculariales, Auriculariaceae (Kirk et al., 2008) is widely adopted in tropical, sub-tropical and temperate zone. To date, 14 species of *Auricularia* were reported in China (Yan et al., 1998), among which, eight (Table 1) were common species while other six species are narrowly distributed in certain region such as *A. xishaensis* that only exists in Paracel Islands. In those 14 species, *A. auricula-judae* (Bull.) Quel., also known as wood ear was widely cultivated in China and also won favor around the world for its special nutrition and medicinal value in prevention of diabetes (Kim et al., 2007) and heart attacks (Ma et al., 2010), etc. As the fourth important edible mushroom in the world whose total export value reached 7.6 million dollar in China now, the cultivation and production of *A. auricula-judae* plays an increasingly crucial role in China mushroom industry. China has abundant germplasm resources of *Auricularia* species, but the identification and classification of

Auricularia strain are under confusing circumstance. Rapidly and accurately distinguishing *A. auricula-judae* from other *Auricularia* species is very important for domestication and large-scale cultivation. The conventional way to classify different *Auricularia* species relies on morphological characters such as size, shape and color of the fruiting body. But morphological characters are susceptible to environmental changes and fruiting body cultivation is also time-consuming.

Lowy (1951) described several characteristic zones in the transverse section of basidiocarps of *Auricularia* species, then he suggested that the internal structure of fruiting bodies could be selected as the principal standard to identify different *Auricularia* species. However, Lowy's method is questionable for the reasons that: (1) too much emphasis is placed on the characteristic of the medulla and the length of the abhymenial hairs for identifying species, (2) analysis using different parts of the same fruit body may cause confusing results (Wong and Wells, 1987). Fortunately, the molecular biology techniques based on environmental stable DNA sequence provide rapid and reliable tools for species identification. In recent years, as the structure and function of fungal nuclear ribosomal DNA (rDNA) have been comprehensively

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Table 1. The tested strains used in this study.

Strain name	Source ^a	Species	GenBank accession No. of IGS1 ^b	Length of fragment
APLME	Fujian	<i>A. peltata</i>	JF440700	2528 bp
AC110	Sanming	<i>A. cornea</i>	/	b
AC141	Sanming	<i>A. cornea</i>	/	b
ADFJ	Sanming	<i>A. delicate</i>	JF440701	2677 bp
AD5177	Guangdong	<i>A. delicate</i>	/	~2.7 kb ^c
AD5424	Guangdong	<i>A. delicate</i>	/	~2.7 kb ^c
AMME	Hunan	<i>A. maxima</i>	/	b
ARME	Henan	<i>A. reticulata</i>	/	b
AFJLH	Sanming	<i>A. fuscusucconea</i>	JF440697	2634 bp
AFHP	Shanghai	<i>A. fuscusucconea</i>	/	~2.7 kb ^c
APFJ	Sanming	<i>A. polytricha</i>	/	~2.7 kb ^c
APHP	Henan	<i>A. polytricha</i>	/	~2.7 kb ^c
AP112	Hubei	<i>A. polytricha</i>	JF440699	2662 bp
AP180	Hubei	<i>A. polytricha</i>	/	~2.7 kb ^c
HW5D31	Heilongjiang	<i>A. auricula-judae</i>	JF440694	2479 bp
5L0096	Heilongjiang	<i>A. auricula-judae</i>	JF440696	2477 bp
5L0109	Heilongjiang	<i>A. auricula-judae</i>	JF440695	2479 bp
5L0114	Heilongjiang	<i>A. auricula-judae</i>	/	~2.5 kb ^c

^a The exact sources of the tested strains are as follows: Heilongjiang: The Microbiological Institute of Heilongjiang; Fujian: Edible Mushroom Institute of Fujian Agriculture and Forestry University; Hubei: Huazhong Agricultural University; Guangdong: The Microbiological Institute of Guangdong; Henan: Biological Institute of Henan Academy; Shanghai: Shanghai Academy of Agricultural Sciences; Hunan: The Microbiological Institute of Hunan; Sanming: Edible Mushroom Institute of Sanming, Fujian province. ^b There are no amplification in *A. cornea*, *A. Maxima* and *A. Reticulata* using universal primer combination. ^c This is an estimated band size value for lack of precise sequence result.

studied, internal transcribed space (ITS) domain was widely used in fungal systematics and phylogeny (Thorn et al., 2000). However, Nilsson et al. (2008) found that the average variability of ITS sequence within one species was 2.51%, which indicated that 'ITS' could not define species exactly. Intergenic spacer (IGS) known as the fastest evolved domain of rDNA has enormous varied sites in its DNA sequence among different species (Paule and Lofquist, 1996). It is more useful to analyze phylogenetic relationships and genetic diversity in fungi. So far, IGS1 sequence has not yet been applied in *Auricularia*. According to analysis of IGS1 sequences in different *Auricularia* species, there are several species-specific sites existed in the IGS1 sequence of *A. auricula-judae*.

The aim of the present study was to develop species-specific primers for *A. auricula-judae*, thus laying the foundation for rapid and reliable identification of *A. auricula-judae* on large scale commercial cultivation.

MATERIALS AND METHODS

Strains

Eighteen strains of eight common *Auricularia* species collected from eight fungal institutes of China (Table 1) were used to develop species-specific primers. In addition, another 32 strains of *A. auricula-judae* were utilized to verify the reliability of the developed species-specific primers (Li et al., 2010).

DNA extraction

Mycelia of all the tested strains were incubated on CYM (complete yeast media) liquid broth at 25°C for 2 weeks. Genomic DNA was extracted from freeze-dried mycelia by the method of Sambrook and Russell (2001). The qualities of DNA samples were confirmed by 1.0% (w/v) agarose gel electrophoresis and the DNA concentrations were determined with a BioPhotometer 6131 (Eppendorf, Germany). Then the qualified DNA samples were diluted to 50 ng/μl for PCR amplification.

IGS1 fragment amplification

The complete IGS1 fragments of the tested strains were obtained using the universal primer combination LR12R/M-1 (Bunyard et al., 1996) (Table 2). Each 20 μl PCR reaction mixture contained 1 × *Taq* buffer, 0.25 mM dNTP mixture, 0.4 μM each primer, 1.2 U *Taq* DNA polymerase (TaKaRa, Japan), 2.0 mM MgCl₂ and 150 ng DNA template. The PCR program consist of an initial denaturation of 4 min at 94°C; followed by 37 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 3 min; and a final extension of 10 min at 72°C. DNA fragments were separated by 1.5% agarose gels and purified using Sureclean gel extraction kit (Bioline, China). The extracted DNA fragments were then ligated into pMD18-T vector (TaKaRa, Japan) for sequencing by ABI 373 DNA sequencer.

Species-specific primers design and specific fragment amplification

Alignment of IGS1 sequences was carried out with Clustal W software (Thompson et al., 1994) and the primers specific to IGS1 region of *A. auricula-judae* were designed via the web-derived

Table 2. Primer combinations used to amplify genomic DNA of *A. auricula-judae*.

Primer character	Primer name	Primer sequence 5'-3'	Ta* (°C)	Product Size (bp)
Universal primer combination	LR12R	CTGAACGCCTCTAAGTCAGAA	56	2479
	M-1	AACCACAGCACCCAGGATTCCC		
Species-specific primer combination	HMEF	TGTCCCTGCGATGTATTT	52	1544
	M-1	AACCACAGCACCCAGGATTCCC		
	IGS1-5F	TATGTCCC GCATGTGTTAGT	55	2301
	IGS1-3R	GCGTCTATAAGGCGTAACTA		

* Ta: Annealing temperature of PCR amplification.

software "Primer 3" (<http://frodo.wi.mit.edu/primer3/>). For PCR amplifications using species-specific primer, tested samples were amplified five times with the same protocol as for the universal primer except for a different annealing temperature (Table 2).

RESULTS

Except for four strains (No. 2, 3, 7 and 8) which belonged to *A. cornea*, *A. maxima* and *A. reticulata*, all the other fourteen strains can produce a bright and reproducible fragment about 2500 bp by the universal primer combination LR12R/M-1 (Figure 1a). Sequence indicated that the length of complete IGS1 region has remarkable variation among different species (Table 1). Comparison of IGS1 sequence showed that some species-specific sites existing in *A. auricula-judae* that were distinctly different from other species (Figure 2). Based on the IGS1 sequence, three internal primers (IGS1-5F, IGS1-3R and HMEF) were designed. Primer IGS1-5F was non-specific while the other two were specific to *A. auricula-judae* strains. Finally, two primer combinations (HMEF/M-1 and IGS1-5F/IGS1-3R) were tried as the species-specific primer combinations of *A. auricula-judae* (Table 2). Expected size of amplification DNA fragments forecasted by Primer 3 were observed in all the four *A. auricula-judae* strains only, while not in any other fourteen *Auricularia* species strains (Figure 1b and c). Further sequence results confirmed the actual sizes of those fragments were 1544 and 2301 bp, respectively (Table 2). In addition, the reliability of the two species-specific primer combinations was verified by other 32 main cultivars of *A. auricula-judae* in China (Li et al., 2010). As predicted, the two primer combinations produced different fragments with expected size among the 32 cultivars (Figure 3).

DISCUSSION

IGS domain is rich in variations among different species, it has been successfully applied to study the phylogenetic

relationships and genetic diversity in edible mushrooms such as *Agaricus* (Bunyard et al., 1996), *Pholiota nameko* (Matsumoto et al., 2003), *Pleurotus eryngii* (Kawai et al., 2008), *Pleurotus nebrodensis* (Zhang et al., 2006) and *Armillaria mellea* (Terashima et al., 2006). Among different domains of rDNA region, the variation significance was 28S 3'end < 28S 5' end < IGS1 in *Agaricus* (Bunyard et al., 1996) and nLSU (nuclear large subunit ribosomal) < ITS < IGS1 in *Armillaria* species in North America (Kim et al., 2006). Satio et al. (2002) revealed that IGS domain of *Lentinula edodes* contained intricate sub-repeat regions (SR1 and SR2) and the DNA fingerprinting targeting those regions were a useful tool for discriminating different *L. edodes*. Since IGS domain was variable at species or strain level, their variation sites could be used to develop specific primers for species or strain authentication. It was the first time to analyze the IGS1 domain of *Auricularia*. However, the universal primer combination (LR12R/M-1) could not generate any amplification in *A. cornea*, *A. maxima* and *A. reticulata*. The failure to amplify IGS1 sequence in three *Auricularia* species could be attributed that the universal primer combination cannot appropriately match the template DNA. The universal primer combination (LR12R/M-1) was developed from some species of Agaricales (Bunyard et al., 1996), however, there is a relatively far genetic distance between Agaricales and Auriculariales which may result in the unfitness for amplify all the species of Auriculariales. After sequence analysis, variable sequences within IGS1 domain of *A. auricula-judae* were proven to be highly effective in designing specific primers for species identification. Another 32 strains of *A. auricula-judae* were then used to confirm the validity of the two specific primer combinations which guaranteed the accuracy and reliability of the combinations when employing in a large strain population. In practice, the integrated utilization of the two primer combinations would enhance the reliability and stability of *A. auricula-judae* authentication. This study established the foundation for further development of species-specific primers based on IGS1 sequence in fungi.

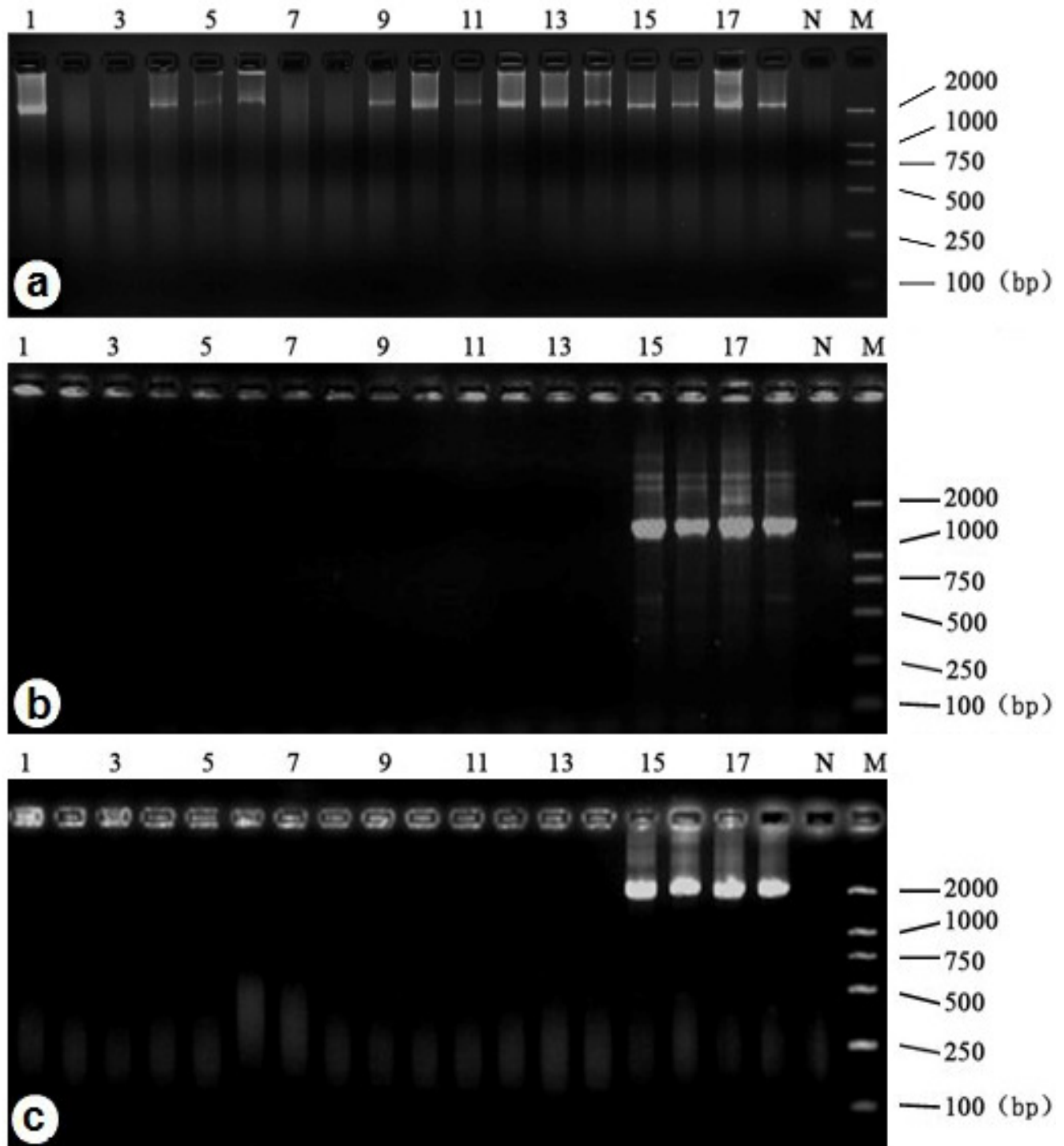


Figure 1. PCR profile amplified by universal primer combinations and species-specific primer combinations Lane N: water blank control; lane M: DL2000 molecular marker (TaKaRa, Japan). Lanes 1 to 18 correspond to the strains number listed in Table 1. (a) PCR amplification using LR12R/M-1; (b) PCR amplification using HMEF/M-1 and (c) PCR amplification using IGS1-5F/IGS1-3R.

A. auricula-judae is an economic mushroom with great nutrient and medicinal value which plays a significant role in the development of mushroom industry in China. It seems that distinguishing *A. auricula-judae* from other

morphologically similar species is especially important before large-scale commercial cultivation. Our study provides a rapid, cheap and reliable diagnostic tool for identifying *A. auricula-judae*. This is also the first report

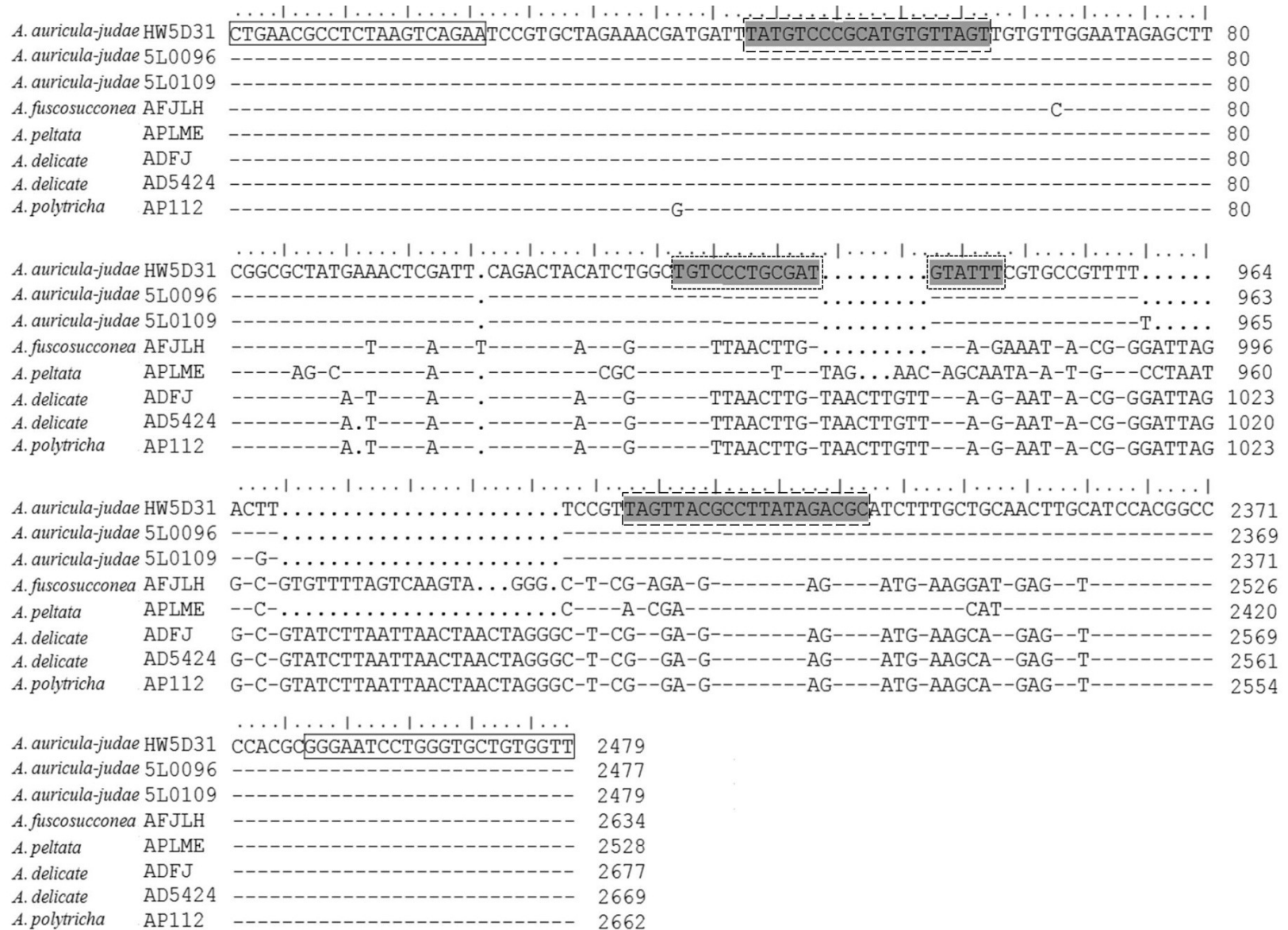


Figure 2. Sequences comparison of IGS1 domain in *Auricularia* sequence sites corresponding to the universal primer pair LR12R/ M-1 are boxed with solid line (nt 1-21 and 2458-2479 respectively); corresponding to the species-specific primer pair IGS1-5F/IGS1-3R are boxed with long dashed line (nt 43-62 and 2324-2343 respectively) and corresponding to primer HMEF(nt 936-953) are boxed with short dashed line.

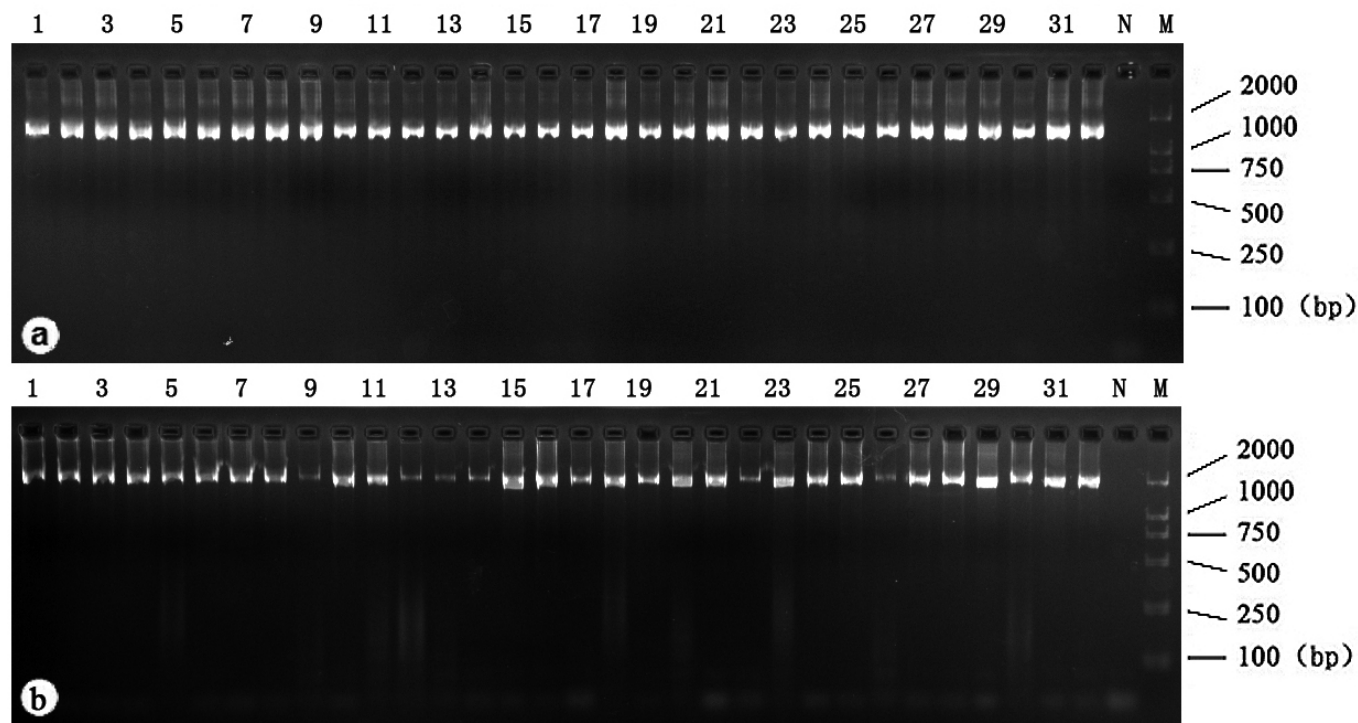


Figure 3. PCR profile amplified by species-specific primer combinations in *A. auricula-judae*. Lane N: water blank control; Lane M: DL2000 molecular marker (TaKaRa, Japan). Lanes 1 to 32 correspond to the strains number listed in Li et al. (2010). (a) PCR amplification using HMEF/M-1 and (b) PCR amplification using IGS1-5F/IGS1-3R.

on application of IGS1 sequence in jelly fungi and may facilitate further utilization of IGS1 domain in other research fields, like analysis of genetic diversity and phylogenetics.

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