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Identification and characterization of common walnut using sequence-related amplified polymorphisms (SRAP) markers

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ABSTRACT

Common walnut is one of the oldest cultivated and economically important nut trees. This study was aimed to analyze 40 walnut germplasms in Xinjiang, China using 12 nut phenotypic traits and 18 sequence-related amplified polymorphism (SRAP) markers. Samples included 22 native cultivars and 18 naturally seeded trees. Statistical results of the phenotypic traits were consistent with those in previous studies regarding walnut germplasm diversity. The SRAP marker system generated 178 alleles, of which 134 were polymorphic. SRAPs could effectively distinguish the genotypes tested and explain the phylogenetic relationships among the cultivars to a certain extent. The genetic similarity levels of nut phenotypes and SRAP markers among the walnut genotypes varied within the range of 54%–95% and 62%–88%, respectively, based on the Jaccard similarity coefficient. Four nut-shape traits that are easy to evaluate (namely the shape of the longitudinal section perpendicular to the suture, the prominence of the apical tip, the shape of the apex perpendicular to the suture, and the shape of the base perpendicular to the suture) and 18 SRAP makers with a high distinguishing power were used to fingerprint the 40 walnut germplasms. The fingerprints could distinguish the tested genotypes. Two-dimensional graphical DNA barcodes were generated for rapid and accurate identification of the genotypes to protect intellectual property and assist in breeding studies.

Key words: *Juglans regia*, nut phenotype, two-dimensional graphical barcode, marker-assisted breeding.

INTRODUCTION

Common walnut (*Juglans regia* L.) is one of the oldest and most extensively cultivated economically important nut trees worldwide. The nut has a high oil and protein content and is rich in minerals, vitamins, and antioxidants that benefit human health and may decrease the risk of heart disease and certain cancers (Yerlikaya *et al*., 15). Accordingly, the Food and Agriculture Organization (FAO) considers walnut to be a primary fruit. The global annual walnut production is 3,747,549 tons, of which 2,530,896 tons are produced in Asia. Indeed, China is one of the largest walnut producers, with an annual output of 1,785,879 tons according to the FAO. In particular, Xinjiang in northwestern China has a long history of walnut cultivation and produces various walnuts of high quality, particularly in the Aksu prefecture. Importantly, a large population of non-grafted walnut trees exists in Xinjiang, which provides a rich resource base and ample opportunities to breed elite lines.

The primary breeding methods for walnut include seed selection and hybrid breeding, with germplasm identification as an important link. Genetic markers are important tools for germplasm identification and characterization. Walnut breeders use morphological, biochemical, and molecular approaches to identify walnut germplasms. Phenotypic markers are traditionally used to distinguish genotypes and are the most common tools used for germplasm characterization. In recent years, the application of phenotypic markers for walnut germplasm resource identification has been limited; although the method is simple and rapid, its ability to identify germplasms is limited (Lázaro *et al*., 7). Molecular markers are extensively used because they can accurately identify germplasms and are unaffected by environmental factors, unlike phenotypic markers. Various researchers have proposed a strategy of combining phenotypic markers with molecular markers for germplasm identification, and fingerprints of several plant species have been successfully constructed (Lázaro *et al*., 7)However, to date, no study has used a combination of phenotypic markers and sequence-related amplified polymorphism (SRAP) markers to identify walnut germplasms. Here, we used an integrated approach to identify genotypes using both morphological characterization and sets of SRAP data and construct a unique fingerprint for 40 tested genotypes. These results can provide a resource for the rapid and effective identification of

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walnut cultivars and naturally seeded individuals with excellent agronomic traits in Xinjiang.

MATERIALS AND METHODS

In total, 40 walnut germplasms were collected from different counties and cities around the Aksu prefecture in Xinjiang based on a comprehensive survey of native cultivars, including 22 native cultivars and 18 naturally seeded individuals (Table 1). To establish distinctness among germplasms, each genotype was measured and described for each of 12 traits (Table 2), according to the International Union for the Protection of New Varieties of Plants (UPOV) descriptors at each harvest season in 2017 and 2018: to assess distinctness and stability, ten nuts of each genotype were observed each year.

Genomic DNA was extracted from young leaves using a plant genomic DNA kit (Thermo Fisher Scientific, Wilmington, DE, USA), according to the manufacturer's instructions. DNA was quality-checked and quantified using a protein/nucleic acid analyzer (Thermo Fisher Scientific, Wilmington, DE, USA) and agarose gel electrophoresis. A set of 18 SRAP markers was selected based on their degree of polymorphisms, reproducibility using polymerase chain reaction (PCR) amplification, and ease of interpretation among 100 SRAP markers developed to fingerprint the 40 sample germplasms. Primer sequences are listed in Table 3. Markers were amplified using a 96-well Veriti Applied Biosystems thermocycler (Applied Biosystems) in a reaction mixture comprising 0.4 µL of dNTP (2.5 mM) and 0.5 of U Taq DNA polymerase (TransGen Biotech, Beijing, China). The PCR heat cycling protocol involved an initial denaturation step at 94°C for 5 min; followed by four cycles at 94°C for 1 min, 33°C for 1 min, and 72°C for 1 min; 39 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 10 min. PCR products were separated on 8% nondenaturing polyacrylamide gels (acrylamide: methylene bis-acrylamide 29:1) that were run in 1×Tris/borate/EDTA buffer at 200 V for 1 h, and visualized by silver staining (Bassam *et al*., 2). The appropriate SRAP markers were identified based on the size of the PCR product relative to a 100-bp DNA ladder.

Fingerprinting to identify the walnut germplasms included a combination of nut phenotypes and SRAP markers. In the fingerprint code of each germplasm, the phenotypes are represented by the nut-shape types (e.g., the prominence of the apical tip [PAT]-1), whereas the SRAP markers were digitally coded as "0" or "1" based on its primers. The fingerprinting string code obtained for each tested germplasm was transposed onto a two-dimensional barcode graph using Caoliao Two-Dimensional Code Management (https://cli.im).

In the current study, we only considered strong and reproducible polymorphic fragments that were clearly distinguishable among all tested accessions. Fragments were recorded as 1 representing the presence of a fragment, 0 representing the absence of a fragment, and 9 representing missing data. The discrimination power (DP) of each primer was calculated as follows: $DP = 1 - \Sigma(P_i)^2$, where P_i represents the frequency of each genotype (Smith *et al*., 12). Morphological and SRAP-derived molecular data were coded as binary variables in a matrix (1 = present, 0 = absent), and the Jaccard similarity coefficients for the two sets of markers were calculated separately. An unweighted pair-group method with arithmetic mean (UPGMA) phenogram was generated from both similarity matrices using DARwin version 6.0 (Perrier and Jacquemoud-Collet, 9). A bootstrapping value of 1,000 replicates was used.

RESULTS AND DISCUSSION

Morphological variability is a key factor associated with plant breeding and selection. However, the environment modulates the expression of these traits in most cases. Therefore, several researchers consider that the inclusion of morphological traits can influence the results of genetic analyses, including the degree of genetic variation(Adjebeng-Danquah *et al*., 1). Highly penetrant characteristics such as kernel and nut traits are considered to be efficient markers for differentiating genotypes (Fatahi *et al*., 4). Therefore, recording and analyzing walnut phenotypic data is can be a useful reference for researchers and contribute to the identification, breeding, selection, and introduction of new cultivars (Chen *et al*., 3)

Differences in nut morphological traits were observed among the tested germplasms. Table 4 provides a summary of the metrics (mean, standard deviation [SD], range, and percentage coefficient of variation, CV%) for each trait. Nut width and diameter showed the lowest variation (CV% values of 7.28 and 9.84, respectively), whereas nut weight and shell thickness had the highest variation (CV% values of 22.56 and 20.09, respectively). The descriptive statistics for these phenotypic traits were within the ranges reported in previous studies regarding the diversity of walnut germplasm resources (Rezaei *et al*., 10; Hussain *et al*., 5). The CV% of the quantitative traits indicates a moderate degree of variation among the genotypes under investigation.

In forest economics, nut shape is important for germplasm identification and is a stable and heritable trait (Krška *et al.,* 6). The descriptive

statistics of nut shapes are presented in Table 5. The most common shape in cross-section (SCS) was oblate (SCS-2), accounting for 80.0% of individuals. The shape of the longitudinal section perpendicular to the suture (SLSPS), PAT, shape of the apex perpendicular to the suture (SAPS), and shape of the

Table 1. Summary of the 40 walnut germplasms analyzed.

NO.	Genotypes	Type	Origin
JR01	Xinfeng	Cultivar	Selected from progenies of Xinjiang walnuts
JR02	Xinguang	Cultivar	Selected from progenies of Xinjiang walnuts
JR03	Louren	Cultivar	Selected from progenies of Xinjiang walnuts
JR04	Xinpai04	Cultivar	Selected from progenies of Xinjiang walnuts
JR05	Wen185	Cultivar	Selected from progenies of Xinjiang walnuts
JR06	Wuhuo03	Cultivar	Selected from progenies of Xinjiang walnuts
JR07	Kuxi01	Cultivar	Selected from progenies of Xinjiang walnuts
JR08	Alin11	Cultivar	Selected from progenies of Xinjiang walnuts
JR09	Ashi04	Cultivar	Selected from progenies of Xinjiang walnuts
JR10	Kashi11	Cultivar	Selected from progenies of Xinjiang walnuts
JR11	Hechun06	Cultivar	Selected from progenies of Xinjiang walnuts
JR12	Ashi14	Cultivar	Selected from progenies of Xinjiang walnuts
JR13	Ashi01	Cultivar	Selected from progenies of Xinjiang walnuts
JR14	Wushi91	Ungrafted individual	Selected from walnut population in Xinjiang
JR15	Wushi90	Ungrafted individual	Selected from walnut population in Xinjiang
JR16	Wushi86	Ungrafted individual	Selected from walnut population in Xinjiang
JR17	Wushi89	Ungrafted individual	Selected from walnut population in Xinjiang
JR18	Wushi85	Ungrafted individual	Selected from walnut population in Xinjiang
JR19	Wushi83	Ungrafted individual	Selected from walnut population in Xinjiang
JR20	Wushi88	Ungrafted individual	Selected from walnut population in Xinjiang
JR21	Wushi87	Ungrafted individual	Selected from walnut population in Xinjiang
JR22	Wushi81	Ungrafted individual	Selected from walnut population in Xinjiang
JR23	Wushi76	Ungrafted individual	Selected from walnut population in Xinjiang
JR24	Wushi79	Ungrafted individual	Selected from walnut population in Xinjiang
JR25	Wushi78	Ungrafted individual	Selected from walnut population in Xinjiang
JR26	Wushi72	Ungrafted individual	Selected from walnut population in Xinjiang
JR27	Wushi75	Ungrafted individual	Selected from walnut population in Xinjiang
JR28	Wushi74	Ungrafted individual	Selected from walnut population in Xinjiang
JR29	Wushi73	Ungrafted individual	Selected from walnut population in Xinjiang
JR30	Wushi77	Ungrafted individual	Selected from walnut population in Xinjiang
JR31	Wushi71	Ungrafted individual	Selected from walnut population in Xinjiang
JR32	Xinjufeng	Cultivar	Selected from progenies of Xinjiang walnuts
JR33	K-8	Ungrafted individual	Selected from walnut population in Xinjiang
JR34	Xincuifeng	Cultivar	Selected from progenies of Xinjiang walnuts
JR35	Heyue05	Cultivar	Selected from progenies of Xinjiang walnuts
JR36	Ashi05	Cultivar	Selected from progenies of Xinjiang walnuts
JR37	Heyue10	Cultivar	Selected from progenies of Xinjiang walnuts
JR38	Wen179	Cultivar	Selected from progenies of Xinjiang walnuts
JR39	K-10	Ungrafted individual	Selected from walnut population in Xinjiang
JR40	Hechun05	Cultivar	Selected from progenies of Xinjiang walnuts

Table 2. The characterization and taxonomic values of morphological traits of dragon walnut nut.

Code	Traits	Characterization
1	Shape in longitudinal section through suture (SLSTS)	1 circular; 2 triangular; 3 ovoid; 4 elliptic; 5 trapezoid
2	Shape in longitudinal section perpendicular to suture (SLSPS)	1 circular; 2 oblate; 3 ovoid; 4 elliptic; 5 oblong; 6 trapezoid
3	Shape in cross section (SCS)	1 circular; 2 oblate; 3 elliptic; 4 rectangle
4	Prominence of apical tip (PAT)	1 concave; 2 flat; 3 convex; 4 sharp
5	Shape of apex perpendicular to suture (SAPS)	1 concave; 2 flat; 3 convex; 4 sharp
6	Shape of base perpendicular to suture (SBPS)	1 concave; 2 flat; 3 convex; 4 sharp
7	Nut length (NL)	Average of 10 Nut length
8	Nut width (NWi)	Average of 10 Nut width
9	Nut thickness (NT)	Average of 10 Nut thickness
10	Nut weight (NW)	Average of 10 Nut weight
11	Shell thickness (ST)	Average of 10 Shell thickness
12	Kernel percentage (KP)	Average of 10 Kernel percentage

The table shows descriptions for each of the nut morphological traits assessed in this paper in the form trait-characteristic. For example, SLSPS-1 translates to longitudinal section perpendicular to suture with circular shape and PAT-4 represents sharp prominence of apical tip.

Table 3. Description of the SRAP primers used for genetic diversity analyses of 40 walnut germplasms.

Primer name	Forward primer (5′–3′)	Primer name	Reverse primer (5′–3′)
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAC
Me2	TGAGTCCAAACCGGAGC	Em3	GACTGCGTACGAATTGAC
Me3	TGAGTCCAAACCGGAGG	Em4	GACTGCGTACGAATTGCA
Me4	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTTGA
Me6	TGAGTCCAAACCGGAAT	Em6	GACTGCGTACGAATTCAA
Me7	TGAGTCCAAACCGGACC	Em8	GACTGCGTACGAATTCAG
Me8	TGAGTCCAAACCGGTAG	Em10	GACTGCGTACGAATTGCT
Me9	TGAGTCCAAACCGGACA	Em11	GACTGCGTACGAATTCCA
Me13	TGAGTCCAAACCGGACG	Em13	GACTGCGTACGAATTTCA
Me14	TGAGTCCAAACCGGTCA	Em14	GACTGCGTACGAATTTAG

Table 4. The descriptive statistics for nut and kernel traits in 40 walnut germplasms from Xinjiang.

Trait (units)	Min.	Max.	Mean	SD	CV (%)
Nut length (mm)	34.06	56.61	41.67	5.23	12.55
Nut width (mm)	29.47	43.93	35.52	3.49	9.84
Nut thickness (mm)	29.15	39.03	34.11	2.48	7.28
Nut weight (g)	9.33	22.37	15.43	3.48	22.56
Shell thickness (mm)	0.89	2.69	1.67	0.37	22.09
Kernel percentage (%)	36.61	64.44	50.08	6.30	12.58

base perpendicular to the suture (SBPS) are more commonly observed and are easier to evaluate than other index types.

Sequence-related amplified polymorphisms (SRAPs) have been extensively used and have proven to be effective for genetic diversity analysis, species identification, variety identification, genotyping, and gene cloning in different plant species, such as *Brassica oleracea*, *Paeonia* spp., *Hibiscus cannabinus*, *Coffea arabica* and *Petunia* × *hybrida* (Uzun *et al*., 13). In the current study, the 18 selected primers generated 100 distinguishable and reproducible amplified fragments, resulting in 178 bands. Each primer generated between four (M7/E5)

Table 5. The description and frequency (%) of nut morphological traits for walnut.

Nut shape traits	1	2	3	4	5	6
Shape in longitudinal section through suture (SLSTS)	35	0	0	30	35	/
Shape in longitudinal section perpendicular to suture (SLSPS)	32.5	17.5	12.5	30	5	2.5
Shape in cross section (SCS)	17.5	80	0	2.5	/	/
Prominence of apical tip (PAT)	10	67.5	17.5	10		
Shape of apex perpendicular to suture (SAPS)	32.5	42.5	15	32.5		
Shape of base perpendicular to suture (SBPS)	25	47.5	22.5	25		

/ = not detected

and 23 distinct bands (M4/E13), with an average of 9.72 bands per primer (Table 6). The scored bands ranged from 100 bp to 1500 bp in length, whereas the percentage of polymorphic bands (PPB) varied from 50% (M7/E5) to 100% (M3/E4) with an average of 75.53%, with 134 polymorphic bands in total (Table 6). DP among the tested genotypes varied from 0.19 (M2/E4) to 0.47 (M3/E10; Table 6). Following our application of SRAP markers to different walnut genotypes in Xinjiang, the average PPB obtained using a single primer combination was 75.53%. This indicates that the polymorphism observed using SRAP markers is relatively higher than that observed using other markers, such as randomly amplified polymorphic DNA (RAPD) or inter simple sequence repeats that have been used to detect genetic diversity in walnut germplasm (Salieh *et al*., 11).

Table 6. List of SRAP primers, number of polymorphic bands, percentage of polymorphic bands, and discrimination power (DP).

Primer	number of polymorphic bands	percentage of polymorphic bands	DP
M4/E4	7	70.00	0.20
M3/E10	6	100.00	0.47
M7/E5	2	50.00	0.22
M3/E13	6	85.71	0.30
M14/E4	8	66.67	0.21
M2/E11	5	83.33	0.21
M3/E4	9	100.00	0.28
M2/E4	3	50.00	0.19
M12/E13	5	71.43	0.24
M7/E1	5	62.50	0.23
M7/E3	12	63.16	0.25
M13/E4	10	83.33	0.29
M14/E13	8	72.73	0.31
M1/M3	8	80.00	0.38
M2/M5	7	77.78	0.28
M4/E13	17	73.91	0.32
M3/E11	6	85.71	0.30
M3/E14	10	83.33	0.29
Total	134		
Mean	7.44	75.53	0.28
Standard Error	3.45	14.09	0.07

A genetic similarity matrix among all the tested walnut germplasms was generated from amplified fragments of SRAP markers and nut morphological traits using Jaccard similarity coefficients separately. Based on the results of the nut morphological trait analysis, the genetic similarity between walnut germplasms was observed to range from 0.54 to 0.95. Figure 1A shows an UPGMA dendrogram that presents the genetic relationships based on nut morphological traits. Based on SRAP markers, the genetic similarity among the genotypes were observed to range from 0.49 to 0.87. The UPGMA dendrogram summarizing the genetic relationships using the SRAP markers among the 40 walnut germplasms is presented in Figure 1B, and all genotypes were distinguishable from one another. Our results are consistent with those of previous studies (Uzun *et al*., 13), indicating that the SRAP method is effective for identifying diversity relationships among walnut germplasms.

The findings from morphology-based clustering of accessions were inconsistent with those derived from the SRAP-based analysis of DNA polymorphisms, showing that the two marker methods differed in their ability to detect differences in genetic diversity. Our findings were consistent with those of previous studies (Liu *et al*., 8). Nut morphological traits may be more susceptible to environmental factors than molecular markers, thus explaining the small proportion of genetic variation captured by morphological traits. In contrast, SRAP analysis provides extensive coverage of the genome and is unaffected by the environment. On the other hand, there was no obvious clustering between cultivars and natural seeding individuals, but clusters generally occurred in a mixed fashion.

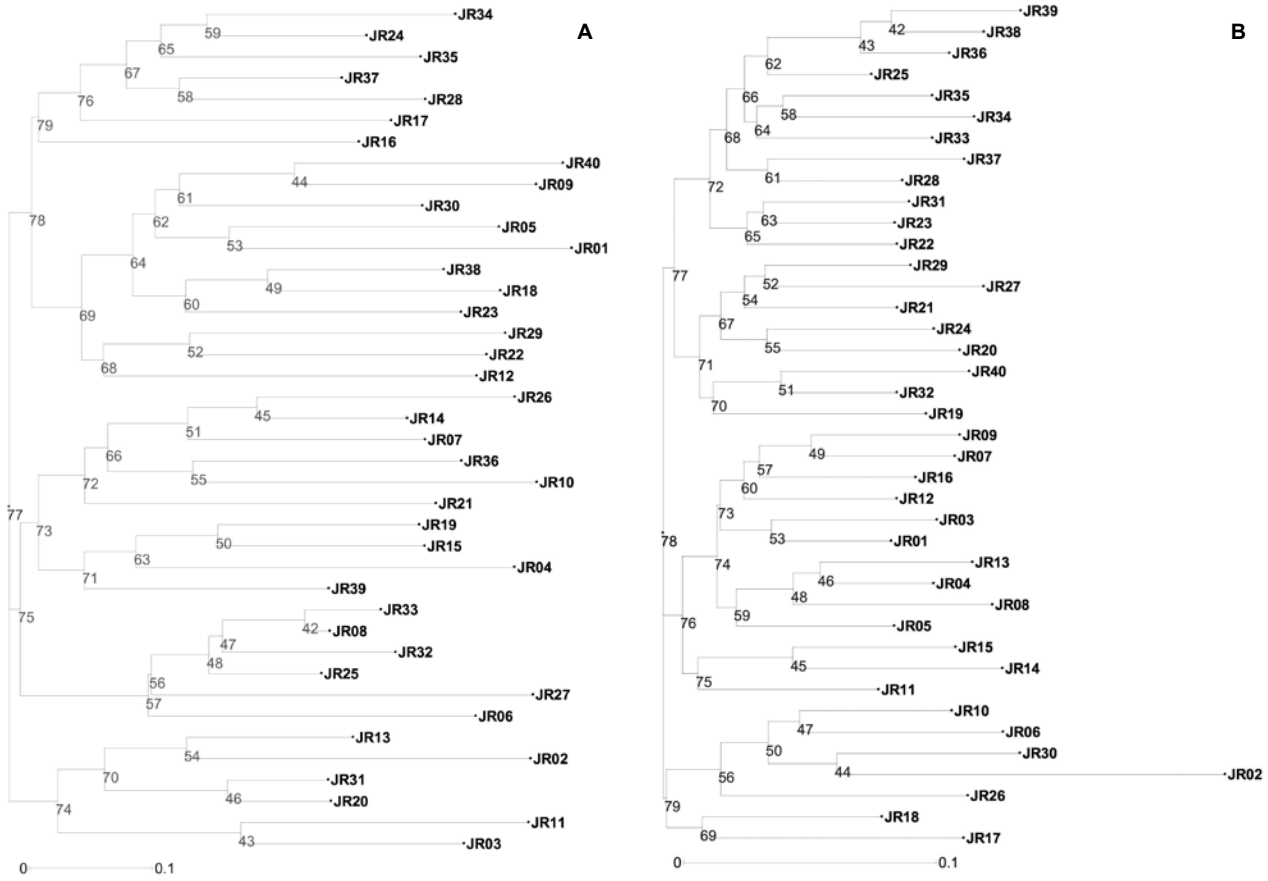

Fig. 1. Dendrograms showing the clustering patterns of 40 walnut accessions from Xinjiang, China, based on nut morphological traits (A) and SRAP makers (B), using the Jaccard similarity coefficient. The green and red numbers represent bootstrap confidence limits for 1000 replicates.

The accessions may correspond to closely-related individuals; therefore, the germplasm resources may be located geographically close and were not affected by geographical isolation and adaptation.

The genetic diversity of walnut has been the focus of various studies. Other studies have been conducted using different morphological and molecular markers such as simple sequence repeats (SSR), RAPDs, and amplified fragment length polymorphisms to assess the genetic diversity and relationships among walnuts. These studies support the following points by comparing morphological traits and molecular markers as indicators of genetic diversity. First, because of their considerable variability, morphological traits cannot accurately estimate the genetic relationship between walnut accessions. Second, germplasm selection and breeding in horticulture is based on the identification and classification of plant genotypes. Third, identification and classification methods have progressed with developments in science and technology; however, a single optimal and standard method has not yet been established.

In the current study, to establish a rapid and simple method of germplasm characterization, the tested genotypes were differentiated using a combination of four nut-shape traits and data regarding SRAP primer pairs (Figure 2). This strategy included traits, such as SLSPS, PAT, SAPS, and SBPS, and SRAP primer pairs M3/E10, M1/E3, and M4/E13 (all primers with high DP values). Other studies have been conducted using 12 SSR markers and 22 nut-shape traits to distinguish and identify 35 cultivars of walnut grown in China. Similar methods have been employed in other plant species(Chen *et al*., 3).

To collate the genetic data for each walnut genotype, fingerprints were transformed into a graphical two-dimensional barcode containing the nut-shape traits, primer name, and the resulting DNA fingerprint. These codes (Figure 3) are readable by electronic devices such as smartphones. The barcode has multiple applications as a display label: it can be used for field display and in breeding to identify high-quality genotypes, or it can be used

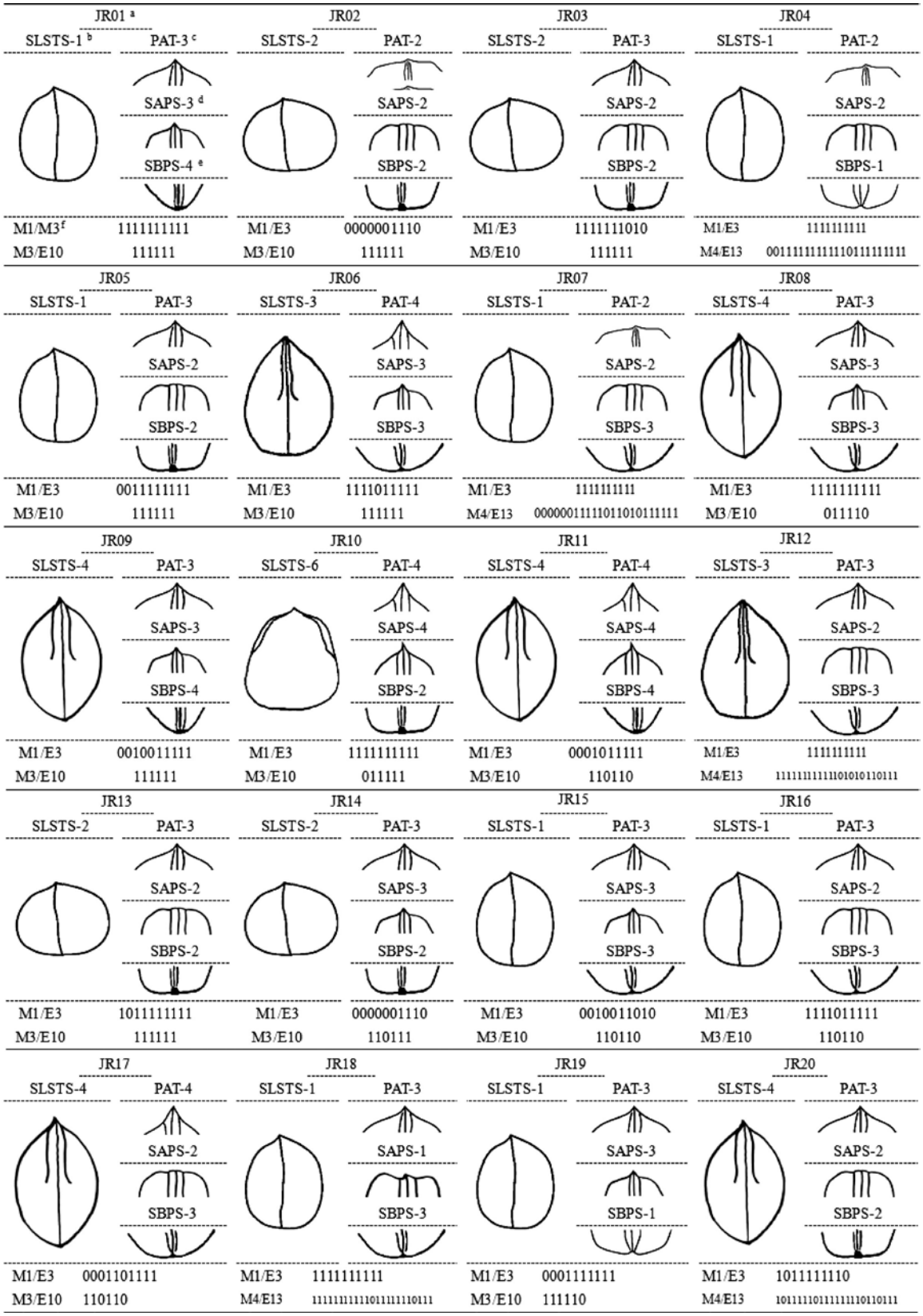

Figure 2. (Continued)

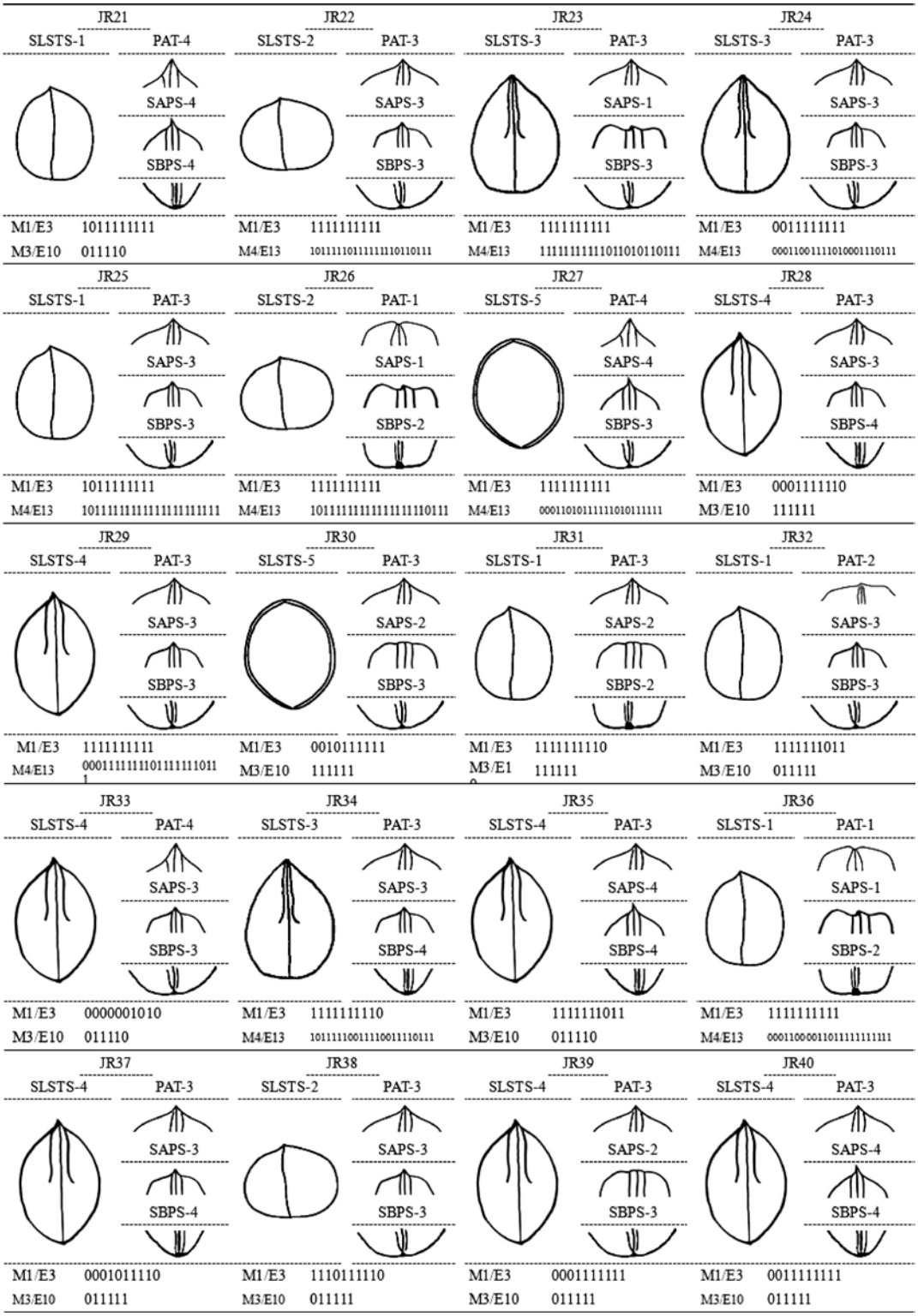

Fig. 2. Identification codes of 40 walnut genotypes. Each genotype contained nut-shape characteristics and SRAP information. a cultivar name; b~e The description of nut phenotype traits as listed in Table 2, e.g.the SLSTS-1 represents the shape in longitudinal section through suture is circular; PAT-3 represents the prominence of apical tip is convex. The sketches of nut shape were drawn according to UPOV (1999). SRAP primer designations and binary coded data derived from amplification results.

Fig. 3. Two-dimensional graphical barcodes for three walnut genotypes (JR02, JR17, and JR23) using SRAP markers.

as an identifier for cargo and external distribution. Studies have shown that two-dimensional barcodes can accurately capture fingerprints and provide an effective and simple method to identify individual plant genotypes and verify their authenticity (Wei *et al*., 14)for enhancing market expansion and farm management.

A combination of molecular characteristics and morphological traits has been used in previous studies to identify and classify variations in different germplasms, including *Juglans regia* (Chen *et al*., 3) and *Lactuca sativa* (Liu *et al*., 8). In the current study, using a combination of nut phenotypes and SRAP markers, we could successfully genotype various walnut germplasms. Moreover, we have demonstrated the feasibility of this technique for identifying individual walnut germplasms. Screening for informative markers is extremely important for effective selection and collection of germplasm resources. Moreover, this method can be further enhanced by increasing the number of phenotypes and molecular markers, thereby facilitating the selection of additional walnut genotypes.

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