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GENETIC DIVERSITY AND CHARACTERIZATION OF *PSEUDERANTHEMUM LATIFOLIUM* BY RAPD AND ISSR MOLECULAR MARKERS

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ABSTRACT

Pseuderanthemum palatiferum is a commonly used ornamental plant in Vietnam. It is getting more attention due to its medicinal values. In this study, the genetic composition of 16 *P. palatiferum* accessions and relatives collected from Vietnam were analyzed using 16 RAPD and ISSR molecular markers. The obtained results show that both RAPD and ISSR produced a high number of polymorphic bands. The similarity coefficient varies among RAPD and ISSR, from 0.53 – 0.95 and 0.32 – 0.91, respectively. Mantel test also shows poor correlation between RAPD and ISSR markers. Both data from RAPD markers or combination of RAPD and ISSR markers could differentiate *P. palatiferum* from its relatives. The obtained results reveal the large variation in genetic composition of *P. palatiferum* population in Vietnam. The findings could be beneficial in classification, identification, and conservation efforts of *P. palatiferum*.

Key words: Genetic diversity, ISSR, molecular markers, *Pseuderanthemum palatiferum*, RAPD,
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INTRODUCTION

Pseuderanthemum palatiferum or Hoan-ngoc in Vietnamese, belongs to Acanthacea family, is a medicinal plant native to Vietnam. This plant is distributed in whole Vietnam and neighbor countries such as Thailand, China, Cambodia, Laos, Malaysia and Myanmar. Aside its use as an ornamental plant, *P. palatiferum* possesses high medicinal values such as preventing stomach pain, colitis, high blood pressure, nephritis and diarrhea in both human and animals. All parts of the tree such as roots, stems, leaves, flowers can be used as medicine. The extract was proven to treat Alzeimer disease effectively and inhibits the activity of the enzyme acetylcholinesterase in the hippocampus in rat (Buncharoen *et al*., 2010), reduces inflammatory condition in murine macrophage cells (Sittisart and Chitsomboon, 2014). The leaf extract shows strong antibacterial activity against several bacteria and fungi (Nualkaew *et al*., 2015). Chemical analysis also shows that the fresh leaves are rich in Ca, Mg and Fe (Dieu, 2008) and bioactive ingredients (Nualkaew *et al*., 2015).

At present, in Vietnam, studies on *P. palatiferum* are mainly focused on determining the chemical composition and extract utilization in the treatment of a number of diseases such as diarrhea, hypertension, rheumatoid arthritis, and pharyngitis (Padee, 2009). Currently, the leaves are processed into functional foods with many different forms such as instant tea, tea bags, and capsules or combined with many other ingredients to increase the benefits of the products. At the present, the identification of *P. palatiferum* is commonly based on morphological characteristics which are easily influenced by several factors such as environment, developmental stage and personal experience of identifier. Especially, *P. palatiferum* is hard to distinguish to other species of Acanthacea family, especially *Clinacanthus nutans* and *Pseuderanthemum bracteatum*. Thus, study genetic diversity to distinguish *P. palatiferum* with other plants for conservation, selection, and propagation to meet developing requirements of sustainable pharmaceutical processing industry is necessary.

Numerous DNA-based methods have been utilized to evaluate genetic diversity of plants such as Restriction Fragment Length Polymorphism (RFLP), Polymorphic DNA Random Amplification (RAPD), Inter Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR), and Simple Nucleotide Polymorphism (SNP). RAPD and ISSR are highly preferred since they are PCR-based indicators and have general advantages such as simplicity, speed, economy, minimum laboratory skill requirement, and quantity requirement. RAPD has been commonly applied to analyze genetic diversity in plants (Kawa *et al*., 2009, Khan *et al*., 2010), to identify the genetic modification (Martin *et al*., 2002) and to determine species classification (Rossi *et al*., 2000). ISSR technique has also been applied on many plants, such as mangosteen (Mansyah *et al*., 2010), mango (Rocha *et al*., 2012), and *Jewel orchid* (Zhang *et al*., 2010). No study using molecular marker has been implemented to study genetic composition and classification of *P. palatiferum.* This study was carried out to evaluate the effectiveness of two most common markers using for genetic diversity analysis consisting of RAPD and ISSR to characterize

genetic composition of *P. palatiferum* genotypes and its relatives in Vietnam. The obtained results could be potential for classification, identification and conservation purposes of *P. palatiferum*.

MATERIALS AND METHODS

A total of 16 samples, consisted of 14 *P. palatiferum* genotypes and two of its close relatives were collected from different provinces in Vietnam and presented in Table 1.

The clear amplification bands from gel electrophoresis were used in the analysis. Products were encoded in binary method: position with bar band were indicated as "1", and position not appearing were indicated as "0". Phylogenetic tree was performed by using UPGMA method in the SAHN module of NTSYSpc 2.1 software (Rohlf, 2000).

Table 1. List of plant samples used in this study.

No.	Sample ID	Collection site	Species
1	BT	An Hiep ward, Ba Tri district, Ben Tre province	*P. palatiferum*
2	TG	Than Cuu Nghia Ward, Chau Thanh district, Tien Giang province	*P. palatiferum*
3	LA	Khanh Hung ward, Vinh Hung district, Long An province	*P. palatiferum*
4	SG1	Ward 6, Go Vap district, Ho Chi Minh city	*P. palatiferum*
5	SG2	Ward 7, Binh Thanh district, Ho Chi Minh city	*P. palatiferum*
6	SG3	Tang Nhon Phu ward, District 9, Ho Chi Minh city	*P. palatiferum*
7	SG4	Tan Son Nhi ward, Tan Phu district, Ho Chi Minh city	*P. palatiferum*
8	TN1	Cha La ward, Duong Minh Chau district, Tay Ninh province	*P. palatiferum*
9	BP1	Loc Hiep ward, Loc Ninh district, Binh Phuoc province	*P. palatiferum*
10	HN1	Thach Ban ward, Long Bien district, Ha Noi city	*P. palatiferum*
11	HN2	Hoang Liet ward, Hoang Mai district, Ha Noi city	*P. palatiferum*
12	DB	Muong Thanh ward, Dien Bien city, Dien Bien province	*P. palatiferum*
13	PT1	Yen Kien ward, Doan Hung district, Phu Tho province	*P. palatiferum*
14	TH1	Tinh Gia town, Tinh Gia district, Thanh Hoa province	*P. palatiferum*
15	HND	Tien Hai town, Tien Hai district, Thai Binh province	*P. bracteatum*
16	XK	An Hiep ward, Ba Tri district, Ben Tre province	*Clinacanthus nutans*

DNA was extracted from fresh sample leaves following Sika *et al*. (2015). DNA quality was then electrophoresized on 1.5% agarose gel stained 0.5 µg/ml GelredTM and observed under ultraviolet light using Quantum gel reader - ST4 3000 (Montreal- Biotech, Canada). Spectrophotometer (Optima SP3000 nano UV-VIS, Japan) was used to determine and calculate DNA concentration. A total of 16 RAPD and ISSR primers were used and shown in Table 2 (Levi *et al*., 2004; Yonemoto *et al*., 2006; Shukla *et al*, 2017).

Table 2: Sequences of RAPD and ISSR markers used in this study.

RAPD	Sequence (5'-3')	ISSR	Sequence (5'-3')
D41	GAGACCCGTCGA	UBC880	GGAGAGGAGAGGAGA
D53	GCCGCGGAACTA	UBC825	ACACACACACACACACT
D29	GACCCGGAACGA	UBC841	GAGAGAGAGAGAGAGACTC
D12	CTGGTCTCTGGG	UBC855	ACACACACACACACACCTT
C31	GAGTTGCCCGGA	UBC809	AGAGAGAGAGAGAGAGG
C82	ATCGTCACCCCG	UBC811	GAGAGAGAGAGAGAGAC
C11	AGGTACGCCCGA	UBC810	GAGAGAGAGAGAGAGAT
A39a	CCTGAGGTAGCT	UBC826	ACACACACACACACACC

PCR reactions were performed according to Ho and Tu (2019) 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 RAPD cycle conditions consisted of pre-denaturation step at 95 °C for 2 min; then 35 cycles of 30 sec at 95 °C for denaturation, 30 sec at 35 °C for primer annealing, and 1 min at 72 °C for primer extension. Finally, 5 min at 72 °C was added to complete the reactions. The ISSR cycle conditions were similar to those of RAPD reactions, except that temperature annealing for primers was set at 54 °C. PCR reactions were run on SureCycler 8800 Thermal Cycler (Agilent, USA). PCR amplifications were separated using 1% agarose gel and the product size were estimated based on 1 kb DNA ladder (Bioline, UK).

This software was also utilized to determine the Jaccard's coefficient showing the genetic relatedness among accessions. The cut-off values of the dendrograms were determined based on calculation method described by Jamshidi and Jamshidi (2011). Information on the quality of primers is determined by the coefficient PIC

(Polymorphism Information Content) according to the formula of Chesnokov and Artemyeva (2015). The Mantel test at a significant level of 1% in 1.000 permutations was conducted to identify the correlation between similarity matrices of two markers by using program Mantel test of Microsoft Excel 2010 (Mantel, 1967).

RESULTS AND DISCUSSION

RAPD analysis: Initially, genetic compositions of studied samples were investigated by RAPD markers. The results showed that the amplified bands appeared clearly on the agarose gel (Figure 1A). All primers were suitable for *P. palatiferum* studies through high amplification band numbers and high polymorphism rates and information index (PIC) of the primer (Table 3). All primers have PIC values as high as 0.62 to 0.90. This means that all these primers applied are suitable for studying genetic diversity connecting to the classification of Botstein and colleagues: very high information if PIC ≥ 0.5; information if 0.5> PIC ≥ 0.25 and little information if PIC <0.25 (Botstein *et al*., 1980). The ability of RAPD primers to form multiple polymorphic bands is important in genetic diversity analysis because they could provide more detail information about the genetic makeup of the individuals being analyzed. Up to 76/76 bands of polymorphic DNA accounted for 100% with an average of 9.5 bands of polymorphism appear on the sample.

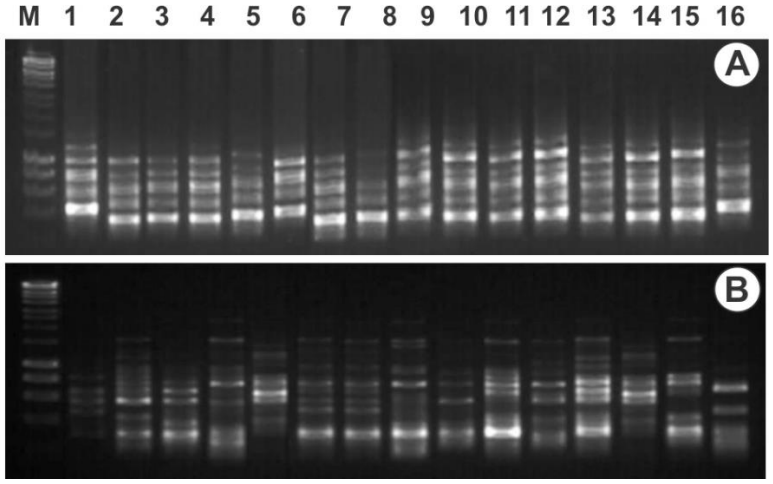

Figure 1. Representative result of RAPD marker D84 primer (A) and ISSR maker with UBC855 primer (B). (The number corresponds to the sample number in Table 1; M: DNA marker).

Table 3. Characteristics of DNA profiles generated by RAPD and ISSR markers..

RAPD markers	SB	NPB	PPB (%)	PIC	ISSR markers	SB	NPB	PPB (%)	PIC
D41	8	8	100	0.62	UBC880	6	6	100	0.77
D53	16	16	100	0.70	UBC825	4	4	100	0.48
D29	8	8	100	0.82	UBC841	13	13	100	0.72
D12	12	12	100	0.76	UBC855	12	12	100	0.70
C31	10	10	100	0.67	UBC809	9	9	100	0.78
C82	9	9	100	0.65	UBC811	2	2	100	0.28
C11	7	7	100	0.65	UBC810	12	12	100	0.87
A39a	6	6	100	0.90	UBC826	5	5	100	0.56
Sum	76	76	-	-		63	63	-	-
Average	9.5	9.5	100	0.72		7.9	7.9	100	0.64

(SB: Total scored bands for each marker; NPB: number of polymorphic bands for each marker; PPB: percentage of polymorphic bands based on the ratio of NPB to SB; PIC: polymorphism information content).

To calculate genetic similarity coefficients and construct phylogenetic diagrams, the results of RAPD-PCR were encoded and phylogenetic trees were constructed using NTSYSpc version 2.1 software with UPGMA clustering method in SAHN program. The results showed that there was a large variation in the similarity coefficients between samples, ranging from 0.53-0.95: the highest similarity coefficient was found in the TN and HN1 samples (0.95) and the lowest was from LA and XK (0.53). Clustering analysis shows that of all

of 14 *P. palatiferum* accessions are grouped together and its relatives consisting of HND (*P. bracteatum*) and XK (*Clinacanthus nutans*) were separated in a distant branch (Figure 2A).

ISSR analysis: The ISSR reaction results are shown in Figure 1B; a total of 63 bands were generated from 8 primers, ranging from 200 to 2000 bps, and all of them are polymorphic. In particular, the UBC841 primer gives the highest number of bands (13 bands), while the UBC811 primer gives the least number of bands (2 bands). The PIC coefficient of the six primer pairs ranged from 0.28 (UBC811) to 0.87 (UBC810) (Table 3) with total average of 0.64. Thus, 6 out of 7 markers are informative for study except for UBC811. The result from ISSR phylogenetic analysis was not corresponding to that of RAPD markers (Figure 2A) in which two distant relatives consisting of HND (*P. bracteatum*) and XK (*Clinacanthus nutans*) are grouped with other samples, whereas TH samples was sorted out into a distinct group.

Several studies have reported the higher capacity of RAPD than ISSR in different plants such as *Vigna umbellate* (Muthusamy *et al*., 2008) and pistachio (Tagizad *et al*., 2010). The reasonable explanation for this result could be the difference between the obtained data generated by two marker types where RAPD and ISSR amplify the non-repetitive and repetitive regions of plant genome, respectively. Furthermore, Mantel test of genetic matrices of RAPD and ISSSR were 0.23 ($p<0.01$) showing the low relevance of these two markers indicating a low fit of the two matrices and low correlation of the two markers. As classification of Lalhruaitluanga and Prasad (2009), the marker is considered fit if r>0.8, from 0.7 to .8 is poor fit and very poor fit if r< 0.7. However, high correlation between RAPD and ISSR markers were also reported from studies on different plant namely sweet potato (Moulin *et al*., 2012); Saccharina (Cui *et al*., 2017); and rice bean (*Vigna umbellate*) (Muthusamy *et al*., 2008). The low correlation of two markers in this study could be due to low number of samples and marker number; the greater number of markers and samples could increase the precise estimation of genetic relatedness.

The data from RAPD and ISSR markers were then merged for UPGMA analysis. The obtained dendrogram from this combination is similar to that of RAPD (Figure 2C). This tree is also able to distinguish HNA and XK from another sample and made into a discrete group. This data is in line with previous studies in different organisms such as *Jatropha curcas* (Gupta *et al*. 2008), and wormwood capillary (Shafie *et al*., 2011).

This study shows that both RAPD and ISSR markers are potential for evaluating the genetic variation of *P. latifolium* genotypes. RAPD slightly outperforms ISSR because of its high percentage of polymorphism and reproducibility. Furthermore, RAPD-built dendrogram is potential to classify *P. latifolium* with two species in Acanthacea family consisting of *Clinacanthus nutans* and *Pseuderanthemum bracteatum*. In the future, the development of the Sequence Characteristics Amplification Area (SCAR) marker from polymorphic RAPD and ISSR bands should be considered to provide specific primers to increase the accuracy of *P. latifolium* authentication.

Table 4: Simple matching coefficients of similarity among 14 *P. palatiferum* accession and two relatives with 8 RAPD primers (below diagonal) and 8 ISSR primers (above diagonal).

	BT	**TG**	**LA**	**SG1**	**SG2**	**SG3**	**SG4**	**TN**	**BP**	**HN1**	**HN2**	**BD**	**TH**	**PT**	**HNĐ**	**XK**
BT		0.57	0.73	0.48	0.55	0.50	0.64	0.55	0.55	0.45	0.52	0.59	0.66	0.57	0.57	0.52
TG	0.82		0.66	0.77	0.84	0.80	0.75	0.80	0.89	0.70	0.82	0.66	0.41	0.64	0.68	0.77
LA	0.82	0.82		0.57	0.64	0.68	0.82	0.64	0.68	0.68	0.61	0.64	0.57	0.57	0.66	0.61
SG1	0.76	0.91	0.84		0.84	0.84	0.66	0.89	0.80	0.84	**0.91**	0.57	0.45	0.64	0.59	0.73
SG2	0.69	0.76	0.84	0.75		**0.91**	0.73	0.82	0.77	0.77	0.75	0.59	0.43	0.61	0.66	0.75
SG3	0.75	0.82	0.89	0.80	0.91		0.73	0.86	0.77	0.82	0.80	0.59	0.48	0.57	0.70	0.70
SG4	0.84	0.84	0.73	0.78	0.75	0.76		0.68	0.73	0.68	0.70	0.59	0.57	0.57	0.61	0.66
TN	0.73	0.87	0.80	0.89	0.78	0.84	0.82		0.77	0.77	0.84	0.59	0.43	0.66	0.61	0.70
BP	0.71	0.82	0.75	0.91	0.73	0.78	0.87	0.87		0.82	0.89	0.68	0.48	0.66	0.70	0.70
HN1	0.75	0.85	0.82	0.87	0.80	0.89	0.84	**0.95**	0.89		0.84	0.59	0.48	0.66	0.70	0.70
HN2	0.71	0.89	0.75	0.87	0.73	0.78	0.84	0.91	0.89	0.89		0.57	0.50	0.64	0.59	0.68
DB	0.65	0.65	0.69	0.67	0.64	0.65	0.64	0.67	0.65	0.65	0.62		0.48	0.57	0.70	0.61
TH	0.75	0.82	0.85	0.84	0.76	0.85	0.73	0.84	0.78	0.85	0.78	0.73		0.36	0.41	**0.32**
PT	0.76	0.87	0.80	0.85	0.75	0.80	0.82	0.82	0.84	0.80	0.87	0.71	0.87		0.64	0.68
HNĐ	0.58	0.58	0.58	0.60	0.60	0.58	0.60	0.64	0.58	0.62	0.62	0.60	0.55	0.56		0.77
XK	0.56	0.60	**0.53**	0.58	0.55	0.60	0.62	0.58	0.60	0.56	0.64	0.58	0.56	0.62	0.73	

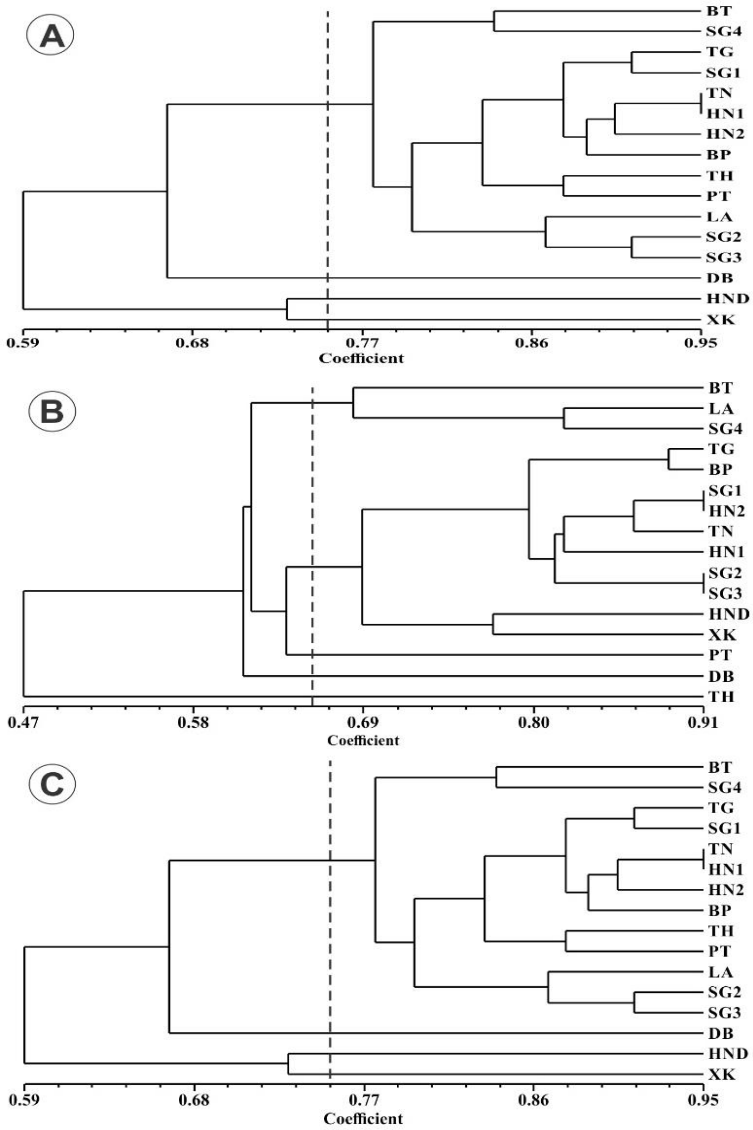

Figure 2. The dendrograms produced by using with UPGMA method based on Jaccard's coefficient by using 8 RAPD primers (A); 8 ISSR primers (B) and 8 RAPD + 8 ISSR primers (C). The vertical lines indicate the cut-off values of each dendrogram and the scale shown at the bottom is the measure of genetic similarity through Jaccard's coefficient.

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