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Full Length Research Paper

Genetic diversity of wild *Auricularia polytricha* in Yunnan Province of South-western China revealed by sequence-related amplified polymorphism (SRAP) analysis

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As an economically and medically important fungus, *Auricaularia polytricha* has been collected and cultivated widely in China, however, its diversity has not yet investigated previously. The genetic diversity of 20 wild *A. polytricha* strains from Yunnan Province and four cultivated strains from Henan and Sichuan Province were profiled by using 10 pairs of sequence-related amplified polymorphism (SRAP) primers. A dendrogram tree was constructed by unweighted pair-group method with arithmetic means (UPGMA) method according to the similarity coefficient among strains. A total of 425 (P=99.8%) and 37 (P=40.7%) polymorphic loci were detected in the wild and cultivated strains respectively, and the 24 strains were classified into six groups with the similarity coefficient of 0.74. The genetic diversity of the wild strains was much higher than that of the cultivated strains, and SRAP marker was an effective method to evaluate the genetic diversity of *A. polytricha*.

Key words: A total of 425 (*P*=99.8%), 37 (*P*=40.7%) polymorphic loci.

INTRODUCTION

Auricularia polytricha (Mont.) Sacc. belongs to Auriculariaceae, Basidiomycota, and it is known as wood ear, Jew's ear, or red ear (Yu et al., 2008). It has a world-wide distribution from the temperate to the tropics growing on living and dead broad-leaved trees, decayed stumps or logs (Imazeki and Hongo, 1965; Reichard et al., 2005). *A. polytricha* is one of the most important medicinal fungi in China, and it has been reported to have several medicinal functions, such as promoting blood circulation, treating hemorrhoids, and having analgesic, antitumor, immuno-stimulating, hypolipidaemic and hypocholesterolaemic effects (Dai et al., 2009, 2010; Sheu et al., 2004; Yang et al., 2002). Recently a fraction of polysaccharide (APPIIA) from *A. polytricha* with significant anti-tumor effect was isolated; it has been found to activate the macrophage dramatically, induce the release of nitric oxide (NO), promote secretion of cytokines tumor necrosis factor (TNF) -*α*, interleukin (IL)-1*β*, and IL-6, and enhance phagocytosis of macrophage in several stages (Yu et al., 2009).

In addition, *A. polytricha* is a famous edible fungus in China; it has been widely used in soups and dishes in oriental cuisine because of their rich in fibers, proteins, microelements, and low fat content. Because of the mentioned beneficial characters above the consumption of the fungus has increased year by year. *A. polytricha* is widely cultivated in China, but the strains for cultivation were isolated from limited resources, and with the expanding of cultivation area, the code name of strain was confusional in the long term mushroom production, and the phenomenon of the same strain with different names and different strains with the same name was very serious. Meanwhile, aged and degenerate strains were introduced to the production, so the depression is a common phenomenon in cultivated strains. Yu et al.

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(2008) reported that different *A. polytricha* strains do not only vary in their output, cultivation cycle, shape and taste, but also in the pharmacological effects.

The evaluation of genetic diversity would promote the efficient use of genetic variations in the breeding (Paterson et al., 1991) and pharmacological programs. To make mushroom highly productive, it is very necessary to have more novel improved strains with good characteristics. So it is important to establish the genetic diversity analysis of natural *A. polytricha* strains.

Yunnan Province is situated on the Yunnan-Guizhou Plateau in China's south-western frontier region, and with complex-geographical conditions and various types of climate and environment, it is considered as one of the biodiversity hotspots in the world, so it is possible that the genetic diversity of *A. polytricha* in Yunnan may be higher. Some efforts have been made to study the genetic diversity of *A. polytricha* by using random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers (Yan et al., 2004; Yu et al., 2008; Zhang et al., 2006, 2007a). Most of these studies, however, are mainly on cultivated strains, but almost no study on the diversity among wild strains.

SRAP is a novel molecular marker, being firstly introduced by Li and Qurios (2001), it had been applied extensively in genetic linkage map construction, germplasm identification, gene tagging and mapping, genetic diversity analysis, and comparative genetics of different fungal species and other fields (Li and Qurios, 2001; Ferriol et al., 2003; Sun et al., 2006; Li et al., 2003). The method has been used for the identification of cultivars of several medicinal and edible fungi (Fu et al., 2010; Sun et al., 2006; Tang et al., 2010; Yu et al., 2008), but only few reports estimated the genetic diversity of wild strains (Chen et al., 2009).

In the present study, SRAP markers were used to detect variation among 20 wild strains collected from Yunnan Province and four cultivated strains from Henan and Sichuan Province. The purpose of this study was to evaluate the genetic variations among wild *A. polytricha* strains and to compare them with that of cultivated strains, in order to obtain some information for further study on pharmacology and cultivation programs.

MATERIALS AND METHODS

Samples

Twenty natural strains of *A. polytricha* in Yunnan Province, southwestern China, and four cultivated strains from Henan and Sichuan Province were isolated by tissue isolation (Table 1), and were deposited at the herbarium of the Institute of Microbiology, Beijing Forestry University (BJFC).

DNA extraction

Strains of each collection were grown in liquid medium, containing 25 ml malt extract dextrose broth and incubated at 26°C for 10 days. The mycelia were harvested and rinsed with distilled water, then dried with filter paper. ZR Fungal/Bacterial DNA Kit™ procedure was used to extract total genomic DNA. The concentration of DNA was estimated by Smart Spec™ Plus (Bio-rad, USA) and quality was checked by 0.8% agarose gel electrophoresis. DNA samples were diluted to working solutions of 25 ng/µl, confirmed by ultraviolet (UV) absorption on a Smart Spec™ Plus (Bio-rad, USA) visible spectrophotometer, and stored at -20°C until use.

SRAP amplification and examination

SRAP is designed to amplify open reading frames (ORFs) (Li and Qurios, 2001) based on two special primer pairs. The primers are 17 or 18 nucleotides long (Table 2). Core sequences, which are 13 to 14 bases long, where the first 10 or 11 nucleotides at the 5' end is a random sequence of nucleotides ("filler" sequences), similar to primers used to amplify RAPD. Followed by the sequence CCGG in the forward primer (me1-me10) results in this primer preferentially amplify exonic regions, while the AATT domain in the reverse primer (em2-em13) causes preferential annealing to introns and regions with promoters. The core is followed by three selective nucleotides at the 3′ end of each primer. The observed polymorphism fundamentally originates in the variation of the length of these introns, promoters, and spacers, both among individuals and among species (Li and Qurios, 2001).

A total of 130 different primer pairs were assembled using 10 forward and 13 reverse primers. Of which 10 pairs produced clear and reproducible bands, and were selected for the subsequent experiments (Table 2). SRAP-PCR amplifications were performed in Mastercycler PCR System (Eppendorf, Germany) with the cycling program: 5 cycles of 94°C for 3 min, 35°C for 1 min, and 72°C for 1 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 8 min. PCR amplification was carried out in 25 µl volumes: 2.5 µl 10 x PCR buffer, 25 ng template DNA, 0.48 mM dNTPs, 1.5 U *Taq* DNA polymerase, 1.5 mM Mg^{2+} and 0.4 µM primer, ddH_2O 14.7 µl. 10 µl amplification products were electrophoretically separated on 1.5% agarose gels with 1 x TAE buffered. A DNA ladder was used as a size standard (D 2000), electrophoresis runs 80-90 min at 80 V. Gels were stained with ethidium bromide and bands were visualized and photographed under ultraviolet light using the Gel Doc XR system (Bio-rad, USA).

Data analysis

Bands were scored as present (1) or absent (0); the fragments produced by each primer pairs were numbered sequentially and entered into a binomial matrix. DNA fragments which bigger than 2000 bp and lower than 100 bp were not adopted for data analysis. The resulted 0, 1 data matrix was analyzed using NTSYS pc version 2.10e (Rohlf, 2000). Dice's similarity coefficients between strains were calculated and used to create unweighted pair-group method arithmetic means (UPGMA) dendrogram. Bootstrap analysis using UPGMA search with 1000 replicates was performed to obtain the confidence of the tree. Finally, a principal coordinate analysis (PCoA) was performed based on the genetic similarity matrix of the tested strains using EIGEN programs.

RESULTS

Ten primer combinations chosen for analysis produced a total of 426 and 91 SRAP loci in the wild and cultivated

Table 1. Sample data of *A. polytricha* strains.

Strain no.	Collection no.	Location and colour of the cultivated basidiocarps
1	Dai 11000	Yunnan Province, Kunming, Kunming Bot. Garden
2	Cui 8009	Yunnan Province, Tengchong, Gaoligong Mountain
3	Cui 8066	Yunnan Province, Tengchong, Gaoligong Mountain
4	Cui 8604	Yunnan Province, Xishuangbanna, Shangyong Nat. Res.
5	Cui 8065	Yunnan Province, Tengchong, Gaoligong Mountain
6	Cui 8067	Yunnan Province, Tengchong, Gaoligong Mountain
7	Cui 8479	Yunnan Province, Mengla, Menglun, Green Shilin Park
8	Cui 8381	Yunnan Province, Mengla, Xishuangbanna Tropic Bot. Garden
9	Cui 8158	Yunnan Province, Baoshan, Gaoligong Nat. Res.
10	Cui 8312	Yunnan Province, Tengchong, Yinghua Vale
11	Cui 8478	Yunnan Province, Mengla, Menglun, Green Shilin Park
12	Cui 8159	Yunnan Province, Baoshan, Gaoligong Nat. Res.
13	Cui 8477	Yunnan Province, Mengla, Menglun, Green Shilin Park
14	Cui 8597	Yunnan Province, Mengla, Xishuangbanna, Wangtianshu Park
15	Cui 8061	Yunnan Province, Tengchong, Gaoligong Mountain
16	Cui 8008	Yunnan Province, Tengchong, Gaoligong Mountain
17	Cui 8064	Yunnan Province, Baoshan, Gaoligong Nat. Res.
18	Cui 8473	Yunnan Province, Mengla, Menglun, Green Shilin Park
19	Cui 8474	Yunnan Province, Mengla, Menglun, Green Shilin Park
20	Cui 8475	Yunnan Province, Mengla, Menglun, Green Shilin Park
21	Cui 8693	Cultivated in Henan Province, basidiocarp brown to dark brown
22	Cui 8694	Cultivated in Henan Province, basidiocarp white
23	Cui 8695	Cultivated in Sichuan Province, basidiocarp white
24	Cui 8696	Cultivated in Sichuan Province, basidiocarp brown to dark brown

Table 2. The primer sequences of SRAP.

Forward primer	Sequence (5'-3')	Reverse primer	Sequence (5'-3')
me1	TGAGTCCAAACCGGATA	em2	GACTGCGTACGAATTTGC
me2	TGAGTCCAAACCGGAGC	em3	GACTGCGTACGAATTGAC
me3	TGAGTCCAAACCGGAAT	em4	GACTGCGTACGAATTTGA
me7	TGAGTCCAAACCGGTCC	em5	GACTGCGTACGAATTAAC
me8	TGAGTCCAAACCGGTGC	em8	GACTGCGTACGAATTGGT
me9	TGAGTCCAAACCGGTGT	em9	GACTGCGTACGAATTCGG
me10	TGAGTCCAAACCGGTCA	em10	GACTGCGTACGAATTCAG
		em11	GACTGCGTACGAATTCCA
		em13	GACTGCGTACGAATTCAA

strains of *A. polytricha*, of which 425 and 37 loci were polymorphic, an average of 43 and 3.7 loci were amplified per primer pairs, the percentage of polymorphic loci were 99.8 and 40.7% respectively (Table 3). So the polymorphism in the wild strains was distinctly higher than those in cultivated strains. The size of DNA fragments ranged from 100 to 2000 bp, and few bands were more than 2000 bp and lower than 100 bp (Figure 1).

The similarity coefficient matrix among 20 wild strains varied from 0.666 with the least similarity between strains 6 and 15 to 0.796 with the greatest similarity between strains 16 and 17. However, they are from 0.930 to 0.965 among four cultivated strains (Table 4), so the cultivated strains seem to be closely related genetically. A UPGMA dendrogram (Figure 2) was made based on similarity coefficient among 24 strains (genotypes), and it showed all strains could be classified into six groups with a similarity coefficient value of 0.74. Group I comprised 10 strains, demonstrated the 10 strains are closely related. Group II composed four cultivated strains alone. Group III and IV comprised three (12, 13, 14) and five strains,

Table 3. Polymorphism obtained by SRAP from wild and cultivated strains of *A. polytricha*.

SRAP primer combination	Bands from wild strains			Bands from cultivated strains		
	Total	Polymorphic	Percentage of polymorphism (*P*) (%)	Total	Polymorphic	Percentage of polymorphism (*P*) (%)
m1-em11	39	39	100	6	3	50
m2-em3	41	41	100	8	2	25
m2-em4	46	46	100	10	5	50
m2-em11	44	44	100	10	0	0
m3-em9	42	42	100	13	5	38.5
m7-em2	46	46	100	8	5	62.5
m8-em13	38	37	97.4	12	7	58.3
m10-em5	42	42	100	8	6	75
m10-em11	44	44	100	11	3	27.3
m10-em13	44	44	100	5	1	20
Total	426	425	99.8	91	37	40.7
Mean	42.6	42.5	99.8	9.1	3.7	40.7

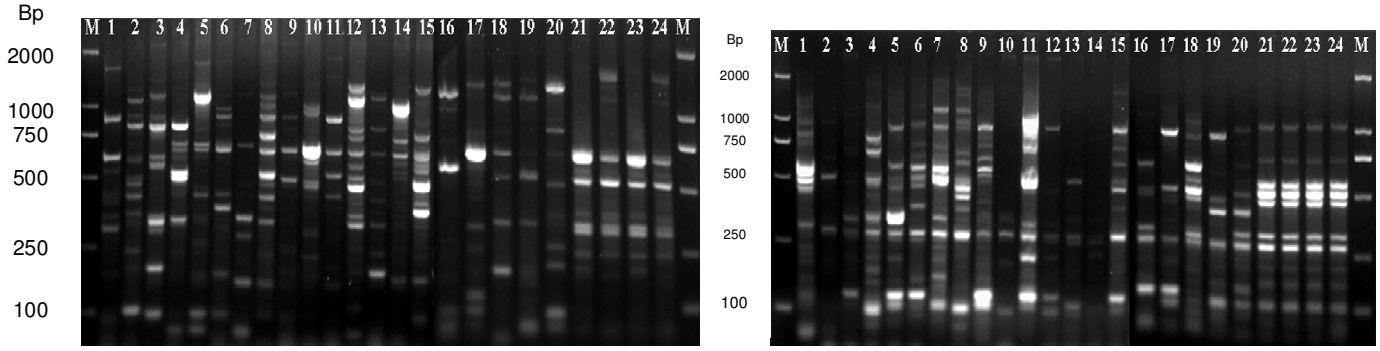

Figure 1. SRAP amplification profiles of 24 *A. polytricha* strains generated by using the primer combinations m2-em4 (left), m2-em11 (right). Lane M: D 2000 marker. Lanes 1-24: corresponding to the strains listed in Table 1.

respectively. Group V and VI comprised a single strain respectively, and they appeared to be distinct from all the other genotype. A principal coordinate analysis (PCoA) (Figure 3) was consonant with the clustering data of Figure 2, and confirmed the genetic distinctiveness of genotypes 15 and 6.

DISCUSSION

Analysis of genetic diversity is very important in fungi in practical pharmacology and cultivation programs, and SRAP technique is a powerful tool to analyze the genetic diversity among *A. polytricha* strains. In this study, all the used primer combinations could distinguish the wild strains, and the percentage of polymorphic loci of nine primer pairs was 100%, although it was 97.4% from the primer combination m8-em13. However, the greatest percentage of polymorphic loci in cultivated strains was 75% amplified by m10-em5. The primer combination m2-em11 could not distinguish the four cultivated strains (Figure 1); this indicated that the genetic diversity of cultivated strains is lower.

In the present study, the number of loci amplified by different SRAP primer pairs varied from 38-46 in wild strains and 5-13 in cultivated ones. An average percentage of polymorphic loci in wild strains were 99.8%, which was over two times higher than that in the cultivated strains (40.7%). So the genetic diversity of wild *A. polytricha* is higher than that in the cultivated strains, this is consistent with the results on the genetic study on *Agaricus bisporus* (J. E. Lange) Imbach and *Lentinula edodes* (Berk.) Singer (Loftus et al., 1988; Moore et al., 2001; Zhang et al., 2007b).

It is demonstrated herein that the SRAP analysis was so sensitive that it could detect a slight genetic difference among strains and also sensitive to the cultivated strains from different localities with the same cultivated method.

Table 4. Similarity coefficient matrix of SRAP markers in 24 strains of *A. polytricha*

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1.000	0.745	0.756	0.719	0.712	0.701	0.738	0.735	0.763	0.747	0.759	0.731	0.752	0.740	0.742	0.717	0.726	0.701	0.747	0.703	0.715	0.712	0.729	0.726
2		1.000	0.784	0.738	0.726	0.733	0.761	0.745	0.754	0.733	0.740	0.689	0.724	0.717	0.705	0.708	0.717	0.724	0.715	0.694	0.710	0.703	0.701	0.698
3			1.000	0.763	0.756	0.726	0.768	0.761	0.766	0.726	0.719	0.715	0.735	0.733	0.735	0.733	0.719	0.735	0.754	0.729	0.745	0.724	0.740	0.742
4				1.000	0.747	0.680	0.759	0.738	0.738	0.735	0.747	0.724	0.731	0.733	0.684	0.738	0.715	0.703	0.726	0.701	0.740	0.733	0.749	0.738
5					1.000	0.742	0.784	0.735	0.749	0.738	0.726	0.708	0.724	0.689	0.705	0.698	0.694	0.691	0.715	0.694	0.724	0.722	0.733	0.717
6						1.000	0.735	0.677	0.752	0.726	0.710	0.687	0.703	0.682	0.666	0.682	0.696	0.671	0.712	0.677	0.708	0.691	0.694	0.696
7							1.000	0.752	0.761	0.754	0.747	0.724	0.749	0.742	0.712	0.715	0.715	0.740	0.740	0.756	0.773	0.761	0.763	0.761
8								1.000	0.745	0.724	0.773	0.712	0.733	0.708	0.705	0.694	0.712	0.701	0.738	0.708	0.738	0.740	0.747	0.735
9									1.000	0.784	0.749	0.731	0.766	0.740	0.715	0.731	0.717	0.691	0.756	0.735	0.742	0.722	0.742	0.745
10										1.000	0.752	0.705	0.740	0.747	0.712	0.719	0.710	0.689	0.735	0.733	0.735	0.719	0.722	0.729
11											1.000	0.735	0.766	0.726	0.733	0.703	0.703	0.696	0.729	0.689	0.729	0.717	0.724	0.726
12												1.000	0.761	0.735	0.710	0.694	0.708	0.677	0.705	0.680	0.710	0.712	0.729	0.722
13													1.000	0.752	0.726	0.715	0.710	0.712	0.731	0.710	0.731	0.715	0.745	0.742
14														1.000	0.719	0.680	0.680	0.691	0.738	0.694	0.738	0.726	0.756	0.745
15															1.000	0.696	0.715	0.698	0.708	0.691	0.703	0.677	0.694	0.696
16																1.000	0.796	0.742	0.738	0.731	0.724	0.703	0.705	0.703
17																	1.000	0.752	0.752	0.754	0.719	0.703	0.710	0.698
18																		1.000	0.773	0.756	0.708	0.705	0.712	0.710
19																			1.000	0.761	0.754	0.756	0.768	0.756
20																				1.000	0.775	0.759	0.752	0.740
21																					1.000	0.942	0.930	0.937
22																						1.000	0.965	0.949
23																							1.000	0.951
24																								1.000

Among the four cultivated strains, strain 22 and 23 were from different province, but they have the greatest similarity (96.5%). This indicated that the strains producing white basidiocarps were closely related although they come from different areas. This study indicated that the genetic diversity of wild *A. polytricha* in Yunnan Province was very high and distinct, so it has an ability to adapt to different environments. In order to broaden genetic bases and improve genetic depression of *A. polytricha*, it is necessary to introduce wild strains for developing high yield of

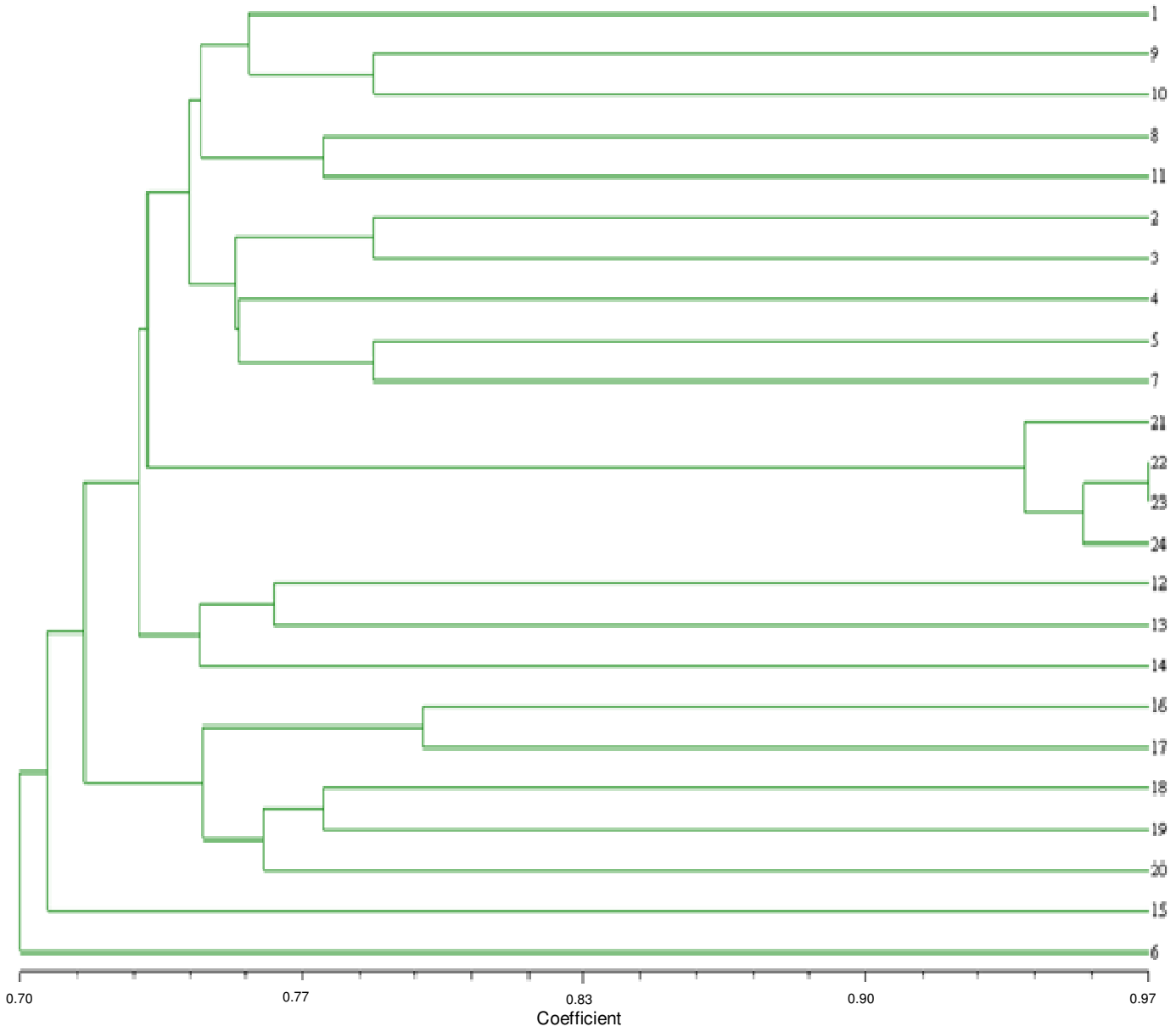

Figure 2. A dendrogram constructed by UPGMA based on the genetic similarity coefficient among SRAP profiles of 24 *A. polytricha* strains. 1-24 corresponding to the strains listed in Table 1.

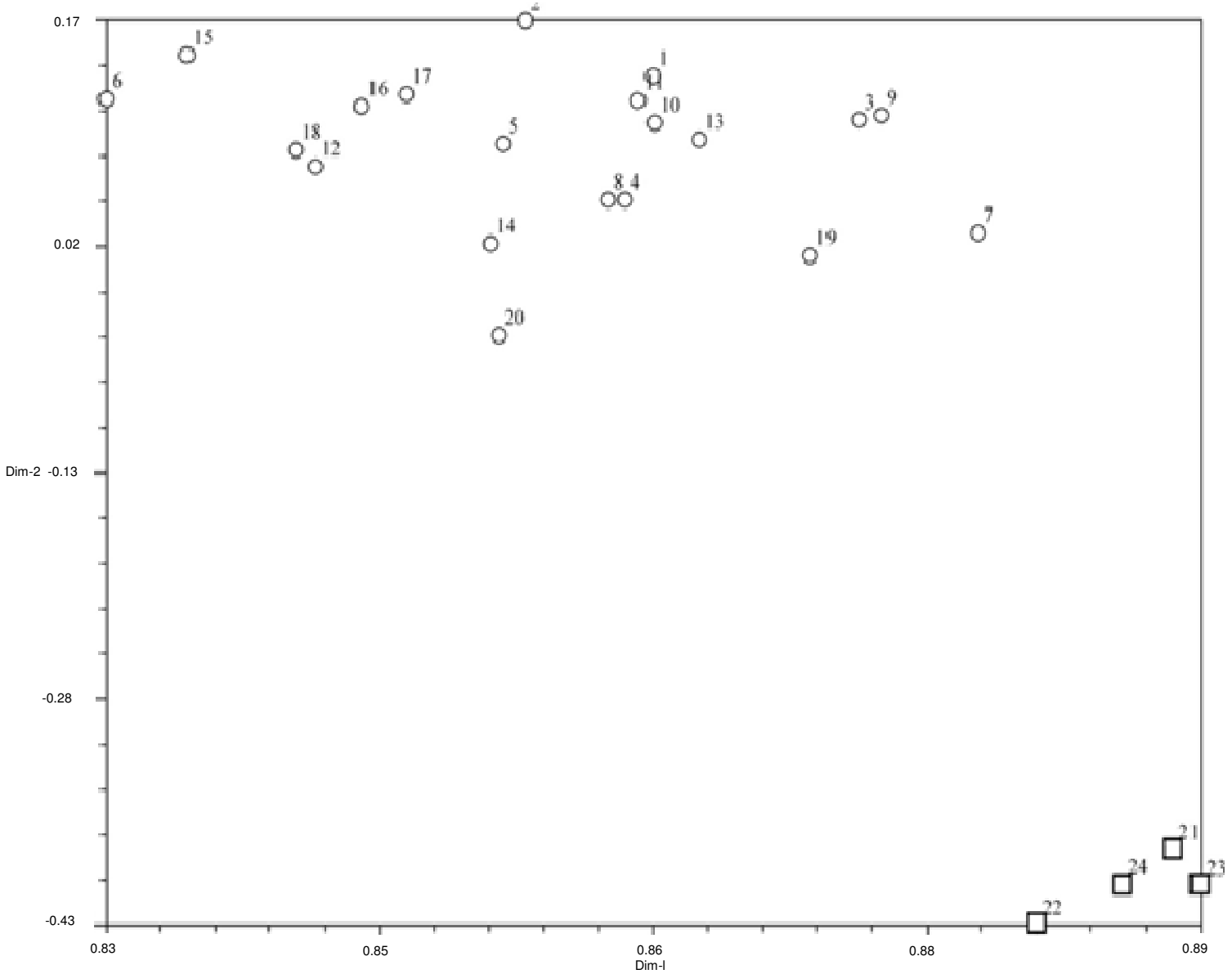

Figure 3. The relationships among 24 *A. polytricha* strains visualized by principal coordinate analysis (PCoA) of SRAP-based genetic similarities. Coordinate 1 and 2 were used to analyze the relationships. € = cultivated strains (21-24) and o = wild strains (1-20).

polysaccharide, and search new active substance in pharmacology.

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