Application of ISSR markers in detection of genetic variation among Chinese yam (*Dioscorea opposita* Thunb) cultivars[☆]

Yanqing Zhou[*], Chune Zhou, Huanling Yao, Yanju Liu, Rongtao Tu

College of Life Sciences, *Henan Normal University*, *Xinxiang*, *Henan* 453007, *China*

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Abstract

Chinese Yam (*Dioscorea opposita* Thunb) is a kind of rare and famous Huai Chinese traditional medicine. ISSR markers were used to assess the genetic diversity and relationships of 28 cultivars of yam. Out of 44 ISSR primers detected, seven were selected for their reproducibility, reliability and high polymorphism. Seven ISSR primers amplified 65 bands with 54 (83.01%) polymorphic ones. The genetic diversity, estimated by Shannon' index, was 0.3191, revealing a quite high level of genetic variation in them. The dendrogram with the within-group linkage indicated that 28 cultivars could be divided into four main clusters: Cluster 1 (Ribenbai, Huashanyao and Ribenyuan), Cluster 2 (Xiaoyeshanyao), Cluster 3 (No.1 Songye) and Cluster 4 (other 23 cultivars). PCA (principal component analysis) was employed to evaluate the resolving power of markers to differentiate among them. The results suggested that these cultivars of Chinese yam have a valuable source of genes to be exploited, and provided a good method for evaluating genetic diversity of Chinese yam using ISSR markers and valuable information to assist parental selection in current and future yam breeding programmes. [Life Science Journal. 2008; 5(4): 6 – 12] (ISSN: 1097 – 8135).

Keywords: *Dioscorea opposita* Thunb; genetic diversity; ISSR; Shannon' index

1 Introduction

Yams (*Dioscorea* species) are perennial trailing rhizome plants, which belong to *Dioscoraceae* family. The crops have been used widely in traditional Chinese medicine to promote human health and have been provided for functional foods. They serve as an important staple food in many parts of the world[1]. However, the major food species of yams occur in three isolated centers: West Africa, Southeast Asia and tropical America, which are also considered areas for independent yam domestication, and represent considerable diversity[2]. Its tubers could be a source of energy, primarily, as their dry material predominantly consists of carbohydrates. Its tubers contain vitamin C, musin (glycoprotein), minerals (K, P, Ca, Mg, Fe, Cu, Co), phytosterols and steroidal saponins (furostanol and spirostanol glycosides), etc. Its tubers are processed into various types of food, including pounded yam, boiled yam, roasted or grilled yam, fried yam slices, yam balls, mashed yams, yam chips, and yam flakes. Fresh yam tubers are also peeled, chipped, dried, and milled into flour that is used to prepare a dough called amala or telibowo[3]. Furthermore, the crude protein, crude fat, crude fiber and ash contents of yams are in the range of 6.7% – 7.9%, 1.0% – 1.2%, 1.2% – 1.8%, and 2.8% – 3.8%, respectively.

There are more than 600 species of yam (*Dioscorea* spp.) in the world, 93 of which are found in China. Chinese yam (*Rhizoma dioscoreae*, *Dioscorea opposita*) has been used in traditional Chinese medicine for many years, to strengthen stomach function, alleviate anorexia, and cure diarrhea, and used as a delicious food in Chinese diets. Chinese yam is produced in Hebei, Shanxi and Shandong, with the best from Xinxiang county of Henan Province (containing Wenxian). It contains many chemical components such as mannan, allantoin,

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[*]Corresponding author. Tel: 86-373-3326340; Email: luckyqing2004@126.com

dopamine, batatasine, phytic acid, abscisin II, amino acids, glucoprotein, choline, ergosterol, campesterol, saponins, starch, non-starch polysaccharides, minerals (K, S, Ca, Mg, Fe, Zn, Cu, Mn) and so on[4]. Up to date, yam has become not only an international medicinal and edible crop but also important special vegetables that China exports. *Dioscorea opposita* Thunb from Xinxiang county of Henan Province is rare and known as one of the four famous Huai Chinese traditional medicines with some special traits such as heavy tubers, oily quality, less tendon, sweet soothing, high medicinal effect, boiling tolerance and so on. In yam cultivation and marketing, farmers and merchants prefer high and stable yield of marketable tubers with acceptable quality, i.e., dry matter content, cooking texture, taste, dormancy and rate of enzymatic browning[1]. Therefore, many studies have already been covered in various aspects of yam plants[5]. However, Breeding and selection of yam cultivars with novel or improved characteristics currently suffers from the fact that traditional cultivars have not been adequately characterized. Moreover, considerable linguistic variation exists in the nomenclature of yam cultivars, with each locality having its own unique series of names for the different cultivars. This seriously hampers the reliable identification of yam cultivars for germplasm management and improvement.

DNA fingerprinting has become an important tool for cultivar identification in plant breeding and for germplasm management. A number of different DNA molecular assays have been applied in yam, including RAPD[6–9], RFLP[10], SSR[8,9], AFLP[9,11] and 18S rRNA gene sequencing[12]. Nevertheless, there is no report on the use of inter-simple sequence repeats (ISSRs) to assess genetic relationships in yams. ISSR is a kind of simple and quick technique, permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple repeats, and possesses some advantages of stability and reproducibility, rich polymorphism, reliability[13–14], much larger numbers of fragments per primer and relatively low cost, have been widely used for DNA fingerprinting, population genetics and phylogenetic studies and so on in field crops, fruit trees and herbs[15] and was described as a powerful technique to assess genetic diversity[16–18,1] and to detect similarities between and within species levels[19]. The objectives of this study are to establish ISSR marker system, and to assess the level of genetic diversity among different cultivars of *Dioscorea opposita* Thunb, and to determine their genetic relationships in order to lay the foundation for the development of strategies for genetic analysis and crop improvement of Chinese yam.

2 Materials and Methods

2.1 Plant materials

The analysis was carried out on twenty-eight yam cultivars from Wenxian Institute of Agricultural Sciences, Henna Province, China, which are as follows: 1. Huashanyao; 2. Baiyu; 3. Ribenbai; 4. Shatanyuan; 5. Qinyuanyeshanyao; 6. Yeshanyao; 7. Shanxiyongji; 8. Hebeishanyao; 9. No. 3 Songye; 10. Zongbowen; 11. Jiangxinancheng; 12. No. 1 Songye; 13. Jiaxiangxichangmao; 14. Xiaoyeshanyao; 15. Baitie; 16. Taigunanyang; 17. Ribenyuan; 18. Tiegun; 19. Wenxishangguan; 20. No. 2 Yanye; 21. No. 2 Wenke; 22. D. Maoshanyao; 23. No. 47; 24. Jinanmi; 25. Huaishanyao; 26. Taiguqinyang; 27. No. 3 Wenke; 28. No. 1 Huaiqing.

2.2 DNA extraction

Total DNA was isolated from freeze-dried powdered young leaf tissue of yam cultivars according to the CTAB method[20] with minor modifications: if there was not (or little or gelatinous) DNA pellet in solution in DNA precipitation, (1) one mol/L ammonium acetate was added into the DNA solution at a final concentration of 10 mmol/L and mixed thoroughly, then added 1 – 2 volume isopropylalcohol; (2) stored for 15 min at room temperature after mixed thoroughly; (3) centrifuged for 10 min at 10000 rpm; (4) DNA pellet was resuspended in TE buffer (pH8.0); (5) repeat (3); (6) repeat (1) to (5) if DNA pellet was still gelatinous. After that, DNA was quantified by Beckman Du530 DNA/protein Analyzer and adjusted to 10 – 20 ng/μl for PCR amplification, and DNA quality and purity were evaluated by electrophoresis on 0.8% agarose gel and the ratio of A260/A280.

2.3 PCR-ISSR procedures

A total number of 44 ISSR primers, purchased from the University of British Columbia, were screened using a few DNA samples. PCR amplification was performed in a 9700 PE thermocycler (Applied Biosystems, Warrington, UK). An initial denaturation period of 5 min at 94 ºC was followed by 45 cycles of 60 s at 94 ºC, 45 s at 53 ºC, 90 s at 72 ºC, and then 7 min at 72 ºC for final extension. Reaction was carried out in a total volume of 25 μl containing about 60 ng of template DNA, 200 mM each of dATP, dTTP, dCTP and dGTP, 3.5 mM $MgCl_2$, 60 pg of primers, 2.5 μl 10 × Taq DNA polymerase buffer, 2% deionization formamide and 1.5 U Taq

DNA polymerase. To reduce the possibility of cross-contamination in the amplification reactions, a control reaction was used. It consisted of the reaction mixture excluding any DNA matrix. The amplification products was analyzed by electrophoresis on 1.7% agarose gel in 1 × TAE buffer (pH 8.3) and detected by ethidium bromide staining[15]. 200 bp DNA Ladder was used to determine the size of the ISSR fragments. From the preliminary screening, 17 primers that could amplify visible bands were selected for further examination. Different anneal temperature were examined to optimize the amplification condition for the 17 selected primers. Eventually, 7 ISSR primers that produced clear and reproducible bands were selected for the amplification of all samples. The gel was photographed with Polaroid MP-4 Land Camera.

2.4 Statistical analysis

Amplified products were scored as present (1) or absent (0) to form a binary matrix. The computer software SPSS 11.0 generated Jaccard's genetic similarity matrix with Jaccard method, drawed the dendrogram with the within-group linkage and performed a principal components analysis (PCA) using Quartimax with normalization. Shannon's information index (I), effective number of alleles (Ne) and the percentage of polymorphic loci were calculated by POPGENE32 software.

3 Results

3.1 ISSR polymorphism

Of the forty-four primers screened, seven ISSR primers which produced clear and reproducible bands were selected for amplifying 28 yam's cultivar's DNA samples (amplifying three times with each primer). A total of 65 fragments were amplified by seven primers, with an average of 9.29 bands per primer and the band size ranged from 50 bp to 850 bp, and 54 polymorphic bands were found. Out of these ISSR primers, gel electrophoresis pattern obtained using primers $GGA(GTG)_4$, $(CA)_8RG$ and $(AC)_8C$ were illustrated in Figure1, Figure 2 and Figure 3, respectively. The oligonucleotide sequences of these primers and the resultant multiple band patterns were summarized in Table 1.

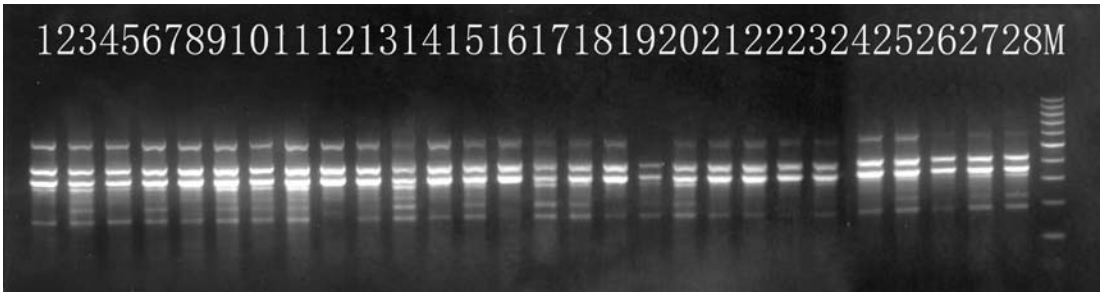

Figure 1. ISSR patterns of *Dioscorea opposita* Thunb generated by primer ISSR14. Lanes 1 – 28 and M stand for No. 1 – 28 yam cultivar shown in 2.1 plant materials and 200 bp ladder, respectively.

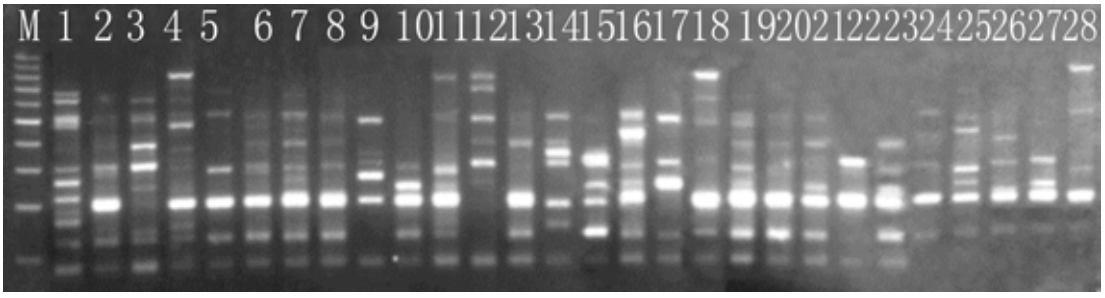

Figure 2. ISSR patterns of *Dioscorea opposita* Thunb generated by primer ISSR23. Lanes 1 – 28 and M stand for No. 1 – 28 yam cultivar shown in 2.1 plant materials and 200 bp ladder, respectively.

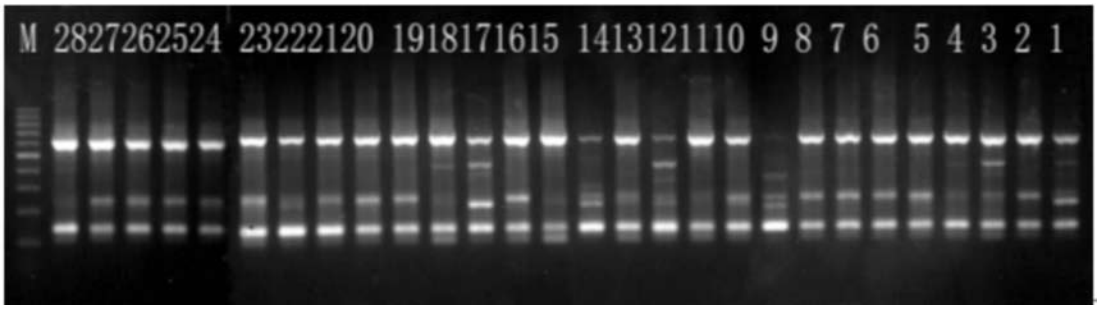

Figure 3. ISSR patterns of *Dioscorea opposita* Thunb generated by primer ISSR25. Lanes 1 – 28 and M: No. 1 – 28 yam cultivar shown in 2.1 plant materials and 200 bp ladder, respectively.

Table 1. ISSR primers used in this study and the parameters of genetic diversity

Sequence (5' – 3')	Annealing temperature (ºC)	Total bands and sizes (No. and bp)	Polymorphic bands (No.)	PPB (%)	Shannon index
$GSG(GT)_6$	55	11, 120 – 750	9	81.82	
$CCA(GTG)_4$	53	6, 190 – 600	4	66.67	
$GGA(GTG)_4$	50	7, 180 – 600	5	71.43	
$BDB(TCC)_5$	55	7, 180 – 500	5	71.43	
$(CA)_8RG$	55	17, 50 – 850	17	100	
$(AC)_8C$	50	8, 100 – 600	6	75	
$(AG)_8G$	50	9, 150 – 490	8	88.89	
Total		6, 550 – 850	54	83.01	0.3191

Note: S stands for G and C residues; B for C, G and T residues; D stands for A, G and T; R for A and G residues.

3.2 Genetic diversity of yam cultivars

The parameters of genetic diversity of the cultivars analyzed were listed in Table 1. The analysis of data showed that the percentage of polymorphic band (PPB) ranged from 66.67% to 100% with a mean value of 83.01% and 7.71 polymorphic bands per primer, and that the overall Shannon index is 0.3191. The PPB suggested that ISSR markers were polymorphic markers suitable to detect the genetic diversity of these cultivars of Chinese yam at the DNA level. The overall Shannon index was relatively high, indicating that a relatively great genetic diversity lies in the cultivars of Chinese yam.

3.3 Genetic relationships among yam cultivars

In order to estimate the genetic distances among these cultivars, the similar matrix was computed with Jaccard method. The obtained matrix (Table 2) showed that Jaccard's coefficient of similarity values (GS) ranging from 0.33 to 0.96, with a mean of 0.6246 revealing a high level of genetic diversity (GD) within these 28 cultivars. Because GD = 1 – GS, the smallest similarity value (0.33) suggested the high divergence between Huaishanyao and Xiaoyeshanyao, and the maximum similarity value (0.96) was scored between Jinanmi and Taiguqinyang indicating that both cultivars were the most similar. In addition, the standard deviation of 0.1499 detected that the yam cultivars exist higher polymorphic and richer genetic diversity. Figure 4 showed the dendrogram for 28 yam cultivars that revealed four main clustering clusters. Two monophyletic branches correspond to Cluster II: Xiaoyeshanyao and Cluster III: No. 1 Songye. The third cluster (Cluster I) included Ribenbai, Huashanyao and Ribenyuan. Cluster IV was composed of 23 cultivars and furthermore divided into two subgroups: one for No. 3 Songye and the other for the 22 remaining cultivars.

In addition, KMO test showed that the KMO value of 0.906 (generated from ISSR data) was bigger than 0.5 indicating the PCA (Figure 4) was applied to the data matrix. The percentage of variability revealed that the first three principal components absorbed 75.46 % of the total Initial Eigenvalues. Component 1, Component 2 and Component 3 explained 60.730%, 9.731%, 4.342% of Initial Eigenvalues, respectively. The graphic representation of the cultivars dispersion (Figure 4) showed the existence of an important diversity within analyzed cultivars (Figure 5). The derived clusters are similar to those identified in the dendrogram.

4 Discussion

There were a number of published reports on the use of other molecular marker techniques than ISSR markers to analyze *Dioscorea* spp. in the past as stated above. Because ISSR markers have a better reproducibility and a much greater number of total, polymorphic and discriminant fragments than RAPDs[21,22], and lower relative costs compared with RAPD[23], are of easier

Table 2. A similarity matrix among 28 yam cultivars using Jaccard Measure

Cult-ivar	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28
S1	1.000																											
S2	0.444	1.000																										
S3	0.750	0.524	1.000																									
S4	0.467	0.718	0.512	1.000																								
S5	0.488	0.824	0.538	0.658	1.000																							
S6	0.422	0.457	0.465	0.650	0.794	1.000																						
S7	0.447	0.816	0.523	0.591	0.757	0.838	1.000																					
S8	0.455	0.912	0.500	0.692	0.848	0.939	0.789	1.000																				
S9	0.421	0.447	0.432	0.400	0.500	0.500	0.415	0.500	1.000																			
S10	0.442	0.800	0.488	0.641	0.844	0.722	0.737	0.771	0.486	1.000																		
S11	0.422	0.757	0.465	0.692	0.694	0.778	0.659	0.829	0.543	0.676	1.000																	
S12	0.545	0.468	0.558	0.429	0.477	0.447	0.469	0.447	0.450	0.467	0.447	1.000																
S13	0.442	0.703	0.525	0.684	0.735	0.676	0.650	0.722	0.486	0.714	0.722	0.435	1.000															
S14	0.564	0.409	0.579	0.370	0.487	0.386	0.413	0.419	0.500	0.405	0.386	0.512	0.439	1.000														
S15	0.489	0.732	0.533	0.674	0.634	0.628	0.682	0.667	0.395	0.659	0.628	0.510	0.744	0.367	1.000													
S16	0.575	0.641	0.590	0.585	0.714	0.615	0.634	0.658	0.514	0.694	0.615	0.457	0.694	0.463	0.605	1.000												
S17	0.706	0.475	0.676	0.429	0.528	0.450	0.442	0.487	0.548	0.514	0.450	0.590	0.514	0.528	0.524	0.583	1.000											
S18	0.415	0.686	0.462	0.765	0.774	0.657	0.590	0.706	0.455	0.750	0.706	0.442	0.750	0.375	0.600	0.629	0.486	1.000										
S19	0.452	0.879	0.500	0.750	0.871	0.848	0.757	0.906	0.500	0.844	0.794	0.444	0.788	0.415	0.675	0.714	0.486	0.774	1.000									
S20	0.429	0.794	0.475	0.676	0.900	0.818	0.684	0.875	0.563	0.871	0.765	0.455	0.758	0.425	0.610	0.686	0.500	0.800	0.900	1.000								
S21	0.442	0.750	0.488	0.641	0.844	0.824	0.692	0.824	0.576	0.818	0.722	0.500	0.714	0.439	0.581	0.694	0.514	0.750	0.844	0.933	1.000							
S22	0.375	0.647	0.421	0.541	0.733	0.667	0.595	0.667	0.552	0.656	0.571	0.439	0.606	0.405	0.564	0.588	0.441	0.633	0.677	0.700	0.656	1.000						
S23	0.381	0.735	0.425	0.579	0.774	0.758	0.722	0.706	0.500	0.806	0.611	0.442	0.647	0.375	0.561	0.629	0.444	0.677	0.774	0.800	0.806	0.750	1.000					
S24	0.372	0.765	0.415	0.605	0.806	0.735	0.703	0.735	0.531	0.839	0.639	0.465	0.676	0.366	0.585	0.611	0.472	0.710	0.806	0.833	0.781	0.724	0.893	1.000				
S25	0.432	0.730	0.409	0.625	0.714	0.703	0.718	0.703	0.432	0.794	0.615	0.396	0.649	0.333	0.605	0.722	0.425	0.629	0.765	0.735	0.694	0.636	0.781	0.813	1.000			
S26	0.381	0.788	0.425	0.622	0.833	0.758	0.722	0.758	0.500	0.867	0.657	0.442	0.697	0.375	0.600	0.629	0.444	0.733	0.833	0.862	0.806	0.750	0.926	0.963	0.839	1.000		
S27	0.419	0.824	0.429	0.615	0.813	0.794	0.757	0.794	0.457	0.788	0.649	0.444	0.686	0.381	0.634	0.667	0.447	0.667	0.813	0.781	0.735	0.733	0.833	0.867	0.875	0.897	1.000	
S28	0.357	0.657	0.400	0.686	0.688	0.676	0.605	0.676	0.516	0.667	0.676	0.452	0.719	0.350	0.575	0.600	0.457	0.759	0.742	0.710	0.667	0.714	0.700	0.793	0.697	0.759	0.742	1.000

detection and at lower costs than AFLPs[16], and simpler to use than the SSR technique[24,25] and less restrictive than RFLPs, and may offer considerable variation among species[26], ISSR markers are increasingly applied since 2000[16,17,27,28]. Therefore, ISSR technique was employed in the present study. To our knowledge this is the first report on the assessment of genetic variation in this medicinal plant.

Evaluation and identification of germplasms using ISSR markers are playing an important role in studies of genetics and breeding. In this paper, seven ISSR primers were used for fingerprinting and estimating genetic diversity of yam cultivars. Using these primers, 65 discernible DNA fragments were generated with 54 polymorphic ones. The present study revealed quite high polymorphism (83%) in yam cultivars based on the statistical data. The high percentage of polymorphism is common for ISSR amplified products. Other workers obtained similar results in olive (Latin No) and other plants: 100%[29], 90%[30], 93%[31], 96%[32], 62.2%[33] and 77.4%[34].The high level of polymorphism of ISSR technique suggests that inter simple sequence repeat amplification techniques is a useful and potentially powerful technique for genotypic studies in yams. Hamrick *et al*[35] suggested, several factors are important level. Geographic range is strongly associated with in determining levels of genetic diversity at the species the

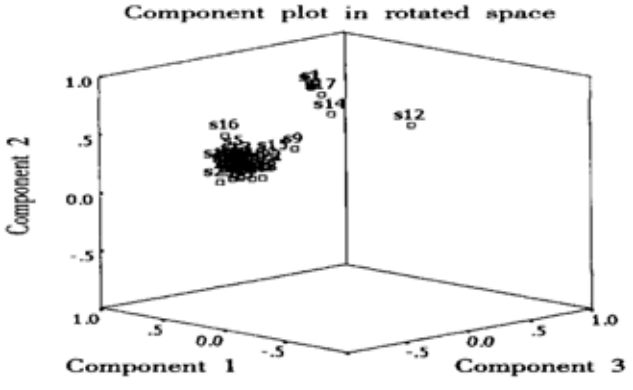

Figure 4. Associations among 28 yam cultivars of *Discorea opposita* Thunb revealed by PCA using ISSR markers and Quartimax with Kaiser normalization. S1 – 28 is the same as No. 1 – 28 shown in 1.1 materials.

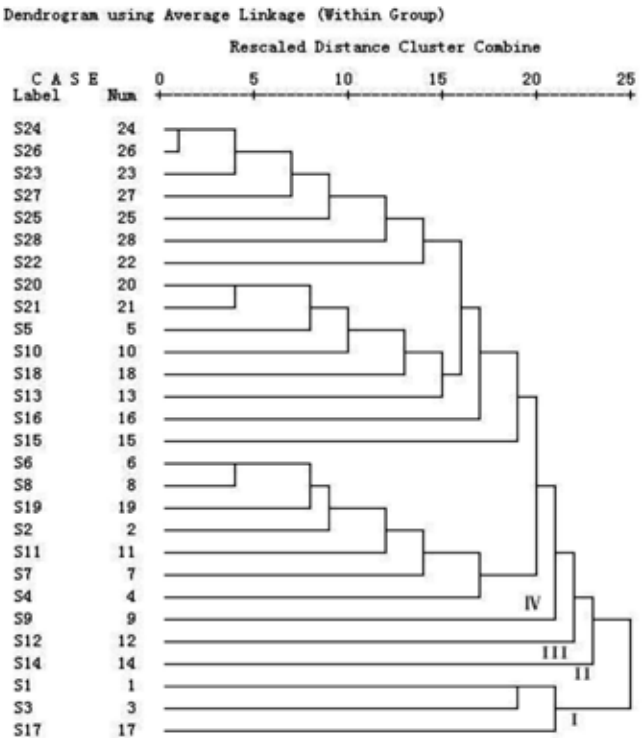

Figure 5. Dendrogram of the relationship among 28 yam cultivars of *Dioscorea opposita* Thunb. S1 – 28 was the same as No. 1 – 28 shown in 1.1 materials.

level of variation maintained at the species level. Generally endemic species have lower genetic diversity than widespread species. Other factors such as breeding systems, vegetative reproduction, dispersal pattern, sample size, etc., also significantly influence the genetic diversity of a species. The level of genetic diversity in the cultivars of Chinese yam in our study (Shannon index, I = 0.3191) is higher than the average values for long-lived perennial herbs (0.116) and widespread distributed species (0.202)[36]. On the other hand, the dendrogram consisted of four clusters. Cluster II and Cluster III were two monophyletic branches (Xiaoyeshanyao and No. 1 Songye), indicating the bigger differences from each other and 26 remaining cultivars. Cluster I included three ones such as Ribenbai, Huashanyao and Ribenyuan, divided into two subgroups and revealing the different relations to each other. Cluster IV was composed of 23 cultivars and furthermore divided into two subgroups: one for No. 3 Songye and the other for the 22 remaining cultivars. It was due to possible association with their parents for crossing.

5 Conclusion

The results provided an optimized method for evaluating the genetic diversity of Chinese yam using ISSR markers which was useful for further investigation. ISSR markers can be successfully employed to assess the level of polymorphism and diversity in yam cultivars. The great genetic diversity found among yam cultivars supports the idea that Wenxian Institute of Agricultural Sciences has a valuable source of yam genes to be exploited and provides valuable information to assist parental selection in current and future yam breeding programmes.

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