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Genetic relationship and identification of *Dioscorea polystachya* cultivars accessed by ISAP and SCAR markers

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Abstract: A survey of intron sequence amplified polymorphism (ISAP) in conjunction with sequence characterized amplified region (SCAR) was carried out to examine the genetic relationships among 14 *Dioscorea polystachya* cultivars and identify the most popular cultivar 'Tiegun'. Our results revealed that there is a high level of polymorphism among these cultivars. Furthermore, in this study, ISAP markers were consistent with the morphological characters of *Dioscorea polystachya* cultivars and previous hypotheses on the classification of these cultivars into 2 groups via leaf and tuber shapes have been confirmed. Based on morphological characters and molecular data, we show for the first time that *D. doryphora* might be a single species and another progenitor of these cultivars. An ISAP fragment specific to the 'Tiegun' cultivar was converted into a SCAR marker. This marker could be used to discriminate the 'Tiegun' cultivar from the other 13 cultivars. Overall, the results of our study provide the foundations for subsequent breeding programs or conservation actions.

Key words: *Dioscorea polystachya*; Genetic relationship; Identification; ISAP; SCAR

INTRODUCTION

Dioscorea L., the largest genus of the family Dioscoreaceae, consists of some economically important tuber crops in Southeast Asia and equatorial Africa [1,2]. These crops are a leading source of calories for millions of people in many developing countries because their tubers are rich in carbohydrates, proteins and vitamins [3,4]. In East Asia, *D. polystachya* Turcz. (Chinese yam) is the most important *Dioscorea* species used as food and in traditional herb medicine [5]. It belongs to the section *Enantiophyllum*, which also includes economically important species such as *D. alata* and *D. rotundata* [6]. It is believed that this species originated in China and was domesticated by farmers in the Song Dynasty dating back about 1000 years [2]. Nowadays, there are mainly 14 cultivars on the Chinese market [7,8].

Previous research showed there were several morphological variations among these cultivars. For example, two tuber shapes, 'oblong' and 'round', were reported among those cultivars [9]. Furthermore, two leaf types (shallow/deeply lobed) were found among those cultivars [10]. A further question is whether these morphological variations correctly reflect genetic relationships among the cultivars. To date, genetic studies have had some limitations for addressing this question. For example, some genetic studies focused on limited cultivars from local regions such as the Fujian and Henan provinces and their obtained phylogenetic resolution was relatively low [11]. To the best of our knowledge, there has been no research directly comparing the genetic variations and morphological characters of *D. polystachya* cultivars. Therefore, the genetic relationships among those cultivars must have been well established.

Among these cultivars, 'Tiegun' possesses more nutritional and pharmaceutical value than other cultivars so that it is the most popular landrace on both local and foreign markets [12]. Unfortunately, it is not easy to identify for both consumers and researchers this elite cultivar by its morphological characters due to the limited variability among cultivars [13]. Thus, it is necessary to develop a rapid and reliable technique for cultivar-specific identification of *D. polystachya*.

Molecular methods have been used for estimating genetic relationships among yam accessions and species, such as the genetic structure and diversity of cultivated and wild yams in Nigeria [14], systematic relationships of Guinea yams [15] and the genetic relatedness among some edible *Dioscorea* species [16]. However, reports have shown that it is difficult to study the phylogenetic relationships with markers such as simple sequence repeats (SSR), inter simple sequence repeat (ISSR), and random amplified polymorphic DNA (RAPD), because these markers are usually not consistent with morphological characters [14,17,18]. Recently, intron sequence amplified polymorphism (ISAP) has been developed. This molecular marker used the conservative consistent sequence of intron splicing sites as its core sequence of amplification, and its high degree of allelic variation and abundant unique fragments have proven to be especially useful in the assessment of genetic variation in cultivated species, such as wheat, chili and tobacco [19].

In the case of species-, region- and cultivar-specific identification, traditional molecular techniques may be ambiguous, pose time constraints and are costly [20,21]. To overcome this, traditional molecular markers can be converted into sequence characterized amplified region (SCAR) markers [22-24]. Cultivar-specific identification using SCAR markers has been demonstrated in rice, coconut and wheat [25-27].

Our aim was to evaluate the genetic relationships of 14 cultivars of *D. polystachya* mainly cultivated in China using ISAP markers, and to develop a robust tool for cultivar-specific identification of 'Tiegun' with the SCAR marker.

MATERIALS AND METHODS

Plant materials and sampling design

A total of 14 cultivars of *D. polystachya* were collected from Henan, Shanxi, Jiangsu, Shandong, Sichuan and Guizhou provinces. Collections of silica gel-dried leaf material were obtained from 10 individuals from each cultivar randomly, resulting in a total of 140 putative individuals for ISAP study (Table 1). Another 10 individuals per cultivar were assayed for SCAR marker validation, with 140 individuals in total. The samples were identified by Professor Yueyu Hang of the Jiangsu Institute of Botany, Chinese Academy of Sciences.

DNA extraction

Leaf samples from the 10 individuals from each cultivar were mixed in equal volume. Then the mixture was extracted as described for ISAP profiling [28]. Genomic DNAs of another 10 randomly selected individuals from all 14 cultivars were extracted for SCAR validation.

Table 1. Plant materials of *D. polystachya* cultivars.

Cultivar	Voucher No.	Locality	Individuals	Morphological characteristics
'Tiegun'	PB2012001	Wenxian, Henan	5	Round tuber, Shallow lobed leaf, red fleck, bulbils
	PB2012002	Taiyuan, Shanxi	5	
'Taigu'	YY2012003	Wenxian, Henan	5	Round tuber, shallow lobed leaf, red fleck, bulbils
	YY2012004	Taiyuan, Shanxi	5	
'Xiaobaizui'	CX2012004	Wenxian, Henan	10	Oblong tuber, deeply lobed leaf, no red fleck, bulbils
'Wujiashuangbao'	SX2012005	Xuzhou, Jiangsu	10	Round tuber, shallow lobed leaf, no red fleck, bulbils
'Mishanyao'	HZ2012006	Xuzhou, Jiangsu	10	Round tuber, shallow lobed leaf, no red fleck, bulbils
'Xichangmao'	PB2012007	Wenxian, Henan	10	Round tuber, shallow lobed leaf, no red fleck, bulbils
'Huaying'	PB2012008	Guangan, Sichuan	10	Round tuber, shallow lobed leaf, no red fleck, bulbils
'Huazi'	PB2012009	Wenxian, Henan	5	Oblong tuber, deeply lobed leaf, no red fleck, no bulbils
	XM2021009	Xuzhou, Jiangsu	5	
'Cuniutui'	XH2012010	Wenxian, Henan	10	Oblong tuber, deeply lobed leaf, no red fleck, bulbils
'Jiujinghuang'	PB2012011	Wenxian, Henan	10	Oblong tuber, deeply lobed leaf, no red fleck, bulbils
'Baiyu'	PB2012012	Heze,Shandong	10	Oblong tuber, deeply lobed leaf, no red fleck, bulbils
'Caoshanyao'	PB2012013	Heze,Shandong	10	Round tuber, shallow lobed leaf, no red fleck, bulbils
'Anshui'	PB2012014	Guiyang, Guizhou	10	Round tuber, shallow lobed leaf, no red fleck, bulbils
'Mashanyao'	PB2012015	Wenxian, Henan	10	Round tuber, shallow lobed leaf, no red fleck, bulbils

Primer selection and ISAP profiling

Based on an earlier report [19], 31 pairs of ISAP primers were synthesized. Subsequently, the DNA of 3 cultivars of *D. polystachya* was randomly selected for PCR amplification. Then primers based on the presence of highly polymorphic and good resolution bands were chosen to amplify the genomic DNA of all 14 cultivars. PCR amplifications were performed in a 20-µl reaction volume containing 25 ng of genomic DNA, 0.6 mM of each primer, 0.2 mM of $MgCl_2$, 0.3 mM of dNTPs and 1.0 U of Taq DNA polymerase. PCR amplifications were carried out with a thermal cycler using the following program: 5 min of denaturing at 94°C, followed by 5 cycles of 1 min at 94°C (denaturing), 1 min at 36°C (annealing) and 1 min at 72°C (elongation). For the next 35 cycles, the annealing temperature was increased to 50°C, with a final elongation step of 10 min at 72°C.

ISAP data analysis

The data were entered into a binary matrix by means of the presence or absence of amplification fragments from each ISAP primer pair. PopGen software (Available from: http://www.ualberta.ca/-fyeh/fyeh) was used to calculate the observed number of alleles (Na), the effective number of alleles (Ne), Nei's genetic diversity (H), and Shannon's information index (I). A phylogenetic tree was obtained for genetic relationship among cultivars using NTSYS2.10 software (Available from: http://ntsyspc.software.informer.com). Principal coordinate analyses (PCoA) were calculated by NTSYS2.10 software

Morphological character analysis

In order to assess the congruence between molecular and morphological characters, four morphological traits in cultivars of *D. polystachya* were optimized onto the tree generated from the ISAP data. The traits included (i) tuber shape (round or oblong), (ii) red flecks on the tuber skin (present or absent), (iii) leaf type (shallow or deeply lobed) and (iv) bulbils (present or absent).

SCAR marker development and validation

The presence of an ISAP fragment in the 'Tiegun' cultivar, which is absent in all the other cultivars,

Table 2. List of primers of ISAP and the amplification results.

Primers	Sequence (5'→3')	Total bands	Polymorphic bands	Percentage of polymorphic bands	Amplification range (bp)
F2R4	F2:GCATGAATGCAAAGGTAA R4:CTGCAAGTGAGAACACCC	12	12	100.00	120-1200
F1R1	F1:CGATATAAGCAAAGGTAA R1:CTGCAATTAAGCAAGAAC	9	9	100.00	100-1100
F2R1	F2:GCATGAATGCAAAGGTAA R1:CTGCAATTAAGCAAGAAC	6	6	100.00	150-1100
F4R2	F4:ACGAAGATGGAAAGGTAA R2: CTGCAATGTCCCATAGAT	10	9	90.00	200-1400
F4R5	F4:ACGAAGATGGAAAGGTAA R5: CTGCAAAATTCAATAGTT	6	6	100.00	250-1400
F6R4	F6:CGTCCGATGAAAAGGTAA R4:CTGCAAGTGAGAACACCC	9	8	88.89	100-1000
F1R7	F1:CGATATAAGCAAAGGTAA R7: CTGCAAGGGTTAACCAGT	7	7	100.00	200-900
F8R1	F8: AGCCGTTTATACAGGTAA R1:CTGCAATTAAGCAAGAAC	12	11	91.67	100-1200
F7R7	F7: ATCAGCTGCTGCAGGTAA R7: CTGCAAGGGTTAACCAGT	9	8	88.89	100-1300
F7R1	F7: ATCAGCTGCTGCAGGTAA R1:CTGCAATTAAGCAAGAAC	9	9	100.00	250-1000
F1R8	F1:CGATATAAGCAAAGGTAA R8:CTGCAATAACCACATGAA	6	6	100.00	100-1100
Total	--	95	91	--	
Average	--	8.64	8.27	95.79	

was designated as the cultivar-specific marker. The cultivar-specific fragment of 'Tiegun' was cloned into a pMD18-T vector, then propagated by transferring the recombinants into *E. coli* cells and sequenced by Majorbio Co. (Shanghai, China). Primers were designed from this fragment using Primer Premier 5.0 software for the cultivar-specific amplification [29]. The primer pairs were amplified in 10 randomly selected individuals from each cultivar for validation using the following program: 5 min of denaturing at 94°C followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 58°C, extension for 2 min at 72°C and a final extension for 10 min at 72°C.

RESULTS

Eleven highly polymorphic primers were selected from the 31 ISAP primer pairs to amplify the 14 cultivars, and a total of 95 fragments were generated, ranging from 120 bp to 1400 bp, with an average of 8.64 bands per primer pair for ISAP. The number of polymorphic bands for ISAP was 91, with an average of 8.27 polymorphic bands per primer pair. The percentage of polymorphic bands of ISAP was 95.79% (Table 2). The highest number of bands was obtained from the primer pair F2R4 (12 bands, all of which were polymorphic) and F8R1 (12 bands, 11 of which were polymorphic), whereas the lowest was found with the primer pair F2R1 (6 bands, all of which were polymorphic) and F1R8 (6 bands, all of which were polymorphic).

Significant genetic difference and high genetic diversity were observed among the cultivars: within the 11 primer pairs, the number of alleles (Na) was from 1.8999 to 2.0000, with an average of 1.9651; the effective number of alleles (Ne) ranged from 1.1529 to 1.4271, with an average of 1.3018; the difference between Na and Ne was from 0.5729 to 0.8471, with an average of 0.6983. Nei's genetic diversity index (H) was from 0.1327 to 0.2673, with an average of 0.2002. Shannon's information index (I) was from 0.2011 to 0.5832, with an average of 0.3102 (Table 3).

A dendrogram was generated with NTSYS2.10 software using the unweighted pair group method with arithmetic mean (UPGMA) method. The 14 cultivars could be completely separated with the 11 primer pairs (Fig. 1). Furthermore, it was possible to identify into 2 well-defined clusters. Cluster I included 9 cultivars: 'Tiegun', 'Taigu', 'Caoshanyao', 'Mashanyao',

Table 3. Genetic diversity of *D. polystachya* among 14 cultivars.

Primers	Sequence (5'→3')	*Na*	*Ne*	*Na-Ne*	*H*	*I*
F2R4	F2:GCATGAATGCAAAGGTAA R4:CTGCAAGTGAGAACACCC	2.0000	1.4271	0.5729	0.2673	0.5832
F1R1	F1:CGATATAAGCAAAGGTAA R1:CTGCAATTAAGCAAGAAC	2.0000	1.3243	0.6765	0.2329	0.3271
F2R1	F2:GCATGAATGCAAAGGTAA R1:CTGCAATTAAGCAAGAAC	2.0000	1.3021	0.6979	0.2113	0.2915
F4R2	F4:ACGAAGATGGAAAGGTAA R2: CTGCAATGTCCCATAGAT	2.0000	1.2977	0.7023	0.1961	0.2635
F4R5	F4:ACGAAGATGGAAAGGTAA R5: CTGCAAAATTCAATAGTT	2.0000	1.3219	0.6781	0.2101	0.3027
F6R4	F6:CGTCCGATGAAAAGGTAA R4:CTGCAAGTGAGAACACCC	1.8999	1.2461	0.7539	0.1419	0.2216
F1R7	F1:CGATATAAGCAAAGGTAA R7: CTGCAAGGGTTAACCAGT	2.0000	1.3661	0.6339	0.2569	0.4329
F8R1	F8: AGCCGTTTATACAGGTAA R1:CTGCAATTAAGCAAGAAC	1.9167	1.2986	0.7014	0.1998	0.2758
F7R7	F7: ATCAGCTGCTGCAGGTAA R7: CTGCAAGGGTTAACCAGT	1.8999	1.1529	0.8471	0.1327	0.2011
F7R1	F7: ATCAGCTGCTGCAGGTAA R1:CTGCAATTAAGCAAGAAC	2.0000	1.2614	0.7386	0.1532	0.2339
F1R8	F1:CGATATAAGCAAAGGTAA R8:CTGCAATAACCACATGAA	2.0000	1.3218	0.6782	0.2007	0.2791
Mean	--	1.9651	1.3018	0.6983	0.2002	0.3102

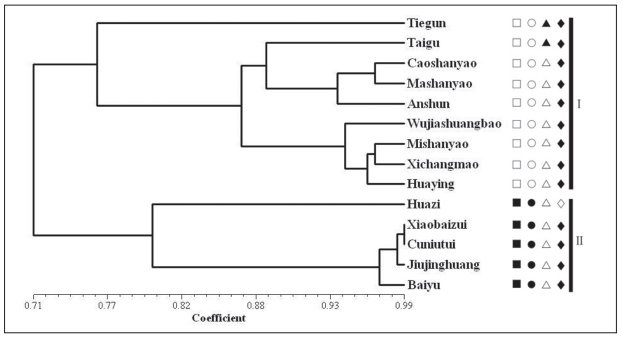

Fig.1. Dendrogram of all 14 cultivars (the bar under the tree is the coefficient). □ – shallow lobed leaf, ▪ – deeply lobed leaf; ○ – round tuber, ● – oblong tuber; ▲ – red fleck on the tuber skin present, Δ – red fleck on the tuber skin absent; ◊ – bulbils absent, ◆ – present bulbils.

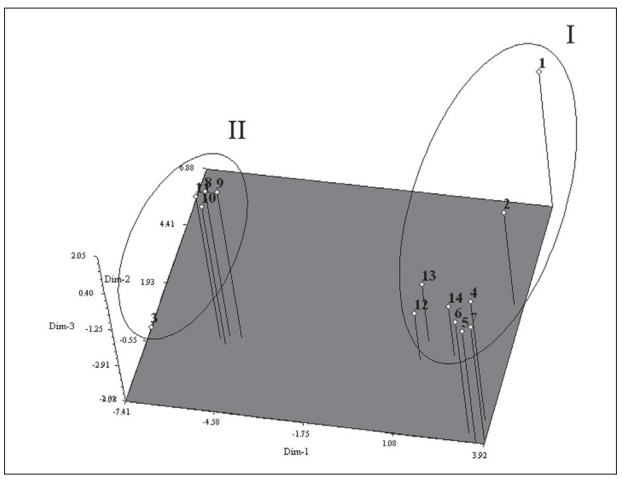

Fig.2. Principal coordinate analysis of all the cultivars revealing 2 distinct groups.

'Anshun', 'Wujiashuangbao', 'Mishanyao', 'Xichangmao' and 'Huaying', whereas Cluster II included 5 cultivars: 'Huazi', 'Xiaobaizui', 'Cuniutui', 'Jiujinhuang' and 'Baiyu'.

The morphological characters of each cultivar were observed (Table 1) and optimized on the tree generated from ISAP bands. The result showed that the samples with round tuber and shallow lobed leaf were observed only in Cluster I, whereas those with oblong tuber and deeply lobed leaf were found only in Cluster II. The red fleck on the tuber skin was present in 'Tiegun' and 'Taigu', and absent in other cultivars. Bulbils were observed in all cultivars except 'Huazi'.

Principle coordinate analysis (PCoA) using Euclidean similarity indices also clearly separated all the samples into two groups (Fig. 2).

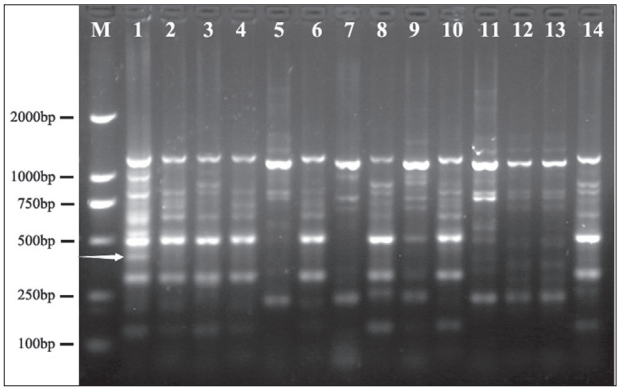

Fig.3. ISAP polymorphism in 14 cultivars with primer pairs F2R4. M: 2000 maker; 1-14: 'Tiegun', 'Taigu', 'Wujiashuangbao', 'Mishanyao', 'Baiyu', 'Caoshanyao', 'Xiaobaizui', 'Huaying', 'Anshun', 'Mashanyao', 'Xichangmao', 'Cuniutui', 'Jiujinghuang' and 'Huazi'. The arrow showed the specific fragment for 'Tiegun' with a length of 435 bp.

Fig.4 The specific sequence of 'Tiegun' cultivar (underlined area represents binding sites of SCAR marker).

A specific amplicon at approximately 400 bp was observed in 'Tiegun' and was missing in all the other cultivars from the amplification using the primer pair F2R4 (Fig. 3). Subsequently, this specific amplicon was cloned and sequenced. The BLAST result of sequence data (435 bp) in GenBank showed there was no homology sequence in other species. A SCAR primer pair specific for 'Tiegun' identification (F: 5′-GTAATGATTGGAGATAAGTGTTCGTG-3′;R: 5′-AATTTACAAGTTGTGCGACCAAG -3′ was constructed based on Primer Premier 5.0 software (Fig. 4).

This primer pair was amplified in 140 individuals of all the 14 cultivars. All 10 individuals of 'Tiegun' had the cultivar-specific band at 399 bp, which was absent in the other 13 cultivars (Fig. 5).

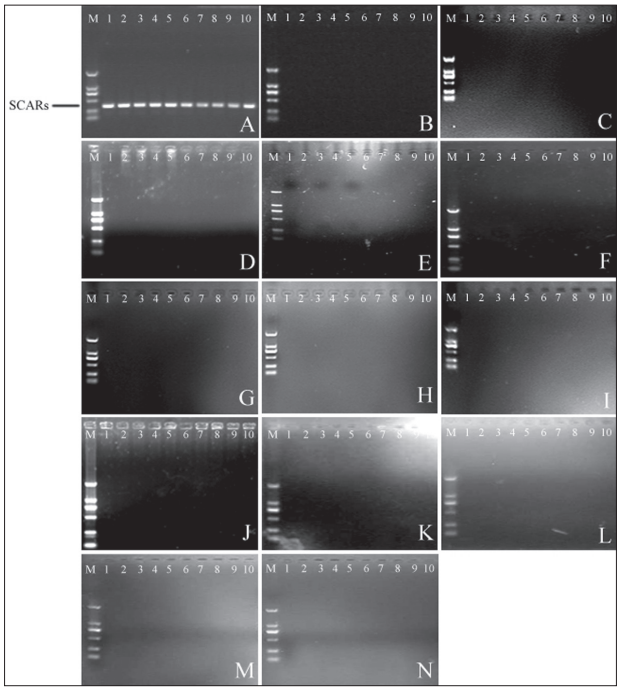

Fig.5. The SCAR marker validation on individuals of different cultivars. (A: 'Tiegun'; B: 'Taigu '; C: 'Wujiashuangbao'; D: 'Mishanyao'; E: 'Huazi'; F: 'Xichangmao'; G: 'Cuniutui'; H: 'Huaying'; I: 'Caoshanyao'; J: 'Anshun'; K: 'Xiaobaizui'; L: 'Jiujinghuang'; M: 'Baiyu'; N: 'Mashanyao'. 1-10: individuals of each cultivar; M: 2000 bp DNA ladder).

DISCUSSION

Understanding the genetic relationship of cultivars is vital to breeding programs and conservation strategies [30-33]. Unfortunately, *D. polystachya* has not received enough attention, unlike other *Dioscorea* species that have received much more attention. At the molecular level, the percentage of polymorphic band (PPB), the effective number of alleles (Ne), Nei's genetic diversity (H) and Shannon's information index (I) are essential for evaluating the genetic diversity of germplasm [34,35]. In this study, high levels of genetic diversity among *D. polystachya* cultivars were detected with these parameters. Our results also indicted that ISAP markers detected a higher degree of variation among *D. polystachya* cultivars compared with RAPD markers (PPB: 85.16%) [11]. The high genetic diversity among the cultivars may be explained by the domestication process of *Dioscorea* tuber crops [36,37]. Primarily, traditional farmers collected and grew clones of varieties for the special characteristics they preferred. Subsequently, other varieties were subject to selection by farmers to meet different demands of the market. As a result, these varieties of *D. polystachya* were maintained and this species could avoid genetic erosion.

Some studies showed there was no agreement between genetic clustering and morphological traits of yam cultivars using molecular markers such as RAPD, SSR and ISSR [14,18,31,38]. This might be because the loci amplified by these markers did not target the genes of morphological characteristics [39]. These incongruent results may also explain the reason why there are not enough improvements in yam breeding programs, clonal selection and genetic conservation as in other tuber crops. In the present research, the phylogenetic tree generated by ISAP data was consistent with morphological characteristics: the cultivars with round tuber and shallow lobed leaf were gathered into an independent cluster, whereas cultivars with oblong tuber and deeply lobed leaf made an independent cluster (Cluster I and Cluster II, respectively). As for the phylogenetic relationships of *D. polystachya* cultivars, some researchers have suggested that these cultivars should be divided into two groups based on tuber shape: a round tuber group and an oblong tuber group [10]. Moreover, two ploidy levels among cultivars have been reported (2n = 100, 140): cultivars with deeply lobed leaves were 2n=140 while those with shallow lobed leaves were 2n=100 [40]. Finally, all the available data are consistent with the hypotheses that the cultivars of Chinese yam should be divided into 2 groups.

Despite the morphological variability found within this species, the intraspecific phylogeny remains somewhat enigmatic. Due to many morphological characters in common, the species *D. doryphora* has been considered as a synonym of *D. polystachya* [2]. In subsequent decades, *D. polystachya* was thought to be the only wild progenitor of all the cultivars. But recently, *D. polystachya* and *D. doryphora* could be distinguished from each other with chloroplast markers [41]. It is interesting to notice that the morphological differences in only two characters (tuber and leaf morphology) between *D. doryphora* (oblong tuber and deeply lobed leaf) and *D. polystachya* (round tuber and shallow lobed leaf) [41] were consistent with our molecular results. It seems that *D. doryphora* might be classified as a single species once more. Meanwhile, deductions could be made from morphological and molecular evidence that the cultivars in Cluster I were probably domesticated from

D. polystachya, and *D. doryphora* may be the wild progenitor of the cultivars in Cluster II. However, further research is needed to verify the history of domestication of these cultivars and the systematic relationship between *D. polystachya* and *D. doryphora*.

The cultivar 'Tiegun' is a landrace cultivar native to China, and possesses more nutritional and pharmaceutical value than other cultivars [42,43]. Due to its high popularity in China, many other cultivars are sold as 'Tiegun' in order to acquire a higher price. In the market, it is not easy to identify 'Tiegun' morphologically. Therefore, there is an urgent need for the development of identification techniques to distinguish 'Tiegun' from other cultivars. In our ISAP analysis, significant genetic polymorphism was observed among the cultivars and a cultivar-specific SCAR marker was developed for the identification of 'Tiegun'. In order to check its sensitivity and accuracy, this SCAR marker was validated by testing on 140 individuals of 14 cultivars. The result showed that all 10 individuals of 'Tiegun' amplified the cultivar-specific band at 399 bp, which was absent in the other 13 cultivars, confirming the specificity of the SCAR marker for cultivar-specific identification. In summary, an ISAP-SCAR technique proved to be a helpful tool in identifying Chinese yam cultivars. The results of our study indicate that PCR products can be used to design SCAR primers and then used as a DNA barcoding for cultivar identification at a lower cost.

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Authors' contribution: Bin Peng designed and performed the experiments, analyzed the data and wrote the manuscript. Bin Peng, Xiaoqin Sun, Mimi Li, Yanmei Zhang, Jiayu Xue and Yueyu Hang carried out the accession collection.

Conflict of interest disclosure: The authors declare no conflict of interest.

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