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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2021 18(2): 1182-1188. **OPEN ACCESS**

Utilization of ISSR markers to investigate genetic diversity of *Curculigo orchioides* Gaertn. Population in Vietnam

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Curculigo orchioides Gaertn. is a rare and economical medicinal herb used to treat many diseases. Currently, large efforts to protect and sustainably develop this plant are being implemented. However, traditional identification based on plant morphology is low accuracy leading the limited effectiveness of conservation and exploitation programs. In this study, a total of 15 ISSR molecular markers were used to evaluate genetic structure of 14 accessions of *C. orchioides* collected in the northern provinces of Vietnam. Obtained results revealed the large variation in genetic structure of the studied population. The information of this study can be applied to classify and develop into specialized indicators to classify *C. orchioides* for the identification, conservation and development of this plant.

Keywords: *Curculigo orchioides*; Genetic diversity; ISSR; Molecular markers,

INTRODUCTION

Curculigo orchioides Gaertn. is a flowering plant in the Hypoxidaceae family which was first studied by Gaernt in 1788. It is a humid and shade tolerant plant, usually growing on relatively fertile soil in valleys, limestone foothills or upland fields. This plant is found in subtropical Asian regions including China, Laos, Malaysia, Thailand, the Philippines, and India. In Vietnam, *C. orchioides* is scattered in mountainous provinces, from Lai Chau, Tuyen Quang, Cao Bang to the Central Highlands. As important herb of traditional medicine, *C. orchioides* is commonly used to treat impotence, limbs, arthritis and knee arthritis, diarrhoea, and sex stimulant (Nie et al. 2013; Chaturvedi and Briganza, 2016). Experiments show that the ethanol extract of *C. orchioides* has adaptive effects such as enhancing tolerance to high temperatures and hypoxia, it also has sedative, anticonvulsant and androgenic, besides increases the immune activity of the mouse (Chen et al. 1989). The various medicinal compounds such as mucus, phenolic glycosides, saponins and were found in this plant (Chauhan et al. 2010). *C. orchioides* is effective against cyclophosphamide-induced toxicity because of its antioxidant properties and regulation of inflammatory cytokine levels (Murali and Kuttan, 2016) and high potential for liver protection, anti-osteoporosis, antioxidant and anti-cancer (Wang et al. 2017).

Traditionally, *C. orchioides* is identified mostly based on plant features such as leaf and root morphology; root colour. This method is simple and cost effective but low accuracy since the characteristics are highly influenced by environmental conditions and developmental stages of plant (Siew et al. 2018; Angeliena et al. 2019). Recently, Inter-Simple sequence Repeat (ISSR) molecular marker has been intensively applied for characterizing genetic composition and identifying plant origin. This marker have been proven as a potential substitution for morphological identification with several

advantages, such as unlimited in number, unaffected by environment and growing conditions, easy to interpret with reliable repeatable results. Furthermore, this is PCR-based markers and possess several advantages such as simple, require small DNA quantity, high number of fragments in each reaction, and do not require prior knowledge of genetic genome of targeted plants (Wolfe and Liston, 1998) leading to expanded use in genetic diversity research, population genetic studies, genetic markers, crop identification, breeding in different medicinal herbs such as *Ziziphus spina-christi* (Alansi et al. 2016); *Oroxylum indicum* (Rajasekharan et al. 2017); *Cassia auriculata* (Panapitiya and Welikala, 2018). ISSR have also been utilized for genetic studies of *C. orchiodes* in several countries such as China (Li et al. 2012), India (Alagar et al. 2014).

In Vietnam, due to overexploitation *C. orchioides* population is in the danger of extinction in the wild. Therefore, it was included in the Red Book of Vietnam and the Red List of medicinal plants. Numerous studies have been done to propagate this medicinal plant using tissue culture methods. (Vo et al. 2011; Nguyen et al. 2018). However, the selection of starting materials for propagation is still based on personal experience based on morphological characteristics, which can lead to the risk of choosing the starting materials with variable genetic background. Consequently, it could negatively affect to herbal quality as well as the sustainable development of this plant.

In present study, a total of 15 ISSR primers were used to evaluate the genetic relatedness of 14 *C. orchioidieshis* genotypes collected from different provinces in Northern Vietnam. The obtained results could provide scientific information for identification, classification, and propagation purposes of this valuable herbal plant in Vietnam.

MATERIALS AND METHODS

A total of 14 *C. orchioides* accessions were collected from of different provinces in Northern Vietnam (Figure 1). After sampling, leaf samples were dried in silica gel and stored in cool place until use. Total DNA was extracted from dried *C. orchioides* leaves using the cetyltrimethylammonium bromide (CTAB) method described by Doyle & Doyle (1987). 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). DNA samples were stored at -20 °C until use.

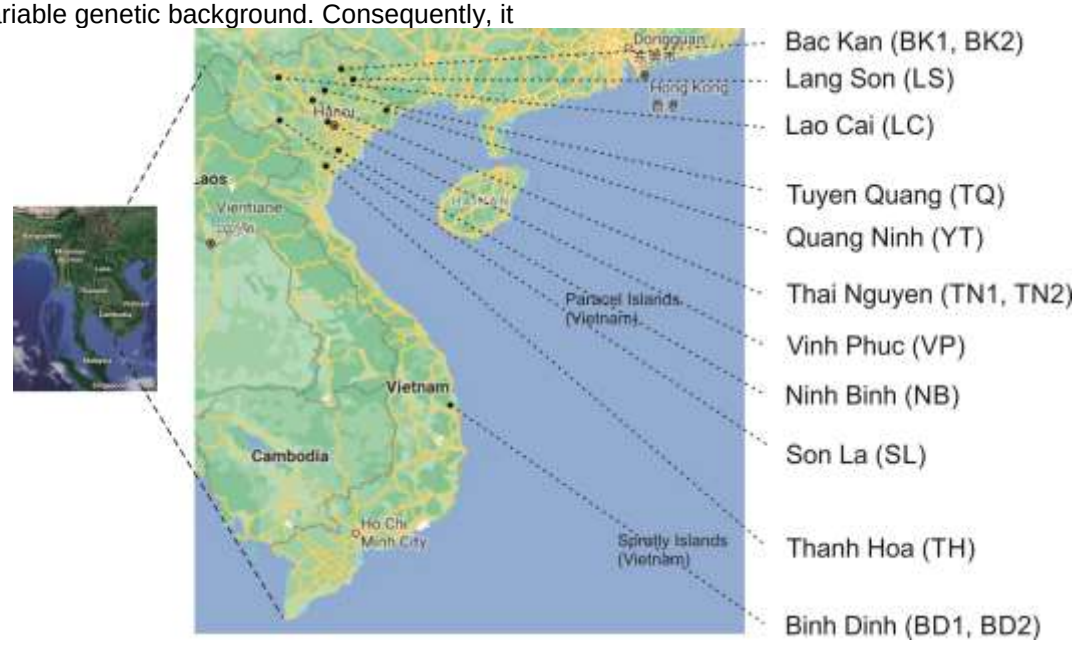

Figure 1: Targeted areas for collecting *C. orchioides* accessions in this study (Samples collected at each location are shown in parentheses).

. **Table 1: Sequences of 15 ISSR markers in this study**

No	Primer	Sequence (5'-3')	Reference
1	UBC880	GGAGAGGAGAGGAGA	Levi et al., 2004
2	UBC825	ACACACACACACACACT	Levi et al., 2004
3	UBC841	GAGAGAGAGAGAGAGACTC	Levi et al., 2004
4	UBC855	ACACACACACACACACCTT	Shukla et al., 2017
5	UBC813	CTCTCTCTCTCTCTCTT	Levi et al., 2004
6	UBC853	TCTCTCTCTCTCTCTCRT	Shukla et al., 2017
7	UBC814	CTCTCTCTCTCTCTCTA	Shukla et al., 2017
8	UBC811	GAGAGAGAGAGAGAGAC	Shukla et al., 2017
9	UBC810	GAGAGAGAGAGAGAGAT	Levi et al., 2004
10	UBC 814- 11	CTCTCTCTCTCTCTCAT	Kumar et al., 2005
11	UBC834	AGAGAGAGAGAGAGACYT	Shukla et al., 2017
12	UBC 840	GAGAGAGAGAGAGAGACTT	Singh et al., 2015
13	UBC 856	ACACACACACACACACCTA	Singh et al., 2015
14	UBC 868	GAAGAAGAAGAAGAAGAA	Kumar et al., 2005
15	UBC 890	ACGACTACGGTGTGTGTTTGTGT	Singh et al., 2015

A total 15 ISSR primers were used and shown in Table 1. The reagent composition of PCR reactions was prepared as follows: 7.5 µL 2X Mytaq Red Mix (Bioline, UK), 20 ng DNA, 0.2 µM primer and PCR water 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 ^{o}C, 30 seconds at 54 ^{o}C, and 1 minute at 72 ^{o}C and 5 minutes to finish reaction at 72 ^{o}C. All reactions were carried out with the SureCycler 8800 Thermal Cycler (Agilent, USA). PCR products were 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 UV transilluminator

After gel electrophoresis of PCR products, the positions with clearly visible ISSR amplified bands were scored as 1, whereas the absent band was scored as 0. The numbers of scored bands (SB), numbers of polymorphic bands (NPB) and percentage of polymorphic bands (PPB) were calculated. The quality information of the primers Polymorphism Information Content (PIC) was determined according to Chesnokov and Artemyeva (2015). Genetic similarity among accessions and phylogenetic tree were developed by NTSYS-pc 2.1(Rohlf, 2000). The cut-off value of the dendrograms was determined based on method described by Jamshidi and Jamshidi (2011). Then Principal Coordinate Analysis (PCA) was carried out based on similarity matrices to construct a three-dimensional diagram using DCENTER module of NTSYS-pc 2.1 program (Ibrahim et al.2017).

RESULTS AND DISCUSSION

In this study, the genetic relatedness of 14 *C. orchioides* genotypes in Northern Vietnam was investigated with ISSR markers. After carrying out 15 ISSR primers, the data showed the successful of PCR reactions with clear and reproducible bands (Figure 2).

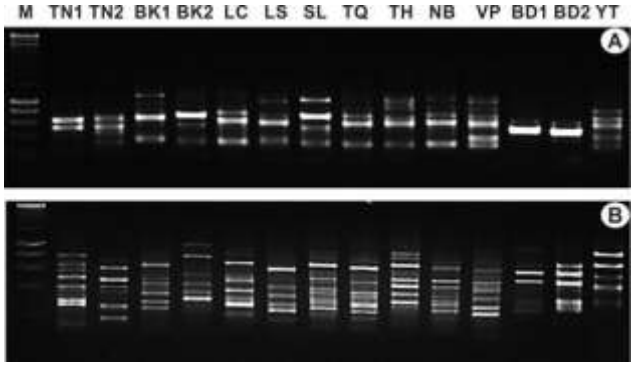

Figure 2: Representative ISSR results of 14 *C. orchioides* genotypes with UBC855 primer (A), and UBC811 primer (B). (M: 1 kb DNA marker, Bioline, UK).

A total of 129 bands were generated in which 119 bands were polymorphic, accounting for 91.65% (Table 2). Most of amplified bands are mostly in range from 100 to 2000 bp. The number of bands varied from 6 (primer UBC855, UBC814-11, UBC834, and UBC840) to 13 (primer UBC811) which is slightly higher than result reported by study in India in which Alagar and colleagues used a set of 10 ISSR primers with 4 to 8 bands per primer (Alagar et al. 2014). The average of amplified bands and polymorphic bands per primer were 8.60 and 7.93, respectively. Polymorphic percentage ranged from

66.67 % to 100% with an average of 90.65% which is slightly higher than previous study conducted on 25 *C. orchioides* accession of Li and colleagues in China (Li et al. 2012). ISSR primers showed high PIC values from 0.47 to 0.92 with average of 0.72 meaning that all these primers are suitable for studying genetic diversity of *C. orchioides* according to Botstein and colleagues (1980) as following: highly informative if PIC > 0.5; reasonably informative if 0.5 > PIC > 0.25 and slightly informative if PIC < 0.25. The complex marker patterns of ISSR found in this study suggests the advantages for distinguishing closely related accessions.

Table 2: Characteristics of DNA profiles generated in 14 *C. orchioides* genotypes by 15 ISSR primers.

No	Primer	SB	NPB	PPB (%)	PIC
1	UBC880	9	7	77.78	0.92
2	UBC825	8	7	87.50	0.68
3	UBC841	14	14	100	0.74
4	UBC855	6	5	83.33	0.64
5	UBC813	9	8	88.89	0.67
6	UBC853	9	9	100	0.72
7	UBC814	9	9	100	0.73
8	UBC811	13	13	100	0.80
9	UBC810	8	8	100	0.78
10	UBC 814- 11	6	6	100	0.76
11	UBC834	6	4	66.67	0.70
12	UBC 840	6	4	66.67	0.47
13	UBC 856	9	8	88.89	0.87
14	UBC 868	10	10	100	0.59
15	UBC 890	7	7	100	0.79
Total	-	129	119	-	-
Average	-	8.60	7.93	90.65	0.72

SB: Scored bands; NPB: number of polymorphic bands; PPB: percentage of polymorphic bands; PIC: polymorphism information content).

Electrophoresis gels of ISSR reaction were then documented and the information on the presence or absence of PCR products was converted to a binary matrix. After analysing by NTsyspc software, a similarity matrix indicating the relatedness of 14 *C. orchioides* genotypes was produced and showed in Table 3. Overall, the genetic similarity varied from 0.41 to 0.92. The lowest similarity value (0.41) was observed between NB and TN1. Whereas, the highest similarity value (0.92) was observed between BD1 and BD2. This high similarity is not surprised because two these samples were collected in Binh Dinh province suggesting these two accessions share a common ancestor. The genetic relatedness of samples in this study is more diverse than study performed in China in 2012 when total of 25 accessions were analysed with ISSR markers but the similarity value just from 0.60 to to 0.82 (Li et al. 2012).

The dendrogram was then constructed based on similarity matrix in Table 3. The cluster analysis reveals that there is variation of genetic composition of *C. orchioides* genotypes in Northern Vietnam. At cut-off value of 0.66, the 14 *C. orchioides* genotypes were divided into three groups (Figure 3). Group A and group B consists of only one accession for each group whereas, remaining 12 accessions are bound together in group C. In general, the clustering of the analysed samples did not correspond to the geographic location of the sampling area. Similar result was also previously reported by Hoang and colleagues when analysing 92 *C. orchioides* samples by RAPD method (Hoang et al., 2018). This may be due to sample exchanges between localities or the out-crossing system of plant in nature resulting the mixture of genetic composition of *C. orchioides.*

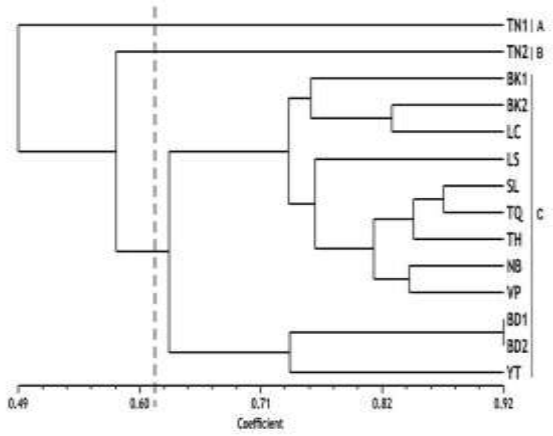

Figure 3: Dendrogram obtained from 14 *C. orchioides* genotypes collected in Northern Vietnam with UPGMA based on Jaccard's coefficient by using 15 ISSR primers.

The relationship among 14 *C. orchioides* accessions was also evaluated by PCoA, the first three coordinates were shown in Figure 4. The result of this analysis is relatively corresponding to UMPMA in Figure 3 with three distinct groups. Thus, PCoA can be used for further confirmation of genetic diversity by using UPGMA method as described previously by Johar et al. (2007).

Table 3: Similarity coefficients among 14 *C. orchioides* genotypes with 15 ISSR markers

	TN1	TN2	BK1	BK2	LC	LS	SL	TQ	TH	NB	VP	BD1	BD2
TN2	0.50												
BK1	0.53	0.56											
BK2	0.54	0.59	0.74										
LC	0.49	0.56	0.76	0.82									
LS	0.46	0.63	0.75	0.72	0.74								
SL	0.49	0.61	0.76	0.73	0.80	0.83							
TQ	0.45	0.59	0.73	0.68	0.79	0.78	0.87						
TH	0.46	0.63	0.72	0.70	0.77	0.73	0.82	0.87					
NB	0.41	0.58	0.73	0.69	0.74	0.74	0.80	0.81	0.80				
VP	0.50	0.57	0.69	0.68	0.75	0.70	0.79	0.82	0.82	0.84			
BD1	0.53	0.51	0.59	0.60	0.61	0.56	0.60	0.59	0.55	0.60	0.65		
BD2	0.51	0.56	0.62	0.63	0.63	0.61	0.61	0.62	0.58	0.61	0.68	0.92	
YT	0.53	0.56	0.65	0.68	0.63	0.63	0.64	0.66	0.66	0.67	0.74	0.73	0.74

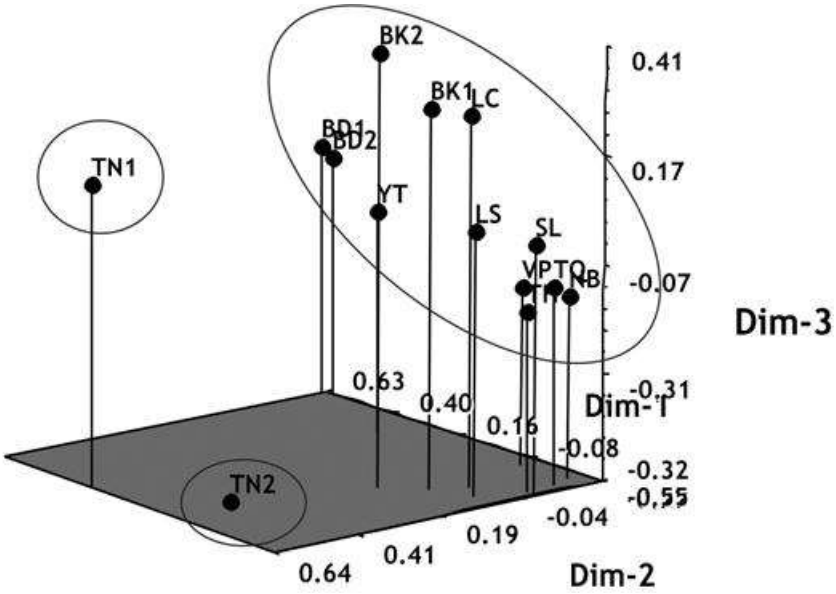

Figure 4: Principle coordinate plot for the first three principal coordinates estimated for 15 ISSR markers of the 14 *C. orchioides* genotypes

CONCLUSION

The data obtained from 15 ISSR markers in this study show the high variation of genetic background among 14 *C. orchioides* genotypes in Vietnam. The data found in this study is important information for identification, conservation and propagation of this plant. In the future, the distinguishable DNA bands of each accession will be developed into specific markers by Sequence-characterized amplified region (SCAR) in order to facilitate the molecular assisted-selection breeding programs.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The authors would like to thank Ho Chi Minh University of Food Industry-Vietnam for supporting laboratory and research equipment.

AUTHOR CONTRIBUTIONS

HVT: Conceptualization, Methodology, Project administration, Supervision, Writing and editing

the manuscript. TQD: Sample collection, Laboratory work, Writing - original draft, THN: Sample collection, Data analysis, Laboratory work, All authors read and approved the final manuscript.

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