Genetic Relationship And Species Identification of *Dioscorea Polystachya* Turcz. In Yams Determined By ISSR, ISAP, SRAP And SCAR Markers

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Abstract

Dioscorea polystachya Turcz. is the authentic herb Yam in Chinese traditional medicine. In the medicine market, some relative species are misused as “Yams”, such as *D. alata* L., *D. exalata* C.T. Ting & M.C. Chang, *D. persimili* Prain et Burkill, *D. fordii* Prain et Burkill and *D. japonica* Thunb. In order to estimate the genetic relationships among those species and develop a rapid and accurate method for *D. polystachya* identification, Inter Simple Sequence Repeat (ISSR), Intron Sequence Amplified Polymorphism (ISAP), Sequence-Related Amplified Polymorphism (SRAP) and Sequence Characterized Amplified Region (SCAR) were conducted in this study. Combining ISSR, ISAP and SRAP markers, a high degree of variation among the *Dioscorea* species was detected. Moreover, a relatively high gene flow ($N_m = 0.3293$) in those species was observed. The main reason for this phenomenon might be there were genepools exchanging in the wild materials. The phylogenetic tree and genetic similarity results showed *D. japonica* had the closest genetic relationship with *D. polystachya*. Considering the lowest inter-species variation observed in *D. polystachya* and the highest genetic divergence within *D. japonica*, we suggested the taxonomy of one species complex *D. polystachya*-*D. japonica* for breeding and conservation purpose. The phylogeny data in this study indicated *D. alata* and *D. persimilis* should be independent species respectively instead of cultigens. Furthermore, a SCAR marker was developed and could be used to discriminate the authentic herb *D. polystachya* from other confused *Dioscorea* species. Overall, the results are useful as guidelines for breeding programs and conservation actions.

Introduction

Yam (*Dioscorea* spp.) is not only an important tuber crop, but also an important pharmaceutical plant used in the traditional Chinese medicine (Karnick, 1969; Hodeba et al., 2008; Peng et al., 2017; Hang and Sun, 2020). As a traditional herb, the pharmaceutical uses of yam are prescribed for treating diabetes, strengthening stomach function and alleviating anorexia (Hang, 1995; Hang and Sun, 2020). In addition, it is also used as a healthy food (Shin et al., 2006). In the Chinese Pharmacopoeia, the authentic herb “Yam” exclusively refers to *Dioscorea polystachya* Turcz. in Dioscoreaceae (Pharmacopoeia of China 2020). In China, due to its high market demand, other species in the genus *Dioscorea*, including *D. alata* L., *D. exalata* C. T. Ting & M. C. Chang, *D. persimili* Prain et Burkill, *D. fordii* Prain et Burkill and *D. japonica* Thunb. are commonly treated as “Yams” to be used as medicine (Lay et al., 2001; Hang et al., 2006; Li Mimi et al., 2012; Hang and Sun, 2020). Given that the nutrients and bioactive compositions quantitatively and qualitatively varied among many *Dioscorea* species (Wu et al., 2016), the confusion and mixed use of the authentic yam has had a great impact on the medicine market, medicinal efficacy and herb planters. Furthermore, there are still some controversies with respect to some edible *Dioscorea* taxonomy. For example, some studies showed *D. persimili* should be a cultivar of *D. polystachya* based on 18S sequence analysis (Liu et al., 2000). It is still a controversial issue whether *D. alata* is a true species or a putative cultigen (Lebot et al., 1998; Wilkin et al., 2005; Mengesha et al., 2013a).

To date, species identification in *Dioscorea* is predominantly dependent on morphological and micromorphological descriptors. For example, leaf morphology, stem twining direction, tuber anatomy

and micromorphology of leaf epidermal cell could be used for identification of *Dioscorea* species (Hang et al., 2006; Li Mimi et al., 2012; Adeniran and Sonibare, 2016; Hang and Sun, 2020). However, morphological identification requires conditionality including the suitable development stage of the plants or sophisticated assessment.

The development of molecular tools, such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeat (ISSR), Microsatellites or Simple Sequence Repeats (SSR), Restriction Fragment Length Polymorphism (RFLP) and so on, have become important means to identify *Dioscorea* species (Lay et al., 2001; Tostain et al., 2006; Mengesha et al., 2013b; Mukherjee and Bhat, 2013). For example, RFLP molecular markers could be used to identify species of the *D. cayenensis*-*D. rotundata* species complex (Mukherjee and Bhat, 2013). Through combined analysis of SSR and ISSR, many edible yam species in West Africa could be identified, including cultivated species of *D. prahensis*, *D. abyssinica* and wild species of *D. sagittifolia* in Nigeria (Nascimento et al., 2013). However, those molecular techniques may be ambiguous, pose time constraints and are costly as PCR amplification depends on multiple reactions (Wu et al., 2009; Sun et al. 2012;). To overcome this, traditional molecular markers may be converted into stable and reliable marker just like Sequence Characterized Amplified Region (SCAR) markers (Devaiah et al., 2011; Al-Qurainy et al., 2018). This approach has been demonstrated in species-identification including *Satureja montana* L., *Cucumis sativus* L., and *Casuarina equisetifolia* L (Ghosh et al., 2010; Marieschi et al., 2011; Saengprajak, 2012). Furthermore, we have developed a SCAR marker to distinguish a landrace *D. polystachya* 'Tiegun' from the other *D. polystachya* varieties (Peng et al., 2017).

In this study, ISSR, ISAP (Intron Sequence Amplified Polymorphism) and SRAP (Sequence-Related Amplified Polymorphism) were used to evaluate the genetic relationships of yam species. Moreover, species diagnostic SCAR marker would be developed for the authentic herb "Yam" *D. polystachya* identification.

Materials And Methods

Plant material.

Here, we collected not only cultivated *Dioscorea* materials, but also many wild *Dioscorea* materials in China (Table 1). Healthy young leaves from *Dioscorea* species were collected to extract genomic DNA. The wild *D. zingiberensis* C. H. Wright was selected as outgroup. Another 6–10 individuals per species were assayed to validate the reliability of SCAR marker (Double Blind Test). When the validation finished, the experimental materials were disclosed (Table 2). All the samples in this study were identified by Professor Yueyu Hang (Jiangsu Institute of Botany, Chinese Academy of Sciences).

Table 1
Plant materials for genetic relationship among *Dioscorea* species

Spices	Code	Habit/cultivated name	Locality
<i>D. polystanchya</i>	SY1	Wild	Jiangsu jurong
	SYHZ	Cultivate/'Huazi'	Jiangsu Xuzhou
	SYWJSB	Cultivate/'Wujiashuangbao'	Jiangsu Xuzhou
	SYMI	Cultivate/'Mishanyao'	Jiangsu Xuzhou
	SYXCM	Cultivate/'Xichangmao'	Henan Wenxian
	SUHY	Cultivate/'Huaying'	Sichuan Guangan
	SYCSY	Cultivate/'Cao'	Shandong Heze
	SYMA	Cultivate/'Ma'	Henan Wenxian
	SYAS	Cultivate/'Anshun'	Guizhou Guiyang
	SYXBZ	Cultivate/'Xiaobaizui'	Henan Wenxian
	SYCNT	Cultivate/'Cuniutui'	Henan Wenxian
	SYJJH	Cultivate/ 'Jiujinghuang'	Henan Wenxian
	SYBY	Cultivate/ 'Baiyu'	Shandong Heze
	SYTG	Cultivate/'Tiegundun'	Shanxi Taiyuan
	SYTG-2	Cultivate/'Tiegundun'	Jiangsu Xuzhou
	SYTG-1	Cultivate/'Tiegundun'	Hennan, wenxian
	SYTG-5	Cultivate/'Tiegundun'	Shandong Jining
	SYTG-6	Cultivate/'Tiegundun'	Hebei Cangzhou
	SYTG-7	Cultivate/'Tiegundun'	Hunan Liuyang
	SYTG-3	Cultivate/'Tiegundun'	Sichuan Nanchong
	SYTG-4	Cultivate/'Tiegundun'	Guangdong Dongguan
<i>D. persimilis</i>	HB-1	Cultivate	Fujian mingi
	HB-2	Wild	Guangdong shaoguan
	HB-3	Wild	Yunnan lijiang
	HB-4	Wild	Fujian wuyishan

Note: "Unknown" means the material were collected from the wild, but there was no cultivation in this area.

Spices	Code	Habit/cultivated name	Locality
	HB-5	Wild	Jiangxi lushan
	HB-6	Wild	Guangxi nanning
<i>D. alata</i>	SS-1	Cultivate	Guangdong shaoguan
	SS-2	Cultivate	Hainan lingshui
	SS-3	Cultivate	Hunan hengshan
	SS-4	Cultivate	Yunnan lijiang
	SS-5	Unknown	Jiangxi lushan
	SS-6	Unknown	Yunan jinghong
<i>D. exalata</i>	WC-1	Cultivate	Guangxi guilin
	WC-2	Cultivate	Yunnan lijiang
	WC-3	Wild	Guangxi,guilin
	WC-4	Wild	Yunan jinghong
	WC-5	Wild	Yunnan,lincang
	WC-6	Wild	Guangxi,longzhou
<i>D. fordii</i>	SHANS-1	Cultivate	Fujian,shanming
	SHANS-2	Wild	Guangxi guilin
	SHANS-3	Wild	Yunan jinghong
	SHANS-4	Wild	Hainan,limushan
	SHANS-5	Wild	Guangdong,dinghushan
	SHANS-6	Wild	Yunnan, kunming
<i>D. japonica</i>	RB-1	Wild	Zhejiang, linan
	RB-2	Wild	Jiangxi, lushan
	RB-3	Wild	Hunan, yongshun
	RB-4	Wild	Fujian wuyishan
	RB-5	Wild	Guangdong lianshan
	RB-6	Wild	Anhui guniujiang
Note: "Unknown" means the material were collected from the wild, but there was no cultivation in this area.			

Table 2
Plant materials for validation of developed SCAR marker

Spices	Individuals	Habit	Location
<i>D. polystanchya</i>	2	Cultivate	Henan Wenxian
	2	Cultivate	Jiangsu Xuzhou
	2	Cultivate	Shanxi Taiyuan
	2	Cultivate	Henan Wenxian
	2	Wild	Jiangsu Nanjing
<i>D. persimilis</i>	10	Cultivate	Jiangsu Yixing
<i>D. alata</i>	10	Cultivate	Jiangsu Yixing
<i>D. fordii</i>	10	Cultivate	Jiangsu Yixing
<i>D. exalata</i>	1	Cultivate	Guangxi guilin
	1	Cultivate	Yunnan lijiang
	1	Wild	Guangxi,guilin
	1	Wild	Yunan jinghong
	1	Wild	Yunnan,lincang
	1	Wild	Guangxi,longzhou
<i>D. japonica</i>	1	Wild	Zhejiang, linan
	1	Wild	Jiangxi, lushan
	1	Wild	Hunan, yongshun
	1	Wild	Fujian wuyishan
	1	Wild	Guangdong lianshan
	1	Wild	Anhui guniujiang

DNA extraction.

Genomic DNA extraction was the same as those described previously (Peng et al., 2017). The extracted DNA was detected by agarose gel electrophoresis to confirm that it was intact, and stored in a refrigerator at -20°C for later use.

ISSR, ISAP and SRAP reaction system.

Preliminary assays were conducted to determine the optimum primers for *Dioscorea* genetic relationship analysis. Finally, 10 highly polymorphic ISSR primers were chosen to for PCR reaction (Supplemental

Table S1). PCR amplifications were performed in a 20 μ L reaction volume containing 1 μ L DNA (20 ng $\cdot$ μ L⁻¹), 2 μ L 10 $\times$ Buffer (Mg²⁺), 1.6 μ L dNTP (2.5 mM), 0.8 μ L (10 mM) primer, Taq DNA polymerase 0.2 μ L (5 U $\cdot$ μ L⁻¹), ddH₂O up to 20 μ L. PCR amplifications were carried out with T1 Thermocycler (Biometra, Germany) using the following program: 1 min at 94°C; 35 cycles of 45 s at 94°C, 50 s annealing at 50°C, 75 s extension at 72°C; with final extension at 72°C for 5 min. Then, 11 ISAP primer pairs were chosen to for PCR reaction based on high polymorphism and good reproducibility (Supplemental Table S2). The ISAP analysis was performed according to those described previously (Peng et al., 2017). Further, a total of 21 highly polymorphic primer pairs were selected for SRAP analysis (Supplemental Table S3). PCR was carried out with a 20 μ L reaction mixture containing 25 ng DNA as template, 0.7 mM primer, 0.2 mM Mg²⁺, 0.3 mM dNTPs, and 1 U Taq DNA polymerase. The PCR reaction program consisted of: 94°C for 1 min; 5 cycles of 94°C, 45 s; 36°C, 3 s; 72°C, 60 s; 35 cycles of 94°C, 45 s, 50°C, 30 s; 72°C, 75 s; 72°C for 5 min. All the primers used in this study were synthesized by Invitrogen Trading (Shanghai) Co., Ltd.

Genetic relationship analysis

All clearly detectable polymorphic and monomorphic bands were scored for the analysis, and were scored as present (1) or absent (0) in each sample. POPGENE software was used to analyze the genetic relationship of species, and calculate the indices of Number of alleles (N_a), the number of effective alleles (N_e), Nei's gene diversity index (H), the Shannon Index (I), Gene differentiation coefficient (G_{st}) and gene flow (N_m) (Yeh et al., 1999). The observed total heterozygosity (H_t) and the average heterozygosity within the species (H_s) were calculated using GenAlEx v. 6.5 (Peakall and Smouse, 2012). Principal coordinate analyses (PCoA), genetic similarity coefficient and genetic distance were obtained by using the NTSYS 2.10e software (Rohlf, 2000). The intra-specific similarity coefficient (F_{is}) and inter-specific variation coefficient (F_{st}) were calculated by Artificial Bee Colony (ABC) algorithm (Foll et al., 2008).

SCAR identification system

In order to screen the specie-specific bands conveniently, all individual DNAs of each species (Table 1) were mixed into one gene pool respectively. The presence of a specific fragment in the *D. polystachya*, which is absent in all the other species was designated as the identification marker. The specific fragment of *D. polystachya* was obtained by Agarose Gel Extraction Kit (Takara, Dalian, China), then sequenced by Majorbio Co. (Shanghai, China). According to the results of DNA sequence analysis, a pair of specific primers were designed for SCAR reaction using Primer Premier 5.0 (Lalitha, 2000). PCR was carried out with a 20 μ L reaction mixture containing 1 μ L (10–25 ng) DNA as template, 13.3 μ L double distilled water, 2 μ L Mg²⁺, 0.3 μ L (10 mmol/L) dNTPs, 2 μ L PCR buffer, 0.6 μ L (10 p mol/L) each primer and 0.2 μ L (1 U) Taq DNA polymerase. The PCR reaction program consisted of: 94°C for 1 min; 30 cycles of 94°C for 50 s, 60°C for 45 s, 72°C for 60 s; 72°C for 5 min. The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide (EB), observed and photographed with a gel imaging system, and a 2000 bp DNA ladder was used as a molecular weight marker.

Results

Genetic relationship between *D. polystachya* and its related species

Combination of ISSR, ISAP and SRAP markers, a total of 523 bands were obtained. The number of polymorphic bands for those markers was 507, with a percentage of polymorphic bands 96.94%. The number of alleles (N_a) of *Dioscorea* species was 1.9963, while the number of effective alleles (N_e) was 1.7724. Nei's gene diversity index (H) was 0.4950, and the Shannon Index (I) was 0.6762. Analysis of the coefficient of genetic differentiation revealed that the total heterozygosity (H_t) of the six species was 0.4821, and the average heterozygosity within the species (H_s) was 0.1769. Gene differentiation coefficient (G_{st}) among species was 0.6029, while gene flow (N_m) among species was 0.3293.

The genetic similarity coefficient among *Dioscorea* species were 0.7126–0.9258, while the genetic distance was 0.0770–0.2825 (Table 3). The closest and farthest genetic relationship associated with *D. polystachya* were *D. japonica* (genetic similarity coefficient 0.8430) and *D. fordii* (genetic similarity coefficient 0.7126) respectively. In addition, *D. exalata* and *D. persimilis* were the closest two species out of the six, with a genetic distance and genetic similarity coefficient of 0.0770 and 0.9258.

Table 3
Genetic similarities and genetic distances among six *Dioscorea* species

species	<i>D. polystachya</i>	<i>D. alata</i>	<i>D. japonica</i>	<i>D. persimilis</i>	<i>D. exalata</i>	<i>D. fordii</i>
<i>D. polystachya</i>	****	0.8282	0.8430	0.8085	0.7954	0.7126
<i>D. alata</i>	0.1885	****	0.8255	0.7539	0.7753	0.8059
<i>D. japonica</i>	0.1708	0.1918	****	0.8738	0.8679	0.8567
<i>D. persimilis</i>	0.2126	0.2825	0.1349	****	0.9258	0.9111
<i>D. exalata</i>	0.2289	0.2545	0.1416	0.0770	****	0.8770
<i>D. fordii</i>	0.3388	0.2158	0.1546	0.0930	0.1312	****
Note: Genetic similarity coefficient (above diagonal) and genetic distance (below diagonal).						

The intra-species similarity coefficient (F_{is}) ranged from 0.2096 to 0.4339, in which the F_{is} of *D. persimilis* was the highest, and the F_{is} of *D. japonica* was the lowest. The inter-species variation (F_{st}) varied from 0.0497 to 0.1862. Compared with other species, *D. fordii* had the highest variability, and *D. polystachya* had the lowest. The P -values of all results were less than 0.05, indicating high confidence (Table 4).

Table 4
The *F*-data in 6 *Dioscorea* species

Species	<i>F</i> -data	Mean	<i>P</i> -value
<i>D. polystachya</i>	F_{is}	0.2611	0.000744
<i>D. persimilis</i>	F_{is}	0.4339	0.002997
<i>D. japonica</i>	F_{is}	0.2096	0.000246
<i>D. exalata</i>	F_{is}	0.3054	0.000255
<i>D. fordii</i>	F_{is}	0.3248	0.000679
<i>D. alata</i>	F_{is}	0.4173	0.000587
<i>D. polystachya</i>	F_{st}	0.0497	0.000889
<i>D. fordii</i>	F_{st}	0.1862	0.004133
<i>D. japonica</i>	F_{st}	0.0756	0.000392
<i>D. exalata</i>	F_{st}	0.0698	0.000487
<i>D. persimilis</i>	F_{st}	0.1088	0.000355
<i>D. alata</i>	F_{st}	0.1255	0.000873

The phylogenetic tree revealed that individuals of each species clustered together respectively. All samples of *D. polystachya* were clustered in one branch, and gathered with all *D. japonica* individuals into a large branch. *D. alata*, *D. persimilis*, *D. exalata* and *D. fordii* clustered together into another large branch, and located at the base of the phylogenetic tree (Fig. 1).

Interestingly, Principle Coordinate Analysis (PCoA) using Euclidean similarity indices could separate all the samples except *D. exalata* and *D. persimilis* (Fig. 2).

Specific identification of *D. polystachya* based on SRAP-SCAR analysis

In this study, a specific band of *D. polystachya* was found in SRAP amplification (Fig. 3).

The sequencing results showed that the length of the specific band in *D. polystachya* was 1228 bp (Fig. 4). The sequence was compared to the GenBank database, and no homologous sequence was found. A pair of SCAR primers (F: 5'-TTGCCCTCAAATGATTGCCC-3'; R: 5'-AGGTGATGGATATGGCCCAAAT-3') were designed according to the primer design principle. Then, a total of 46 *Dioscorea* individuals were prepared for Double Blind Test. The result showed the specific band was observed in 10 individuals (Fig. 5). When the experimental materials were disclosed, all the 10 individuals with the specific band belonged to *D.*

polystachya. Meanwhile, the specific band was not amplified in the individuals of other 5 *Dioscorea* species, indicating that this band is unique to *D. polystachya*.

Discussion

Combination of multiple molecular markers can more accurately determine the genetic relationship among species. For example, RAPD, ISSR, IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposon microsatellite amplified polymorphism) markers revealed genetic diversity and relationship among the *Citrus* species (Biswas et al., 2010). Genetic relationships and modes of *Robinsonia* species were illustrated based on AFLP and SSR markers (Takayama et al., 2015). Combining SSR and ISSR markers could better reflect genetic relationship of *D. trifida* than single marker (Nascimento et al., 2013). At the molecular level, the percentage of polymorphic band, Nei's genetic diversity (H), the effective number of alleles (N_e), Shannon's information index (I), total heterozygosity (H_t) and the average heterozygosity within the species (H_s) are essential for evaluating the genetic diversity of germplasm (Mignouna et al., 2005; Narzary et al., 2009). In this study, ISSR, ISAP and SRAP molecular markers were used to analyse the genetic relationship between *D. polystachya* and its related species, and high levels of genetic diversity among *Dioscorea* species were observed with the above parameters. Our results also indicted that combining the three markers detected a higher degree of variation among *Dioscorea* species compared with single marker as reported previously (Shiwachi et al., 2000; Malapa et al., 2005; Zhou et al., 2008).

It is interesting that there was a relatively high gene flow ($N_m = 0.3293$) among the 6 species involved in this study, which is much higher than previous studies ($N_m = 0.1081$) (Wu et al., 2014). Meanwhile, Gene differentiation coefficient ($G_{st} = 0.6029$) among species was lower than previously reported ($G_{st} = 0.8222$) (Wu et al., 2014). An important reason for this phenomenon might be that the experimental materials involved in this study including many wild individuals. Some studies have indicated that there was more genepool exchange in wild *Dioscorea* species than cultivated crops (Mengesha et al., 2013a; Mengesha et al., 2013b). In previous studies, the samples of *Dioscorea* species were almost cultivated crops. In fact, the main reproductive mode of cultivated *Dioscorea* crops is asexual reproduction, which limits the genepool exchange between species (Takayama et al., 2015). This might be the reason why there was a low gene flow in some *Dioscorea* genetic diversity studies. Furthermore, we also conducted genetic structuring analysis, which is typically assessed using the so-called F -statistics (Foll et al., 2008). The lowest intra-species similarity coefficient (F_{is}) was observed in *D. japonica*, which means that there were the highest degree of intraspecific differentiation and variation in this species. Therefore, it might be suitable for potential breeding materials of *Dioscorea*. The F_{is} value of *D. persimilis* was the highest, indicating that the gene purity in the species was high, and the species may rarely have sexual reproduction. On the other hand, the lowest inter-species variation (F_{st}) was observed in *D. polystachya*. This indicated that there was less reproductive isolation between *D. polystachya* with other *Dioscorea* species, and it was a good parent material for *Dioscorea*. The highest F_{st} value was obtained in *D. fordii*,

which indicating the highest differentiation between *D. fordii* and other five species. Combined with the results of phylogenetic tree, it could be induced that *D. fordii* was the ancestor of those *Dioscorea* species.

The genetic relationship of some edible species in Sect. Enantiophyllum Uline has always been controversial. For example, based on 18S sequence analysis, it was speculated that *D. persimilis* might be a cultivar of *D. polystachya* and should not be classified as an independent species (Liu et al., 2000). However, our result showed that all individuals of *D. persimilis* had a distant relationship with *D. polystachya*. At the same time, phylogenetic tree also showed all the individuals of the two species were located in different branches. The evidence in this study indicated that *D. persimilis* should be an independent species. Another taxonomic problem in *Dioscorea* is whether *D. alata* is a true species or a putative cultigen. Some studies indicated that *D. alata* might be a cultigen of *D. persimilis* (*D. hamiltonii* synonym.) due to the narrow genetic base (Hahn, 1991; Wilkin et al., 2007; Mengesha et al., 2013a). In fact, *D. alata* originates in North and East of Bay of Bengal, and it's found to be naturalized in China (Hang and Sun, 2020). Our results showed all the individuals of *D. alata* clustered together in the phylogenetic tree. It is indicated that *D. alata* might be a true species and not a putative cultigen. This phenomenon was also found in previous studies using isozymes analysis (Lebot et al., 1998). Another interesting phenomenon in this study was the genetic relationship between *D. exalata* and *D. persimilis*. The closest genetic relationship was observed between them. Furthermore, PCoA analysis could not separate the individuals of the two species. Therefore, further work will be required to establish the genetic relationship of the two species.

In this study, the phylogenetic tree and genetic similarity results in this study showed the genetic relationship between *D. japonica* and *D. polystachya* was the closest. Since Song Dynasty, *D. japonica* have been called 'Wild Yam' (Hang and Sun, 2020). The morphological characteristics of *D. japonica* is very similar to *D. polystachya*, and there was no reproductive isolation between them (Araki et al., 1983; Mizuki et al., 2010). Moreover, in pharmacological experiments, unlike other *Dioscorea* species, *D. japonica* and *D. polystachya* had the same efficacy (Hang, 1995). For subsequent breeding programs or conservation actions, we suggested they might be referred to as *D. polystachya*-*D. japonica* complex according to the classification of Guinea yam (*D. cayenensis*-*D. rotundata* complex) (Martin and Rhodes, 1978; Mengesha et al., 2013a; Mengesha et al., 2013b; Loko et al., 2015;)

Identification with SCAR makers.

Nowadays, the specie-identification of the yam with medicinal properties is still obscure. Therefore, there is an urgent need for the development of a fast and robust identification method to distinguish *D. polystachya* from its relative species. In this study, a specie-specific band was obtained from SRAP and developed to a SCAR marker. In order to examine its accuracy and sensitivity, the specie-specific SCAR marker was conducted by Double-Blind Test. The result showed that all the individuals of *D. polystachya* amplified the specie-specific band, which was absent in the other species, confirming the specificity of the SCAR marker for specie-specific identification. In short, a converted-SCAR technique proved to be a

helpful tool in identifying *Dioscorea* species. The results in this study indicated that PCR products can be used to covert SCAR marker and then used as a DNA barcoding for specie identification at a lower cost.

Declarations

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figures

Figure 1

Phylogenetic tree of *D. polystachya* and its 5 relatives (Each terminal node described by a corresponding code was given in Table 1).

Figure 2

Three-dimensional PCoA plot of all samples based on ISSR, SRAP and ISAP.

Figure 3

SRAP polymorphism in six *Dioscorea* species with primer SF3SR7 (The arrow showed the specific band of *D. Polystachya*). M indicates the marker. 1-6: *D. polystachya*, *D. persimilis*, *D. alata*, *D. japonica*, *D. exalata*, *D. fordii*.

5'-TTGACATAACGCTCTGATATCAAAATGATCATTGGGGGCGAGTTCGAATATTCCCCCTTCCCTG
 TCTCTTCATCCTTTTGTGCGCGGTTGGGTATATAATTATATATATGTGACTAAACAGAAGTCAATAAA
 AATGCAATAGGACACTTGATGAAAGTTATTGATGAACTGTGACTCAAGTGAAGATGTCTTTGGG
 CTTTCATTGATCTTCAAAGGTACAAAGGGAGTGAATGGGTGGATGGATGGAAAATGACTAGTCT
 GACAACAACCTTTTTGCCCTCAAATGATTGCCCAGTCAGTGAAACATCAATCACTAAATGAGA
GAAAGAGAGGAGAGAATGGGGCCACTTGTGCAAACATTGGCTTTGCATAAAAAGGATGAA
 TATATATGTGTGCTTGTGTCATGCTATGCTGTTGATGTTTGTACCGGCTGACCCCCCTATGG
 AATGGGTCCACCGTGCGCGCACTGAGCATGCCTGACATAACATGGTGTGGACATTGCAAA
 CCTATCGGGCTGGACGGCACAACGATGTTATATATTCCTGCTCTCTATTCTTCCCAATGC
 CTGCACTCTTTACCCAATCTCATCCTTTCTTTCTCTCTCTTTTCGATTGACTCCCACTGAGTG
 TGGTATGATTTGAGGGTTTAAAGTGGTTGGTGACTATTACTGCTGTATCACTTTGACCATT
 CGGTCCCTTAATGATTATGAATCCAAAAGAAAACACAAAATGTGTCACTTAACTCAGTAT
 TCATCAATAACTCTTTCAAGGGTCTGATTTGCACTAACTGACATCTGTGAACTCGTTATATA
 TGTGTATATACATACTGAGAGACTGATACTGGAAGAGCGAACAGAGTGGGATCCTTACACA
 CACTCACTTCTGATCGGTCTATGAGCCTCAGAGTGATATCTCAACATACCCACTGCCACAC
 CCAGAGTGACCCATATTTGGGGCCATATCCATCACCTCTCTCCCTTCACAACRAAAATGCGGC
 3'-TAAACCCGGTATAGGTAGTGGA-5'|
 AGGCTTCTCAGCTGACCCAACCTTTCTTTGCATGGTCCTTGGCTTTTGATACAGACTATACCTTACC
 TTCCTTTTCATCGTTCTTTCAAATCTGGGACTAGAGAATCCATTGCAGATCATGAAAGTGTTGTGC
 ATCATTTTCACCTTCCAGATGAACTGAAATGTTTCATTCTTGGCACTTTTGGAGTTGCTCCTTTC
 TTGCAGAGATCACATGGCTAAGTCTGGTCTTATTAC-3'

Figure 4

Sequence of the specific band of *D. polystachya* (the underlined parts were the primer sequences).

Figure 5

The SCAR marker validation on individuals of different species. A: *D. Polystachya*; B: *D. alata*; C: *D. persimilis*; D: *D. fordii*; E: *D. exalata*; F: *D. japonica*. M: marker (2000bp).

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