Hue University Journal of Science: Agriculture and Rural Development
pISSN: 2588-1191; eISSN: 2615-9708
Vol. 134, No. 3D, 2025, P. 5–16, DOI: 10.26459/hueunijard.v134i3D.7774

GENETIC DIVERSITY OF *ANOECTOCHILUS ROXBURGHII* IN HUE CITY, VIETNAM, BASED ON RAPD MARKERS

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(*Received: March 22, 2025; Accepted: April 8, 2025*)

Abstract. *Anoectochilus roxburghii* (Wall.) Lindl, a valuable medicinal plant, is under threat due to overexploitation and adverse growing conditions. Therefore, genetic research is necessary to preserve and create hybrid varieties for breeding. In this study, we used RAPD markers to assess genetic differences among 17 *A. roxburghii* accessions collected in Hue City. The results showed that 17 RAPD selected primers were suitable for evaluating genetic polymorphism due to the high percentage of polymorphic bands (76.105%) and the n_a, n_e, h, and I indices gave high values, reaching 1.762, 1.428, 0.249 and 0.373, respectively. Among these 17 primers, UBC#405, UBC#427, UBC#446, UBC#469, UBC#476 and UBC#498 were the six most optimal primers for RAPD marker development with the highest PIC, Rp, MI, and EMR indices. Additionally, the UPGMA tree was separated into three clusters, which showed the genetic grouping among accessions in the population of *A. roxburghii*. The similarity coefficients ranged from 0.635 to 0.930. HUIB_AR10 was most similar to HUIB_AR06 while it was most different from HUIB_AR01.

Keywords: *Anoectochilus roxburghii*, genetic diversity, jewel orchid, RAPD, Vietnam

1 Introduction

Anoectochilus roxburghii (Wall.) Lindl , a herbarceous plant belonging to the family Orchidaceae, is widely used in traditional medicine in China, Japan, India, Laos, Myanmar, Indonesia, Thailand and Vietnam [1, 2]. It is known as "the king of medicine" because of health benefits such as antidiabetic, blood vessel protection, liver protection, antioxidant, antibacterial, anti-inflammation, anti-hyperlipidemia and anticancer effects [3, 4]. It contains bioactive compounds such as kinsenoside, polysaccharides, alkaloids, steroidal compounds, flavonoids, terpenoids, volatile oils, organic acids and glycosides [3, 5]. In addition to its medicinal value, it is also cultivated as an ornamental plant due to its diverse leaf colors and vein patterns [2, 6].

The wild population of *A. roxburghii* has declined in recent years due to severe destruction of habitat, slow growth, low germination rate, increasing demand and overexploitation [7, 8]. It is considered a vulnerable species in the Convention on International Trade in Endangered Species of Wild Fauna and Flora [9] and an endangered species in group IA according to Decree 84/2021/ND-CP of the Vietnam Government dated September 22nd, 2021 [10]. Therefore,

developing breeding and conservation plan for this species is urgently needed. The scientific basis for this is to ensure genetic uniformity during the breeding process. However, at a later stage, when *A. roxburghii* needs to have diverse sizes, colours, and vein patterns to meet market demand, more genetic variations must be created from hybrid materials. Genetic diversity is investigated because it is independent of environmental influence and can reveal differences at the whole genome level [11, 12].

Among molecular markers, Random Amplified Polymorphic DNA (RAPD) is a PCR-based marker, widely used in detecting genetic variations and diversity assessment and identifying germplasm in several plant species [12]. This method is simple and efficient because it requires only a minimal amount of genomic DNA without sequence and can cover a large part of the genome. Besides, RAPD analysis is fast, cost-effective and simple with the arbitrary sequence of the primers [13, 14]. RAPD was successfully utilized to evaluate the genetic structure of different species of jewel orchid in Vietnam, including *Anoectochilus*, *Goodyera* and *Ludisia* [1, 15]. Furthermore, the genetic diversity of *Anoectochilus calcareus* in Quan Ba District, Ha Giang Province, was explored by the study of Nguyen et al. [16]. Meanwhile, studies on the genetic characteristics of *A. roxburghii* in Hue City are limited.

Based on the aforementioned background, the present study aims to assess the genetic relatedness characterization of 17 accessions collected in Hue City, Vietnam. The results will be useful for genetic conservation and breeding processes. Additionally, by finding markers tightly linked to specific accessions, useful scientific bases will be provided for classification, conservation and protection of *Anoectochilus*.

2 Materials and Methods

Plant materials, genomic DNA and primers

Seventeen samples of *Anoectochilus roxburghii* (Wall.) Lindl. were collected in Hue City, Vietnam (Table 1). Then, the genomic DNA was extracted from young leaves by CTAB method (cetyl trimethyl ammonium bromide) and purified with a ratio A260:A280 from 1.8 to 2.0 [17]. UBC RAPD primers (University of British Columbia) were used to amplify plant DNA as previously described [18].

Table 1. List of seventeen collected accessions of *Anoectochilus roxburghii*

No.	Accession code	Collection site	Coordinates
1	HUIB_AR1 (AR1)	Loc Tri, Phu Loc, Hue City	16°11'36.2"N 107°50'56.5"E
2	HUIB_AR2 (AR2)	Loc Thuy, Phu Loc, Hue City	16°13'45.4"N 107°50'53.2"E
3	HUIB_AR3 (AR3)	Huong Phu, Phu Loc, Hue City	16°11'29.4"N 107°44'26.1"E
4	HUIB_AR4 (AR4)	Huong Phu, Phu Loc, Hue City	16°11'26.7"N 107°45'04.1"E
5	HUIB_AR5 (AR5)	Huong Phu, Phu Loc, Hue City	16°11'09.4"N 107°45'21.5"E
6	HUIB_AR6 (AR6)	Huong Phu, Phu Loc, Hue City	16°11'08.1"N 107°45'26.4"E
7	HUIB_AR7 (AR7)	Thuong Lo, Phu Loc, Hue City	16°04'58.1"N 107°45'02.4"E
8	HUIB_AR8 (AR8)	Thuong Lo, Phu Loc, Hue City	16°04'45.9"N 107°45'23.2"E
9	HUIB_AR9 (AR9)	Huong Loc, Phu Loc, Hue City	16°08'12.4"N 107°50'49.0"E
10	HUIB_AR10 (AR10)	Huong Loc, Phu Loc, Hue City	16°07'55.4"N 107°51'09.0"E
11	HUIB_AR11 (AR11)	Huong Loc, Phu Loc, Hue City	16°07'54.2"N 107°51'12.3"E
12	HUIB_AR12 (AR12)	Huong Loc, Phu Loc, Hue City	16°07'53.1"N 107°51'26.4"E
13	HUIB_AR13 (AR13)	Loc Tri, Phu Loc, Hue City	16°11'34.5"N 107°50'44.2"E
14	HUIB_AR14 (AR14)	Huong Loc, Phu Loc, Hue City	16°07'27.9"N 107°47'16.4"E
15	HUIB_AR15 (AR15)	Huong Phu, Phu Loc, Hue City	16°13'04.1"N 107°43'53.6"E
16	HUIB_AR16 (AR16)	Huong Phu, Phu Loc, Hue City	16°13'03.9"N 107°44'50.9"E
17	HUIB_AR17 (AR17)	Huong Phu, Phu Loc, Hue City	16°12'06.5"N 107°44'50.6"E

RAPD analysis

Firstly, we randomly chose genomic DNA of two accessions (HUIB_AR14 and HUIB_AR16) to screen with 100 UBC RAPD primers. Then, only primers that showed polymorphism between these 2 accessions were used to amplify DNA from 4 accessions (HUIB_AR14, HUIB_AR15, HUIB_AR16 and HUIB_AR17) to identify primers that showed polymorphism in all four accessions (Table 2). These RAPD primers were finally used to amplify all 17 accessions and evaluate the genetic characteristics.

PCR (10 μL) contained 50 ng DNA, 5 μL of 2X MyTaq Mix (Meridian Bioscience), 1.675 mM added $MgCl_2$, 0.67 μM of primer, 167.5 μM of each dNTP. PCR amplification was conducted as follows: initial strand separation at 95 °C for 5 minutes; 40 cycles of 1 minute at 95 °C, 1 minute at 35 °C, 2 minutes at 72 °C; and a final extension for 10 min at 72 °C. PCR products were then separated on 1% agarose gel (in 0.5X TAE buffer) and stained with a 1:10000 dilution of SYBR Green I (Invitrogen). Agarose gel was visualized under ultraviolet light (Vilber).

Table 2. List of selected polymorphic primers

No.	Primer name	Sequence	T_m	No.	Primer name	Sequence	T_m
1	UBC#405	CTCTCGTGCG	34	10	UBC#447	CAGGCTCTAG	32
2	UBC#406	GCCACCTCCT	34	11	UBC#448	GTTGTGCCTG	32
3	UBC#411	GAGGCCCGTT	34	12	UBC#452	CTAATCACGG	30
4	UBC#412	TGCGCCGGTG	36	13	UBC#458	CTCACATGCC	32
5	UBC#427	GTAATCGACG	30	14	UBC#469	CTCCAGCAAA	30
6	UBC#429	AAACCTGGAC	30	15	UBC#471	CCGACCGGAA	34
7	UBC#440	CTGTCGAACC	32	16	UBC#476	TTGAGGCCCT	32
8	UBC#441	CTGCGTTCTT	30	17	UBC#498	GACAGTCCTG	32
9	UBC#446	GCCAGCGTTC	34				

Data analysis

To begin the analysis of the RAPD data, a binary matrix was created based on the electrophoretograms. Specifically, the number "1" was assigned to DNA bands (clear, unaltered bands), the number "0" was assigned to the absence of DNA bands (or too faint), and their sizes were estimated via the GeneRuler 1 kb DNA Ladder Mix (Thermo Scientific). Then, it was used to calculate evaluation indicators including: total number of bands (TB), number of polymorphic bands (PB), number of monomorphic bands (MB), percentage of polymorphic bands (PPB (%)), polymorphism information content (PIC), resolving power (Rp), marker index (MI) and effective multiplex ratio (EMR) [19]. In addition, the matrix was also analyzed using POPGENE 1.32 software to find diversity indices like Nei's gene diversity (h), Shannon's information index (I), observed number of alleles (n_a) and effective number of alleles (n_e). Finally, the matrix data was put into NTSYS software version 2.10 to calculate genetic similarity coefficients and develop a UPGMA clustering tree [18, 20].

3 Results and Discussion

Information on genetic diversity indicators

From the 17 selected RAPD primers, 244 DNA bands were generated with 186 polymorphic bands, accounting for 76.105%. This polymorphism rate was much higher than the results of Nguyen and David [16, 21]. PPB% of UBC#446 and UBC#427 were the highest, reaching 100% and 92.308%, respectively, followed by UBC#498 and UBC#469. The primer possessing the fewest polymorphic bands was UBC#406 with 5 bands (33,333%) (Table 3, Figure 1). Furthermore, the mean number of amplified RAPD loci was 14.353, which indicated a very high allelic richness.

These results indicated the genetic pool of this germplasm is more diverse than previously reported germplasm [1, 15]. The number of polymorphic bands in the study of Ho et al. and the study of Tran et al. were 9.05 and 8.0, respectively.

A useful molecular marker can detect genetic differences existing in a group of individuals. Quantitatively, the level of polymorphism can be assessed through genetic diversity indices. Among them, polymorphism information content (PIC) played the most important role, and it was similar to heterozygosity in dominant markers such as RAPD [19, 22]. The study results showed that only 6 RAPD primers had high PIC values (greater than 0.25), namely UBC#405, UBC#427, UBC#446, UBC#469, UBC#476 and UBC#498. The remaining primers brought medium PIC indices (0.1< PIC < 0.25), ranging from 0.116 to 0.235. Overall, the mean PIC values for all primers were only moderate (PIC = 0.209) (Table 3), but it was similar to that reported in some other studies. Specifically, the average value of PIC reached 0.20-0.21 in the studies of Diallo et al. and Saengprajak et al. [22, 23].

MI, Rp and EMR were important indicators for evaluating a molecular marker. MI suggested that applying a technique to evaluate amplified bands with a large number was more effective than relying only on polymorphic bands [24]. Rp expressed the correlation between genotypes and DNA molecular markers; the higher the Rp, the more effective the molecular marker was in classifying genotypes and vice versa [25]. Furthermore, the EMR index indicated the effectiveness of the primer [26]. The average values of the three indices (MI, Rp and EMR) were 2.039, 5.225 and 8.742, respectively. They were most prominent in primers UBC#405, UBC#427, UBC#446, UBC#469, UBC#476 and UBC#498. These were also the primers with the largest PIC values (Table 3). From this, it can be concluded that these 6 RAPD primers are the most effective in assessing genetic diversity in *Anoectochilus* populations. In addition, the 17 primers used to survey 17 *A. roxburghii* accessions provided high n_a, n_e, h, and I values, reaching 1.762, 1.428, 0.249 and 0.373, respectively (Table 4). These h index and I index were higher than the analytical results in *A. burmannicus*, *A. albolineatus*, *A. roxburghii* and *A. elwesii* reported by Khemkladngoen et al. [27]. This showed the effectiveness of using these 17 RAPD primers in demonstrating allelic and gene polymorphism in populations.

Table 3. Evaluation indicators of RAPD analysis

No.	Primer name	TB	PB	PPB (%)	PIC	Rp	MI	EMR
1	UBC#405	18	14	77.778	0.296	7.294	3.218	10.889
2	UBC#406	15	5	33.333	0.116	3.059	0.194	1.667
3	UBC#411	15	12	80.000	0.235	5.882	2.259	9.600
4	UBC#412	14	11	78.571	0.120	2.471	1.034	8.643
5	UBC#427	13	12	92.308	0.274	7.412	3.030	11.077
6	UBC#429	12	10	83.333	0.208	5.176	1.737	8.333
7	UBC#440	8	6	75.000	0.117	2.941	0.528	4.500
8	UBC#441	10	8	80.000	0.175	4.353	1.120	6.400
9	UBC#446	14	14	100.000	0.295	7.647	4.126	14.000
10	UBC#447	15	10	66.667	0.186	4.706	1.243	6.667
11	UBC#448	15	7	46.667	0.118	2.706	0.386	3.267
12	UBC#452	12	10	83.333	0.180	4.118	1.499	8.333
13	UBC#458	19	16	84.211	0.204	4.941	2.753	13.474
14	UBC#469	16	14	87.500	0.286	7.412	3.501	12.250
15	UBC#471	13	7	53.846	0.161	4.235	0.608	3.769
16	UBC#476	17	14	82.353	0.276	6.941	3.182	11.529
17	UBC#498	18	16	88.889	0.299	7.529	4.250	14.222
	Mean	**14.353**	**10.941**	**76.105**	**0.209**	**5.225**	**2.039**	**8.742**

Note: Total number of bands (TB), number of polymorphic bands (PB), number of monomorphic bands (MB), percentage of polymorphic bands (PPB (%)), polymorphism information content (PIC), resolving power (Rp), marker index (MI), effective multiplex ratio (EMR).

Table 4. Genetic diversity indicators in RAPD analysis

Indices	n_a	n_e	h	I
	1.762	1.428	0.249	0.373
SD	0.427	0.380	0.196	0.272

Note: Nei's gene diversity (h), Shannon's information index (I), observed number of alleles (n_a), effective number of alleles (n_e); SD: Standard deviation.

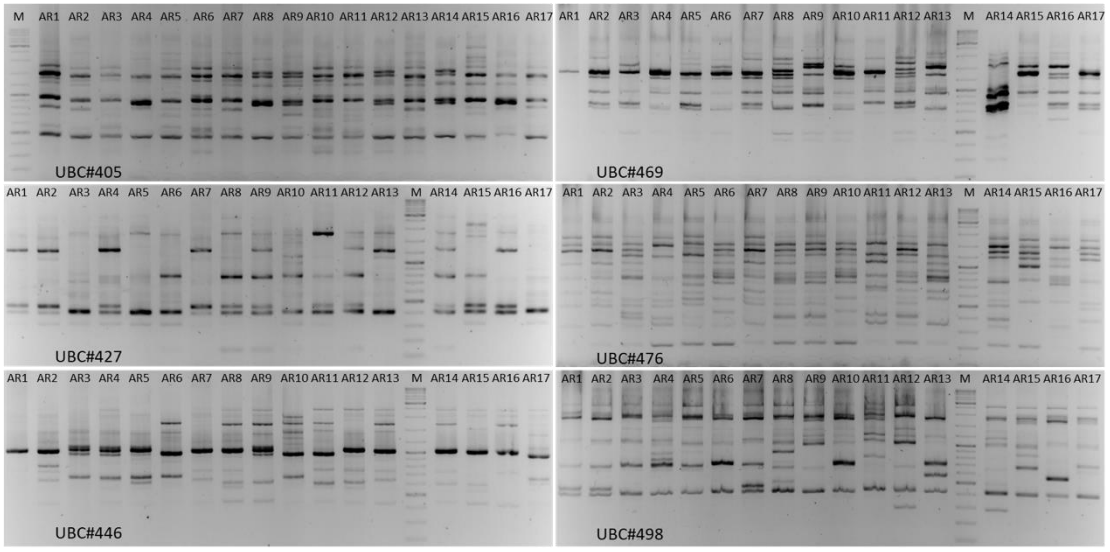

Figure 1. Electrophoresis results of the 6 most polymorphic RAPD primers (UBC#405, UBC#427, UBC#446, UBC#469, UBC#476 and UBC#498). M: GeneRuler 1 kb DNA Ladder

Genetic relationship among studied accessions

Genetic diversity studies played important roles for species conservation and breeding programs [28]. In this analysis, similarity coefficients are employed to assess genetic similarity. The closer the species were genetically, the closer the value of these similarity coefficients was to 1. A higher coefficient showed a closer genetic relationship and greater similarity between individuals, while a lower one addressed greater genetic diversity [29]. In this study, the pairwise comparison of accessions indicated relative genetic similarity between accessions ranging from a maximum of 0.930 to a minimum of 0.635. While HUIB_AR10 (AR10) was closely related to HUIB_AR06 (AR06), it had the greatest genetic distance from HUIB_AR01 (AR01) (Table 5). Overall, accessions were quite genetically similar, as their genetic similarity coefficients were large. This may be because there were not many geographical variations in the collected accessions.

To quantify the polymorphism level among the genotypes, Jaccard's similarity matrix was used to generate a dendrogram using the unweighted pair group method with arithmetic average algorithm (UPGMA) in NTSYS software. The resulting dendrogram revealed that all 17 accessions were grouped into three major clusters at a similarity coefficient of 72.0%. Cluster I comprised 6 accessions: HUIB_AR1 (AR1), HUIB_AR2 (AR2), HUIB_AR4 (AR4), HUIB_AR7 (AR7), HUIB_AR13 (AR13) and HUIB_AR16 (AR16). Cluster II consisted of six other accessions: HUIB_AR8 (AR8), HUIB_AR9 (AR9), HUIB_AR11 (AR11), HUIB_AR12 (AR12), HUIB_AR14

(AR14) and HUIB_AR15 (AR15). The remaining five accessions were assigned to cluster III (Figure 2). This clustering showed that genetic differences existed among accessions. This genetic variation is likely due to the fact that *Anoectochilus* reproduces sexually via seeds, which inherently generates genotypic variation [30, 31].

Table 5. Genetic similarity coefficients among 17 *A. roxburghii* accessions

	AR1	AR2	AR3	AR4	AR5	AR6	AR7	AR8	AR9	AR10	AR11	AR12	AR13	AR14	AR15	AR16	AR17
AR1	1.000																
AR2	0.898	1.000															
AR3	0.738	0.750	1.000														
AR4	0.734	0.787	0.766	1.000													
AR5	0.684	0.713	0.791	0.754	1.000												
AR6	0.648	0.709	0.811	0.717	0.709	1.000											
AR7	0.852	0.889	0.730	0.758	0.668	0.705	1.000										
AR8	0.680	0.717	0.672	0.775	0.676	0.697	0.738	1.000									
AR9	0.717	0.730	0.750	0.754	0.697	0.717	0.742	0.840	1.000								
AR10	**0.635**	0.705	0.783	0.697	0.680	**0.930**	0.693	0.709	0.730	1.000							
AR11	0.697	0.725	0.705	0.709	0.668	0.697	0.721	0.721	0.750	0.701	1.000						
AR12	0.693	0.738	0.717	0.754	0.672	0.734	0.742	0.807	0.803	0.746	0.775	1.000					
AR13	0.713	0.791	0.754	0.799	0.750	0.795	0.795	0.770	0.775	0.791	0.762	0.775	1.000				
AR14	0.680	0.693	0.697	0.734	0.676	0.672	0.689	0.811	0.799	0.676	0.721	0.824	0.770	1.000			
AR15	0.676	0.705	0.684	0.730	0.689	0.709	0.709	0.725	0.713	0.689	0.717	0.746	0.750	0.725	1.000		
AR16	0.734	0.754	0.725	0.779	0.705	0.717	0.775	0.717	0.697	0.697	0.717	0.738	0.824	0.734	0.738	1.000	
AR17	0.689	0.709	0.811	0.783	0.807	0.779	0.713	0.713	0.750	0.766	0.705	0.725	0.795	0.746	0.750	0.775	1.000

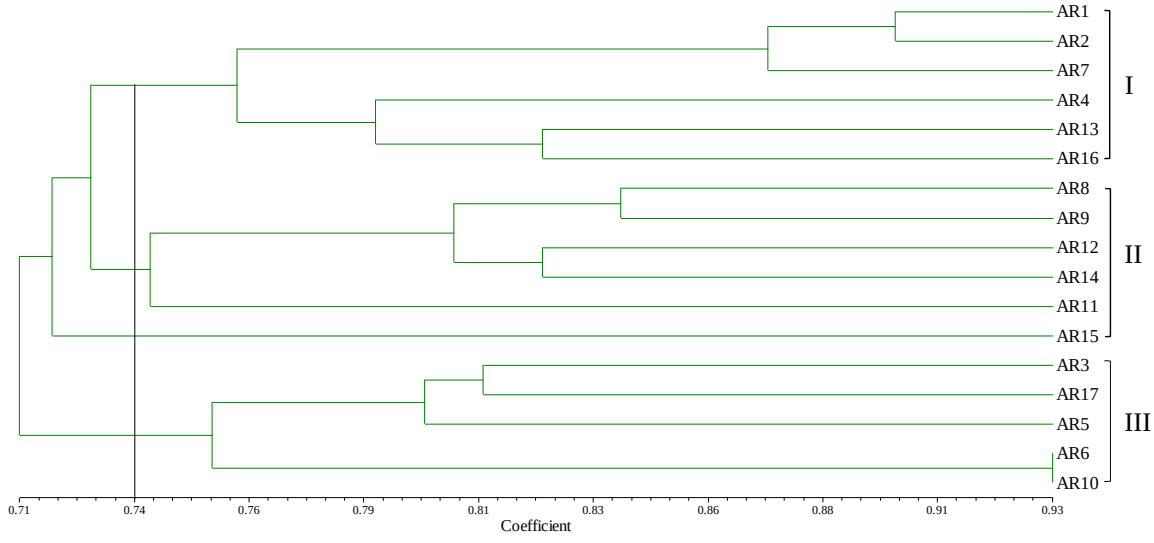

Figure 2. UPGMA clustering of 17 *A. roxburghii* accessions based on RAPD data

3 Conclusion

In this study, we evaluated the genetic diversity of *A. roxburghii* accessions collected in Hue City, Vietnam. Seventeen polymorphic RAPD primers were selected to amplify the DNA of 17 accessions. These primers yielded a relatively high proportion of polymorphic bands (76.105%) and high polymorphism indices (n_a, n_e, h, I). UBC#405, UBC#427, UBC#446, UBC#469, UBC#476, and UBC#498 were the six best primers for assessing genetic diversity because they possessed the highest PIC, Rp, MI and EMR indices. In addition, dendrogram analysis also revealed a pretty high genetic differentiation among accessions in the *A. roxburghii* population. Three clusters were separated based on similarity coefficients. Among them, HUIB_AR01 (AR01) and HUIB_AR10 (AR10) had the largest genetic distance.

Acknowledgements

Nhi Thi Hoang Ho was funded by the Master, PhD Scholarship Programme of Vingroup Innovation Foundation (VINIF), code VINIF.2023.ThS.099. This study was partially supported by the Core Research Program (NCTB.DHH.2024.03), which covered article processing charges.

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