INITIAL APPLICATION OF RAPD MOLECULAR MARKERS TO EVALUATE THE GENETIC DIVERSITY OF JEWEL ORCHID (*Anoectochilus* spp.) ACCESSIONS

Ho Viet The*, Vo Thi Thu Duyen, Pham Lai Phuc
Ho Chi Minh City University of Food Industry
*Email: *thehv@hufi.edu.vn*
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ABSTRACT

Jewel orchid (*Anoectochilus* spp.) is an important herbal plant in Vietnam which is at risk of exhaustion due to overexploitation. About of 15 different species of this plant has been reported in Vietnam with the variation in morphological features and medical value. The identification of jewel orchid is mainly based on personal experience relying on morphological traits leading the difficulty of genetic conservation of this plant. In this study, genetic richness and relativeness of 10 jewel orchid accessions collected from Ho Chi Minh City and nearby region were evaluated by using 20 Random Amplified Polymorphic DNA (RAPD) primers. Our results reveal that there is a large variation of genetic background among studied jewel orchid accessions. The combination of different RAPD markers found in this study could help to identify four jewel orchid genotypes. The results from this project could provide valuable information, which is necessary for classifying, identifying plant origins, breeding and conserving programs of jewel orchid in Vietnam.

Keywords: *Anoectochilus* spp., genetic diversity, molecular markers, RAPD.

1. INTRODUCTION

Jewel orchid (or Lan gấm, lan kim tuyến in Vietnamese) is a rare medicinal species with high economic value. Jewel orchid in Vietnam currently lists 15 species, which is known not only for its ornamental value, but also for its medicinal value. Jewel orchid could enhance health, helps blood circulation. This plant is also used as a medicine to treat tuberculosis, rheumatism, arthralgia, chronic gastritis [1]. By using liquid chromatography, column chromatography and spectroscopy techniques, the chemical structure and biological activity of some compounds in jewel orchid were determined. These compounds have strong biological activity, capable of reducing free radicals in the body, so they have a very good ability to prevent disease. Especially, there are two organic acids, olenolic acid and ursolic acid, which have anti-cancer, blood cholesterol lowering, anti-hypertensive, and antibacterial activities.

Jewel orchid distribute mainly in different countries in Asia such as China, India, Laos and Indonesia. In Vietnam, this plant is commonly found in Lao Cai, Ha Giang, Yen Bai, Vinh Phuc, Quang Tri, Kon Tum, and Gia Lai provinces. Due to the small population, scattered and over-exploited, the natural jewel orchid plant is in danger of extinction. At present, jewel orchid is included in the list of endangered species belonging to group IA of Decree 32/2006/CP, banned for commercial exploitation and classified as endangered forest plant group in the Red Book. In recent years, few research projects focusing on *in vitro* propagation of this orchid has been carried out [2-4]. However, the identifying and choosing study materials of this plant are mainly depended on morphological characteristics such as

shape, color, size and texture of leaves. The morphological characteristics could show several advantages due to easiness to observe and classifying based on analytical statistics. Nevertheless, morphological observations are highly dependent on the environmental conditions and developmental stage of plant [5]. Furthermore, if the specimens are not intact or damaged, the identification process could be more challenging [6].

Taking advantages of molecular development, Random Amplified Polymorphic DNA (RAPD) technique has been utilized intensively to characterize genetic composition of different organism. RAPD does not require the genome information of the target audience and can be applied to different species with common primers. Moreover, the RAPD technique is simple and easy to implement, less expensive and fast because of its simplicity, which requires a minimum amount of DNA. In 1998, a research group from Taiwan used RAPD to distinguish 20 samples of jewel orchid belonging to species namely *Anoectochilus formosanus* and *Anoectochilus koshunensis*, and 8 RAPD primer with 19 specific amplification bands where are able to distinguish two species [7]. In Vietnam, RAPD was also utilized to assess the genetic diversity of *Anoectochilus calcareus* in Quan Ba district, Ha Giang province [8]. The present study is focusing on characterization of genetic relatedness of ten jewel orchid accessions collected from different places. The obtained results in this study will be useful for genetic conservation and breeding purposes. Furthermore, the finding markers tightly linking to specific accessions will also pave the way for classification, conservation and protection of this plant.

2. MATERIALS AND METHODS

2.1. Sample collection, DNA extraction and RAPD reaction

Total of 10 jewel orchid accessions were collected from different places (Table 1). Leaf samples were dried and stored in silica gel until use. DNA was extracted with CTAB method (Cetyl Trimethyl Ammonium Bromide) as described by Madhou *et al.* [9]. PCR reactions were performed in a total volume of 20 µL containing 30 ng DNA, 1X reaction buffer, 2 mM $MgCl_2$, 0.3 µM of each primer, 200 µM of each dNTP, 1 unit of *Taq* polymerase and sterile water to the final volume. PCR amplification was carried out as follows: initial denaturation at 95 °C for 3 minutes; after that followed by 40 cycles of 30 s at 95 °C, 30 s at 36 °C, 1 minute at 72 °C and final extension for 5 min at 72 °C. PCR amplification was then separated by electrophoresis in 1.5% agrarose gel in 1X TAE buffer, and stained with 0.5 µg/mL Gelred TM loading bufer then visualized under ultra violet light.

Table 1. Samples collected for genetic characterization in the present study

Number	Sample code	Collected location
1	CNSH	Biotechnology Center, Ho Chi Minh City
2	BC1	Plant tissue culture laboratory, Binh Chanh district, Ho Chi Minh City
3	HUFI	Ho Chi Minh City University of Food Industry
4	KT1	Kon Tum province
5	KT2	Kon Tum province
6	CC1	Agricultural Hi-tech Park of Ho Chi Minh City
7	CC2	Agricultural Hi-tech Park of Ho Chi Minh City
8	CC3	Agricultural Hi-tech Park of Ho Chi Minh City
9	BC2	Plant tissue culture laboratory, Binh Chanh district, Ho Chi Minh City
10	CC4	Agricultural Hi-tech Park of Ho Chi Minh City

The primers for PCR reactions were chosen as described by Yonemoto *et al*. [5] and Mei *et al.* [10]. All primers are shown in Table 2.

Table 2. List of RAPD primers used to analyze genetic diversity of 10 jewel orchid accessions

No.	Primer	Primer sequence	No.	Primer	Primer sequence
1	Z1**	CCGGTGCCTTCT	11	C31*	GAGTTGCCCGGA
2	F10a**	CAGGCCGAAGTC	12	C82*	ATCGTCACCCCG
3	F44a**	GGTGTCTTGCGG	13	C16**	CGCCCTGCAGTA
4	D41*	GAGACCCGTCGA	14	C62a**	CCATCCGCACGA
5	D50**	GACTCGCGGTCT	15	C11**	AGGTACGCCCGA
6	D38*	AAGCTCGACGGG	16	A39a*	CCTGAGGTAGCT
7	D53**	GCCGCGGAACTA	17	C59a*	CGCGTTCGTGGA
8	D29*	GACCCGGAACGA	18	A58*	GTCATGCCTGGA
9	D84**	AGACACACGGGC	19	A62*	TCGTCCGGAGAT
10	D12*	CTGGTCTCTGGG	20	A18**	GACTCGCGATCT

(* and ** symbols indicate the primers from Yonemoto *et al*. [5] and Mei *et al.* [10], respectively).

2.2. Data analysis

After electrophoresis, only clear and reproducible bands with size from 200 to 1,500 bp were considered for data analysis. The quality of RAPD markers is evaluated through polymorphism information content (PIC) value described by Chesnokov and Artemyeva [11] as the formula:

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

Where i is i-th allele of the j-th marker, n is the number of the j-th marker's alleles, P is allele frequency. PCR products were scored as "1" for the presence and "0" for absence in specific position. The dendrogram was built based on the unweighted pair group method with arithmetic mean and the algorithm (UPGMA) was produced by using SAHN module in NTSYSpc 2.1 package [12]. Principal Coordinate Analysis (PCoA) was performed based on RAPD data to have better understanding about similarity among accessions by using PCoA package in NTSYS-pc 2.1 [13].

3. RESULTS AND DISCUSSION

Total of 10 jewel orchid accessions were characterized with 20 RAPD primers. The results show that all primers used in this study generated high polymorphism among different accessions with clear amplification and high reproducibility after two replications (Figure 1). Tested primer generated from 6 to 13 amplifications ranging from 200 to 1500 bp with large variation of total and polymorphic bands from 85.71 to 100%. All primers show PIC value from 0.61 to 0.80 (Table 3), meaning that all of these used primer are suitable for genetic characterization of jewel orchid collected in research areas. As PIC classification of Botstein *et al*. [14] as following: highly informative if PIC > 0.5; reasonably informative if 0.5 > PIC > 0.25 and slightly informative if PIC < 0.25. The average ratio of polymorphic bands in this study is up to 98.04%, which is higher than the previous study of Nguyen Thi Tho *et al.* where the highest ratio of polymorphic bands was only 79.79% [8].

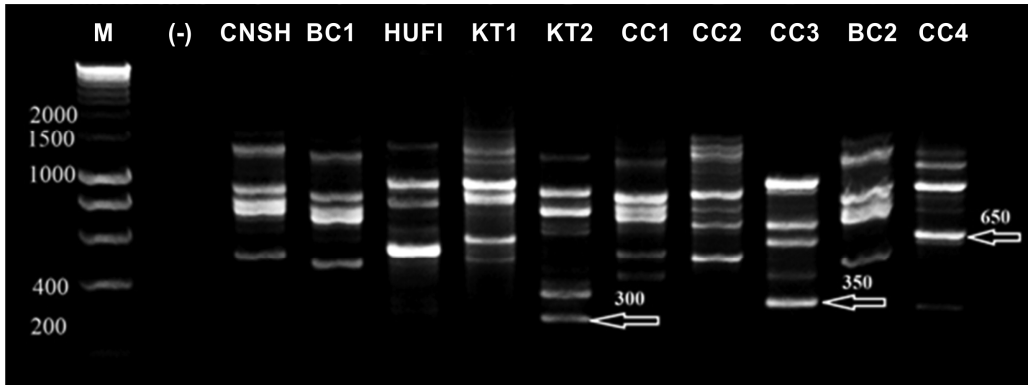

Figure 1. Representative RAPD result with D12 primer.
(M: 1 kb DNA ladder (Bioline, UK); (-): negative control without DNA).

Table 3. Test results of RAPD primers used in the present study

Primer	Total bands	Polymorphic bands	Polymorphism (%)	PIC value
Z1	11	11	100	0.76
F10a	12	11	91.67	0.63
F44a	13	13	100	0.75
D41	12	12	100	0.76
D50	6	6	100	0.59
D38	12	12	100	0.62
D53	8	8	100	0.71
D29	6	5	83.33	0.55
D84	7	7	100	0.77
D12	12	12	100	0.77
C31	7	6	85.71	0.71
C82	6	6	100	0.64
C16	7	7	100	0.61
C62a	11	11	100	0.70
C11	9	9	100	0.80
A39a	7	7	100	0.67
C59a	9	9	100	0.79
A58	8	8	100	0.64
A62	7	7	100	0.73
A18	13	13	100	0.73
Total	184	181	-	-
Average	9.2	9.05	98.04	0.70

The combination of different amplicons from this study shows the high discrimination capacity of RAPD markers, relying on RAPD data, we could successfully identified several accessions by using specific amplification from either single or combined different primers. The total RAPD amplification which can be utilized to different jewel accessions are presented in Table 4. The ability of RAPD method to differentiate jewel orchid genotypes

early was reported in the previous study of Chen *et al.* [7]. Thus, the specific bands obtained in our study could be used to identify the origin of materials for jewel classification and conservation programs at least in studied areas. In spite of numerous genetic information generated, the RAPD makers used in this study is not able to distinguish completely all jewel accessions. We suggest that the estimation of genetic diversity by RAPD marker is highly influenced by specific genome of selected accession and by the specific RAPD primer examined. In order to get more accurate result from RAPD markers, future studies need to use higher number of marker to cover higher density on genome of plant to measure the genetic variation more exactly. Another reason should be noted that the most samples in this study is from cross-pollinated plants, consequently the obtained genetic variability is highly depended on individual plants.

Table 4. Jewel orchid accessions-specific amplified bands produced by selected RAPD primers

Sample	Primer	Amplification bands (bp)
CC3	Z1	400
CC4	F44a	1200
CC3	D38	300
KTN	D53	300
KTN	D12	300
CC3		350
CC4		650
CC3	C16	200
CC4	C59a	600

Based on obtained RAPD data, the relatedness of 10 jewel orchid accessions was analyzed with NTSYSpc 2.1. Amplification profiles were compared to generate a similarity matrix and showed in Table 5. Overall, the genetic similarity varied from 0.40 to 0.69. The lowest similarity value (0.40) was observed between CC2 and CC2 indicating that they have a distant relationship even though the samples were collected at the same place. Whereas the highest similarity value (0.69) was observed between BC2 and CNSH. This result is reasonable because these two samples were collected from Binh Chanh and district 12, respectively. Geographically, these two districts are approximate, thus these samples could have close origin.

Table 5. Simple matching coefficients of similarity among 10 jewel accessions

	CNSH	BC1	HUFI	KT1	KT2	CC1	CC2	CC3	BC2	CC4
CNSH	1.00									
BC1	0.67	1.00								
HUFI	0.47	0.49	1.00							
KT1	0.62	0.57	0.48	1.00						
KT2	0.51	0.54	0.63	0.57	1.00					
CC1	0.62	0.56	0.52	0.67	0.53	1.00				
CC2	0.59	0.58	0.57	0.63	0.59	0.61	1.00			
CC3	0.48	0.52	0.55	0.48	0.53	0.42	0.40	1.00		
BC2	0.69	0.65	0.47	0.57	0.48	0.62	0.61	0.42	1.00	
CC4	0.50	0.52	0.56	0.55	0.52	0.51	0.48	0.55	0.46	1.00

The dendrogram was then built based on similarity matrix which showed clear two main groups (Figure 2). The first group consists of six accessions consisting of CNSH, BC1, BC2, KT1, CC1 and CC2, the second group consists of four accession, namely HUFI, KT2, CC3 and CC4. It can be seen that the classification does not depend on the geographical location of sample collection. Samples collected from same places such as Kontum province or Agricultural Hi-tech Park of Ho Chi Minh City are divided into separate groups. This could explain that jewel orchid could be exchanged from different places.

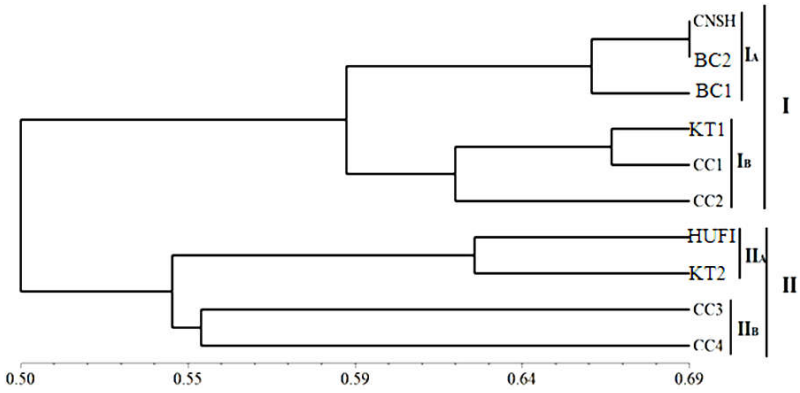

Figure 2. Dendrogram generated by using 20 RAPD markers to show the genetic relatedness of 10 jewel orchid accessions. This dendrogram was developed using UPGMA cluster procedure of NTSYSpc 2.1. The scale shown at the bottom is the measure of genetic similarity.

The relationship among different jewel orchid accessions was also evaluated by PCoA, the first three PCoA were shown in Figure 3. The result of this analysis is relatively corresponding with the dendrogram analyzed by UPGMA at Figure 2. Thus, PCoA can be used for further confirmation of genetic diversity by using UPGMA method as described previously by Johar *et al.* [15].

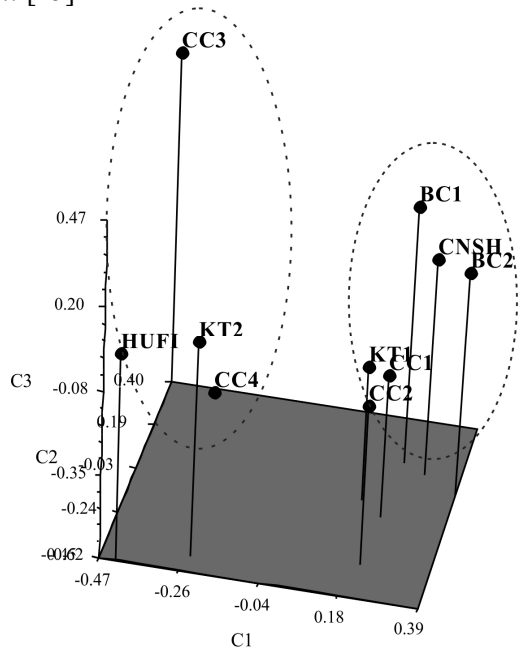

Figure 3. Three-dimensional plot of principle coordinates analysis depicting the genetic relatedness of ten jewel orchid genotypes.

4. CONCLUSION

RAPD is considered as frontline technique to study organism with limited prior genetic knowledge such as jewel orchid. In this project, the usefulness of this technique in investigating genetic diversity of jewel orchid was revealed. By using 20 RAPD primers to analyze ten jewel orchid accessions, the collected samples were divided into two main groups showing the distant relatedness of accessions in different places. Several specific RAPD bands found in this study could be further analyzed to use as specific markers to identify specific jewel orchid accessions. The obtained results in this study will be important information which will be useful for several purposes such as classification, conservation and jewel orchid breeding programs.

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TÓM TẮT

BƯỚC ĐẦU SỬ DỤNG CHỈ THỊ PHÂN TỬ RAPD ĐỂ ĐÁNH GIÁ ĐA DẠNG DI TRUYỀN CỦA MỘT SỐ MẪU LAN KIM TUYẾN (*Anoectochilus* spp.)

Hồ Viết Thế*, Võ Thị Thu Duyên, Phạm Lai Phúc
Trường Đại học Công nghiệp Thực phẩm TP.HCM
*Email: *thehv@hufi.edu.vn*

Lan kim tuyến (*Anoectochilus* spp.) là một loại cây thảo dược quan trọng ở Việt Nam hiện đang có nguy cơ cạn kiệt do khai thác quá mức. Có khoảng 15 loài khác nhau của loại cây này đã được báo cáo ở Việt Nam với sự khác biệt về đặc điểm hình thái và giá trị y học. Hiện nay, việc nhận diện lan kim tuyến chủ yếu dựa vào các đặc điểm hình thái với độ chính xác không cao dẫn đến khó khăn trong việc bảo tồn di truyền của loại cây này. Trong nghiên cứu này, sự đa dạng di truyền của 10 mẫu lan kim tuyến được thu thập từ Thành phố Hồ Chí Minh và các vùng lân cận được đánh giá bằng cách sử dụng 20 đoạn mồi DNA đa hình ngẫu nhiên (RAPD). Kết quả của cho thấy rằng có sự khác biệt lớn về cấu trúc di truyền của các mẫu lan được nghiên cứu. Ngoài ra, nghiên cứu cũng phát hiện được sự kết hợp của các chỉ thị RAPD khác nhau có thể giúp phân biệt được bốn mẫu lan khác nhau. Kết quả của nghiên cứu này có thể cung cấp thông tin có giá trị quan trọng trong phân loại, xác định nguồn gốc, và hỗ trợ các chương trình nhân giống và bảo tồn cây lan kim tuyến ở Việt Nam.

Từ khóa: Anoectochilus spp., chỉ thị phân tử, đa dạng di truyền, RAPD.