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FULL PAPER

Genetic diversity analysis of *Monascus* strains using SRAP and ISSR markers

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Abstract In this study, sequence-related amplification polymorphism (SRAP) and inter-simple sequence repeat (ISSR) were analyzed for accessing the genetic diversity of 37 *Monascus* isolates and 14 control strains. According to the dendrogram produced by SRAP data, all the tested strains were grouped into four clusters at a 78% similarity level. Comparatively, 51 tested strains were divided into four major groups at a similarity level of 74% based on the dendrogram generated via ISSR marker analysis. Based on the two sets of dendrograms, *Monascus aurantiacus*, *M. purpureus*, *M. serorubescens*, *M. anka*, and *M. ruber* were clustered in the same clade; *M. albidus*, *M. fuliginosus*, and *M. barkeri* were clustered with *M. pilosus* in a second clade; and *M. lunisporas* and *M. argentinensis* occurred together in a third cluster distinct from the other *Monascus* species. The cluster result produced by SRAP data shared great similarity with that by ISSR data with minor differences in the subgroups, which is basically in agreement with morphological observations. In general, SRAP and ISSR are more simple, rapid, and efficient, which may provide alternative molecular approaches to studying genetic diversity, classification, and identification of *Monascus* strains.

Keywords Cluster analysis · Molecular marker · Polymorphism

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Introduction

In East Asia, *Monascus* spp. has been widely used for centuries to produce red yeast rice (RYR), also known as Hongqu, red fermented rice (RFR), red Koji, red rice, or anka, a kind of traditional fermentation food and a traditional Chinese medicine. Recent studies showed RYR contained many kinds of useful compounds, such as monacolins, γ-aminobutyric acid, sterols, isoflavones, monounsaturated fatty acids, protease, and a group of edible pigments (Endo 1979; Erdogrul and Sebile 2004; Lin et al. 2008), which could promote the isolation of novel strains for the use in food industries and medicine. Thus, it is necessary to identify *Monascus* isolates either for strain management or for protection of the breeder's rights in practice.

Traditionally, microbial identification is based on the phenotypic characters including morphological, physiological, and biochemical appearances. Based on cultural characters, there are 9 species of *Monascus* that are accepted internationally in the genus (Hawksworth and Pitt 1983; Barnard and Cannon 1987; Hocking and Pitt 1988; Udagawa and Baba 1998; Cannon et al. 1995; Stchigel et al. 2004), which include *M. pilosus*, *M. ruber*, *M. purpureus*, *M. floridanus*, *M. eremophilus*, *M. pallens*, *M. sanguineus*, *M. lunisporas*, and *M. argentinensis*. However, more than 20 species of *Monascus* have been recorded in the literature since the genus *Monascus* was erected in 1884 (Li and Guo 2003). In fact, some of them are synonyms (Park et al. 2004; Chen et al. 2007), so the genetic identities of *Monascus* species are still under debate or are even confusing, which is unfavorable for further development of this edible and medicinal fungi resource.

Molecular markers, independent of the cultural conditions, provide a potential tool for discrimination of

closely related genotypes (Bornet and Branchard 2001; Budak et al. 2004; Liu et al. 2008). Currently, various DNA molecular markers have been used to delimit species boundaries and determine the genetic relationships of different *Monascus* strains, such as random amplified length polymorphic DNA (RAPD) (Lakrod et al. 2000; Shinzato et al. 2009), amplification of DNA sequences of D1/D2 region of the large subunit (Park and Jong 2003), β-tubulin gene, internal transcribed spacer (ITS) of rDNA (Park et al. 2004), and *Monascus* retrotransposon (MRT) (Chen et al. 2007). These methods have their own advantages and disadvantages. For instance, RAPD and ITS are simple to handle, but RAPD has low reproducibility. The ITS region has limited power for resolving the phylogenetic relationships within the genus *Monascus* because of difficulty in alignment across all taxa. Although phylogenetic analysis based on the β-tubulin gene is consistent with alignments postulated by DNA sequences of the D1/D2 region of the large subunit, the resolution of these methods was insufficient for differentiating *M. ruber* and *M. pilosus*. MRT dependent on Southern blotting is a time-consuming process.

The inter-simple sequence repeat (ISSR) marker uses a single primer to amplify DNA fragments between two identical microsatellite repeat (SSR) regions oriented in opposite directions. The primer contains a microsatellite "core" sequence anchored at the 5′- or 3′-end by a set of 2–4 purine or pyrimidine residues. Microsatellites are short regions and dispersed throughout the eukaryotic genomes (Zietkiewicz et al. 1994). In contrast to ISSR, the sequence-related amplified polymorphism (SRAP) technique targets open reading frames (ORFs) in the genome using a unique design of primer pairs. The forward primer targets exons of ORF regions, and the reverse prime anchored promoters and introns. The primers comprise a core sequence of 13 or 14 bases, in which the 10 or 11 nucleotides at the 5′-end are nonspecific ("filler" sequences) and are followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core sequence is followed by three selective nucleotides at the 3′-end of each primer (Li and Quiros 2001). In recent years, ISSR and SRAP have been recognized as useful molecular systems for studying fingerprint and genetic diversity in fungi because of their simplicity, reproducibility, and polymorphism (Grunig et al. 2001; Wang et al. 2005; Gryta et al. 2006; Anh Nghia et al. 2008; Yu et al. 2008; Baysal et al. 2009).

In this study, ISSR and SRAP were analyzed to access the genetic diversity of *Monascus* strains isolated from fermentation foods in China and to provide alternative molecular approaches to differentiating and identifying *Monascus* spp.

Materials and methods

Monascus isolates

The *Monascus* isolates and control strains used in this work are listed in Table 1. 37 *Monascus* isolates were obtained from a variety of food products collected from a wide range of regions in China. The isolation process was as follows: 10 g of solid sample was put into the mortar and ground into powder, which was transferred into 90 ml sterile physiological saline with sterilized glass beads and 1% lactic acid (V/V); then, the suspension was shaken evenly and was spread on potato dextrose agar (PDA; QingDao Hopebio-Technology, Qingdao, China) plates by the dilution plate method. After growing for 72 h at 30°C, the red colonies were inoculated onto PDA slants and repeated as single spore cultures. The single colonies were kept on PDA slants at 4°C.

Morphological observations

To compare the phylogenetic analysis based on morphological characters with those based on the two sets of data produced by ISSR and SRAP, we followed the protocol described earlier (Li and Guo 2003) to observe the morphological characters of colonies. Each of the strains was inoculated separately on CYA (Chapek-yeast extract agar: $NaNO_3$, 3.0 g; K_2HPO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; sucrose, 30.0 g; yeast extract, 5.0 g; agar, 15 g per liter; pH 6.0), WA [Walt agar: wort (15°Bx), 1,000 ml; 15 g agar], and G25N (25% glycerol nitrate agar: $NaNO_3$, 3.0 g; K_2HPO_4, 1.0 g, KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; yeast extract, 5.0 g; agar, 15 g; 250 g glycerol per liter; pH 6.0) at 25°C for 7 days. The morphological characters of colonies (including the size, color, shape, and aerial hypha) and microscopic characters (including hyphae, conidia, cleistothecia, and ascospore) were observed.

SRAP and ISSR amplification

To carry out the SRAP and ISSR amplification, the genomic DNA of *Monascus* strains was extracted according to the following description. Briefly, *Monascus* strains were incubated on PDA plates overlaid with cellophane membranes at 30°C for 7 days, then the mycelia were collected and ground in liquid nitrogen with mortar and pestle, and the total genomic DNA was extracted with cetyltrimethylammonium bromide (CTAB) buffer following Sambrook and Russell (2001). The quality of the extracted DNA was detected by BioPhotometer (Eppendorf 3151, Germany).

SRAP amplification was performed with 30 primer combinations, including 5 of forward primers and 6 of

Table 1 *Monascus* strains used in this study

Strain	Species	Origin
M-1	*Monascus* sp.	Koji of Tuo brand in Sichuan Province, China
M-2	*Monascus* sp.	Koji of Tuo brand in Sichuan Province, China
M-3	*Monascus* sp.	Koji of Tuo brand in Sichuan Province, China
M-4	*Monascus* sp.	RYR[a] of Jiacheng Biotechnology Company, Hubei Province, China
M-5	*Monascus* sp.	RYR of Jiacheng Biotechnology Company, Hubei Province, China
M-6	*Monascus* sp.	Koji of Sichuan Province, China
M-7	*Monascus* sp.	Koji of Sichuan Province, China
M-8	*Monascus* sp.	Koji of Sichuan Province, China
M-9	*Monascus* sp.	Koji of Sichuan Province, China
M-10	*Monascus* sp.	Koji of Sichuan Province, China
M-11	*Monascus* sp.	Koji of Sichuan Province, China
M-12	*Monascus* sp.	Koji of Sichuan Province, China
M-13	*Monascus* sp.	Koji of Sichuan Province, China
M-14	*Monascus* sp.	Koji of Sichuan Province, China
M-15	*Monascus* sp.	Koji of Sichuan Province, China
M-16	*Monascus* sp.	Koji of Sichuan Province, China
M-17	*Monascus* sp.	RYR of Yiwu, Zhejiang Province, China
M-18	*Monascus* sp.	RYR of Henan Province, China
M-19	*Monascus* sp.	RYR of Henan Province, China
M-20	*Monascus* sp.	RYR of Zhejiang Province, China
M-21	*Monascus* sp.	Soybean sauce in Wuhan, Hubei Province China
M-22	*Monascus* sp.	Soybean sauce in Wuhan, Hubei Province China
M-23	*Monascus* sp.	Koji of Lanxi, Zhejiang Province, China
M-24	*Monascus* sp.	Red soybean curd of Huanggang, Hubei Province, China
M-25	*Monascus* sp.	RYR of Xianning Hubei Province, China
M-26	*Monascus* sp.	RYR of Yunnan Province, China
M-27	*Monascus* sp.	RYR of Xishui, Hubei province, China
M-28	*Monascus* sp.	RYR of Hongkang, China
M-29	*Monascus* sp.	RYR of Gutian, Fujian Province, China
M-30	*Monascus* sp.	RYR of Guangxi Province, China
M-31	*Monascus* sp.	RYR of Hubei Province, China
M-32	*Monascus* sp.	RYR of Gutian, Fujian Province, China
M-33	*Monascus* sp.	RYR of Zhejiang Province, China
M-34	*Monascus* sp.	RYR of Fujian Province, China
M-35	*Monascus* sp.	RYR of Pingyi, Shandong Province, China
M-36	*Monascus* sp.	RYR of wuhan, Hubei Province, China
M-37	*Monascus* sp.	RYR of wuhan, Hubei Province, China
OUT 2011	*Monascus purpureus*	Department of Biotechnology of Osaka University in Japan
OUT 2012	*Monascus barkeri*	Department of Biotechnology of Osaka University in Japan
OUT 2014	*Monascus purpureus*	Department of Biotechnology of Osaka University in Japan
OUT 2015	*Monascus purpureus*	Department of Biotechnology of Osaka University in Japan
OUT 2137	*Monascus pilosus*	Department of Biotechnology of Osaka University in Japan
CICC5015	*Monascus aurantiacus*	China Center of Industrial Culture Collection (Beijing, China)
CICC5016	*Monascus serorubescens*	China Center of Industrial Culture Collection (Beijing, China)
CICC5017	*Monascus albidus*	China Center of Industrial Culture Collection (Beijing, China)
CICC5020	*Monascus fuliginosus*	China Center of Industrial Culture Collection (Beijing, China)
AS3.2666	*Monascus anka*	Institute of Microbiology, Chinese Academy of Science (Beijing, China)
AS3.549	*Monascus ruber*	Institute of Microbiology, Chinese Academy of Science (Beijing, China)

Table 1 continued

Strain	Species	Origin
AS3.554	*Monascus anka*	Institute of Microbiology, Chinese Academy of Science (Beijing, China)
CBS113675[b]	*Monascus lunisporas*	Central Bureauvoor Schimmel Cultures
CBS109402[b]	*Monascus argentinensis*	Central Bureauvoor Schimmel Cultures

[a] Red yeast rice

[b] Type strain

reverse primers (Table 2), which were synthesized (AuGCT Biotechnology, Beijing, China) according to the description by Li and Quiros (2001). The polymerase chain reaction (PCR) amplification for SRAP was carried out in a 20-μl volume containing 30 ng template DNA, 40 ng forward primer, 40 ng reverse primer, 1.0 μl dNTPs (2.0 mmol/l each), 2.0 μl $MgCl_2$ (20 mmol/l), 1 U Taq DNA polymerase (Dingguo Biotechnology, Beijing, China), and 1× PCR buffer. The program was carried out using a TGRADIENT 96 thermocycler (Whatman, Brentford Middlesex, UK) including 5 min predenaturation at 94°C for 5 min, 5 cycles of PCR reaction at 94°C for 1 min, 35°C for 1 min, 72°C for 1 min, then followed by an additional 35 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a 10-min final extension at 72°C. The amplified products were analyzed by electrophoresis in 1.5% (w/v) agarose gels (BioWest, Spain) containing 0.5 μg/ml ethidium bromide.

ISSR amplification was performed with 14 ISSR primers (see Table 2), which were synthesized (AuGCT Biotechnology) according to the public ISSR primers by Wolfe Lab of University of British Columbia in Canada. PCR amplification for ISSR was performed in a 20-μl volume containing 30 ng template DNA, 40 ng primer, 2.0 μl dNTPs (2.0 mmol/l each), 2.0 μl $MgCl_2$ (20 mmol/l), 1.5 U Taq DNA polymerase (Dingguo Biotechnology), and 1× PCR buffer. The amplification was carried out using a TGRADIENT 96 Thermocycler (Whatman) according to the following steps: initial denaturation at 94°C for 5 min, 40 cycles at 94°C for 30 s, 52°C for 45 s, 72°C for 90 s, and a 7-min final extension at 72°C before maintaining the mixture at 4°C. The PCR products were analyzed by electrophoresis on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide.

Data analysis

Amplified bands from each reaction of SRAP and ISSR were scored as present (1) or absent (0), and only those reproducible and unambiguous bands were considered. Dice's similarity coefficients between strains were calculated by the NTSYSpc 2.10. Cluster analysis was performed using the UPGMA algorithm, and a dendrogram was produced based on each simple matching matrix.

Table 2 Primer sequences used for sequence-related amplification polymorphism (SRAP) and inter-simple sequence repeat (ISSR) analysis in this study

Primers	Sequence (5′–3′)
SRAP primers	
Forward primers	me1: TGAGTCCAAACCGGATA
	me2: TGAGTCCAAACCGGAGC
	me3: TGAGTCCAAACCGGAAT
	me4: TGAGTCCAAACCGGACC
	me5: TGAGTCCAAACCGGAAG
Reverse primers	em1: GACTGCGTACGAATTAAT
	em2: GACTGCGTACGAATTTGC
	em3:GACTG-CGTACGAATTGAC
	em4: GACTGCGTACGAATTTGA
	em5: GACTGCGTACGAATTAAC
	em6: G-ACTGCGTACGAATTGCA
ISSR primers	
808#	$(AG)_8C$
809#	$(AG)_8G$
810#	$(GA)_8T$
811#	$(GA)_8C$
822#	$(TC)_8A$
834#	$(AG)_8YT$
835#	$(AG)_8YC$
841#	$(GA)_8YC$
842#	$(GA)_8YG$
852#	$(TC)_8RA$
853#	$(TC)_8RT$
854#	$(TC)_8RG$
866#	$C(TCC)_5TC$
868#	$(GAA)_6$

R, (A or G); Y, (C or T)

Results

Morphological observations

Based on macroscopic and microscopic characters, 35 *Monascus* isolates were divided into four groups, which were *M. anka*, *M. purpureus*, *M. pilosus*, and *M. barkeri*,

respectively. The largest group was *M. anka*, consisting of 21 isolates, the second was *M. purpureus*, including 6 isolates, the third was *M. pilosus*, including 5 isolates, and the smallest was *M. barkeri*, consisting of 3 isolates. Neither the macroscopic nor microscopic characters of M-3 and M-26 had similarity to the control strains. It is worth noting that OUT 2015 was more similar to *M. pilosus* than to *M. purpureus* based on its morphological characters, including colony appearance and microscopic morphology. The resulting groups and the morphological traits of *Monascus* isolates are shown in Table 3.

SRAP polymorphism and genetic diversity

Eight primer combinations (me1-em2, me1-em4, me1-em5, me2-em4, me3-em2, me3-em6, me4-em4, and me5-em6) generating distinct bands were chosen from 30 pairs of SRAP primers and used for the estimation of genetic similarities among 51 tested *Monascus* strains. A total of 183 scorable bands were produced, of which 173 were polymorphic (94.5%), and the polymorphism ranged from a maximum of 100% (me3-em6 and me4-em4) to a minimum of 87% (me1-em4). Fingerprints of all tested strains could be amplified from 17 (me3-em6) to 29 (me5-em6) pieces of DNA fragments, which varied in size from 150 to 2,500 bp, and an average of 22.88 bands could be amplified with per primer pair (Table 4). Examples of partial PCR fingerprint profiles generated using the SRAP primers are shown in Fig. 1a.

The fingerprints revealed by eight pairs of selected primers (Fig. 1a) were used for the cluster analysis. The similarity coefficients of among the 51 tested strains ranged from 0.46 to 1.00 (Fig. 2a) based on SRAP polymorphic data using the SIMQUAI program in the NTSYSpc software, and all the tested strains were grouped into four clusters at a 78% similarity level. The first major cluster consisted of 36 strains with four subgroups at the 88% similarity level. The first subgroup consisted of 23 strains having two control strains (AS3.5540, *M. anka*; AS3.2666, *M. anka*) and 21 isolates (M-17–M-37); the second consisted of 10 strains having four control strains (OUT 2011, *M. purpureus*; OUT 2014, *M. purpureus*; CICC 5015, *M. aurantiacus*; CICC 5016, *M. serorubescens*) and six isolates (M-4 to M-6 and M-8 to M-10); the third consisted of two isolates (M-13 and M-11); and control strain AS3.5490 (*M. ruber*) clustered independently. The second major cluster was formed by 13 strains consisting of five control strains (CICC 5017, *M. albidus*; CICC 5020, *M. fuliginosus*; OUT 2012, *M. barkeri*; OUT 2137, *M. pilosus*; and OUT 2015, *M. purpureus*) and eight isolates (M-1 to M-3, M-12, M-14, M-15, M-7, and M-16). Control strains CBS 113675 (*M. lunisporas*) and CBS 109402 (*M. argentinensis*) were each placed in a separate branch of a third and the fourth group, which may be the result of their distant geo-relationship with other strains.

ISSR polymorphism

Seven primers (808#, 810#, 811#, 834#, 835#, 841#, and 842#) screened from 14 ISSR primers could generate more diverse bands (Fig. 1b), with the number of amplified fragments ranging from 11 (811#) to 21 (834#) and which varied in size from 300 to 4,500 bp. A total of 108 bands were yielded with seven primers and with an average of 15 polymorphic fragments per primer. Percentage polymorphism ranged from 92.3% (810#) to a maximum of 100% (811#, 834#, 835#, and 842#), with an average of 97.1% polymorphism (Table 4). Examples of partial PCR fingerprint profiles generated using the ISSR primers are showed in Fig. 1b.

The dendrogram revealed that similarity coefficients ranged from 0.48 to 1.00 according to the SIMQUAI program in the NTSYSpc soft by ISSR fingerprints (Fig. 2b). 51 tested strains were divided into four major groups at a similarity level of 74% based on the dendrogram generated via ISSR marker analysis. Control strains including *M. anka* (AS 3.5540 and AS 3.2666), *M. purpureus* (OUT 2011 and OUT 2014), *M. aurantiacus* (CICC 5015), *M. serorubescens* (CICC 5016), and *M. ruber* (AS 3.5490) and 28 isolates (M-4 to M-6, M-8 to M-11, M-17 to M-37) were clustered into the first group; the second group consisted of five control strains (*M. barkeri* OUT 2012; *M. purpureus* OUT 2015; *M. pilosus* OUT 2137; *M. albidus* CICC 5017; *M. fuliginosus* CICC 5020) and nine isolates (M-1 to M-3, M-7, M-12 to M-16), which was very similar to the results from SRAP data with minor differences in the subgroups.

Discussion

In this work, 37 *Monascus* isolates were divided into five groups according to Li and Guo's morphology-base classification index (2003). About 86.5% of *Monascus* isolates (32 strains) belong to *M. anka*, *M. purpureus*, and *M. pilosus*, respectively, which are usually prepared for RYR-related products in many countries of East Asia, and only three isolates were very similar to *M. barkeri*. Classification by SRAP and ISSR was very similar to those by morphological observations, but there were some divergences. Morphologically, the cultural characters of M-3 was very similar to that of M-26, but they were clustered into different group by SRAP and ISSR data, suggesting that SRAP or ISSR has potential in differentiating morphologically similar strains; M-13 (*Monascus* isolates) morphologically identified as *M. pilosus* was grouped into one clade with *M. pilosus* in the ISSR dendrogram, while it was excluded from the *M. pilosus* clade in the SRAP dendrogram, which seems to indicate that the result of phylogenetic anaysis with ISSR data is basically in

Table 3 The cluster result of *Monascus* strains based on morphological observation

Strain no.	Group	Colony morphology (25°C, 7 days)				Micromorphology on WA medium (25°C, 7 days)			
			Walt agar (WA)	Chapek-yeast agar (CYA)	G25 N	Feature of aerial hyphae	Conidia	Cleistothecia	Ascospore
M-4, M-5, M-17 to M-20, M-22 to M-25, M-27 to M-37	*M. anka*	Diameter (mm)	20–30	±17	±15	Colorless or orange; covered with orange crystal; with septa	Globular or obpyriform; colorless or red; single or straight chain	Globular; red or orange; thick	Oval 6–7 × 4–5 μm
		Color	Orange center with white edge	Pink	Red				
		Aerial hypha	Rare	Rare	Rare				
		Shape	With radiate lines Colony raised at 25 days	Flat	Flat				
M-6, M-8 to M-11, M-21	*M. purpureus*	Diameter (mm)	30–40	±18	±20	Smooth; colorless; with septa	Globular or obpyriform; single or straight chain	Globular; colorless or red; thin	Oval 6–7 × 4–5 μm
		Color	Orange or slight pink	White or rufous	White				
		Aerial hypha	Felty	Dense	Dense				
		Shape	Flat	Flat	Flat				
M-1, M-2, M-7, M-13, M-15	*M. pilosus*	Diameter (mm)	±60	±20	±18	Smooth; colorless or red-lilac; with septa	Globular or obpyriform; single or straight chain	Globular; colorless; thin	Oval 5–8 × 3–3.5 μm
		Color	White or slight pink	Red or rufous	White				
		Aerial hypha	Dense and long	Rare and short	Rare				
		Shape	Flat with radiate line	Flat	Flat				
M-12, M-14, M-16	*M. barkeri*	Diameter (mm)	50–60	±45	±20	Smooth; alutaceous; with septa	Globular or obpyriform; brown; single or straight chain	Globular; brown; thin	Oval Alutaceous 6–8 × 4 μm
		Color	White-pink-olivaceous	White	White				
		Aerial hypha	Fluffy	Short and dense	Abundant				
		Shape	Flat with radiate lines	Flat	Flat				
M-3, M-26		Diameter (mm)	±20	±10	±7	Smooth; colorless; with septa	Globular or obpyriform; straight chain[a] single[b]	Not observed	Not observed
		Color	Orange or brown	Orange	White				
		Aerial hypha	No[a] Rare[b]	No	No				
		Shape	Slightly raised; with reductus	Slightly raised; with reductus	Flat[a] Slightly raised; with reductus[b]				

[a] M-3

[b] M-26

Table 4 Total bands and polymorphic bands resulting from SRAP and ISSR, respectively

Technique	Primer	Total bands	Polymorphic bands	Percentage of polymorphism
SRAP	me1-em2	19	17	89.5
	me1-em4	23	20	87.0
	me1-em5	24	22	91.7
	me2-em4	30	30	100.0
	me3-em2	18	17	94.4
	me3-em6	17	17	100.0
	me4-em4	23	23	100.0
	me5-em6	29	27	93.1
	Total	183	173	
	Average	22.875	21.625	94.5
ISSR	808#	14	13	92.9
	810#	13	12	92.3
	811#	11	11	100.0
	834#	21	21	100.0
	835#	12	12	100.0
	841#	18	17	94.4
	842#	19	19	100.0
	Total	108	105	
	Average	15.43	15	97.1

means number

agreement with those previously inferred by morphological data, whereas SRAP is more sensitive for morphologically undetectable differences. By the combination of morphological observations and dendrograms produced by two sets of data (Table 3; Fig. 2), most *Monascus* isolates from RYR were very similar to *M. anka*, suggesting that they are very close to each other in genetic relationship. We supposed those isolates from RYR may come from the same parent. This result also demonstrates that *Monascus* isolates from RYR have a relatively narrower genetic source, which is in consistent with the conclusion of Lakrod et al. (2000), who used RAPD to investigate the genetic variation within a collection of *Monascus* isolates from RYR and red soybean curd (tofu). Comparatively, 15 isolates from Koji were clustered into different subgroups, respectively. We deduced that the monophyletic cluster of isolates from RYR and polyphyletic cluster of isolates from Koji may result from their roles played in the products; for example, RYR production required that the *Monascus* strain could produce amylase with high activity, whereas the *Monascus* strains in Koji should produce different kinds of esterases that are beneficial to the formation of special flavors in the wine. This result suggests that genetic similarity of *Monascus* isolates has a closer relationship with their origins than with geographic factors. In regard to control strains, there also were some dissimilarities in cluster position; for instance, *M. serorubescens* was classified as *M. pilosus* by Hawskworth and Pitt (1983), but we observed that *M. serorubescens* CICC 5016 was more similar to *M. purpureus*, and that *M. purpureus* OUT 2015 resembled *M. pilosus* OUT 2137, and these results were in line with the cluster analysis by SRAP and ISSR. Therefore, we presumed *M. serorubescens* (CICC 5016) is the synonym or variant of *M. purpureus*; *M. purpureus* OUT 2015 may have been misidentified and should be reconsidered as *M. pilosus*.

Based on dendrograms produced by SRAP and ISSR data, *M. aurantiacus* (CICC 5015), *M. serorubescens* (CICC 5016), and *M. purpureus* were placed into the same subgroup, suggesting that they shared great similarities in genetic background; *M. albidus* (CICC 5017), *M. fuliginosus* (CICC5020), *M. barkeri* (OUT 2012), and *M. pilosus* (OUT 2137) were grouped in the same clade. However, the phylogenetic relationship inferred from the D1/D2 regions of LSU rRNA genes (Park and Jong 2003) was incongruent in regard to *M. albidus*, *M. serorubescens*, and *M. fuliginosus*. Although there are remarkable differences between *M. pilosus* and *M. ruber*, these two species could not be differentiated by molecular markers based on the D1/D2 regions of LSU rRNA genes, the partial β-tubulin gene, and MRT sequence (Park and Jong 2003; Park et al. 2004; Chen et al. 2007), although the two species were clustered into different clades according to the dendrograms by SRAP and ISSR. The incongruent phylogenetic relationships may be caused by these two reasons: (1) the molecular markers are based on different principles, for instance,

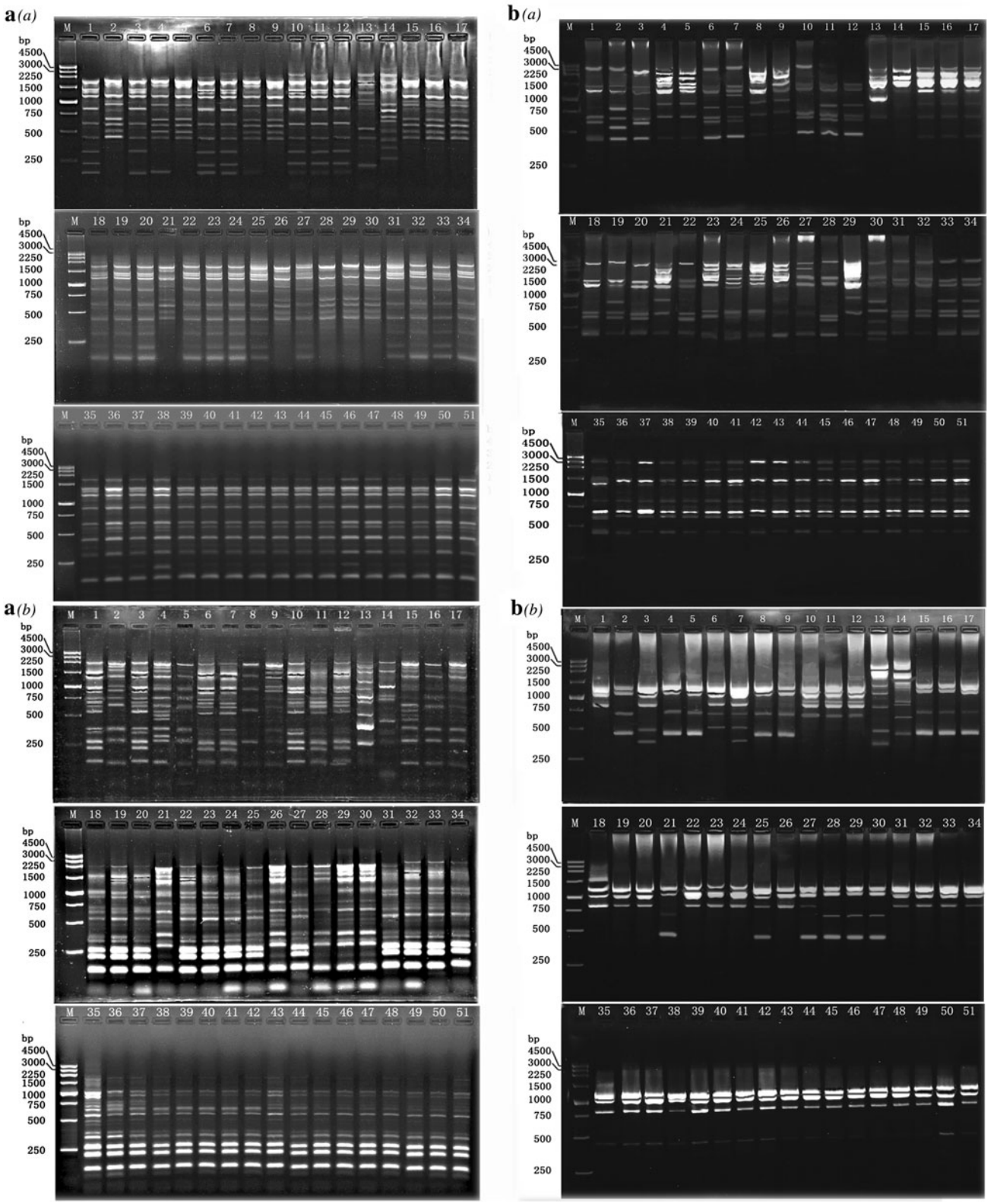

Fig. 1 Amplification products generated from tested strains with different sequence-related amplification polymorphism (SRAP) and inter-simple sequence repeat (ISSR) primers. *1–14*, 14 control strains; *15–51*, 30 *Monascus* isolates from a wide range of regions in China, corresponding to M-1 to M-37. **a** Amplification products generated from tested strains with different SRAP primers: (*a*) amplified fingerprint by me1-em2; (*b*) amplified fingerprint by me1-em4. **b** Amplification products generated from tested strains with ISSR primers: (*a*) amplified fingerprint by primer 808[#]; (*b*) amplified fingerprint by primer 835[#]

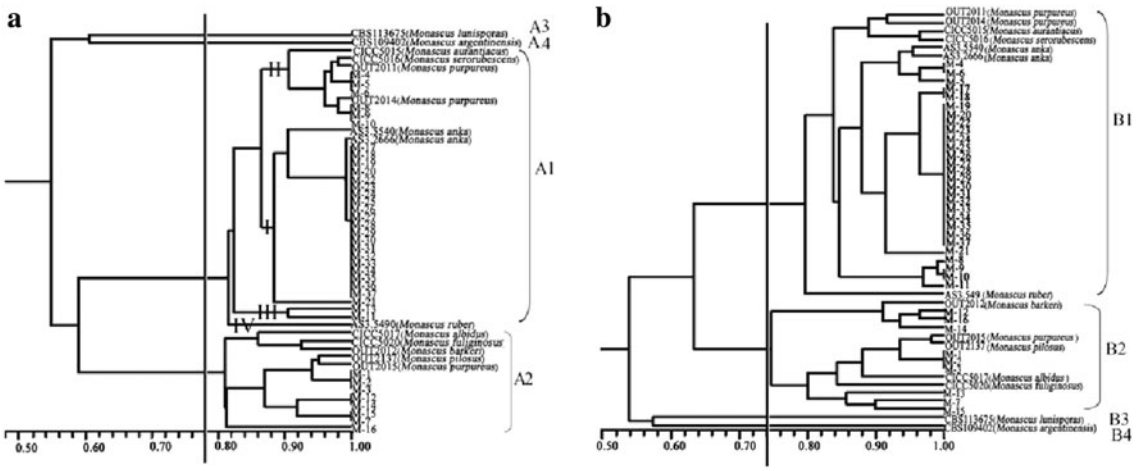

Fig. 2 Dendrograms based on simple matching matrix from SRAP fingerprints and ISSR fingerprints. **a** Dendrogram from SRAP fingerprints: *A1*, *A2*, *A3*, and *A4* represent the four different clusters based on SRAP fingerprints at a 78% similarity level; *I*, *II*, *III*, and *IV* represent the four subgroups in A1 cluster. **b** Dendrogram from ISSR fingerprints: *B1*, *B2*, *B3*, and *B4* represent the four different clusters based on ISSR fingerprints at a 74% similarity level

SRAP and ISSR target the whole genomic DNA sequences, whereas amplification of D1/D2 regions of LSU rRNA genes and partial β-tubulin sequences is based on the comparison of nucleotide sequence; and (2) the strains used in this study were different from those in other studies. The incongruent cluster results may indicate that molecular markers based on different principles had certain scope of applications.

Compared with ISSR and SRAP data (Table 4), SRAP produced more polymorphic bands than ISSR, which may indicate that SRAP is more informative, but the general trend of the two dendrogams by SRAP and ISSR is very similar in spite of minor inconsistencies in the position of some subgroups; this is reasonable because ISSR and SRAP aim to amplify a different region of genome. The results presented here suggested that these two techniques were suitable for genetic polymorphism research and identification of unknown *Monascus* strains. So, SRAP and ISSR would be alternative molecular approaches to the classification and identification of *Monascus* strains on a large scale or used as supplementary approach to traditional classification.

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