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Research Article

High Genetic Diversity of *Dysosma tonkinense* Revealed by ISSR and RAPD Markers

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Abstract

Background and Objective: *Dysosma tonkinense* (Gagnep.) M. Hiroe (Berberidaceae) is a precious medicinal plant that distributes in Vietnam and Southern areas of China. The dried roots and rhizomes of *D. tonkinense* have been used in folk medicine for the treatment of poisonous snake bite, ulcer and cancer. This study aimed to analyze the genetic diversity of 20 accessions of *D. tonkinense* collected from 13 geographical regions of Vietnam through ISSR and RAPD markers. **Materials and Methods:** The 24 ISSR and 11 RAPD primers were examined in this study. Twenty four ISSR primers produced 136 PCR bands, of which 117 were polymorphic with an average of 5.67 polymorphic loci per primer and only 19 loci were monomorphic. **Results:** RAPD marker detected 79 loci, 52 of these markers were polymorphic with an average of 7.18 loci per primer. The similarity coefficient ranged from 0.59-0.85, 0.60-0.98 and 0.62-0.89 for ISSR, RAPD and pooled ISSR-RAPD data, respectively. The UPGMA dendrogram generated using ISSR-RAPD data showed that, at the genetic similarity of 72%, 20 accessions were separated into 3 distinct clusters. **Conclusion:** The present finding indicates that the marker tools ISSR and RAPD combined can be used in determining the genetic relationship between the accessions. It may be concluded that the genetic diversity among the 20 *D. tonkinense* accessions collected in several locations in Vietnam could be utilized for the conservation and breeding programs especially for some endangered species such as *D. tonkinense*.

Key words: *Dysosma tonkinense*, genetic diversity, ISSR marker, RAPD marker, breeding programs

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dysosma Woodson is a small genus that includes 7-10 herb perennial species belong to Berberidaceae family. The genus occurs in China and Vietnam, among them, 6 endemic species in China (FOC). *Dysosma* Woodson has a close relationship with *Diphylleia* Michx. occurring in Western Asia and Northern-America¹. *Podophyllum* L. occurring in Northwestern-America and *Sinopodophyllum* Ying occurring in Western of Himalaya².

Dysosma tonkinense (Gagnep.) M. Hiroe is a fork medicinal plant with most of its distribution occurring in Vietnam and Southern areas of China¹. The species has several synonyms such as *Podophyllum tonkinense* Gagnep, *P. versipelle* Hance subsp. *versipelle*, *Dysosma difformis* (Hemsley and E.H. Wilson) T.H. Wang ex T.S. Ying¹.

In Vietnam, *Dysosma tonkinense* distributes in most of the Northern mountain provinces such as Hoa Binh, Son La, Lai Chau, Dien Bien, Lao Cai, Ha Giang, Tuyen Quang, Yen Bai at an altitude of 470-1.720 m altitude. Kon Tum, a central province of Vietnam, is a new distributional record for *D. tonkinense*, which is likely the southernmost occurrence of the species. The dried rhizomes and roots of *D. tonkinense* have been used in the traditional medicine of Vietnam, call "Bat giac lien"¹. In recent decades, *D. tonkinense* has been threatened by rapid habitat destruction and overexploitation of the forest for timber and medicinal plants. It has been classified as a category "Endangered (EN) species" on the Vietnam Red Data Book³.

Information of genetic resources plays an important role in plant improvement^{4,5}. Moreover, information on genetic diversity is also important for germ plant conservation. Therefore, an analysis of the genetic diversity of *D. tonkinense* accessions collected from 13 geographical regions of Vietnam is necessary.

The assessment and evaluation of genetic diversity by using molecular marker have been very popular since this approach is very powerful in the evaluation of genetic diversity within and among species. Random amplified polymorphic DNA (RAPD)⁶ and inter-simple sequence repeats (ISSR) markers⁷ have been popularly used for evaluation of genetic diversity⁸⁻¹¹ and to identify the relationships between the collected population at the species and cultivar levels¹².

In this study, genetic diversity and relationships among twenty *Dysosma tonkinense* (Gagnep.) M. Hiroe (Berberidaceae) accessions collected from various geographical regions of Vietnam were analyzed using 11 random amplified polymorphic DNA and 24 Inter-Simple Sequence Repeat (ISSR) molecular markers. Besides, the

present study also compared the potential of using RAPD and ISSR markers and their relative efficiency in exploring the genetic diversity of *Dysosma tonkinense* (Gagnep.) M. Hiroe (Berberidaceae).

MATERIALS AND METHODS

Study area: Twenty *Dysosma tonkinense* accessions were collected in several provinces in Vietnam during the flowering season from January, 2016 to December, 2019. The collected accessions were grown in Sapa Medicinal Plant Research Station, National Institute of Medicinal Materials, Vietnam. Molecular experiments were performed from October, 2019 to May, 2020 at the Faculty of Biotechnology, Vietnam National University of Agriculture.

Plant material and DNA extraction: For genetic structure studies, 13 geographically different populations of 19 accessions of *D. tonkinense* from northern and 1 accession of *D. tonkinense* from central Vietnam were sampled. Each accession was positioned by using the Global Positioning System (GPS) tracking with location details listed in Table 1. In the field, fresh and young leaf tissues were collected, dried in silica and transferred to the lab where they were kept at 20°C for further analysis. Total genomic DNA was isolated from leaves according to the Handmade CTAB-based kit¹³. The concentration of DNA was determined by using an Eppendorf Bio Photometer plus spectrophotometer, DNA samples were then diluted to the concentration of 10 ng μL^{-1} and 1 μL of the diluted solution was used as the DNA template for PCR assay.

ISSR and RAPD amplification: All RAPD primers were designed by Operon Tech. Inc. Alameda, CA, USA and UBC_ISSR primers were designed by the University of British Columbia, Canada. ISSR-13 and ISSR-14 and UBC827 were employed in maize (*Zea mays* L.) and *Pitcairnia flammea* (L.) John (Bromeliaceae) ISSR work^{14,15}.

Each 20 μL PCR reaction consisted of iNtRON's2xPCR Master mix solution (i-TaqTM), 0.5 μM primer and 10 ng DNA templates. ISSR amplification was performed using 24 primers in Table 2 in thermocycler instrument Takara PCR Thermal Cycler Personal with the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles: 94°C for 30 sec, annealing at 35-74.3°C (depending on primers on Table 2) for 30 sec and 72°C for 2 min. The final extension was at 72°C for 7 min and hold at 4°C until samples were collected.

RAPD amplifications were performed using 11 randomly primers (Table 2) in thermocycler instrument Takara PCR

Table 1: Details of *D. tonkinense* accessions collected from different regions of Vietnam for genetic diversity analysis

Codes	Accession name	Province	Latitude/longitude	Altitude (m)
M1	D.DB01	Hoa Binh	20°53'38.63"N, 105°12'35.74"E	905
M2	D.NH01	Kon Tum	14°43'56.00"N, 107°32'25.86"E	753
M3	D.SH01	Lai Chau	22°18'15.69"N, 103°13'47.85"E	1,802
M4	D.SH02	Lai Chau	22°18'24.87"N, 103°15'51.85"E	1,815
M5	D.SP01	Lao Cai	22°17'49.74"N, 103°56'19.94"E	1,662
M6	D.BH01	Lao Cai	22°36'35.74"N, 104°15'18.71"E	1,420
M7	D.BH02	Lao Cai	22°36'26.67"N, 104°15'39.25"E	1,669
M8	D.TY03	Yen Bai	21°32'56.46"N, 104°40'34.61"E	1,016
M9	D.VC01	Yen Bai	21°33'58"N, 104°40'03.79"E	902
M10	D.TC01	Dien Bien	21°58'40.52"N, 103°22'55.58"E	1,211
M11	D.VX02	Ha Giang	22°58'4.0"N, 104°53'10.23"E	765
M12	D.CH01	Tuyen Quang	22°15'59.30"N, 105°17'12.88"E	466
M13	D.CH02	Tuyen Quang	22°07'29.24"N, 105°26'6.65"E	520
M14	D.NHg03	Tuyen Quang	22°18'22.57"N, 105°15'55.30"E	577
M15	D.VX01	Ha Giang	22°58'910"N, 104°52'946"E	771
M16	D.TrD01	Lang Son	22°12'33.34"N, 106°26'23.50"E	461
M17	D.NB01	Cao Bang	22°40'23.03"N, 105°48'44.13"E	1,337
M18	D.VN01	Thai Nguyen	21°48'59.47"N, 105°55'23.00"E	643
M19	D.TD01	Vinh Phuc	21°27'50.58"N, 105°35'41.16"E	1,086
M20	D.BV01	Ha Noi	21°04'15.36"N, 105°21'57.44"E	875

Table 2: Polymorphism among 20 *D. tonkinense* accessions revealed by ISSR markers

Primer name	Nucleotide sequence (5'-3')	Annealing temperature (°C)	Total number of loci	Polymorphism (%)	Total number of bands	Number of bands/accession	PIC value	Rp value
ISSR-13	CAG(CA)8	44	3	66.67	49	2.45	0.20	4.90
ISSR-14	CGT(CA)8	44	3	66.67	44	2.20	0.31	4.40
UBC827	(AC)6CG	42	1	0.00	20	1.00	0.00	2.00
UBC_818	(CA)8AG	56	7	71.43	69	3.45	0.28	6.90
UBC_834	(AG)8YT	52.7	4	100.00	43	2.15	0.25	4.30
UBC_843	(CT)8RA	52.7	6	100.00	50	2.50	0.41	5.00
UBC_867	(GGC)6	74.3	4	75.00	45	2.25	0.25	4.50
UBC_879	(CTTCA)3	40.6	8	100.00	72	3.60	0.29	7.20
UBC_829	TAGATCTGATATCTGAATCCC	56.5	15	100.00	73	3.65	0.32	7.30
UBC_895	AGAGTTGGTAGCTCTTGATC	56.4	12	100.00	83	4.15	0.36	8.30
UBC_899	CATGGTGTTGGTCATTGTTCCA	60.3	11	100.00	70	3.50	0.39	7.00
UBC_900	ACTTCCCCACAGGTTAACACA	59.4	16	100.00	103	5.15	0.35	10.30
UBC_807	(AG)8T	49	3	66.67	27	1.35	0.17	2.70
UBC_811	(GA)8C	50	4	50.00	51	2.55	0.18	5.10
UBC_823	(TC)8C	50	6	100.00	65	3.25	0.44	6.50
UBC_827	(AC)8G	56	4	75.00	40	2.00	0.07	4.00
UBC_861	(ACC)6	60.7	6	50.00	94	4.70	0.18	9.40
UBC_864	(ATG)6	45	5	100.00	20	1.00	0.22	2.00
UBC_866	(CTC)6	56	3	66.67	45	2.25	0.17	4.50
UBC_868	(GAA)6	45	2	100.00	20	1.00	0.50	2.00
UBC_872	(GATA)4	35	4	50.00	53	2.65	0.20	5.30
UBC_873	(GACA)4	45	2	0.00	40	2.00	0.00	4.00
UBC_876	(GATA)2(GACA)2	42	4	75.00	73	3.65	0.14	7.30
UBC_808	A(GA)7GC	50	3	100.00	36	1.80	0.36	3.60
Total			136		1,285			
Average/primer			5.67	75.55	53.54	2.68	0.25	5.35
Average/accession					64.25	3.21		

PIC: Polymorphism information content, Rp value: Resolving power of a primer

Thermal Cycler Personal with the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles: 94°C for 30 sec, annealing at 30-32°C (depending on primers on Table 2) for 30 sec and 72°C for 2 min. Final extension at 72°C for 7 min and hold at 4°C.

Agarose gel electrophoresis: The PCR products of ISSR and RAPD were then separated on 1.5% agarose in 1×Tris-Acetate-EDTA buffer (TAE) for 60 min. DNA bands were visualized by staining with Red safe™ and pictured on a UV transilluminator (UVPBio-Doc-It). The size of the amplified

product was estimated using standard size DNA fragments having a known molecular weight (1 kb DNA Ladder-Thermo Fisher, USA). The experiment was repeated twice with each primer and only those were validated as repeatable patterns were selected for further analysis.

Data analysis: Reproducible band was manually scored '1' for presence and '0' for absence. The data were analyzed using NTSYS pc ver. 2.1¹⁶. Similarity coefficient of Sokal-Michener (1958) matrices was used to perform cluster analysis using the unweighted pair group method with arithmetic (UPGMA) and Dendrogram was constructed with the TREE program of NTSYS 2.1.

Polymorphism Information Content (PIC) of each locus was determined by the formula:

$$PIC = 1 - \sum (P_{ij})^2$$

Where:

Pi : Frequency of the ith allele in the genotypes¹⁷

The resolving power (Rp) of a primer is:

$$R_p = \sum I_b$$

where, I_b (band informativeness) takes the value of $1 - [2 \times (0.5 - p)]$.

where, p represents the proportion of the 20 individuals containing the band¹⁷.

Estimation of genetic distance matrix correlation between markers was done using a Mantel Test in XLSTAT package¹⁸.

RESULTS

Molecular analysis using ISSR and RAPD markers: ISSR and RAPD profiles are represented in Fig. 1a and b, respectively. The 24 ISSR markers amplified a total of 136 loci with an average of 5.67 loci per primer, out of which 19 (24.45%) were monomorphic loci and 117 (75.55%) were polymorphic revealing a high degree of polymorphism in Table 2. Moreover, the ISSR primer UBC_829 and UBC_900 produced maximum number of 15 and 16 loci, while UBC827, UBC_868 and UBC_873 generated the minimum number of 1 and 2 loci. The ISSR primers UBC_834, UBC_843, UBC_879, UBC_829, UBC_895, UBC_899, UBC_900, UBC_823, UBC_864, UBC_868 and UBC_808 exhibited the highest polymorphism (100%), while both UBC827 and UBC_873 primers showed the lowest polymorphism (0%) (Table 2). A total of 1,285 bands were detected (53.54 bands per primer and 64.25 bands per accession). The average PIC (Polymorphism Information Content) value for ISSR was 0.25 and the resolving power ranged from 2.0 (UBC827, UBC_864 and UBC_868) to 10.3 (UBC_900) across all accession (Table 2).

RAPD profiles are represented in Fig. 1b, 11 RAPD markers used in the study amplified 79 loci (an average of 7.18 loci per primer), out of which 55 (66.20%) were polymorphic and 24 (33.80%) were monomorphic loci in Table 3. Primer OPC-04

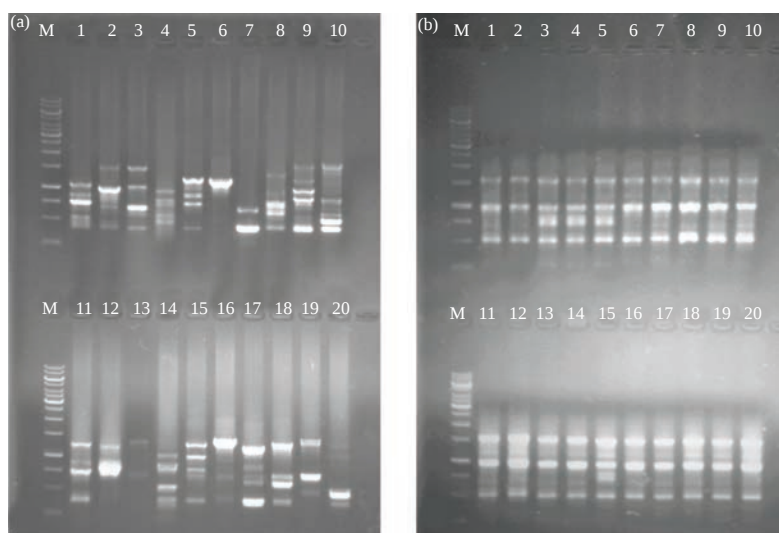


Fig. 1(a-b): ISSR markers were more efficient than the RAPD in the detection of DNA polymorphism of the 20 *Dyosma tonkinense* accessions, (a) ISSR profile obtained with primer UBC829 and (b) RAPD profile obtained with primer OPB05

Lane M: Marker, lane from 1- 20 were amplified PCR products from accession D.DB01, D.NH01, D.SH01, D.SH02, D.SP01, D.BH01, D.BH02, D.TY03, D.VC01, D.TC01, D.VX02, D.CH01, D.CH02, D.NH03, D.VX01, D.TrD01, D.NB01, D.VN01, D.TD01 and D.BV01, respectively

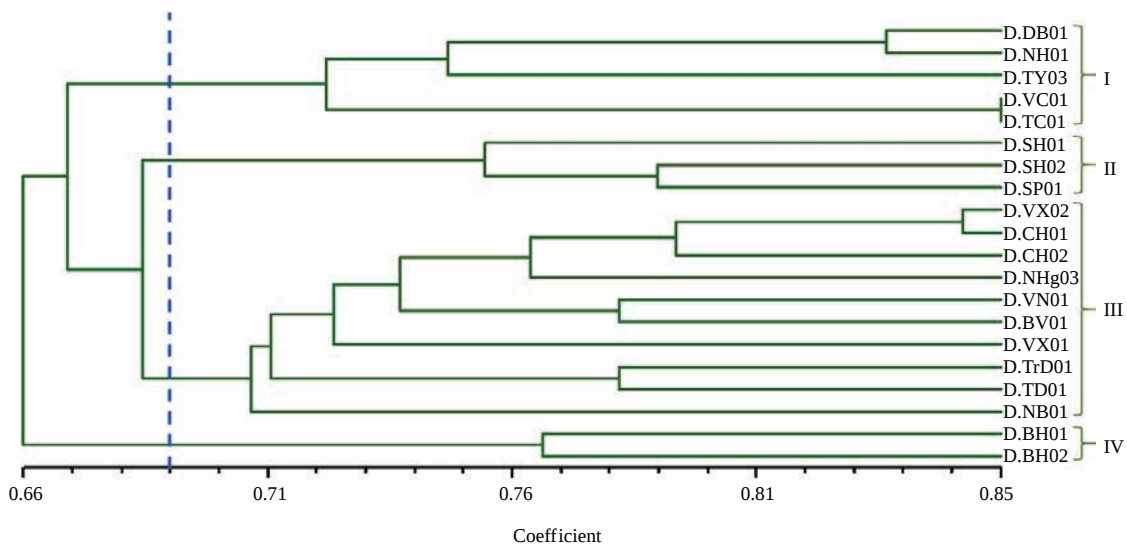


Fig. 2: UPGMA cluster analysis of 20 *Dysosma tonkinense* accessions with a similarity coefficient of ISSR marker

Dash line indicates the mean similarity (0.69). The dendrogram distributed the 20 accessions in four indicated groups (I, II, III and IV)

Table 3: Polymorphism among 20 *D. tonkinense* accessions revealed by RAPD markers

Primer name	Nucleotide sequence (5'-3')	Annealing temperature (°C)	Total number of loci	Polymorphism (%)	Total number of bands	Number of bands /accession	PIC value	Resolving power
OPA04	AATCGGGCTG	30	7	57.14	93	4.65	0.15	9.3
OPB02	TGATCCCTGG	30	9	66.67	102	5.10	0.20	10.2
OPB04	GGACTGGAGT	30	4	75.00	46	2.30	0.17	4.6
OPB05	TGCGCCCTTC	32	6	50.00	88	4.40	0.19	8.8
OPB06	TGCTCTGCCC	32	8	75.00	64	3.20	0.11	6.4
OPC02	GTGAGGCGTC	32	4	50.00	58	2.90	0.16	5.8
OPC04	CCGCATCTAC	30	12	91.67	74	3.70	0.26	7.4
OPC05	GATGACCGCC	32	7	57.14	107	5.35	0.17	10.7
OPD01	ACCGCGAAGG	32	9	88.89	65	3.25	0.32	6.5
OPD02	GGACCCAACC	32	4	50.00	63	3.15	0.19	6.3
OPD03	GTCGCCGTCA	32	9	66.67	117	5.85	0.21	11.7
Total			79		877			
Average/primer			7.18	66.20	79.73	3.99	0.19	7.97
Average/accession					43.85			

generated the maximum number of loci (12 loci), while OPB04, OPC02 and OPD02 amplified the minimum number of loci (4 loci). The RAPD primer OPC04 detected the highest polymorphism, while the lowest polymorphism was recorded by the OPB05, OPC02 and OPD02 primers. A total of 877 bands were detected (79.73 bands per primer and 43.85 bands per accession). The average PIC (Polymorphism Information Content) value for RAPD was 0.19 and the resolving power ranged from 4.6 (OPB04) to 11.7 (OPD03) across all accession.

20 *Dysosma tonkinense* accessions assessed by ISSR marker displayed high genetic diversity, with a mean similarity level of 0.69, varying from 0.59 (D.BH02 vs. D.DB01) to 0.85 (D.TC01 vs. D.VC01 and D.CH01 vs. D.VX02) in Table 4. The generated pairwise similarity matrix was utilized to create a

dendrogram using the UPGMA hierarchical clustering method. At the mean of 69% similarity level, 20 accessions were grouped into four main clusters in Fig. 2. Cluster I comprises 5 accessions including D.DB01, D.NH01, D.TY03, D.VC01, D.TC01. Cluster II comprise 3 accessions including D.SH01, D.SH02, D.SP01. Cluster III comprises 10 accessions including D.VX02, D.CH01, D.CH02, D.NHg03, D.VN01, D.BV01, D.VX01, D.TrD01, D.TD01 and D.NB01. Accessions D.BH01 and D.BH02 were grouped into cluster IV (Fig. 2).

Based on RAPD primers, the pairwise similarity values among 20 accessions fluctuated between 0.60 (D.BH02 vs. D.TY03) and 0.98 (D.VX02 vs. D.CH01) in Table 4 and the mean similarity was 0.79 in Fig. 3. In general, the ISSR marker shows a better capacity to detect variability between accessions than

Table 4: Matrix of genetic similarity among 20 *D. tonkinense* accessions revealed by ISSR markers (above diagonal) and RAPD markers (below diagonal) calculated by similarity coefficient of Sokal-Michener

	D.DB01	D.NH01	D.SH01	D.SH02	D.SP01	D.BH01	D.BH02	D.TY03	D.VC01	D.TC01	D.VX02	D.CH01	D.CH02	D.NHg03	D.VX01	D.TrD01	D.NB01	D.VN01	D.TD01	D.BV01
D.DB01	0.83	0.62	0.69	0.65	0.65	0.62	0.59	0.75	0.74	0.70	0.72	0.70	0.68	0.72	0.70	0.65	0.68	0.74	0.67	0.68
D.NH01	0.89	0.65	0.67	0.63	0.63	0.65	0.63	0.74	0.75	0.69	0.70	0.66	0.67	0.65	0.68	0.68	0.65	0.74	0.72	0.69
D.SH01	0.82	0.81	0.76	0.74	0.74	0.74	0.72	0.60	0.68	0.73	0.72	0.70	0.71	0.72	0.71	0.73	0.65	0.68	0.67	0.73
D.SH02	0.81	0.80	0.82	0.79	0.69	0.69	0.63	0.63	0.67	0.64	0.72	0.74	0.71	0.69	0.67	0.70	0.63	0.64	0.70	0.70
D.SP01	0.78	0.80	0.84	0.86	0.67	0.63	0.63	0.61	0.63	0.62	0.68	0.69	0.64	0.63	0.74	0.69	0.62	0.68	0.68	0.68
D.BH01	0.77	0.81	0.74	0.75	0.82	0.76	0.76	0.68	0.68	0.70	0.71	0.68	0.72	0.63	0.63	0.71	0.65	0.61	0.68	0.70
D.BH02	0.71	0.75	0.68	0.66	0.76	0.69	0.60	0.62	0.67	0.71	0.72	0.67	0.66	0.63	0.63	0.67	0.64	0.61	0.65	0.70
D.TY03	0.72	0.74	0.64	0.68	0.68	0.69	0.60	0.78	0.74	0.71	0.66	0.65	0.66	0.66	0.64	0.65	0.70	0.70	0.68	0.64
D.VC01	0.80	0.83	0.74	0.77	0.75	0.76	0.75	0.78	0.74	0.85	0.71	0.71	0.67	0.65	0.62	0.68	0.69	0.69	0.66	0.69
D.TC01	0.83	0.82	0.82	0.76	0.81	0.82	0.76	0.77	0.84	0.84	0.70	0.69	0.70	0.70	0.65	0.68	0.68	0.66	0.65	0.72
D.VX02	0.78	0.77	0.75	0.74	0.78	0.75	0.74	0.75	0.82	0.81	0.98	0.85	0.78	0.76	0.73	0.70	0.65	0.76	0.73	0.73
D.CH01	0.78	0.77	0.72	0.74	0.78	0.75	0.76	0.77	0.84	0.81	0.94	0.94	0.80	0.74	0.71	0.69	0.69	0.78	0.72	0.75
D.CH02	0.75	0.74	0.71	0.70	0.75	0.71	0.72	0.76	0.83	0.82	0.94	0.87	0.88	0.78	0.74	0.76	0.73	0.70	0.73	0.74
D.NHg03	0.82	0.78	0.76	0.77	0.82	0.78	0.75	0.76	0.83	0.84	0.87	0.87	0.88	0.83	0.74	0.73	0.73	0.70	0.67	0.74
D.VX01	0.75	0.74	0.78	0.80	0.80	0.71	0.65	0.71	0.76	0.82	0.77	0.77	0.81	0.83	0.88	0.68	0.69	0.75	0.69	0.68
D.TrD01	0.82	0.83	0.74	0.82	0.80	0.74	0.68	0.74	0.81	0.80	0.77	0.77	0.76	0.81	0.88	0.83	0.71	0.65	0.78	0.71
D.NB01	0.82	0.78	0.81	0.84	0.82	0.76	0.75	0.71	0.83	0.84	0.84	0.87	0.83	0.83	0.83	0.83	0.93	0.75	0.71	0.72
D.VN01	0.84	0.83	0.81	0.84	0.80	0.78	0.72	0.71	0.83	0.82	0.84	0.84	0.81	0.86	0.81	0.86	0.89	0.78	0.78	0.78
D.TD01	0.81	0.80	0.77	0.81	0.81	0.80	0.71	0.80	0.82	0.86	0.86	0.88	0.84	0.82	0.82	0.84	0.89	0.89	0.78	0.75
D.BV01	0.82	0.76	0.74	0.80	0.77	0.76	0.70	0.76	0.81	0.82	0.84	0.87	0.83	0.81	0.81	0.83	0.88	0.88	0.94	0.94

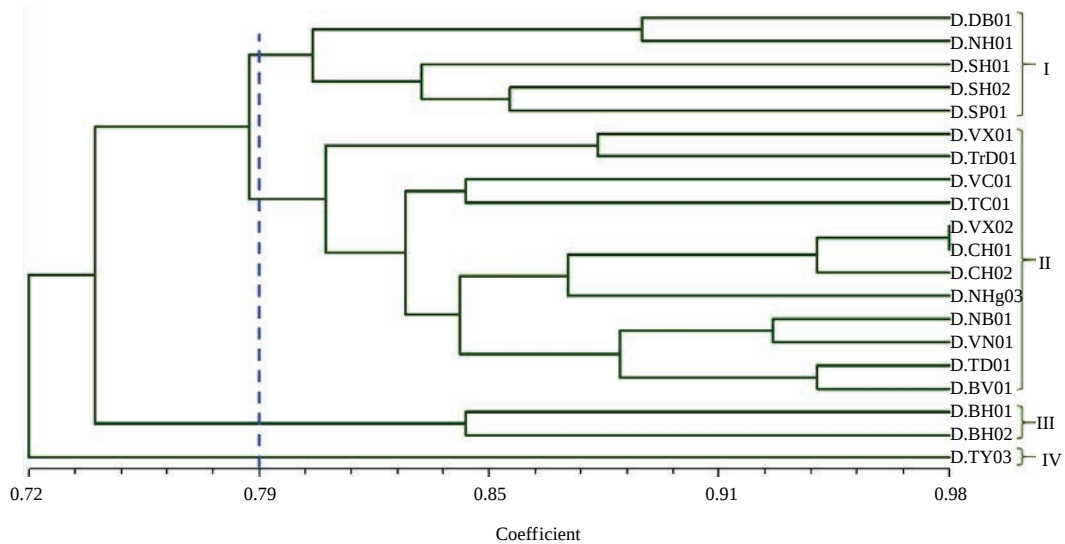


Fig. 3: UPGMA cluster analysis of 20 *Dysosma tonkinense* accessions with a similarity coefficient of RAPD marker
Dash line indicates the mean similarity (0.79). The dendrogram distributed the 20 accessions in four indicated groups (I, II, III and IV)

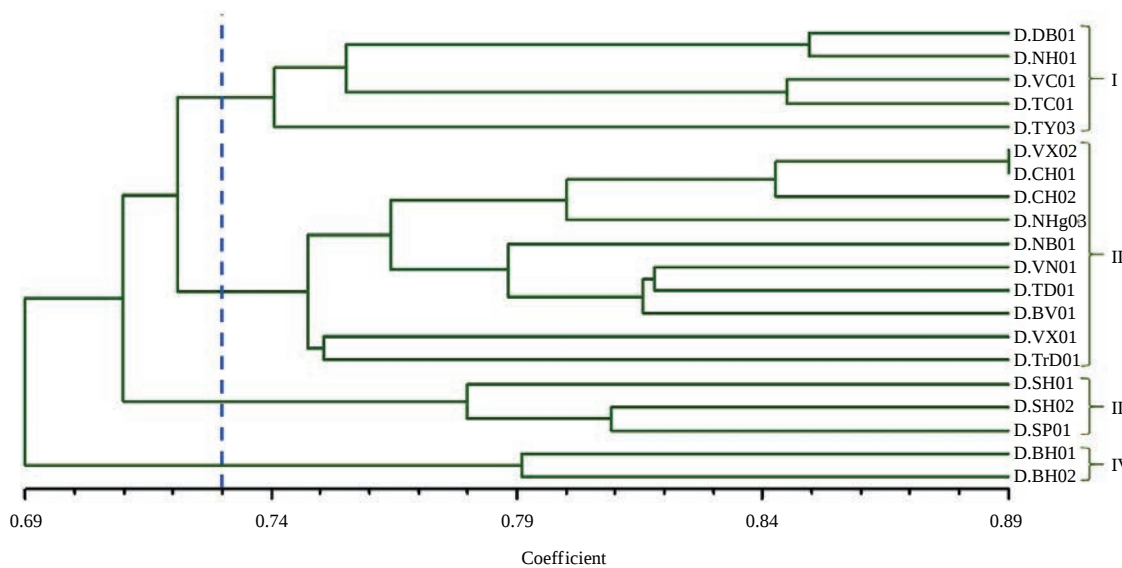


Fig. 4: UPGMA cluster analysis of 20 *Dysosma tonkinense* accessions with similarity coefficient combined ISSR and RAPD markers
Dash line indicates the mean similarity (0.73). The dendrogram distributed the 20 accessions in four indicated groups (I, II, III and IV)

the RAPD marker. For examples, while RAPD marker analysis showed the similarity between D.VX02 vs. D.CH01 was 0.98 while this value was 0.85 when analyzed by ISSR marker Table 4.

A dendrogram based on the UPGMA hierarchical clustering method with RAPD data is shown in Fig. 3. At the average of 79% similarity level, 20 accessions were grouped into four clusters. Cluster I contained 5 accessions including D.DB01, D.NH01, D.SH01, D.SH02 and D.SP01. Cluster II has 12 accessions: D.VX01, D.TrD01,

D.VC01, D.TC01, D.VX02, D.CH01, D.CH02, D.NHg03, D.NB01, D.VN01, D.TD01, D.BV01. Cluster III comprises 2 accessions including D.BH01, D.BH02 and cluster IV has only accession D.TY03.

Combined RAPD and ISSR analysis: To generate a better picture of the genetic diversity between the 20 *Dysosma tonkinense* accessions, data of both the RAPD and ISSR markers were combined to generate a dendrogram by using the UPGMA clustering method in Fig. 4. The dendrogram

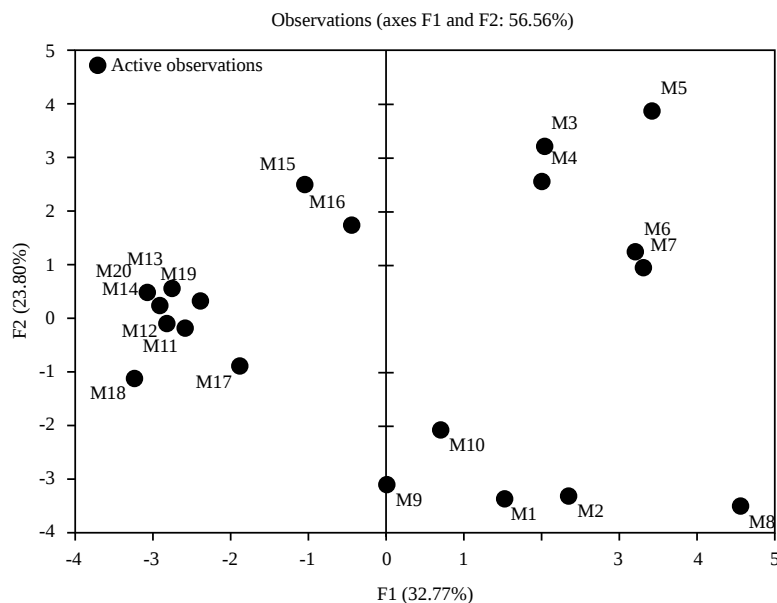


Fig. 5: PCA analysis of 20 *Dysosma tonkinense* accessions based on pooled RAPD and ISSR markers

Table 5: Matrix comparisons of mantel test/two-tailed test between markers

Sokal-Michener matrix	Matrix correlation (r)	p-value (two-tailed)	Alpha
RAPD vs. ISSR	0.496	<0.0001	0.05
RAPD vs. pooled RAPD and ISSR	0.821	<0.0001	0.05
ISSR vs. pooled RAPD and ISSR	0.903	<0.0001	0.05

indicated that, at 73% similarity level, 20 accessions were split into four distinct major clusters (I, II, III and IV) which consisted of 5, 10, 3 and 2 accessions, respectively. Clusters II had 10 accessions, among them, D.VX02 and D.CH01 had a maximum similarity coefficient of 0.89 (Fig. 4). Although dendrograms generated by ISSR, RAPD and combined ISSR with RAPD indicate some variations, D.BH01 and D.BH02 accessions were always assigned in the same cluster. Moreover, accessions D.VX02 and D.CH01 reached the highest similarity level when analyzed by RAPD and combined ISSR and RAPD (Fig. 3 and 4).

In addition to UPGMA clustering analysis, the combined RAPD and ISSR markers were also analyzed with PCA scatter plots which show the first two principal components accounted for 32.77 and 23.8% of the total genetic variation of the total variation, respectively. Moreover, the PCA also indicates the much distance in genetic diversity between accessions in Fig. 5.

Because of the difference in genetic similarity analysis results between accessions when analyzed by ISSR or RAPD were given in Table 4. Moreover, the difference of similarity coefficient between ISSR vs. pooled RAPD and ISSR (Fig. 2 and 4) and RAPD vs. pooled RAPD and ISSR (Fig. 3 vs. 4) were also found, therefore, to estimate the correlation

between matrices can be done. To answer that question, the Sokal-Michener matrices for the Mantel test (Pearson correlation) were used. The result showed a coefficient of correlation (r) between ISSR and RAPD was 0.496 ($p < 0.0001$), which indicated a low correlation between the two markers in Table 5. Genetic matrices of RAPD and pooled RAPD and ISSR resulted from a good correlation ($r = 0.821$, $p < 0.0001$). However, the ISSR matrix exhibited a higher correlation with pooled RAPD+ISSR matrix ($r = 0.903$, $p < 0.0001$) indicating better effectiveness of ISSR over RAPD in determining the genetic diversity. Indeed, ISSR markers showed 75.55% detected loci were polymorphic while it was 66.20% in RAPD markers (Table 2 and 3).

DISCUSSION

For years, many plants have been used as medicine and play an important role in primary healthcare for people all over the world. Demand for medicinal plants is continuously increasing, however, only a very small number of species are cultivated, the main resource is still wildly exploited¹⁹. *Dysosma tonkinense* is a valuable medicinal plant in Vietnam and has been much exploited. Permanent over-exploitation and other causes have led to a serious decline in the reserves

of this medicinal plant. That is why it was included in the Red Data Book of Vietnam and its needs more attention toward the conservation program³.

Molecular markers have been commonly used in genetic diversity evaluation of *Podophyllum* (*Sinopodophyllum*) and *Dysosma* genus. Among them, the RAPD marker was used for *P. hexandrum*²⁰, AFLP marker was used for *D. versipellis*²¹ and *P. hexandrum*²², ISSR marker was used for *D. pleiantha*²³ and *S. hexandrum*²⁴ and both RAPD+ISSD markers were used for *P. hexandrum*²⁵.

A combination of RAPD and ISSR analysis have been popularly used since both markers can provide practical information for germ-plant resources which can provide useful information for classification and breeding^{9,26-28}. To estimate genetic relationships and variation, both RAPD and ISSR markers were used, since pooled data from different types of marker can provide a more informative classification than a single method alone²⁹. These techniques provide complementary information for calculating genetic similarity³⁰.

In this study, the genetic variations among 20 *D. tonkinense* accessions were revealed by both RAPD and ISSR markers. While the pairwise similarity values obtained with the ISSR marker ranged between 0.59 and 0.85, this value ranged between 0.60 and 0.98 when analyzed by the RAPD marker (Table 4). These results show that, although the samples were the same, there were differences in genetic similarity results when 20 accessions analyzed by either ISSR or RAPD. However, many studies have shown different results when the same population analyzed by a different molecular marker such as RAPD and ISSR²⁹.

A combination of RAPD and ISSR analysis found 215 polymorphic loci (with 72.61% were polymorphism) unambiguously discriminated 20 accessions into three major clusters (Fig. 4). To add another layer of data analysis, the relationship among 20 accessions by the Principal Component Analysis (PCA) was determined which was conducted by XLSTAT 2018 software¹⁸. In agreement with the dendrogram result (Fig. 4), 20 accessions when analyzed by PCA were separated into major groups which were comparable with dendrogram analysis (Fig. 5). Moreover, the PCA also shows the far distance between accessions from the others. These results again indicate the high genetic diversity of the collected samples which could be employed in the crossing programs for the improvement of this precious medicinal plant in Vietnam.

The genetic similarity of 20 *D. tonkinense* accessions evaluated by RAPD and ISSR markers showed different results (Table 2 and 3) and the correlation between RAPD and ISSR

matrices was low (Table 5). The low correlation between RAPD and ISSR markers as described in several reports such as black gram (*Vigna mungo* (L.) Hepper) and also in peanut (*Arachis hypogaea* L.)²⁹. ISSR markers were reported more efficient than the RAPD in many analysis such as in *V. mungo*, wheat³¹ and peanut (*Arachis hypogaea*)³². These results also indicate a high level of genetic variability among 20 different accessions of *D. tonkinense* in Vietnam.

CONCLUSION

Dysosma tonkinense (Gagnep.) M. Hiroe has been used as a medicinal plant in Vietnam. ISSR and RAPD markers were used to study the genetic diversity of *D. tonkinense*. The results on genetic variability of 20 *D. tonkinense* accessions collected in Vietnam indicate that RAPD and ISSR markers are effective tools and the combination of the two markers presents better information about the genetic diversity of *D. tonkinense* germ plasm. The high genetic variability among 20 *D. tonkinense* accessions could be used in genetic improvement, sustainable management and germplasm conservation of *D. tonkinense* in Vietnam.

SIGNIFICANCE STATEMENT

This study reveals a high level of genetic variability among 20 different accessions of *D. tonkinense* collected in different provinces of Vietnam by using RAPD and ISSR markers. This result could be useful for the breeding program to develop a new valuable variety of *D. tonkinense* and can be used for genetic conservation and ecological conservation of this important medicinal plant in Vietnam.

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