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Genetic characterization of Gac (*Momordica cochinchinesis*) accessions in Southern Vietnam by ISSR markers

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Abstract. *Ho VT, Le HT, Nguyen TA. 2019. Genetic characterization of Gac (*Momordica cochinchinesis*) accessions in Southern Vietnam by ISSR markers. Biodiversitas 20: 387-392.* Gac (*Momordica cochinchinesis*) plays important roles in Vietnamese cuisine and medicine. It has high nutrient and medicinal values. Demand of gac fruit as ingredient for food processing and raw material for health industry requires accelerated gac cultivation. However, gac quality is variable due to the uncertain available germplasm and its management as breeding stock. Identification of gac cultivars and landraces in Vietnam is mainly based on personal experience relying on morphological traits, thus the conservation and breeding have low impact on productivity and quality of product. Recently, the development of molecular markers has been useful in identifying specific plant(s). In this study, genetic richness and relativeness of 14 gac accessions collected from different provinces in Southern Vietnam were evaluated by using 10 Inter-Simple Sequence Repeats (ISSR) markers. Results revealed large variation in genetic background of studied gac accessions. Seven DNA-based markers with potential to differentiate gac genotypes were recorded. The results provide molecular biological information for classification, identification plant origins, breeding and conserving programs of gac in Vietnam.

Keywords Gac, genetic diversity, ISSR, molecular markers, *Momordica cochinchinesis*

INTRODUCTION

Gac (*Momordica cochinchinesis*) is a herbaceous plant popularly grown in Vietnam. Fruits of this plant are used as material in food processing (Ishida et al. 2004). Glutinous rice cooked with gac fruit (xoi gac) is popular and favorite dish which is used on wedding, birthday and New Year parties. Young gac fruits and shoots are also used as food materials in Thai cuisine (Kubola and Siriamornpun 2011). Gac fruits are important ingredient in traditional medicine. Studies have revealed the high-value compounds, such as lycopene and β-carotene (Vuong et al. 2002), vitamin E (Vuong and King 2003), and high amount of antioxidant with good bioavailability (Burke et al. 2005) in the gac fruits. The gac fruits are valuable for anti-inflammatory (Kha et al. 2012) and anticancer activities (Tien et al. 2005; Zheng et al. 2014), reducing cardiovascular disease (Bazzano et al. 2002). Gac seeds are also important in Vietnam and Chinese medicine (Kubola and Siriamornpun 2011). In order to meet the increasing demand of the market as raw materials for cooking, natural colorant industry, food additive, and functional food, the growing area of gac in Vietnam are expanding lately. However, there is not adequate research on this species due to lack of information on genetic characteristics (Nguyen et al. 2014, Pham et al. 2017).

In recent years, scientists have been approaching closer to adopt DNA markers in evaluating genetic potential as well as the selection of gac in Vietnam. In the central region, Nguyen and her colleagues used molecular biology techniques to select gac genotypes (Nguyen et al. 2014). More recently, Pham and colleagues also assessed the genetic diversity of the gac collection from the Mekong Delta (Pham et al. 2017). However, these two studies mainly employed RAPD (Random Amplification of Polymorphic DNA) technique which is a simple, easy to implement technique and does not require genetic information for the subject. Nevertheless, this marker shows a low repeatability score and results in a high degree of reliance on reactive conditions such as DNA concentrations, the concentration of the components in the PCR reaction, the number of cycles of the reaction (Williams et al. 1990; Mbwana et al. 2006).

Recently, the Inter-Simple Sequence Repeat (ISSR) technique has been focused on the genetic diversity of many plant species such as rice, wheat, millet, grapes, sweet potatoes, apples and *Momordica dioica* (Reddy et al. 2002; Singh et al. 2015; Choudhary et al. 201; Shukla et al. 2017). Because it could eliminate RAPD limitations, leading the expansion of using ISSR in genetic diversity research, population genetic studies, genetic markers, genetic markers, crop identification, source analysis, identification, genetic change identification, and cross-breeding (Nguyen 2014). In this study, total of 10 ISSR primers was used to evaluate the genetic diversity of 14 gac samples in Southern provinces of Vietnam. The obtained results could provide scientific information for identification, classification, propagation, and production of gac in the area.

MATERIALS AND METHODS

Sample collection

Total of 14 gac genotypes was collected from different provinces in Southern Vietnam (Figure 1 and Table 1). After harvesting, leaf samples were dried in silica gel and stored until use.

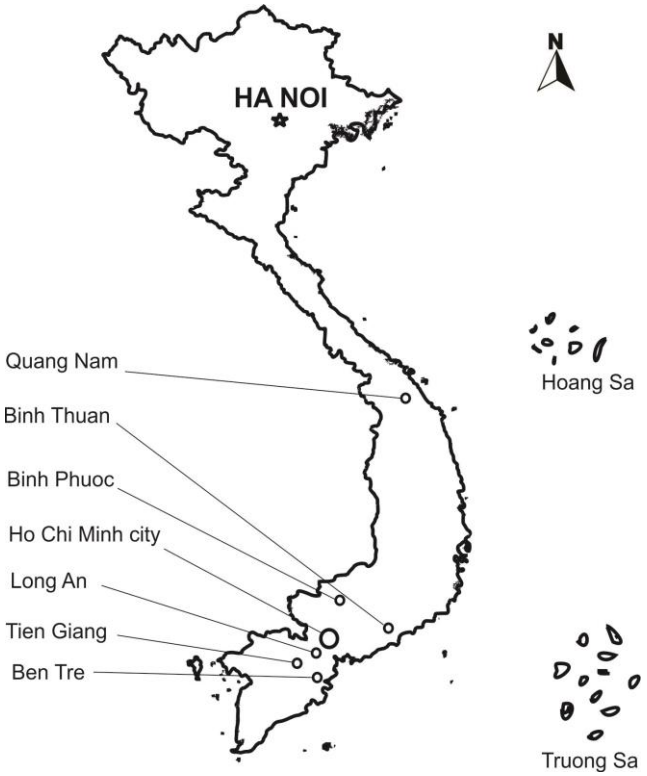

Figure 1. Targeted areas for collecting gac genotypes in Southern Vietnam

Table 1. Gac samples collected for genetic characterization in the present study

No.	Collected location	Sample code
1	Chau Thanh, Tien Giang province	G1
2	Cai Be, Tien Giang province	G2
3	Cai Be, Tien Giang province	G3
4	Bu Dang, Binh Phuoc province	G4
5	Bu Dang, Binh Phuoc province	G5
6	Quang Nam province	G6
7	Mo Cay, Ben Tre province	G7
8	Ham Thuan Nam, Binh Thuan province	G8
9	Thang Binh, Quang Nam province	G9
10	Thang Binh, Quang Nam province	G10
11	Ho Chi Minh city	G11
12	Ben Tre province	G12
13	Duc Hoa, Long An province	G13
14	Ben Tre province	G14

DNA extraction

Total DNA was extracted from dried gac leaves as follows: 0.1 g of gac leaves was ground into fine powder with liquid nitrogen using porcelain pestle and added 1 mL of extraction buffer containing sodium bisulfite 4%. The samples were then transferred to a 2 mL Eppendorf tube and well vortexed, continued adding 250 µL of 10% SDS solution and incubated at 65°C for 1 hour. Then, amount of 600 µL of chloroform: isoamyl alcohol (ratio 24: 1) was added and lightly converted to mix for 1 minute. The sample was next centrifuged 12,000 rpm at 4°C for 10 minutes. The supernatant solution was transferred into a new 2 mL Eppendorf tube. A 1: 1 volume of isopropanol was added, refrigerated for 1 hour at -20°C and centrifuged at 12,000 rpm at 4 °C for 10 minutes. Next, the upper fluid was removed and the pellet was harvested. Amount of 300 µL of 70% ethanol was used to clean the pellet by centrifuging at 8,000 rpm in 4°C for 5 minutes. Ethanol was removed and pellet was dried in air. Finally, 100 µL TE 1X was added and DNA quality was then tested by electrophoresis on 1% agarose gel in TAE 1X buffer and stained with Gelred dye (Biotium, USA). The result was observed under ultraviolet light by Quantum-ST4 3000 gel reader (Montreal-Biotech, Canada). DNA concentrations were determined by spectrophotometer (Optima SP 3000 nano UV-VIS, Japan) and the DNA sample was stored at-20 °C until use.

ISSR reaction

In this study, total of 10 ISSR primers was used to study genetic diversity of gac genotypes (Behera et al. 2007; Shukla et al. 2017). The sequences of primers are shown in Table 2.

The composition of PCR reactions were performed as follows: 7.5 µL 2X Mytaq Red Mix (Bioline, UK), 30 ng DNA, 0.2 µM primer and PCR for final volume of 15 µL. The reaction conditions were as follows: initial denaturation at 95°C for 2 minutes; then 35 cycles of 30 seconds at 95°C, 30 seconds at 35°C, and 54 seconds at 72°C and 5 min finish at 72ºC with the SureCycler 8800 Thermal Cycler (Agilent, USA). PCR amplification was then separated by electrophoresis in 1.5% agarose gel in 1X TAE buffer, and stained with 0.5 µg/mL Gelred TM loading buffer then visualized under ultraviolet light.

Table 2. List of 10 ISSR primers to analyze genetic diversity of 14 gac genotypes in Southern Vietnam

Primer	Primer sequence
UBC880	GGAGAGGAGAGGAGA
UBC825	ACACACACACACACACT
UBC841	GAGAGAGAGAGAGAGACTC
UBC855	ACACACACACACACACCTT
UBC813	CTCTCTCTCTCTCTCTT
UBC853	TCTCTCTCTCTCTCTCRT
UBC809	AGAGAGAGAGAGAGAGG
UBC814	CTCTCTCTCTCTCTCTA
UBC811	GAGAGAGAGAGAGAGAC
UBC810	GAGAGAGAGAGAGAGAT

Data analysis

After electrophoresis of PCR reactions, clear amplification bands were used in the analysis. Only reproducible bands in two PCR replicates were considered for analyzed, the weak signal bands were excluded from final analysis. Since ISSR is a dominant marker, at each locus, the presence of amplified band was interpreted as either a heterozygote or dominant homozygote and the absence of a band in corresponding position as recessive homozygote (Debnath et al. 2008). Clearly visible ISSR amplified bands were scored as 1, whereas the absent band was scored as 0. The numbers of scored bands (SB), number of polymorphic bands (NPB) and percentage of polymorphic bands (PPB) were obtained. The quality information of the primers is determined by the PIC (Polymorphism Information Content) according to the formula of Chesnokov and Artemyeva (2015).

$$PIC_j = 1 - \sum_{i=1}^{n} Pi^2,$$

Where, i is the i th line of the primer j, n is the number of bands of the primer j, P is the frequency of the band.

The ability of primers to differentiate between genotypes was evaluated by their resolving power (RP) value as describe by Prevost and Wilkinon (1999).

$$RP = \sum_{i=1}^{n} BIi,$$

Where, n is the NPB of that primer and BI_i (Informativeness of a band) =1- (2x|0.5-*p*|), p is the proportion of the 14 gac genotypes containing the band, IB value was calculated for 10 ISSR primers.

Phylogeny is built based on the Unweighted Pair Group Method with the Arithmetic mean (UPGMA) and the algorithm with the SAHN module in NTSYSpc 2.1 (Rohlf 2000). Principal Coordinate Analysis (PCO) was performed based on RAPD data to have better understanding about similarity among accessions by using PCO package in NTSYS-pc 2.1 (Ibrahim et al. 2017).

RESULTS AND DISCUSSION

Totally, 14 gac genotypes were characterized with 10 ISSR primers. The obtained results showed that the bands appeared clearly on 1.5% agarose gel (Figure 2, Figure 3, and Figure 4). The tested primers generated from 3 to 12 amplifications per reaction and the length of amplification varies approximately from 150 to 2,000 bp. Our results are consistent with study of Pham and colleagues in 2017, where the genetic correlation of 20 gac samples was characterized by using 10 RAPD primers and resulting 126 bands; however the amplified bands in this study is relatively higher than study of Choudhary and colleagues when they used ISSR to evaluate the genetic homogeneity of different *Momordica dioica* plants with 2-4 bands per primer (Pham et al. 2017; Choudhary et al. 2017). Among those, there were 114 polymorph bands, and an average of 11.4 bands per primer. We also found that all primers were suitable for the study of gac through the number of amplified and high polymorphic bands as well as other parameters of primers (Table 3). All primers showed high PIC values from 0.60 to 0.90. This means that all of these primers are suitable for studying genetic diversity in gac since according to Botstein et al. (1980): Primer for very high PIC if ≥ 0.5; Primer for relative information if 0.5> PIC ≥ 0.25 and little information if PIC <0.25. The resolving power (RP) index was also to assess the ability to differentiate gac genotypes. The RP value varied from 4.57 for primer UBC855 to 8.57 for primer UBC813. In general, the primers showing higher RP value could distinguish more genotypes and shower higher polymorphic bands (Debnath et al. 2008).

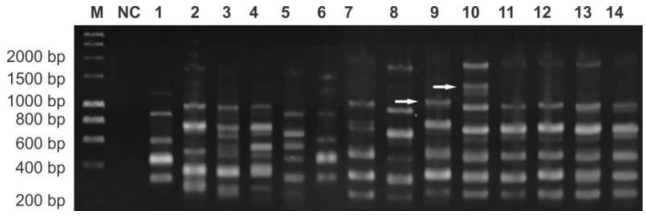

Figure 2. Representative ISSR result with UBC811 primer. (The arrow shows the candidate amplification marker to distinguish specific gac genotype. The number is corresponding to sample number in Table 1; M: DNA marker; NC: Negative control without DNA)

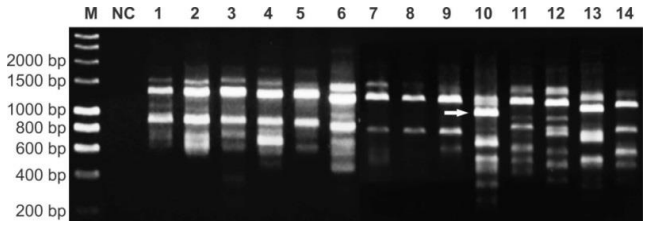

Figure 3. Representative ISSR result with UBC853 primer. (The arrow shows the candidate amplification marker to distinguish specific gac genotype. The number is corresponding to sample number in Table 1; M: DNA marker; NC: Negative control without DNA)

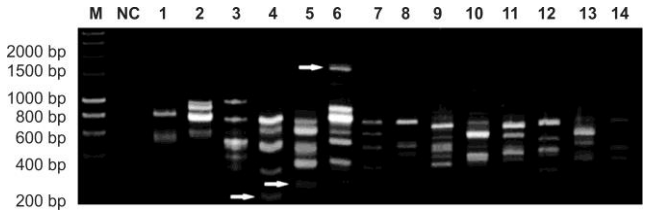

Figure 4. Representative ISSR result with UBC841 primer. (The arrow shows the candidate amplification marker to distinguish specific gac genotype. The number is corresponding to sample number in Table 1; M: DNA marker; NC: Negative control without DNA)

The ability to create multiple polymorphisms of ISSR primers is important in the analysis of genetic diversity as they provide a large amount of information on the genetic structure of the analytical individuals. Out of a total of 126 amplified DNA bands, up to 66 polymorphic DNA bands accounted for 66.2% with an average of 6.6 polymorphic bands appearing on each primer. This finding is consistent with previous study of Rahimi and colleagues when using the same ISSR primer set and found the polymorphism of 69.91% in different ecotypes of sand plantain (Rahimi et al. 2017). This data also demonstrates that the primers used in the study are capable of amplifying in highly genetically distinct regions between gac samples. However, the obtained polymorphism of 66.2% in this study is lower in comparison to previous studies conducted by RAPD markers: such as 90.4% in Pham et al. (2017), 99.43% in Bootprom et al. (2012) and 100% in study conducted by ISSR markers (Wimalasiri et al. 2016). The lower polymorphism of ISSR makers in the comparison with RAPD markers was also previously reported by Indian research group when they characterized genetic composition of *Solanum* L. species (Anil Kumar et al. 2018).

The combination of amplified bands in this study demonstrates the high potential of ISSR primers in identifying gac genotypes. Based on obtained ISSR data, specific gac genotypes could be discriminated by using specific amplified band from either single or combined different primers. For example, the UBC811 primer is capable of detecting samples of gac 9 and 10 with DNA bands of 1,000 bp and 1,500 bp respectively (Figure 2). Gac 10 is distinguished by a 1,200 bp DNA band using the UBC853 primer (Figure 3). For UBC841 primer, there are 3 bands in which the 200 bp band identifies gac 4, 300 bp band identifies gac 5 samples and 1,700 bp band for sample identification gac 6 (Figure 4). The total numbers of DNA bands that can be used to distinguish 14 gac samples in this study are presented in Table 4. This data emphasizes the potential of ISSR markers to identify specific organism and which have been reported in several studies such as distinguish *Eucalyptus* species (Balasaravanan et al. 2005); the invasive of lionfish (Labastida et al. 2015); sex in *Phoenix dactylifera* (Al-Ameri et al. 2016).

To calculate the genetic correlation coefficient and to construct the phylogenetic map, the ISSR results were encoded, and the phylogenetic tree was constructed using NTSYSpc version 2.1 software with the UPGMA clustering method in the SAHN program. The results showed that there was a significant variation in the coefficient of similarity between the samples: the highest correlation coefficient in the sample pair G12-G13 (0.80) and the lowest sample pair G05-G10 (0.45) (Table 5).

Table 3. Characteristics of DNA profiles generated in 14 gac genotypes by using10 ISSR primers

Primer	SB	NPB	PPB (%)	PIC	RP
UBC880	12	9	75.0	0.89	5.29
UBC825	11	7	70.0	0.90	6.29
UBC841	14	6	54.5	0.87	6.57
UBC855	9	5	55.6	0.72	4.57
UBC813	15	8	88.9	0.87	8.57
UBC853	11	7	58.3	0.77	7.57
UBC809	12	6	75.0	0.79	6.00
UBC814	15	7	63.6	0.71	6.43
UBC811	13	5	50,0	0.89	7.86
UBC810	14	6	54.5	0.60	7.00
Sum	126	66	-	-	-
Average	12.6	6.6	66.2	0.80	6.61

Note: SB: Scored bands; NPB: number of polymorphic bands; PPB: percentage of polymorphic bands; PIC: polymorphism information content; RP: resolving power.

Table 4. The ability to combine ISSR primers to identify gac samples

STT	Primer	Sample	Approximate size of candidate amplification (bp)
1	UBC841	G04	200
2	UBC841	G05	300
3	UBC841	G06	1.700
4	UBC811	G09	1.000
5	UBC811	G10	1.500
6	UBC853	G10	1.200
7	UBC814	G11	400

Table 5. Simple matching coefficients of similarity among 14 gac genotypes

	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14
G01	1.00													
G02	0.54	1.00												
G03	0.66	0.74	1.00											
G04	0.63	0.74	0.71	1.00										
G05	0.54	0.56	0.52	0.63	1.00									
G06	0.66	0.61	0.57	0.67	0.56	1.00								
G07	0.51	0.67	0.75	0.67	0.56	0.56	1.00							
G08	0.54	0.63	0.66	0.67	0.57	0.55	0.67	1.00						
G09	0.56	0.63	0.66	0.69	0.54	0.61	0.67	0.75	1.00					
G10	0.52	0.56	0.56	0.56	0.46	0.52	0.52	0.62	0.59	1.00				
G11	0.56	0.67	0.71	0.67	0.50	0.56	0.67	0.74	0.74	0.64	1.00			
G12	0.45	0.55	0.68	0.60	0.52	0.49	0.67	0.69	0.63	0.58	0.63	1.00		
G13	0.45	0.64	0.70	0.62	0.53	0.52	0.71	0.67	0.58	0.58	0.71	0.79	1.00	
G14	0.58	0.60	0.68	0.70	0.58	0.59	0.66	0.64	0.69	0.56	0.63	0.65	0.62	1.00

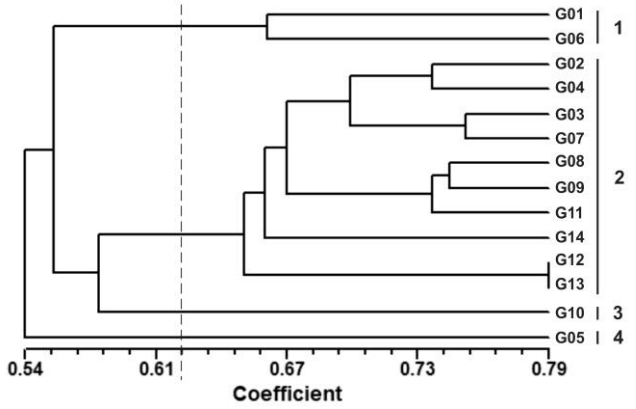

Figure 5. Phylogenetic tree generated by using 10 ISSR markers to show the genetic relativeness of 14 gac accessions collected in Southern Vietnam. The scale shown at the bottom is the measure of genetic similarity

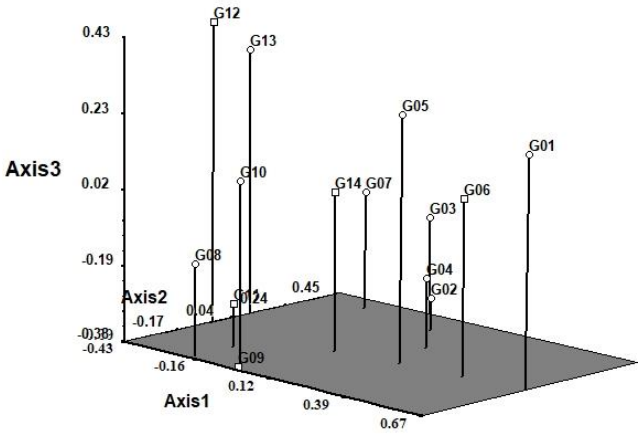

Figure 6. Three-dimensional plot of the principal coordinate (PCO) analysis of distance among 14 gac genotypes designated by codes given in Table 1 by using 10 ISSR markers

Based on the similarity matrix of 14 gac genotypes in Table 5, the phylogenetic tree was constructed and shown in Figure 5. Overall, the constructed phylogenetic tree shows a homology coefficient of studies gac genotypes ranging about 0.54 to 0.79 indicating the high level of genetic variation among genotypes studied. This result is similar to earlier study of Wimalasiri and colleagues when they studied the genetic diversity of different gac accession collected from Vietnam, Thailand, and Australia (Wimalasiri et al. 2016). The cut-off value of the phylogenetic tree was determined at 0.62 based on calculation method described by Jamshidi and Jamshidi (2011). Based on cut-off value, fourteen gac genotypes were divided into four major groups: Group 1, consisting of two samples: G01 (Chau Thanh, Tien Giang) and G06 (Thang Binh, Quang Nam). Group 2 occupied the majority with 10 samples including G02, G03 (Cai Be, Tien Giang), G07 (Mo Cay, Ben Tre), G12, G14 (Chau Thanh, Ben Tre), G08 (Ham Thuan Nam, Binh Thuan), G11 (Cu Chi, Ho Chi Minh City), G13 (Duc Hoa, Long An), G04 (Bu Dang, Binh Phuoc), G09 (Thang Binh, Quang Nam). Meanwhile, group 3 and group 4 has only one accession in each group namely G10 (Thang Binh, Quang Nam) and G05 (Bu Dang, Binh Phuoc), respectively. Interestingly, G05 genotype was collected from Binh Phuoc province together with G04 genotypes but belonging to different groups. G05 genotype also shows the most genetic distant with the remaining 13 samples. This data revealed that the studied gac accessions were not grouped as geographical location where samples were collected. This could be due to the exchange of gac seeds or seedlings from different places which were mentioned by Pham et al. (2017). In addition, the introduction of gac varieties from neighboring countries such as Taiwan, Malaysia also contributes to the genetic difference of the gac varieties increase. Furthermore, the high diversity of collected samples in Vietnam could be owing to different reasons such as out-crossing system, the geographical difference. Different studies also reported the high genetic diversity could cause by the adaptation of plants to specific geographical areas such as wild barley (Dawson et al. 1993); chili (Albrecht et al. 2012); olive (Mousavi et al. 2014). The high genetic diversity of gac accessions collected in Southern Vietnam implies the diverse gene pool in this region. This genetic richness will be useful for gac breeding program in the future.

For better understanding about the grouping of 14 gac genotypes, the principal coordinate analysis was further used to confirm the distribution frequencies of ISSR markers and shown in Figure 6. The plotting present 14.4%, 10.3% and 9.8% of the total variation for the first, second and third component, respectively. This data suggested that the variation was due to characteristics of specific genotype (Debnath et al. 2008). The highest similarity of G12 and G13 genotypes in Figure 5 is also confirmed where they are closely grouped by first axis. Similarly, group 1 consisting G01 and G06 genotypes are distributed together. However, the distribution of G05 genotype is relative changed, which was relocated and near to G02, G04, G06, G07, and G14 genotypes. Thus, the PCO analysis could reveal another level of separation of sample, and it could be benefit to use more than one types of analysis method.

In this study, by using 10 ISSR primers to analyze 14 gac samples collected in different provinces in Southern Vietnam we found that there is large genetic variation among studied genotypes. The collected samples were divided in 4 main groups showing the close relativeness of accessions in different places. We also found significant number of specific ISSR generated bands which could be further analyzed to use as specific markers to identify 14 specific gac accessions. The obtained results in this study will be important information which will be useful for plant breeders to use for several purposes such as classification, conservation, and gac breeding programs in Vietnam. In the future, the DNA bands could be continued to develop into SCAR markers to enhance the accuracy in breeding and conservation of gac in the research region.

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