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Authentication of domestic Taiwan rice varieties based on fingerprinting analysis of microsatellite DNA markers

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ABSTRACT. Microsatellite marker (SSR) analysis was used to differentiate between domestic Taiwan rice varieties and foreign rice cultivars for authentication and traceability. A panel of 32 microsatellite DNA markers and 36 rice varieties from different countries were used for comparative polymorphism analysis. A total of 306 alleles were observed in 32 loci. The number of alleles per locus ranged from 3 to 21, with an average of 9.6. Polymorphic information content (PIC) values varied from 0.205 (RM7023) to 0.926 (RM333), with an average of 0.75. The most useful markers identified for efficient differentiation of domestic Taiwan rice identities were RM21, RM22, RM101, RM333, RM475, RM1387, RM5704, and RM7545. Principle component analysis (PCA) score plot and clustering analysis were sufficient to discriminate between two different groups of rice varieties based on geographic origins. To prevent fraudulent commercial activity, these results provide effective SSR marker sets and workflow for regular genotype verification and premium rice variety purity monitoring in Taiwan. Finally, to ensure the practical use of DNA fingerprinting technology in rice adulteration tests, sensitivity as low as 3 to 7.5 % can be detected by mixing different ratios of Taikeng 9 and Taikeng 16.

Keywords: DNA fingerprinting analysis; Microsatellite markers; Polymorphism; Principle component analysis (PCA); Rice; Simple sequence repeat (SSR).

INTRODUCTION

Rice is one of the world's staple food crops. More than 90% of the population of Asia considers rice their main calorie source. Owing to world population growth, there is a tremendous increase in the demand for rice. The price for rice is not homogenous in the international rice markets due to its variability in quality, variety and processing. To comply with different standards, most rice seed dealers, farmers, millers, food processors and consumers are highly concerned about the authenticity of their rice for its uniformity and quality (Vlachos and Arvanitoyannis, 2009). Thus, it is essential for industry to have a successful strategy to verify rice variety labeling and composition, in other words, its traceability and authenticity. For example, billions of dollar's worth of basmati rice is exported to Europe from India and Pakistan every year. However, by counterfeiting brand names and by deliberately fraudulent labeling, regular-quality rice can be sold as premium-priced basmati rice in order to make profits (Vemireddy et al., 2007; Steele et al., 2008; Colyer et al., 2009). China has recently become the largest importer of Thailand jasmine rice and to meet legal requirements, a standard authentication protocol for jasmine rice was developed (Wu et al., 2009). Furthermore, with the trend of trade liberation and globalization, Taiwan became a member of WTO in 2001, allowing a minimum market access quota of 144,720 tons rice importation. In 2004, Taiwan rice started being to be exported to Japan, the first time in 30 years (Huang, 2001). Imported rice usually has a lower price than domestic rice; therefore, mixing domestic rice with imported rice can earn extra profit. The industry was challenged to develop a valid and rapid rice forensics technique to identify domestic Taiwan rice. Issues including consumer rights protection, ensuring fair competition and market assessment for rice production and for the food industry will soon become critically important (Woolfe and Primrose, 2004).

Over the past decade, morphological characteristics such as height, grain shape, size, etc. were used in rice variety identification. Other biochemical or physiological factors such as starch composition, lipids, seed storage proteins, and alloenzymes were also used to characterize different rice varieties. However, the above parameters are not inherently stable and are easily affected by growth conditions and environment. Recently, DNA profiles based on various molecular markers have been widely applied across different fields (Popping, 2002; Terzi et al., 2005;

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Primrose et al., 2010). These markers include restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), random amplified polymorphic DNA (RAPD) (Williams et al., 1990), amplified fragment length polymorphism (AFLP) (Vos et al., 1995), sequence characterized amplify region (SCAR) (Zhang and Stommel, 2001), microsatellite or simple sequence repeat (SSR) (Tautz, 1989; Chen et al., 1997; McCouch et al., 2002), and single nucleotide polymorphism (SNP) (Hour et al., 2007; Ganal et al., 2009). Among them, SSR markers have several advantages, their co-dominant, stable, and highly polymorphic characteristics have been used intensively for rice cultivar identification (Pal et al., 2004; Singh et al., 2004; Pessoa-Filho et al., 2007; Vemireddy et al., 2007; Bonow et al., 2009; Cirillo et al., 2009; Rahman et al., 2009), genetic diversity evaluation and phylogenetic comparison (Hashimoto et al., 2004; Bajracharya et al., 2006; Giarrocco et al., 2007; Jayamani et al., 2007; Pervaiz et al., 2009; Yuan et al., 2007), germplasm maintenance (Jain et al., 2004; Xu et al., 2004; Lu et al., 2005; Thomposon et al., 2007; Ebana et al., 2008; Agrama et al., 2009), and marker assisted selection. Traditionally, for a small to intermediate laboratory, SSR analyses are done with agarose or polyacrylamide gel electrophoresis. However, routine DNA fingerprinting with large numbers of SSR can be tedious and time consuming, especially when attempting to score correct allele sizes. A capillary gel electrophoresis system, the QIAxcel system-GT12™ genetic analyzer, which replaces traditional, labor-intensive DNA gel analysis, can now be used for fast, accurate, cost-effective and regularly large-scale SSR analysis, gene mapping and population genetic studies (Vemireddy et al., 2007; Archak et al., 2009; Wang et al., 2009).

In Taiwan, rice cultivation covers about 260 thousand acres. From 2005 to 2007, the most popular premium varieties among 30 currently planted commercial Japonica rice varieties, were Tainan 11 (39%), Taikeng 14 (10.8%) and Taikeng 8 (8.3%) (Statistic data from COA, http://www.coa.gov.tw/view.php?catid=17817&print=1). In the past, the introduction of elite germplasm from Japan varieties, such as Sinriki and Kameji, contributed more than 66% of the parentage of Japonica varieties released in Taiwan (Lin, 1991). Through pedigree analysis, Lin (1991) showed that between 1940 and 1987, the 99 Japonica rice varieties released in Taiwan were traceable to 65 ancestors. Among these ancestors, only 11 were from Taiwan, 44 were plant introductions from Japan. Chen et al. (2008) established a Taiwan rice pedigree database (http://ricebreeding.agron.ntu.edu.tw) containing 124 Japonica and 19 Indica varieties or breeding lines developed between 1981 and 1997. Due to the involvement of Japanese introductions in variety improvement, we noticed a narrow genetic base for the Japonica rice varieties, especially for the premium varieties (Taichung 65, Koshihikari or Tainung 67), in Taiwan (Chen et al., 2008). Japan cultivars such as Hokuriku 100, Koshihikari and Kinuhikari continue to be used for breeding parental lines to improve the taste quality of Taiwan Japonica rice varieties (Tainung 71, Hualian 20 and Kaohsiung 145) (Wu and Lin, 2008). Cultivars Hokuriku 100 and Kinuhikari have been used directly as backbone parents to breed Taikeng 9 and Tainung 71, respectively. However, in Japan, Hokuriku 100 and Kinuhikari are genetically closed rice varieties (Hsieh et al., 2007; Chen et al., 2008). It is thus difficult to distinguish the genetic diversity and individual genotypes of domestic Taiwan rice varieties based on traditional agro-morphological traits or isoenzyme analysis. For this purpose, a promising and effective advanced DNA-based marker set, such as SSR with high polymorphism, is absolutely crucial. Meanwhile, with the rice market opening up, Taiwan has begun to import rice from various countries, including the U.S.A, Japan, Australia, Thailand, Egypt, and Vietnam. It is difficult to differentiate between imported and domestic Taiwan rice based on appearance. Hence, this study was aimed at screening and selecting compatible polymorphic microsatellite DNA markers with 36 rice cultivars from different countries according to their allelic diversity and genetic relatedness. To do so, we used the QIAxcel multicapillary electrophoresis gel system. Our final goal is to develop a low-cost, accurate, sensitive and effective DNA fingerprinting method to authenticate domestic Taiwan rice from foreign varieties using SSR markers.

MATERIALS AND METHODS

Plant materials

In total, a collection of 36 rice (*Oryza sativa* L.) accessions were used for microsatellite analysis as listed in Table 1. Twenty three of these were Taiwan rice cultivars from germplasm of TARI (Taiwan Rice Research Institute), the others from separate agricultural research stations. Three commercial American rice varieties were kindly provided by the U.S. Rice Association. Three Japan varieties and six Thai varieties were obtained from the Council of Agriculture in Taiwan. A single sample, Sun-White from Australia, was purchased from Taiwan market.

Genomic DNA extraction

Total genomic DNA was extracted from a bulk of 10 rice grains, randomly selected from each rice cultivar using the CTAB/PTB method (Bernardo et al., 2005). Rice seeds were ground into powder with liquid nitrogen in a mortar. 750 μL of extraction buffer (1% CTAB, 0.02 M EDTA, 1.4 M NaCl and 0.01 M Tris-HCl (pH = 8.0)) was then added and the mixture was incubated at 65°C for 20 min. For protein digestion, 10 μL proteinase K (20 mg/ml) was added and reacted at 65°C for another 30 min. After phenol/chloroform extraction, genomic DNA was precipitated with 2/3 volume of isopropanol. The DNA pellet was washed with 70% EtOH and dissolved in 200 μL of 1X TE buffer (pH = 8.0). After RNase digestion to eliminate RNA residues, the genomic DNA was precipitated with ethanol and dissolved in TE buffer. The concentration of DNA was quantified using Biophotometer (Eppendorff) and then diluted 100X as stock for use in the following PCR reaction.

Table 1. List of rice varieties used in rice authentication study.

Country of origin	Total numbers	Cultivar name
Taiwan	23	*Japonica*: TaiKeng 2 (TK 2), TaiKeng 4 (TK 4), TaiKeng 5 (TK 5), TaiKeng 6 (TK 6), TaiKeng 8 (TK 8), TaiKeng 9 (TK 9), TaiKeng 11 (TK 11), TaiKeng 12 (TK 12), TaiKeng 14 (TK 14), TaiKeng 16 (TK 16), Tainung 67 (TNG 67), Tainung 71 (TNG 71), Kaohsiung 139 (KH 139), Kaohsiung 145 (KH 145), Tainan 11 (TN 11), Taichung 191 (TC 191), Taoyuan 3 (TY 3), Taitung 30 (TD 30), Hualian 19 (HL 19) *Indica*: Taichung Sen 2 (TS 2), Taichung Sen 10 (TCS 10), Tainung 1 (TN 1), Tainung Sen22 (TNS 22)
Thailand	6	*Indica*: Jasmine 85 (J 85), Pathumithani (Path), Hom Mali (Hom), 17032, 17050, KDML
Japan	3	*Japonica*: Koshihikari (Koshi), Koshiibuki (Ibuki), Akitakomachi (Akita)
USA	3	*Japonica*: M 202, M 401 *Indica*: Southern Long Grain (SL)
Australia	1	*Japonica*: SunWhite (Sun)

Microsatellite markers and PCR amplification

Thirty-two microsatellite primer pairs were used for rice variety discrimination in this study (Table 2). These primer sets include RM580, RM1387, RM3412, RM6515, RM7180 (Chr 1); RM3501, RM5862 (Chr 2); RM22, RM2835, RM5755, RM7565 (Chr 3); RM475, RM1359, RM1388, RM3892 (Chr 4); RM1089, RM 7271 (Chr 5); RM276, RM 7023 (Chr 6); RM 1353, RM 8006 (Chr 7); RM72, RM 5428 (Chr 8); RM3912, RM6971 (Chr 9); RM333, RM496, RM3152, RM7545 (Chr10); RM21, RM5704 (Chr11); RM101 (Chr 12) (McCouch et al., 2002). PCR amplification was carried out in a final volume of mixtures (12 μL) containing 15~20 ng of genomic DNA, 0.2 mM dNTP, 0.4 μM of forward and reverse prim-

Table 2. Data on SSR markers used, location on rice chromosomes, polymorphism information content (PIC), number of alleles detected, and allele size range.

SSR marker	Chr. location	SSR motifs	PIC value	Allele number	Size range (bps)	SSR marker	Chr. location	SSR motifs	PIC value	Allele number	Size range (bps)
RM21	11	$(GA)_{18}$	0.809	10	123~169	RM3412	1	$(CT)_{17}$	0.495	7	205~242
RM22	3	$(GA)_{22}$	0.574	5	163~199	RM3501	2	$(CT)_{25}$	0.778	9	181~216
RM72	8	$(TAT)_5C(ATT)_{15}$	0.762	8	157~209	RM3892	4	$(TG)_{14}$	0.901	19	184~359
RM101	12	$(CT)_{37}$	0.875	13	134~309	RM3912	9	$(GT)_{22}$	0.728	9	190~218
RM276	6	$(AG)_8A_3(GA)_{33}$	0.725	7	88~137	RM5428	8	$(TC)_{16}$	0.656	6	90~396
RM333	10	$(TAT)_{19}(CTT)_{19}$	0.926	21	160~287	RM5704	11	$(AAT)_{20}$	0.873	15	160~215
RM475	4	$(TATC)_8$	0.856	12	175~230	RM5755	3	$(ACT)_{26}$	0.752	11	203~261
RM496	10	$(TC)_{14}$	0.724	6	268~297	RM5862	2	$(ATA)_{28}$	0.869	12	144~237
RM580	1	$(CTT)_{19}$	0.77	8	205~266	RM6515	1	$(GCG)_8$	0.329	3	220~227
RM1089	5	$(AC)_{33}$	0.741	11	200~249	RM6971	9	$(TTC)_{13}$	0.775	6	193~232
RM1353	7	$(AG)_{23}$	0.807	9	169~276	RM7023	6	$(AAAG)_{11}$	0.205	4	209~232
RM1359	4	$(AG)_{25}$	0.847	10	114~151	RM7180	1	$(ATAG)_6$	0.662	4	175~219
RM1387	1	$(AG)_{44}$	0.801	8	114~151	RM7271	5	$(ATCT)_8$	0.716	6	197~222
RM1388	4	$(AG)_{46}$	0.889	14	165~240	RM7545	10	$(TATG)_{18}$	0.829	14	167~256
RM2835	3	$(AT)_{36}$	0.781	10	173~267	RM7565	3	$(TCGA)_6$	0.526	3	203~212
RM3152	10	$(CA)_{23}$	0.83	12	232~334	RM8006	7	$(AT)_{94}$	0.9	14	131~310

ers, 1X TAKARA rTaq buffer and 0.25U TAKARA rTaq. PCR reactions were performed on a TAKARA PCR Thermal Cycler Dice Model TP600 (TaKaRa Bio Inc., Japan) for 1 cycle of 5 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30s at annealing temperature, 30 seconds at 72°C, and a final 7-minute extension at 72°C. The optimal annealing temperature for all SSR primer sets was determined by gradient PCR condition conducted using a TAKARA PCR Thermal Cycler.

PCR product separation and allelic size determination

For optimal polymorphic SSR marker screening, PCR products were electrophoresised in 3% Metaphor agarose gel (Cambrex Bio science Rockland Inc., USA) with 1X TBE buffer, stained with EtBr and photographed under UV light. The SSR markers with high polymorphism were further used in SSR fingerprinting analysis and PCR products were analyzed on a QIAxcel multicapillary electrophoresis system (Qiagen; formerly eGene's HDA-GT12 system). Reactions were carried out with QIAxcel DNA high resolution Kit (Cat No. 929002) and the PCR products were separated using the OM750 method in a 12-channel, sieving-gel cartridge (GCK5000). Based on QX Alignment Markers 15 bp/500 bp (Cat. No. 929520) and QX DNA Size Marker 25-450 bp (Cat No. 920555), actual allele sizes were resolved and automatically calculated in bp by BioCalculatorTM software, provided with the QIAxcel system, and shown as a gel image and electrophoregram. To fit into the best resolution capability of the system, the size variation of microsatellite fragments greater than 3 bp were considered as significantly different alleles.

Data analysis

Molecular data from SSR assays, including genetic distance, cluster and principle component analysis (PCA), were analyzed with NTSYS pc ver 2. 2 (Exeter software). PCR products were scored as present (1) and absent (0) for each marker to generate a binary matrix. The genetic similarity of 23 local Taiwan rice cultivars was calculated, and a phylogenetic tree was constructed using the UPGMA (unweighted pair-group method using arithmetic averages) method based on genetic similarity coefficients estimated by Modified Rogers' Distance (RMD) (Goodman and Stuber, 1983; Reif et al., 2003; Reif et al., 2005):

$$d_w = \frac{1}{\sqrt{2m}} \sqrt{\sum_{i=1}^{m} \sum_{j=1}^{n_i} (p_j - q_j)^2}$$

where p_{ij} and q_{ij} are the frequency of the jth allele at the ith marker in two independent cultivars and *m, n* indicate the number of markers. The polymorphic information content (PIC) for each marker was calculated (Botsein et al., 1980) and modified by Anderson et al. (1993) for self-pollinated species as follows:

$$PIC_i = 1 - \sum_{j=1}^{n} p^2{}_j$$

where p_i is the frequency of the i^{th} allele, and *n* is the number of alleles.

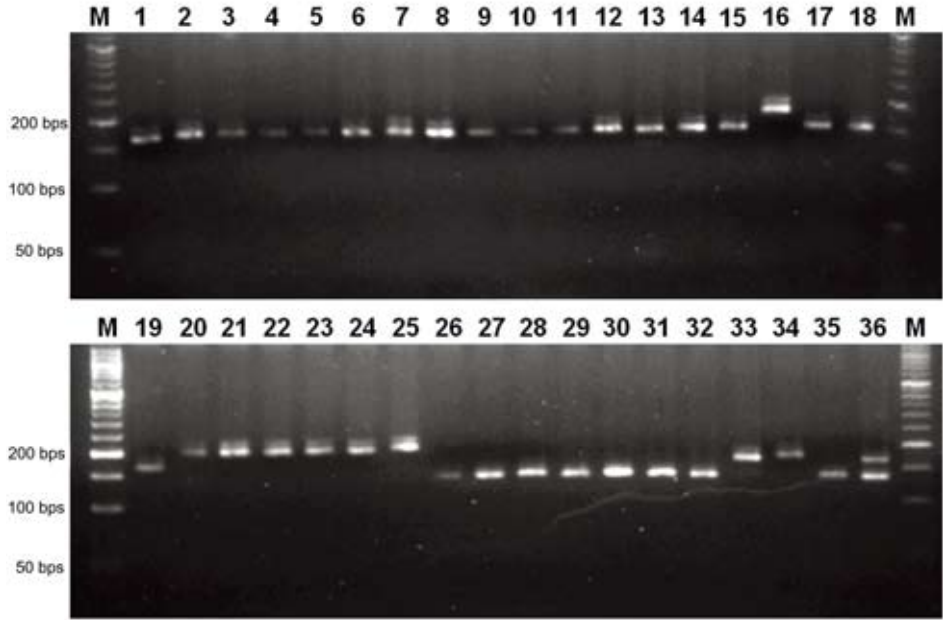

Figure 1. DNA fingerprint analysis of 36 rice varieties from different countries that were amplified by SSR marker (RM1387). The lane (M) represents 50 bp DNA molecule weight markers. The abbreviation name of rice cultivars used for this analysis is the same as Table 1. (1) TK2, (2) TK4, (3) TK5, (4) TK6, (5) TK8, (6) TK9, (7) TK11, (8) TK12, (9) TK14, (10) TK16, (11) TNG67, (12) TN71, (13) KH139, (14) KH145, (15) TN11, (16) TC191, (17) TY3, (18) TD30, (19) HL19, (20) M202, (21) M401, (22) Koshi, (23) Akita, (24) Ibuki, (25) Sun, (26) TS2, (27) TCS10, (28) TN22, (29) TN1, (30) J85, (31) Path, (32) Hom, (33) K17032, (34) K17050, (35) SL, (36) KDML.

Sensitivity of rice adulteration test

To verify the sensitivity of the methodology used for adulterant detection and quantification, standard samples were prepared with a final concentration of 10 ng DNA templates of TK9/TK16 mixture at progressive ratios of 0, 1, 2, 3, 4, 5, 7.5, 10, 50, 90, 92.5, 95, 96, 97, 98, 99 and 100% of each of TK9 in TK16. Adulterant quantification was determined with RM475 using the Qiaxcel capillary electrophoresis system.

RESULTS

SSR polymorphism among rice varieties

To select SSR markers for efficient differentiation of various rice varieties, we conducted a preliminary screening of 120 microsatellite markers (McCouch et al., 2002) by agarose gel analysis using 16 rice accessions from different countries (data not shown). These rice varieties were selected according to the following criteria: (1) where they are currently grown in Taiwan, (2) the higher price available in current rice market, (3) the total trade volume of imported foreign rice and (4) the adulterated rice preferred by customers. Those SSR markers displaying non-specific banding patterns, no polymorphisms or without PCR products were discarded. The identity of either foreign or domestic Taiwan rice varieties was easily determined using RM1387 (Figure 1). In most cases, except for Taichung 191 (number 16), local Taiwan cultivars (number 1 to 19 Japonica rice, and number 26 to 29 Indica rice) exhibited the same DNA fingerprint. All other foreign cultivars, except for Jasmine 85 (number 30), Pathumithani (31), and Hom Mali (32), showed variable characteristic DNA fragments. For high resolution SSR fingerprinting analysis, 32 SSR markers on all 12 chromosomes showing a clear and high polymorphic DNA fingerprinting profile were further used in a genotyping assay of 36 rice cultivars using the QIAxcel multi capillary electrophoresis system (formerly HAD-GT12) (Wang et al., 2009). These 36 commercially-available rice cultivars were obtained from various countries; Japonica (19 from Taiwan, 3 from Japan, 2 from USA and 1 from Australia) and Indica (4 from Taiwan, 6 from Thailand and 1 from USA). Amplification profiles from different primer pairs revealed a considerable difference in their ability to determine genetic variation among these cultivars (Table 2). Some of the primers generated several different alleles across 36 rice varieties but others produced less allelic variation. A total of 306 alleles were detected. The number of alleles per locus ranged from 3 (RM6515, RM7565) to 21 (RM333), with an average of 9.6. The overall allele sizes of DNA ranged from 88 to 396 bp. The polymorphism levels of these 32 markers among the 36 varieties were determined according to the calculated PIC values for each of the SSR loci. The PIC value is an indicator that reflects allele diversity and frequency among various rice varieties. The PIC values varied widely from 0.205 (RM7023) to 0.926 (RM333), with an average of 0.75 per locus (Table 2). The most useful markers for efficient determination of domestic Taiwan rice identities from those of foreign rice are RM21, RM22, RM101, RM333, RM475, RM1387, RM5704, and RM7545. These markers exhibited several distinct bands and showed enough polymorphism to differentiate domestic from foreign rice varieties.

Genetic similarity among rice cultivars

To determine the level of genetic relatedness among the currently studied rice cultivars, pair-wise similarity coefficients were calculated from the SSR-derived data (Table 3). The SSR-based Modified Rogers' Distance (RMD) coefficient between Japonica and Indica rice varieties was

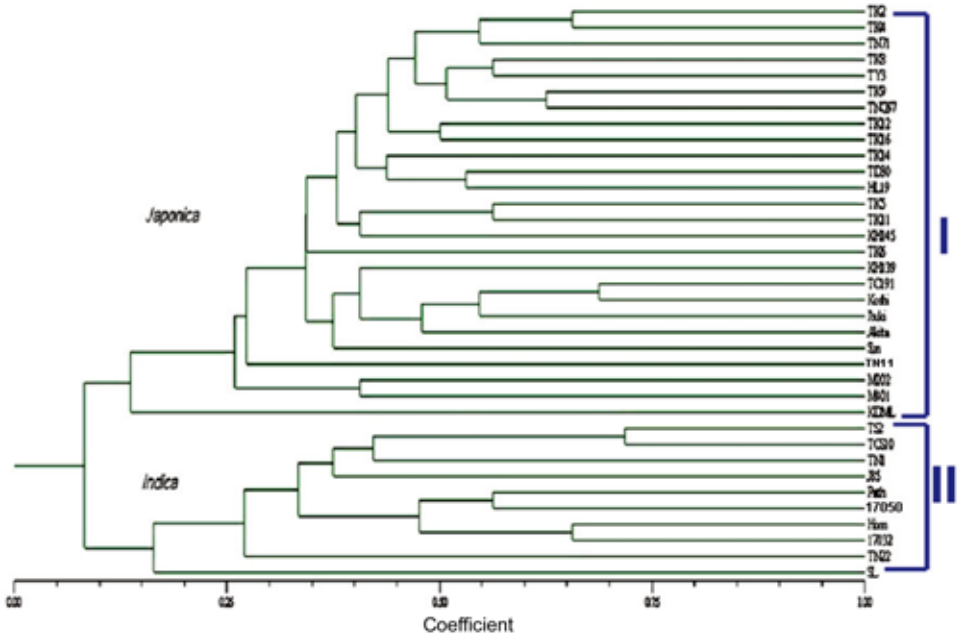

Figure 2. Phylogenetic tree for 36 rice varieties (23 from Taiwan and 13 from foreign countries) from a UPGMA cluster analysis based on 32 SSR markers.

Table 3. Matrix of genetic similarities among pairs of rice cultivars from Taiwan or foreign countries based on SSR fingerprinting analysis.

	TK2	TK4	TK5	TK6	TK8	TK9	TK11	TK12	TK14	TK16	TNG67	TN71	KH139	KH145	TN11	TC191	TY3	TD30
TK2	1.00000																	
TK4	0.46875	1.00000																
TK5	0.31250	0.28125	1.00000															
TK6	0.21875	0.31250	0.15625	1.00000														
TK8	0.34375	0.37500	0.28125	0.15625	1.00000													
TK9	0.31250	0.43750	0.31250	0.21875	0.43750	1.00000												
TK11	0.31250	0.34375	0.53125	0.25000	0.40625	0.53125	1.00000											
TK12	0.31250	0.34375	0.34375	0.21875	0.37500	0.43750	0.43750	1.00000										
TK14	0.18750	0.28125	0.31250	0.06250	0.40625	0.37500	0.43750	0.31250	1.00000									
TK16	0.37500	0.37500	0.28125	0.12500	0.46875	0.37500	0.43750	0.40625	0.37500	1.00000								
TNG67	0.31250	0.46875	0.21875	0.28125	0.40625	0.50000	0.34375	0.37500	0.28125	0.34375	1.00000							
TN71	0.31250	0.34375	0.28125	0.21875	0.31250	0.28125	0.25000	0.37500	0.25000	0.21875	0.34375	1.00000						
KH139	0.18750	0.28125	0.21875	0.25000	0.18750	0.18750	0.15625	0.21875	0.18750	0.18750	0.18750	0.34375	1.00000					
KH145	0.25000	0.21875	0.37500	0.18750	0.21875	0.34375	0.28125	0.18750	0.21875	0.18750	0.18750	0.25000	0.28125	1.00000				
TN11	0.21875	0.18750	0.18750	0.12500	0.15625	0.18750	0.15625	0.06250	0.18750	0.15625	0.15625	0.12500	0.09375	0.21875	1.00000			
TC191	0.21875	0.34375	0.28125	0.25000	0.21875	0.28125	0.25000	0.34375	0.25000	0.31250	0.31250	0.28125	0.40625	0.25000	0.18750	1.00000		
TY3	0.18750	0.40625	0.28125	0.18750	0.37500	0.37500	0.25000	0.34375	0.34375	0.28125	0.34375	0.31250	0.25000	0.28125	0.21875	0.37500	1.00000	
TD30	0.18750	0.40625	0.25000	0.15625	0.28125	0.40625	0.34375	0.28125	0.37500	0.31250	0.31250	0.21875	0.18750	0.25000	0.21875	0.28125	0.31250	1.00000
HL19	0.21875	0.40625	0.34375	0.21875	0.25000	0.37500	0.34375	0.25000	0.34375	0.21875	0.28125	0.25000	0.28125	0.31250	0.31250	0.43750	0.46875	0.37500
M202	0.21875	0.25000	0.25000	0.21875	0.15625	0.28125	0.25000	0.21875	0.18750	0.21875	0.21875	0.15625	0.18750	0.18750	0.18750	0.28125	0.18750	0.21875
M401	0.15625	0.25000	0.12500	0.12500	0.15625	0.25000	0.18750	0.21875	0.18750	0.15625	0.21875	0.18750	0.18750	0.18750	0.09375	0.15625	0.18750	0.15625
Koshi	0.21875	0.28125	0.31250	0.28125	0.18750	0.34375	0.34375	0.37500	0.25000	0.25000	0.28125	0.21875	0.37500	0.34375	0.12500	0.59375	0.31250	0.31250
Akita	0.15625	0.18750	0.21875	0.18750	0.18750	0.12500	0.18750	0.25000	0.25000	0.18750	0.21875	0.25000	0.31250	0.25000	0.18750	0.37500	0.21875	0.18750
Ibuki	0.25000	0.34375	0.15625	0.28125	0.09375	0.15625	0.15625	0.21875	0.18750	0.21875	0.28125	0.34375	0.34375	0.18750	0.12500	0.43750	0.28125	0.15625
Sun	0.18750	0.15625	0.15625	0.06250	0.15625	0.15625	0.15625	0.18750	0.18750	0.21875	0.09375	0.18750	0.18750	0.12500	0.15625	0.28125	0.12500	0.15625
TS2	0.06250	0.12500	0.03125	0.00000	0.03125	0.09375	0.06250	0.09375	0.06250	0.06250	0.09375	0.06250	0.12500	0.03125	0.00000	0.09375	0.06250	0.06250
TCS10	0.06250	0.18750	0.09375	0.03125	0.06250	0.15625	0.12500	0.09375	0.12500	0.09375	0.09375	0.09375	0.12500	0.09375	0.09375	0.12500	0.09375	0.09375
TN22	0.03125	0.06250	0.09375	0.00000	0.03125	0.06250	0.06250	0.03125	0.09375	0.03125	0.03125	0.03125	0.03125	0.06250	0.03125	0.03125	0.03125	0.00000
TN1	0.06250	0.09375	0.09375	0.03125	0.12500	0.06250	0.06250	0.09375	0.06250	0.06250	0.06250	0.06250	0.06250	0.09375	0.03125	0.06250	0.12500	0.03125
J85	0.06250	0.06250	0.06250	0.06250	0.06250	0.03125	0.03125	0.06250	0.03125	0.03125	0.06250	0.03125	0.03125	0.06250	0.06250	0.06250	0.06250	0.06250
Path	0.06250	0.09375	0.06250	0.03125	0.06250	0.09375	0.03125	0.09375	0.03125	0.09375	0.06250	0.06250	0.06250	0.03125	0.03125	0.09375	0.12500	0.06250
Hom	0.03125	0.06250	0.06250	0.06250	0.06250	0.03125	0.03125	0.06250	0.03125	0.03125	0.03125	0.03125	0.03125	0.03125	0.03125	0.03125	0.06250	0.03125
17050	0.06250	0.09375	0.09375	0.06250	0.06250	0.09375	0.06250	0.06250	0.06250	0.09375	0.06250	0.03125	0.03125	0.06250	0.09375	0.09375	0.09375	0.03125
17032	0.03125	0.09375	0.03125	0.06250	0.03125	0.06250	0.06250	0.03125	0.06250	0.06250	0.06250	0.03125	0.06250	0.06250	0.03125	0.06250	0.03125	0.06250
KDML	0.06250	0.09375	0.06250	0.03125	0.12500	0.12500	0.12500	0.09375	0.15625	0.15625	0.12500	0.06250	0.06250	0.03125	0.00000	0.12500	0.03125	0.12500
SL	0.12500	0.12500	0.09375	0.03125	0.09375	0.12500	0.09375	0.09375	0.12500	0.09375	0.09375	0.09375	0.12500	0.15625	0.09375	0.18750	0.15625	0.12500

Table 3. (Continued)

	HL19	M202	M401	Koshi	Akita	Ibuki	Sun	TS2	TCS10	TN22	TN1	J85	Path	Hom	17050	17032	KDML	SL
HL19	1.00000																	
M202	0.28125	1.00000																
M401	0.15625	0.21875	1.00000															
Koshi	0.46875	0.28125	0.15625	1.00000														
Akita	0.34375	0.18750	0.15625	0.40625	1.00000													
Ibuki	0.31250	0.21875	0.15625	0.46875	0.34375	1.00000												
Sun	0.18750	0.21875	0.12500	0.21875	0.31250	0.12500	1.00000											
TS2	0.09375	0.09375	0.06250	0.09375	0.06250	0.09375	0.03125	1.00000										
TCS10	0.15625	0.12500	0.09375	0.12500	0.09375	0.12500	0.03125	0.56250	1.00000									
TN22	0.06250	0.03125	0.03125	0.03125	0.03125	0.06250	0.03125	0.12500	0.12500	1.00000								
TN1	0.06250	0.06250	0.06250	0.09375	0.09375	0.06250	0.03125	0.28125	0.28125	0.09375	1.00000							
J85	0.03125	0.06250	0.03125	0.06250	0.06250	0.06250	0.00000	0.31250	0.25000	0.12500	0.31250	1.00000						
Path	0.09375	0.06250	0.06250	0.06250	0.03125	0.06250	0.00000	0.34375	0.31250	0.12500	0.25000	0.34375	1.00000					
Hom	0.03125	0.03125	0.06250	0.06250	0.03125	0.03125	0.00000	0.18750	0.18750	0.12500	0.28125	0.28125	0.40625	1.00000				
17050	0.06250	0.09375	0.09375	0.06250	0.06250	0.06250	0.00000	0.15625	0.21875	0.12500	0.18750	0.28125	0.37500	0.31250	1.00000			
17032	0.06250	0.06250	0.09375	0.06250	0.06250	0.06250	0.03125	0.18750	0.21875	0.15625	0.18750	0.21875	0.31250	0.43750	0.31250	1.00000		
KDML	0.06250	0.15625	0.06250	0.09375	0.09375	0.09375	0.06250	0.12500	0.09375	0.06250	0.06250	0.03125	0.03125	0.03125	0.06250	0.06250	1.00000	
SL	0.12500	0.12500	0.09375	0.15625	0.06250	0.09375	0.06250	0.12500	0.12500	0.09375	0.06250	0.09375	0.09375	0.03125	0.06250	0.03125	0.03125	1.00000

0.17. The genetic similarity coefficients among foreign and domestic Taiwan Japonica rice ranged from 0.0625 to 0.59375, with an average of 0.11. For Taiwan domestic Japonica varieties, TK 9 and TK 11 showed the closest similarity index, 0.53125, and TK 12 and TN 11 had little similarity, 0.0625. For foreign rice varieties, the genetic similarity coefficient was the highest (0.46875) for two Japonica rices from Japan, Koshihikari and Koshiibuki. On the other hand, rice cultivars from the U.S., Australia and Japan (M401 and SunWhite; Koshiibuki and SunWhite) showed the least similarity, 0.125. We also calculated the similarity coefficients among all Indica rices, which ranged from 0.03125 to 0.56250. Two domestic Taiwan Indica varieties, TS 2 and TCS 10, shared high similarity, 0.5625. A distant genetic relationship was found in Khao Dowk Mali 105 (KDML, Thailand Jasmine rice) and Pathumithani; Hom mali and Southern Long Grain (SL); Southern Long Grain, 17032 and Hom mali, as shown with similarity coefficient 0.03125.

Cluster and principle component analysis among rice varieties

The genetic diversity data were used to group various rice varieties into different clusters and to construct a dendrogram using of NTSYS software UPGMA method. The genotypes which derived from genetically similar ancestors were clustered together as shown in the dendrogram. The 36 rice cultivars were divided into two major groups (Figure 2). In the first group, there are 19 Japonica rice varieties from Taiwan, three from Japan (Koshihikari (Koshi), Koshiibuki (Ibuki), Akitakomachi (Aki)), one from Australia (SunWhite) and two medium rice grains, M 202 and M 401, from U.S. All other Indica cultivars, except for KDML, form a distinct cluster from Japonica as cluster II. A principle component analysis (PCA) was also performed with the 36 rice varieties, which is listed in Table 1. With this statistical method, a large set of variables are replaced by a smaller number of orthogonal variables (called principle components (PC)). Typically, these relationships are represented as a linear function to account for the variance in the whole dataset. The contribution of the first and second principle component (PC1 and PC2) to the multivariate variation was 17.61% and 6.11%, respectively, and the cumulative contribution was 23.72%. The first and second components provided the correlation between various genotypes of rice cultivars and the corresponding geographical origins. Most Taiwan Japonica varieties formed a separate group apart from the rest of U.S. and Japan Japonica, except for Ibuki. Meanwhile, except for KDML and SL, Taiwan and Thailand Japonica rice varieties were easily distinguishable as belonging to separate groups.

Work flow of domestic Taiwan rice authentication

Market-based incentives are one way to encourage Taiwanese consumers to consume local rice and maintain the integrity of the Taiwanese rice industry. Thus, to pre-

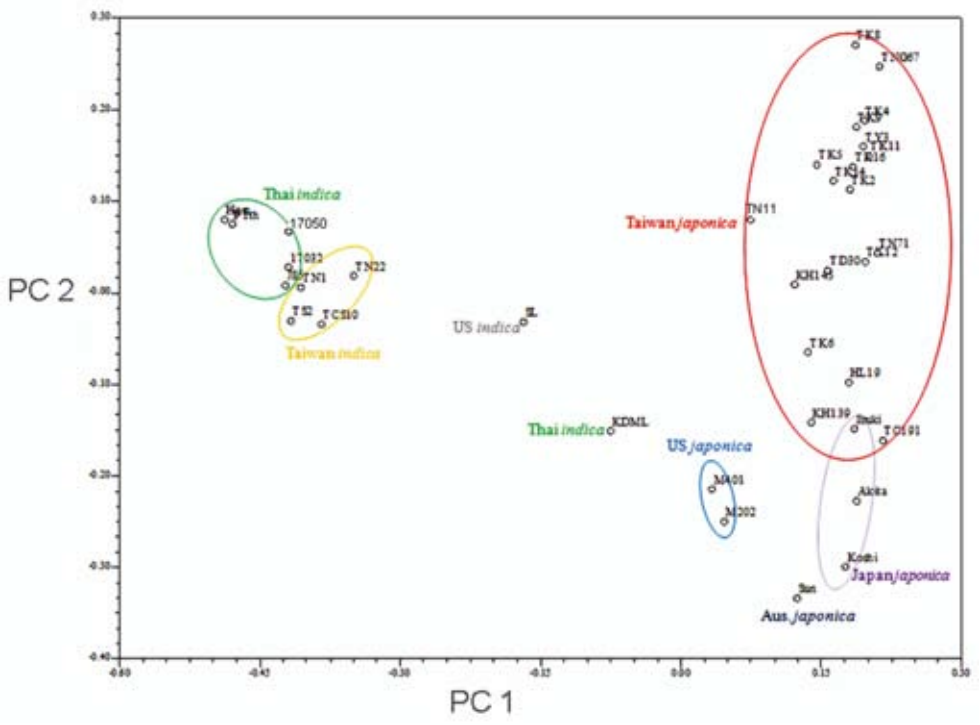

Figure 3. Principle component analysis of 36 rice cultivars using genetic diversity data of 306 alleles at 32 SSR loci. The contribution of PC1 and PC2 was 17.61% and 6.11%, respectively. The cumulative contribution was 23.72%.

vent the adulteration of rice grains and to distinguish local Taiwan rice from other foreign varieties, we developed a workflow of Taiwan rice authentication using SSR DNA fingerprinting analysis (Figure 4). Based on the allelic diversity of SSR fingerprinting, 8 core SSR markers, including RM21, RM22, RM101, RM333, RM475, RM1387, RM5704, and RM7545, were chosen to separate 16 rice accessions in sequential order (Figure 4). The RM1387 was the most efficient core SSR primer to easily distinguish Taiwan rice varieties from foreign rice, except for Taichung 191. The SSR marker RM475, can be further applied to separate foreign rice groups into three subgroups: Koshihikari and Taichung 191, M 401, and SunWhite. Finally, with marker RM101, the identity of TC 191 can be confirmed. Other domestic rice accessions can be distinguished from each other using the different SSR sets shown in Figure 4. Because of the close genetic relationship among local Taiwan Japonica rice varieties during the breeding and selection process, more numbers of polymorphic markers were required to characterize and separate them efficiently. It would be interesting to develop a multiplex SSR genotyping platform based on current SSR markers for economic, cost-effective and sensitive local Taiwan rice variety identification in the near future.

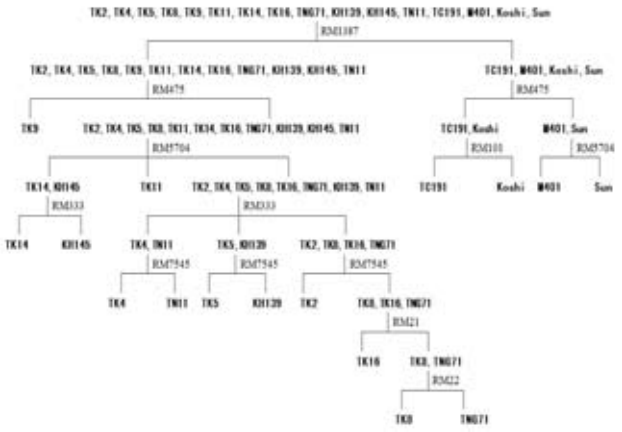

Figure 4. Workflow of domestic Taiwan rice variety authentification by DNA fingerprinting profile analysis based on SSR makers.

Quantification of adulteration with different admixtures of TK9 and TK16

It is possible that the premium Taiwan rice variety in the market is inadvertently mixed with other rice samples in the field, in storage, or even worse, intentionally adulterated with low-priced foreign rice for profit. Thus, in attempting to develop an efficient method for characterizing the identity of a domestic Taiwan rice cultivar for commercial purpose, we worked out a standard workflow of microsatellite markers (Figure 4) and microsatellite allele profiles of the corresponding rice varieties (Data not shown). To validate the sensitivity of our methodology, we first extracted pure DNA from TK 9 and TK16 rice grains and prepared different ratios of standard sample by mixing DNAs from TK9 and TK16. As shown in Figure 5, as low as 4% to 7.5% of impurities admixture in which TK9 or TK16 could be detected with microsatellite marker RM475 by QIAxcel multicapillary electrophoresis gel system. It would be interesting to explore further developments using multiplex SSR genotyping technology by increasing the resolution for adulteration detection.

DISCUSSION

In the past, elite rice varieties from Japan have been introduced to continuously improve the taste quality, increase the genetic diversity of and improve the disease resistance or other agronomical traits of local Taiwan rice varieties. Many studies of pedigree analysis revealed that Japan and Taiwan varieties share high genetic similarities and might be developed from common ancestors (Chen et al., 2008; Lin, 1991; Wu and Lin, 2008). How to keep in-

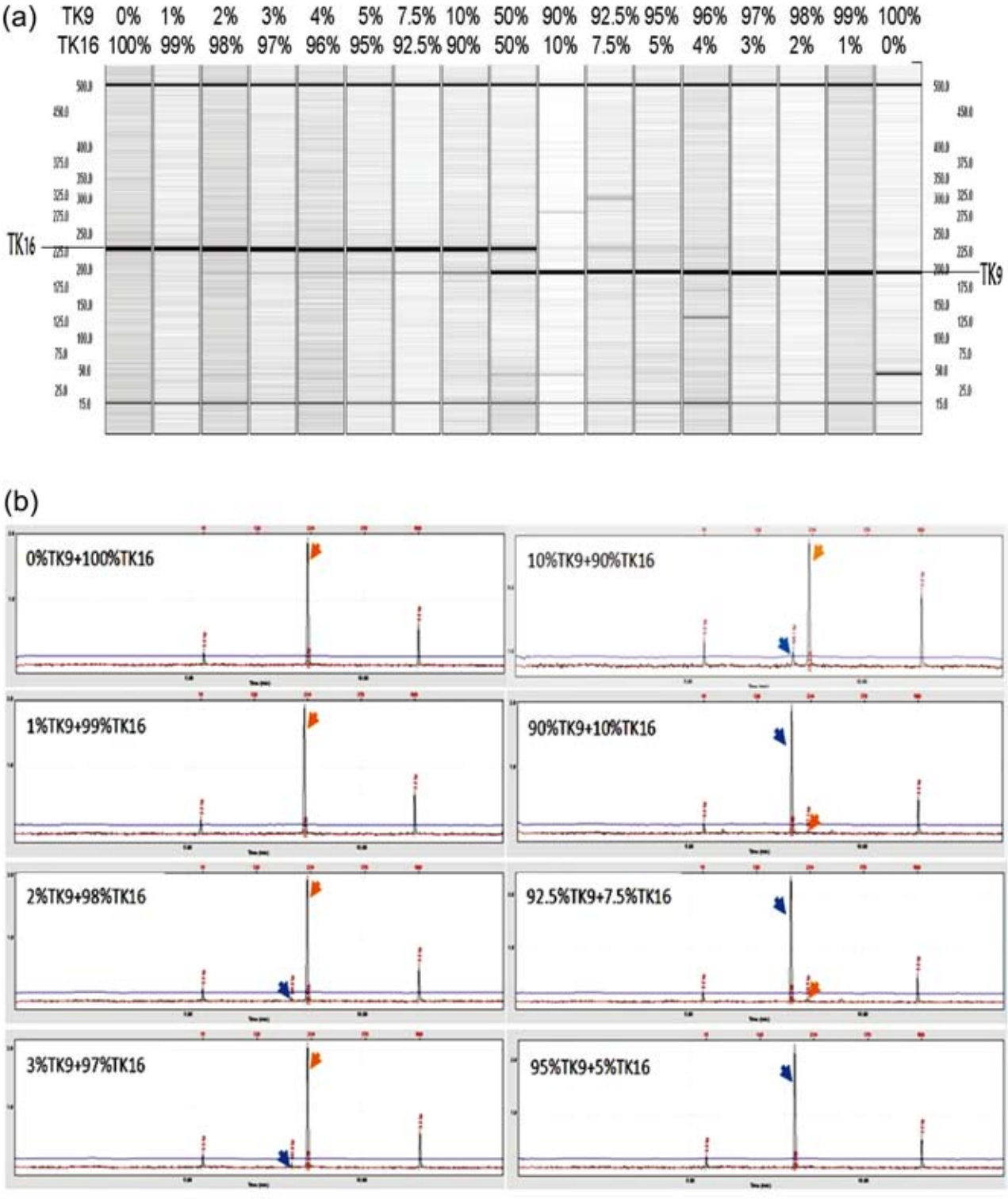

Figure 5. Adulteration quantification using RM475 with QIAxcel capillary electrophoresis system (a) gel image (b) electropherogram. The admixture of TK16/TK9 rice is based on the corresponding DNA in a final concentration of 10 ng. The corresponding peaks from the amplified products of TK9 are shown as blue arrows, while those from TK16 are shown as orange arrows.

creasing the genetic diversity of local Taiwan rice varieties to adapt to future climate change and to preserve the integrity of high quality rice varieties in the market remains a big challenge for breeders. Assessing genetic similarity by SSR fingerprinting among Taiwan and foreign rice varieties is thus not only important for facilitating parent selection in hybridization breeding programs but also for authentication and traceability in the domestic rice market. Although pedigree analysis can provide basic information about the genetic relatedness among rice varieties, a precise and complete pedigree data set related to the hybridization process and ancestors used is needed. Owing to self-crossing and selection, genetic drift and random crossover may also cause ambiguities in interpreting the genetic similarity between rice varieties. The recent deciphering of the rice genome and the development of various DNA markers provides new opportunity for polymorphism discovery, identification and high-resolution rice genetic linkage mapping. Theoretically, with enough specific and polymorphic DNA markers, the differences in genetic compositions between any two rice varieties should not require a sequenced genome. In this study, we preliminarily screened out 32 highly polymorphic primer pairs from 120 microsatellite markers and applied genetic similarity analysis to 36 rice varieties from different countries. These markers were evenly distributed across all 12 rice chromosomes. With SSR-derived data, we were able to determine the level of genetic similarity between domestic Taiwan and foreign rice varieties. Some of these shared the same similarity coefficients (Table 3) suggests that more markers may still be needed for further discrimination and characterization of various rice genotypes.

In this study, a UPGMA dendrogram-based clustering analysis was used to access the genetic relationship of 36 rice varieties from different countries. In group I, Taiwan Japonica rice cultivars were closely linked genetically. Twelve out of sixteen (75%) rice varieties shared "TNG67" as one of the parents in their pedigree: TK2 (25%), TK5 (12.5%), TK8 (37.5%), TK11 (6.25%), TK14 (28.125%), TK16 (37.5%), TNG71 (12.5%), TN11 (18.75%), TY3 (12.5%), HL19 (18.75%) and TD30 (12.5%). Eight out of sixteen (50%) were inherited from the parent of KH139: TK4 (12.5%), TK5 (12.5%), TK6 (12.5%), TK11 (37.5%), TK12 (3.125%), KH145 (50%), TY3 (6.25%) and TD30 (6.25%). Values in parentheses indicate the relative genetic contribution of TNG 67 as a breeding parent (Lu C.T. and H. Y. Lu, 2010). We also noticed that five rice accessions (5/7 = 71.4%) including KH139, TC191, Koshi, Ibuki, Akita, KH145 were pedigree-related to Koshihikari. The Japonica rice from Japan, such as Koshihikari and Kinuhikari had been selected as the parental line for breeding TC191 (56.25%) and TNG 71 (50%); KH145 (50%), respectively. To our understanding, Koshiibuki (Ibuki) and Akitakomachi (Akita) were all pedigrees related to Koshihikari. In addition, we found that TK 9 shares the highest genetic similarity with TNG67, and this may ascribe to their mutual inheritance from Koshihikari. From our SSR data, two Indica rice varieties, KDML in group I and Southern Long Grain Rice (SL) in group II, tended to be more genetically related to Japonica (Figure 2) and were not easily classified and separated into different groups using PCA (Figure 3). This could be due to the number of SSR markers used in the study or the bias of genetic similarity estimation conducted by the UPGMA-based method. KDML is a popular non-basmatic aromatic landrace rice variety developed from a high-yield potential (HYP) program and consumed in the Thai rice market. This Jasmine rice cultivar is well known for its unique appearance, cooking quality and aroma. Southern Long Grain Rice (SL) represents the most common brand of table rice consumed in the U.S. Several varieties of long grain Indica-type rice are grown in different U.S. states, including Arkansas, Mississippi, Missouri, Louisiana, and Texas. Some varieties are consumed with special processing, such as parboiling. Since there is no pedigree data available regarding their hybridization or breeding processes, it is difficult to deduce the putative genetic relationships they have with the current Japonica rice varieties.

Finally, though the number of foreign rice genotypes analyzed in our study was low, the structure of clusters illustrated by PCA clearly indicated that the grouping trends of most rice varieties are geographical-related; the Taiwan rice cultivars in particular. Shu et al. (2009) analyzed 313 Japonica varieties from 20 countries using 34 SSR primers and their results also showed a clear genetic similarity among various Japonica rice varieties that were closely linked to their geographical distribution. Consequently, microsatellite allele profiles can be a convenient, efficient and economic technique for measuring the genetic diversity and the differentiation of rice cultivars having different origins.

CONCLUSION

The competiveness of the domestic rice market or of the international rice trade demands the identification of rice varieties in a milled sample to maintain purity and quality standards. For this purpose, many DNA fingerprinting methods previously applied in rice variety identification and authentication used milled grains. In this study, we took advantage of the high resolution DNA banding pattern of the QIAxcel capillary gel electrophoresis system to measure the genetic diversity of 36 rice verities using 32 SSR markers. Taken together, the microsatellite markers identified in this study can be used for efficient genetic diversity analysis to separate Taiwan rice cultivars from other foreign rice. Moreover, according to the unique profiles of rice genotypes, we selected a SSR marker and tried to establish a workflow for domestic Taiwan rice variety authentication. The results from this study can be extended and further applied to tracing and monitoring adulteration in rice agric-products and agric-food markets. Capillary electrophoresis (CE)-based microsatellite DNA fingerprinting technology holds a promise for highly efficient, sensitive and economic adulterants quantification and can

benefit both farmers' and consumers' communities by preserving the integrity of premium rice varieties in Taiwan.

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利用微衛星 DNA 標誌鑑別台灣市售米常見品種

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爲追溯、偵測並防止混米之目的，本研究利用微衛星標誌 (SSR) 來鑑別台灣常見市售國產米及國外進口食米品種。共使用 36 個國內外食米品種及 32 組微衛星標誌進行水稻多型性之 DNA 指紋分析。結果於 32 個基因座上共得到 306 個不同之等位基因。每個基因座可有 3 至 21 個片段，平均每個基因座爲 9.6 個片段。多態性訊息含量 Polymorphic information value (PIC), 介於 0.205 (RM7023) 至 0.926 (RM333)，平均爲 0.75。其中 RM101, RM333, RM475, RM1387, RM1388, RM2835, RM5428 及 RM5704 微衛星標誌可有效區別台灣國產米。經由主座標 principle component analysis (PCA) 及集群分析 (clustering analysis)，發現來自不同國家之食米品種可分爲 2 群。最後爲防範混米，本研究也建立了台灣良質米品種鑑定及純度檢測流程表。在實際檢測應用上，以不同比例之台粳九號及台粳十六號進行混米檢測，其檢出之靈敏度約爲 3% - 7.5%。

關鍵詞：DNA 指紋分析；微衛星標誌；多型性；水稻；簡單重覆序列。