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Morphological, anatomical and genetic diversity of *Nanhaia speciosa* (Champ. ex Benth.) J.Compton & Schrire from Northern Vietnam

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Abstract. *Ong PX, Cao CB, Khang DT, Huy TG, Cao PB, Chu HD, Nguyen DV, La HV. 2024. Morphological, anatomical and genetic diversity of* Nanhaia speciosa (*Champ. ex Benth.*) *J.Compton & Schrire from Northern Vietnam. Biodiversitas 25: 1174-1184. Nanhaia speciosa* (Champ. ex Benth.) J.Compton & Schrire has been considered a captivating plant species known for its remarkable attributes and contributions to ecological systems and human use. This medicinal plant originated from tropical Southeast Asia regions. However, the investigation of this species in Vietnam has been still lacking. This study comprehensively analyzed *N. speciosa* samples obtained in Vietnam; the morphological and anatomical characteristics of *N. speciosa* leaflets and flower samples collected from different regions were analyzed. As a result, the structural components of the flower were slightly similar between these samples, whereas the leaflet shape exhibited variations in the leaflet base, petiole, and leaflet apex. According to the ISSR and RAPD analysis, we demonstrated that *N. speciosa* found in ND genotypes (Tan Yen, Bac Giang) exhibited clearly different genetic backgrounds from CSS (Loc Binh, Lang Son) and CSD (Son Dong, Bac Giang) genotypes. Based on the clustering diagram of ISSR markers, 15 plant genotypes were classified into 2 large clusters, A and B, with genetic similarity ranging from 0.62 to 0.90. The RAPD dendrogram illustrated two main clusters, C and D, with genetic similarity ranging from 0.57 to 0.92. Therefore, this study could provide a solid foundation for identifying and characterizing *N. speciosa* species in Vietnam and a direction for advanced research on conservation, sustainable breeding, and exploiting pharmaceutical values.

Keywords: Genetic diversity, ISSR, *Nanhaia speciosa*, RAPD

INTRODUCTION

Nanhaia speciosa (Champ. ex Benth.) J. Compton & Schrire (2019) was a climbing-woody perennial plant of the Fabaceae family and was distributed mainly in South China and Vietnam (Wei and Pedley 2010). 'Cat Sam,' also called 'Sam Nam' in Vietnamese, is another name for *N. speciosa*. It is well-known for its ability to treat various illnesses, including hepatitis, arthritis, cough, numbness in the wrists or knees, low blood flow, anemia, tuberculosis, chronic bronchitis, and chronic hepatitis (Zhao et al. 2015a, b; Fu et al. 2016; Huang et al. 2020; Yao et al. 2021). The use of this plant in traditional medicine is because *N. speciosa* contains a lot of bioactive compounds such as saponins, flavonoids, phenolic glycosides, polysaccharides, and formononetin (Yu and Liang 2019; Huang et al. 2020).

There has been debate concerning the classification of *N. speciosa*, classified as *Millettia speciosa* Champ. ex Benth and named the Callerya group of the Millettieae tribe (Wei and Pedley 2010). Due to differences in its larger flower, persistent floral bracts, gibbosities, and densely pubescent ovaries, it has now been reassigned to the new genus *Nanhaia* within the tribe Wisterieae with two species (Compton et al. 2019). According to Compton et al. (2019), the morphological characteristics suggest a close relationship with the genus Wisteriopsis. *N. speciosa* is most closely related to *Wisteriopsis reticulata* of the tribe Wisterieae based on the phylogenetic analysis using a matrix of 69 protein-coding genes. Additionally, the entire genome chloroplasts of *N. speciosa* measured 132,551 bp, with only one copy of the Inverted Repeat (IR), which encoded 110 genes (Xiao et al. 2022).

N. speciosa is a plant with significant medicinal potential due to its various phytochemical constituents. Among them, triterpenoids have the highest contents and are essential for several biological processes. Notably, this plant often contains two bioactive components: maackiain and formononetin (Zhao et al. 2017). In addition, flavonoids are one of the bioactive compounds that contribute to their therapeutic properties. The flavonoid content varies among different extracts. For instance, the petroleum ether extract exhibited the highest flavonoid content (47.50%) (Nasiruddin et al. 2020). Flavonoids are known for their antioxidant and other health-promoting effects. Besides triterpenoids and flavonoids, *N. speciosa* also contains alkaloids, terpenoids, phenylpropanoids, volatile oils, and phytosterols. These

diverse constituents contribute to its medicinal value (Zhao et al. 2017). Recent research has identified nitrogen-containing heterocyclic compounds from the roots of *N. speciosa*, including uridine, 2-(β-D-glucopyranosyl)-3-isoxazolin-5-one, adenosine, and hypaphorine (Dao et al. 2021).

Nanhaia speciosa Polysaccharides (NP) supplementation alleviated diabetic biomarkers, including weight gain, hyperlipidemia, liver steatosis, and adipocyte hypertrophy. It inhibited hepatic de novo lipogenesis, stimulated adipocyte lipolysis, and attenuated hepatic inflammation. In a study by Lam et al. (2022), the ethyl acetate extract fractionation from *N. speciosa* root demonstrated more antioxidant properties than the control drug (butylated hydroxytoluene). Moreover, the researchers observed that several fractional extracts of *N. speciosa* significantly suppressed the survival ability of myeloma cell lines (U266 and KMS11), mantle cell lymphoma (Mino), and the noncancerous LCL cell line (Lam et al. 2022). A crude extract of NP demonstrated anti-obesity effects in a mouse model fed a high-fat diet. Another study also showed that flavonoids extracted from *N. speciosa*, similar to polysaccharides, help prevent obesity by regulating thermogenesis and lipid metabolism in mice (Wang et al. 2022a). NP restored the balance of gut microbiota, becoming a promising prebiotic for managing obesity (Li et al. 2022). NP consisted of glucose and xylose, which strongly exhibited immunomodulatory potential (Huang et al. 2020). *N. speciosa* is considered a clinically significant anti-rheumatoid arthritis medicine. Researchers have developed novel strategies for quality control of MSC. Ultra-high-Performance Liquid Chromatography (UPLC) analysis identified bioactive components absorbed into MSC serum, and Network pharmacology analysis highlighted potential quality markers, such as lenticin (Nong et al. 2023). The aqueous extract of *N. speciosa* was separated into total polysaccharides and supernatant; both exhibited an anti-fatigue effect and showed potential health benefits (Zhao et al. 2015b). According to Chen et al. (2024), *Cyprinus carpio* was consumed as a dietary supplement or diet, which also improved the animals' immune systems, intestinal health, and antioxidant capacity (Chen et al. 2024). It may be an herb additive for aquaculture production. Due to the medicinal value of *N. speciosa*, exploitation in the wild is very common, putting this species in danger of extinction. To preserve and exploit the medicinal values of this species sustainably and to reduce pressure on wild populations, some studies have attempted to produce it in artificial culture conditions through the induction of *N. speciosa* hairy roots with *Agrobacterium rhizogenes* LBA9402, the study also showed the highest polysaccharide content in hairy root cultures when grown on ½ MS medium supplemented with 30 $g.L^{-1}$ sucrose (Yao et al. 2016). In another study, Huang et al. (2017) developed a protocol for the regeneration of *N. speciosa* through anther culture, achieving a high percentage of embryos and plants (Huang et al. 2017). In addition, it is necessary to research the current status and genetic structure of *N. speciosa* to support conservation and breeding programs.

Genetic variation helps organisms adapt to changing environments. The amount of alleles present at different genomic locations relies on various environmental conditions. Evaluation of genetic diversity structure helps conserve and construct strategies for exploiting genetic resources (da Silva Júnior et al. 2020). Inter-Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) are two molecular biology techniques commonly used separately or combined to assess genetic diversity within or between species, such as *Enhalusacoroides* (Lf) Royle (Pharmawati et al. 2021), *Clerodendrum* species of North East India (Gogoi et al. 2020), *Lathyrus* species (Osman and Ali 2020), *Embelia ribes* Burm f. (Kamble et al. 2023). However, their efficacy depended on species, type of explants, DNA quality, and experimental conditions. Osman and Ali (2020) investigated the genetic diversity of five *Lathyrus species* using molecular markers such as RAPD, ISSR, and SCoT (Start Codon Targeted). These species included *L. articulatus, L. hierosolymitanus, L. latifolius, L. pseudocicera*, and *L. tuberosus*. The results revealed that ISSR markers exhibited the highest polymorphism (96.81%), followed by SCoT markers, while RAPD markers showed slightly lower values. Both RAPD and ISSR can effectively be used to determine the genetic diversity of endangered rare *Dalbergia cochinchinensis* (Fabaceae) genotypes in Vietnam (Vu and Dinh 2012). Understanding the genetic diversity of *N. speciosa* populations is crucial for implementing efficient conservation and sustainable management strategies. However, no study has been published on this topic in Vietnam.

This study aimed to record foliar and flower specimens' morphological and anatomical characteristics and evaluate the genetic diversity and relationship of *N. speciosa* specimens collected in northern Vietnam using RAPD and ISSR markers. These results were used to establish a baseline to assist future conservation, breeding programs, and product development of this species. Also, we aim to report the usefulness of RAPD and ISSR for assessing genetic diversity and relationships among *N. speciosa* specimens.

MATERIALS AND METHODS

Materials

Explants collection

Based on previous studies and local people's observations, *N. speciosa* is distributed in some northern provinces of Vietnam, especially in Bac Giang and Lang Son provinces; their tuberous roots were commonly exploited for curing or supplying nutritious elements. We selected these two northern locations in Vietnam to investigate. A total of 15 genotypes of *N. speciosa* were collected from 15 locations that belong to Bac Giang and Lang Son Provinces. Representative trees were obtained in each location; they included 5 individuals from Tan Yen District, Bac Giang Province (denoted as ND), 4 individuals from Son Dong District, Bac Giang Province (denoted as CSD), and 6 individuals from Loc Binh District, Lang Son Province (denoted as CSS) (Table 1).

Table 1. Information on collected samples of *Nanhaia speciosa*

Genotypes	Sampling site	Coordinates	
		Latitude	Longitude
ND01	Dong Sen, Viet Lap, Tan Yen, Bac Giang	21°21'05.7"	106°10'07.4"
ND02	Kim Trang, Viet Lap, Tan Yen, Bac Giang	21°20'52"	106°08'28"
ND05	Am Vang, Viet Lap, Tan Yen, Bac Giang	21°19'07"	106°09'10"
ND07	Um Ngo, Viet Lap, Tan Yen, Bac Giang	21°20'59"	106°09'14"
ND09	Lien Chung, Tan Yen, Bac Giang	21°21'40"	106°10'01"
CSD03	Rong, An Lac, Son Dong, Bac Giang	21°18'32.8"	106°52'37.6"
CSD06	Dong Bai, An Lac, Son Dong, Bac Giang	21°20'00"	106°55'41"
CSD08	Na O, An Lac, Son Dong, Bac Giang	21°20'53"	106°57'29"
CSD10	Kim Bang, An Lac, Son Dong, Bac Giang	21°21'20"	106°57'38"
CSS06	Keo Co, Loi Bac, Loc Binh, Lang Son	21°38'41.2"	107°00'04.7"
CSS08	Ban Chanh, Loi Bac, Loc Binh, Lang Son	21°40'23"	106°56'47"
CSS10	Phai Vai, Loi Bac, Loc Binh, Lang Son	21°38'43"	106°58'58"
CSS14	Na Lang, Loi Bac, Loc Binh, Lang Son	21°39'00"	107°01'07"
CSS15	Na Phi, Loi Bac, Loc Binh, Lang Son	21°40'42"	106°57'01"
CSS16	Na Mu, Loi Bac, Loc Binh, Lang Son	21°36'32"	107°00'24"

Methods

Description of morphological and anatomical characters of leaves

The collected leaves were washed, and five mature leaves from each sample were randomly selected. Then, thin cross-sectional slices were cut at the following positions: leaf stalk (petiole), main vein (vein), leaf apex (Apex), and soaked in distilled water. The iodine-potassium iodide staining is performed in the following steps: Samples were removed from the distilled water and immersed in a Javen solution for 15 minutes. Samples were washed three times and immersed in inorganic acetic acid for 5 minutes to neutralize any remaining Javen's solution. The samples were rinsed with water and immersed in an iodine-potassium iodide staining solution for 7-10 minutes. The samples were rinsed again, immersed in distilled water, and mounted with glycerin. The basic tissues were identified by observing the anatomical sections under a microscope. Cells with a cellulose wall will appear pink, and cells with lignified walls will appear green. Five cross-sectional petiole, main vein, and leaf apex slices are randomly selected for each sample to enhance reliability. Subsequently, the tissue structures were identified, and their dimensions were measured using Toupview software following the principles of Strock et al. (2022). Petiole: Measure the cross-sectional petiole's length and width, and the cortex tissue's thickness. Main vein: Measure the area of the vascular bundle, the parenchyma tissue's thickness, and the collenchyma tissue's thickness. Leaf apex: Count the number of conducting bundles and measure the thickness of the parenchyma tissue and the collenchyma tissue.

Genetic diversity of samples

DNA isolation

Healthy leaves were sterilized with 70% alcohol to remove dust and microbes on the surface. Liquid nitrogen was utilized to grind leaves samples into homogenized powder. The DNA extraction was conducted following the CTAB-based protocol (Rogers and Bendich 1998) with appropriate modifications. The quantity and quality of DNA were measured by Nanodrop One spectrophotometer (Thermo Scientific, USA). DNA integrity was performed by 1% agarose electrophoresis at 100V in TAE 1X.

Amplification of ISSR and RAPD markers

Ten ISSR and five RAPD primers were applied for genetic diversity characterization. The 15 µL reaction consisted of 4.5 µL deionized water, 7.5 µL Mytaq mix 2X (Meridian Bioscience, USA), 1 µL primer (20 µM), and 2 µL DNA template (equivalent 100 ng). Amplifications were performed by a Mastercycler X50s (Eppendorf, Germany) with the thermal cycle followed: an initial denaturation of 95°C in 4 minutes, 40 cycles included three steps; 94°C in 30 seconds, an appropriate annealing temperature (Ta) (Table 2) in 1 minute, 72°C in 2 minutes; a final extension of 72°C in 10 minutes. PCR products were then stored at 4°C until use. Amplified products were tested by 2% agarose gel electrophoresis (25V, TAE 1X) in 1 hour and 30 minutes. Band patterns were visualized under UV light in Gel doc. XR system (Bio-rad, USA).

Table 2. Information on ISSR and RAPD markers utilized in this study

Primer	Nucleotide sequences (5'-3')	Ta (°C)
ISSR825	$(AC)_8T$	46
ISSR855	$(AC)_8CT$	46
ISSR866	$(CTC)_6$	52
ISSR827	$(AC)_8G$	50
ISSR811	$(GA)_8C$	45
ISSR813	$(AC)_8CA$	51
ISSR818	$(GA)_8CT$	44.7
ISSR823	$GAA(GT)_7$	51
ISSR826	$(AC)_8C$	46
ISSR889	$ATG(AC)_7$	52
OPAE-11	AAGACCGGGA	37
OPAE-12	CCGAGCAATC	37
OPAE-13	TGTGGACTGG	36
OPAE-14	GAGAGGCTCC	35
OPAE-15	CCAGCACTTC	36
OPAE15G	TGCCTGGACCG	38
OPAE15A	TGCCTGGACCA	36
OPAE15T	GGACACAGAGT	34
OPAB14	AAGTGCGACC	37
OPL11	ACGATGAGCC	33

Data analysis

Scored bands were transferred into binary data with Microsoft Excel software version 2021; the presence and absence of a band were scored as 1 and 0, respectively. The dendrogram was constructed by the Unweighted Pair Group Method with the Arithmetic mean (UPGMA) method using NTSYS-pc 2.1 software. Correspondence analysis was conducted by Biodiversity Pro software. Genetic diversity indices of each marker include PB: Polymorphic Band, PIC: Polymorphism Information Content, EMR: Effective Multiplex Ratio, MI: Marker Index, D: Discriminating power, and R: Resolving power were calculated by iMEC web-based program (Amiryousefi et al. 2018).

RESULTS AND DISCUSSION

Morphological and anatomical characters of *N. speciosa* leaves and flowers from different regions

The morphological features of *N. speciosa* samples collected from various regions are meticulously documented in Figure 1 and Table 3. Notably, the leaf shape of ND specimens from Tan Yen District, Bac Giang Province, diverges significantly from that of leaves in other regions, particularly in terms of leaflets. While most samples of ND exhibit 3 to 5 leaflets, those collected at the remaining two locations display a broader range of 7 to 11 leaflets. This may be a morphologically important trait to distinguish and identify the characteristics of *N. speciosa* populations in Tan Yen Bac Giang. Additionally, the ND leaves base typically assumes a round form. In contrast, the apex of the leaves is arrow-shaped, while the leaves base of CSD and CSS specimens is often rounded with bilobed forms at the apex, although occasional exceptions occur. Lastly, leaf petioles and young branches in these samples are sparsely covered with hairs compared to those of the CSD and CSS samples. In terms of the geographical location where samples were collected, Tan Yen District Bac Giang Province is quite far away from the other two districts, while Son Dong District, Bac Giang Province is close to Loc Binh District, Lang Son Province. It can be seen that geographical location and climate conditions affect the morphology of genotypes (Wang et al. 2022b).

Table 3. Morphological characters of *Nanhaia speciosa* leaflets from different regions

Characters \ Collected regions	Explants from Tan Yen, Bac Giang province	Explants from Son Dong, Bac Giang province, and Loc Binh, Lang Son province	
Upper leaf			
The number of leaflets	3-5	7-11	
Leaflet apex	Arrow	Two-lobed apex	Two-lobed apex
Leaflet edge		raw edge, no lobes	
Leaflet base	Round	Oblique	Oblique
Young branches and petiole	Covered less hairy	Covered densely hairy	Covered densely hairy

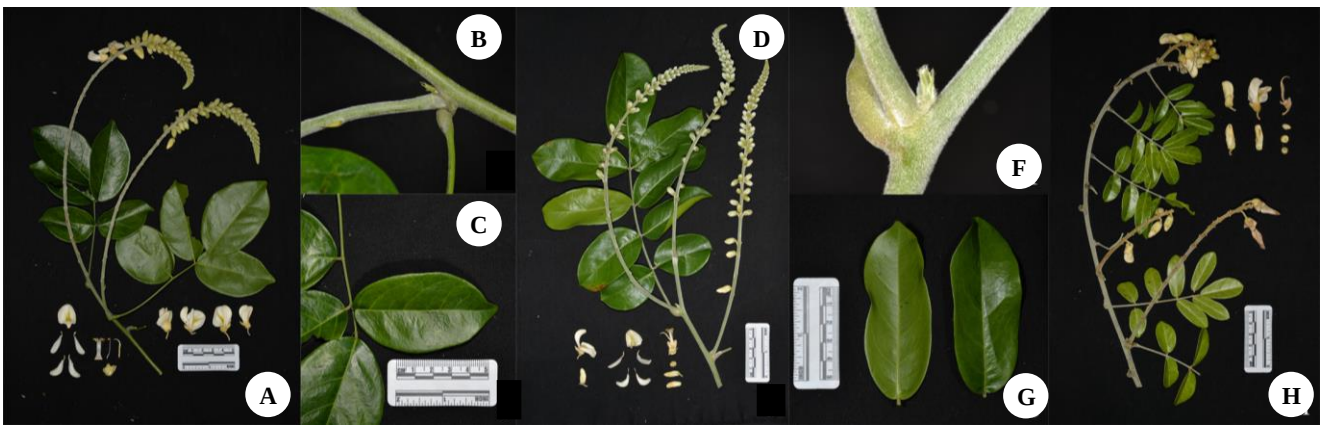

Figure 1. Morphology of branches with leaves of *Nahaia speciosa* from different regions. A-C: ND explants from Tan Yen (Bac Giang), D-H: CSD, and CSS explants from Son Dong (Bac Giang) and Loc Binh (Lang Son)

The morphological descriptive results were consistent with that of *Nahaia speciosa* (Sirichamorn et al. 2016; Compton et al. 2019). They are characterized by their prostrate or climbing twining vines, reaching heights of 1-5 meters and often intermingling among rocks and scrub. The stems exhibit shades of green or brown, featuring a pubescent, terete structure. The evergreen leaves, arranged in an imparipinnate manner, consist of 5-17 leaflets and can be either glabrous or sparsely hairy beneath, with a rachis extending from 3 to 30 centimeters. The anatomy of cross-sectional specimens of leaf petioles, main vessels, and apex of leaves of *N. speciosa* samples is shown in Figure 2. The results are similar to the results of their morphology, which are samples collected in Tan Yen, Bac Giang (Figure 2A) show the same pattern in size and tissue arrangement, while samples collected from Son Dong, Bac Giang, and Loc Binh, Lang Son show both patterns (shown in Figures 2B-C), the size of the petiole and main veins are smaller and larger than the samples collected in Tan Yen, Bac Giang.

Leaves are functional organs of plants and are directly influenced by their surroundings, such as soil humidity, temperature, etc., leading to changes in size and shape within and between species to support plant survival (Hovenden and Vander Schoor 2006; Bussotti and Pollastrini 2015). In this study, the leaf morphology of *N. speciosa* showed significant differences. This result may be due to the samples collected from different locations, and it is also consistent with previous studies stating that altitude above sea level, latitude, etc., especially the sampling location, greatly affects leaf morphology (Jahdi et al. 2020; Desmond et al. 2021).

In botanical research, flowers serve as reproductive organs and are often used for identification and classification because they are less affected by environmental conditions. In this study, the morphological and anatomical characteristics of *N. speciosa species* flowers were depicted in Figure 3.

Moreover, not much difference was observed in the structural components of the flower in the study samples (Figure 3). However, some variations in the shape and size of the petals, as well as the length and arrangement of the stamens, can be observed. These distinctive features may be attributed to genetic variation or environmental influences, and therefore, further investigation is needed with a larger and more statistically significant sample size.

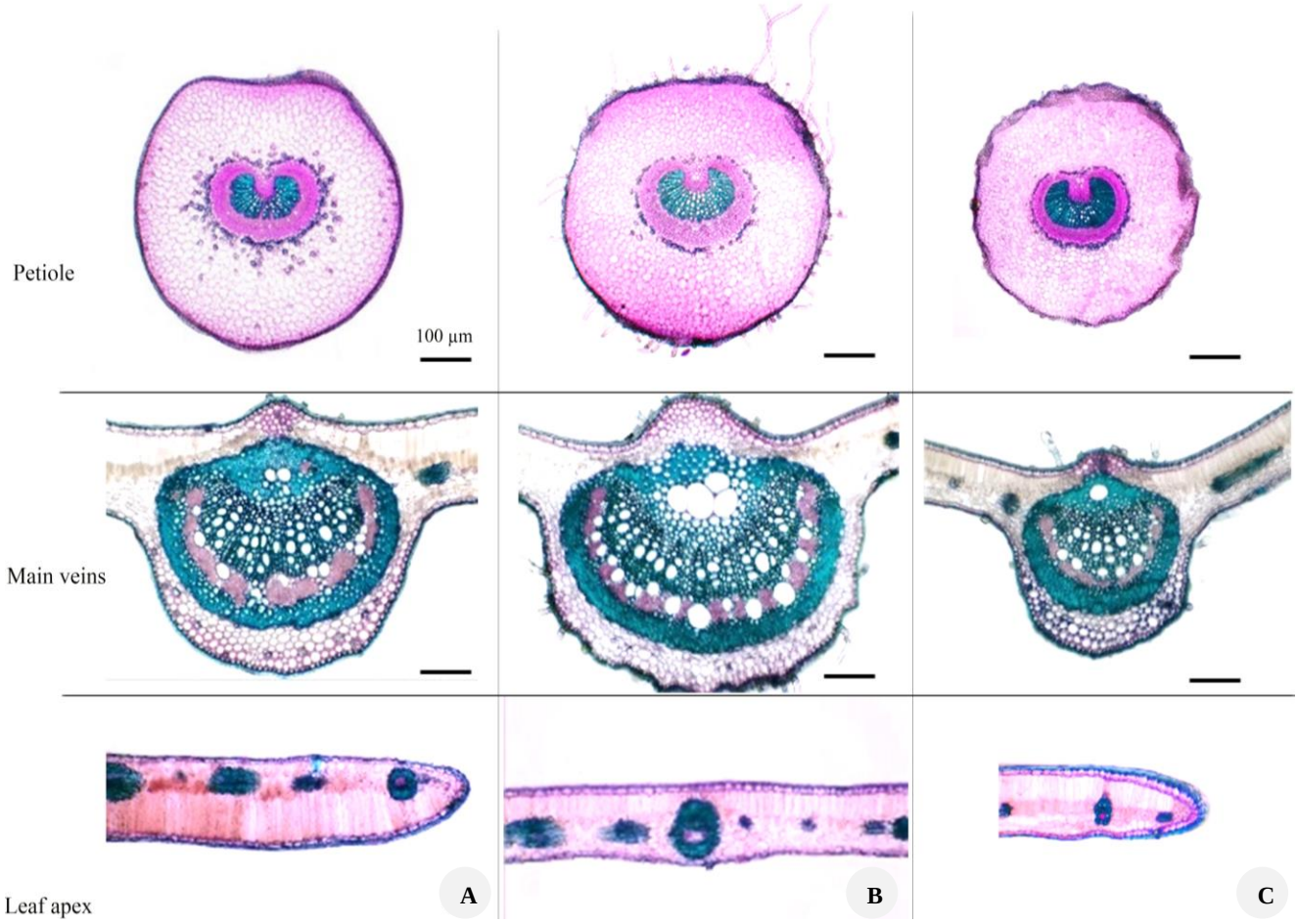

Figure 2. Anatomical characters of *Nanhaia speciosa* leaflets from different regions. The scale bar (in *bold black*) represented a length of 100 µm

Figure 3. Morphological and anatomical characters of *Nanhaia speciosa* flower. A: ND explants from Tan Yen (Bac Giang); B-C: CSD, and CSS explants from Son Dong (Bac Giang) and Loc Binh (Lang Son)

Genetic diversity

DNA profiles

DNA profiles are bands or fragments generated during PCR amplification of specific genomic regions. The presence or absence of bands in the profiles indicates polymorphisms in the studied DNA regions, allowing researchers to assess the level of genetic diversity among individuals or populations. Based on the band patterns on 2% agarose gel, the amplicons from 10 ISSR (Figure 4) markers and 5 RAPD markers (Figure 5) were generated. Such bands were clear and informative for binary scoring, and no band was visualized in non-template samples, indicating the false-positive was controlled.

Polymorphism of ISSR and RAPD markers

The PCR-based markers RAPD and ISSR are simpler and quicker than other markers because they need small DNA samples and do not use radioactive labels. The nucleotide repeats (Inter-Simple Sequence Repeats), dispersed across the genome and may have discriminatory capacity, have been used to measure genetic variation in clonal plant species. These marker systems have previously been used to examine genetic relatedness, resolve intra- and intergenomic relationships, and assess the genetic diversity of plant populations and cultivars (Kamble et al. 2023). In this study, both molecular markers were used to investigate the genetic diversity of *N. speciosa*.

Figure 4 shows the gel pattern displayed distinct and vivid bands corresponding to the ISSR profiles. A total of 86 bands were amplified by 10 ISSR primers for 15 plant genotypes, with an average band count for each marker of 8.6. Among such 86 bands, there were 69 polymorphic bands accounting for 80.23%, with an average value of 8.02% for each marker (Table 4). Furthermore, PCR product sizes ranged from 200 to about 1,000 bp. Among the 10 ISSR markers, ISSR827 and ISSR855 showed the highest polymorphism (100%), whereas ISSR 823 showed the least (44.44%). The marker ISSR825 expressed the maximum Rp value (8.00), whereas ISSR826 showed the smallest (1.86). The maximal PIC (0.37) for the markers ISSR813, 825, 827, 855, 866, 889, and the minimum (0.23) for ISSR826. After analyzing 10 ISSR markers, it was determined

that nine were informative and significant, with PIC values measuring from 0.25 to 0.50, and only the ISSR826 marker was less informative due to its PIC values lower than 0.25. Based on other parameters such as EMR, MI, and Rp, the ISSR825 marker was considered the effective marker for polymorphism estimation (Chesnokov and Artemyeva 2015; Amiryousefi et al. 2018).

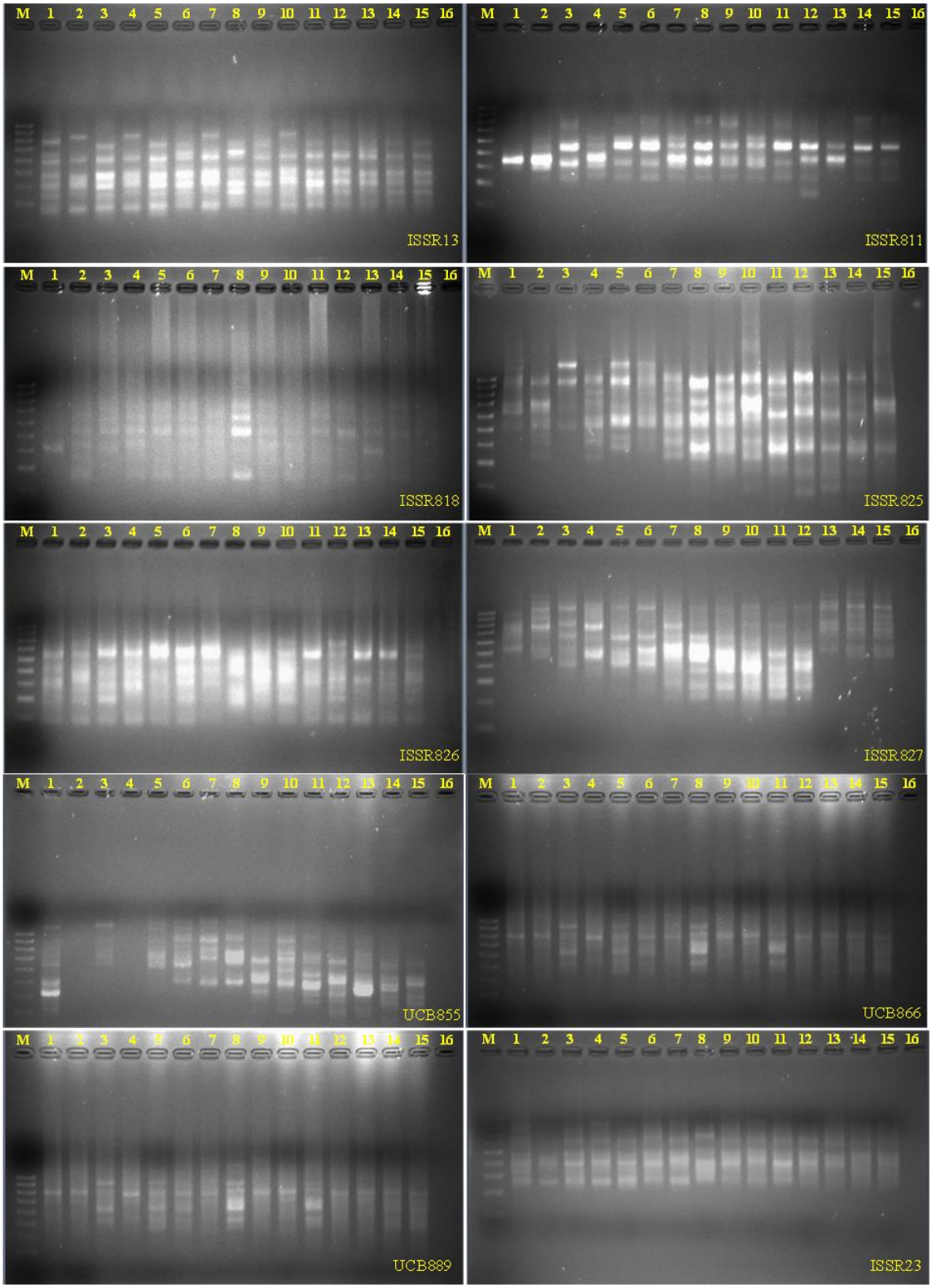

Figure 4. Gel pattern of 10 ISSR markers under 2% agarose electrophoresis. Note: M-100 bp DNA ladder (Nippon Genetics, Europe), 1. ND01, 2. ND02, 3. ND05, 4. ND07, 5. ND09, 6. CSD03, 7. CSD06, 8. CSD08, 9. CSD10, 10. CSS06, 11. CSS08, 12. CSS10, 13. CSS14, 14. CSS15, 15. CSS16

RAPD

According to Moghaieb et al. (2017), RAPD is highly effective in spotting genetic differences and is used for diversity evaluation and identifying germplasm in various plant species, bacteria, and microorganisms. Along with ISSR markers, RAPD was also effective in investigating the genetic diversity of Fabaceae species (Phong et al. 2011). This molecular marker was also used in the present study to study *N. speciosa*. A total of 47 loci were obtained with 5 RAPD markers, with an average of 9.4 loci per marker (Table 5). Of these 47 loci, 45 were polymorphic, corresponding to 95.7% polymorphism, with an average of 9 polymorphic loci per marker. The size of amplified products varied from 200 to 2,500 bp (Figure 5). Among the 5 RAPD markers, four markers, OPAE15G, OPAE15T, OPAB14, and OPL11, showed maximum polymorphism (100%), whereas OPAE15A showed minimum (83.33%). The marker OPAE19T came up with the maximum Rp value (5.86), whereas OPAB14 had with minimum (3.57). The PIC value was maximum (0.37) for the markers OPAE15A and OPL11, whereas minimum (0.32) for 15G. All five RAPD markers were reasonably informative, with PIC values >0.25 and <0.50.

Moreover, regarding polymorphic detection, ISSR and RAPD illustrated the beneficial effects of genetic variation studies in leguminous plants. The two markers have also been employed in several legumes in recent times for studies on diversity and population structure as well as for germplasm characterization of *Dalbergia oliveri* (Fabaceae) genotypes (Phong et al. 2011), Faba bean (Ammar et al. 2015), winged bean (Chen et al. 2015). Those studies indicated that many leguminous plants are now the subjects of genotyping with molecular markers.

Cluster analysis

The ISSR method

ISSRs were effective, highly polymorphic, affordable, and rapid for genetic diversity analysis and are useful for determining the genetic diversity in the *Brassicaceae* family (Maraş-Vanlioğlu et al. 2020). Regarding Fabaceae species, RAPD and ISSR markers for assessing genetic diversity reveal that both are effective, with ISSR markers generally being more efficient (Phong et al. 2011). Based on the dendrogram, 15 plant samples were classified into 2 large clusters at 62% genetic similarity, A and B, with genetic similarity ranging from 62% to 90% (Figure 6). Cluster A consisted of 7 accessions, including ND and CSD genotypes; the genetic similarity between ND and CSD was approximately 65%. Particularly, samples ND02 and ND05 showed the highest similarity at 90%. ND genotypes tend to concentrate into a distinct group, indicating the unique genetic characteristic of ND genotypes, which suggests that ND genotypes may carry specific genes or valuable variations that help ND genotypes adapt to the environment. This is a suggestion for further research to explore the specific genetic characteristics as well as potential applications of this genotype; Cluster B with CSS and CSD genotypes and 1 individual of ND genotypes. The genetic similarity of this cluster was about 66%, nearly the same as cluster A. Two samples, CSS15 and CSS16, showed 90% of identity level.

Table 4. Polymorphism indices of 10 ISSR markers

Primer	Band size	Total band	Polymorphic band	% Polymorphic	PIC	E	MI	Rp
ISSR813	250-900	9	7	77.78	0.37	3.86	0.015	2.00
ISSR811	250-900	8	6	75	0.36	4.93	0.02	4.14
ISSR826	200-800	6	3	50	0.23	5.07	0.016	1.86
ISSR818	250-800	6	5	83.33	0.32	4.28	0.02	2.00
ISSR825	200-800	12	10	83.33	0.37	6.57	0.019	8.00
ISSR827	200-800	13	13	100	0.37	5.79	0.016	7.29
ISSR823	250-900	9	4	44.44	0.34	6.21	0.02	1.86
ISSR855	200-800	11	11	100	0.37	4.57	0.014	7.43
ISSR866	450-900	6	5	83.33	0.37	3.07	0.018	4.14
ISSR889	350-1000	6	5	83.33	0.37	3.50	0.02	2.71
		86	69					

Table 5. Polymorphism indices of 5 RAPD markers

Primer	Band size (bp)	Total band	Polymorphic band	% Polymorphic	PIC	E	MI	Rp
OPAE15G	200-2000	10	10	100	0.32	3.07	0.01	3.86
OPAE15A	300-2500	12	10	83.33	0.37	4.93	0.01	5.57
OPAE15T	300-1200	12	12	100	0.36	4.64	0.01	5.86
OPAB14	400-1600	6	6	100	0.36	2.21	0.01	3.57
OPL11	500-2500	7	7	100	0.37	3.29	0.02	5.43
		47	45	95.7				

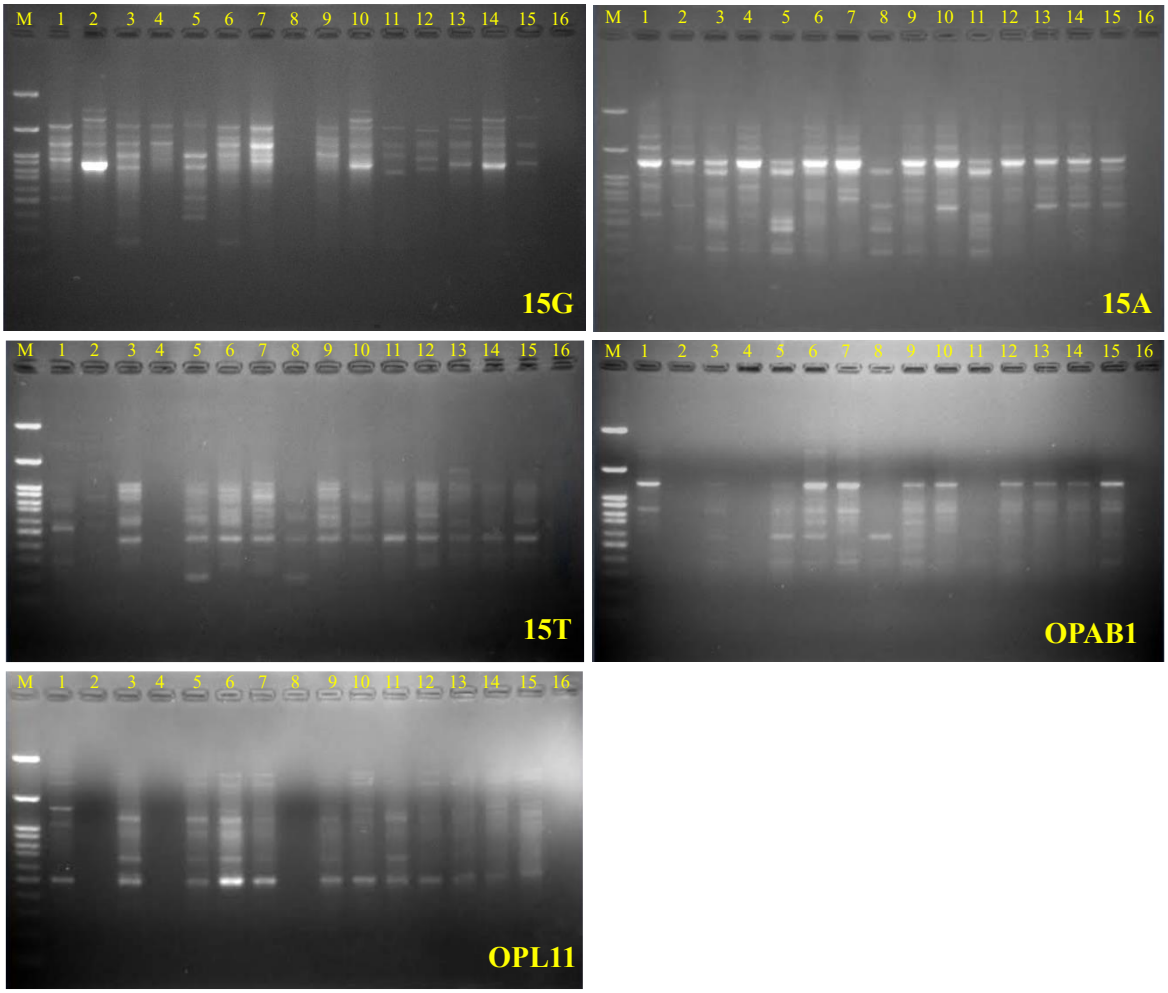

Figure 5. Gel pattern of 5 RAPD markers under 2% agarose electrophoresis. Note: M-100 bp DNA ladder (Nippon Genetics, Europe), 1. ND01, 2. ND02, 3. ND05, 4. ND07, 5. ND09, 6. CSD03, 7. CSD06, 8. CSD08, 9. CSD10, 10. CSS06, 11. CSS08, 12. CSS10, 13. CSS14, 14. CSS15, 15. CSS16

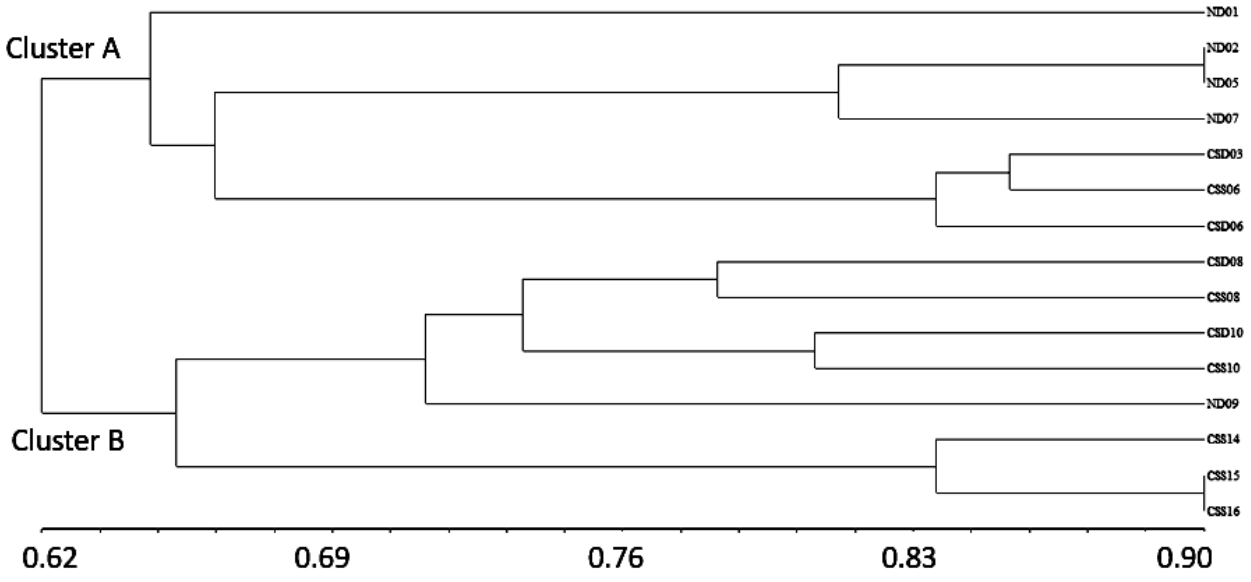

Figure 6. The dendrogram of 10 ISSR markers expressed the similarity coefficients for markers among 15 *Nanhaia speciosa* genotypes

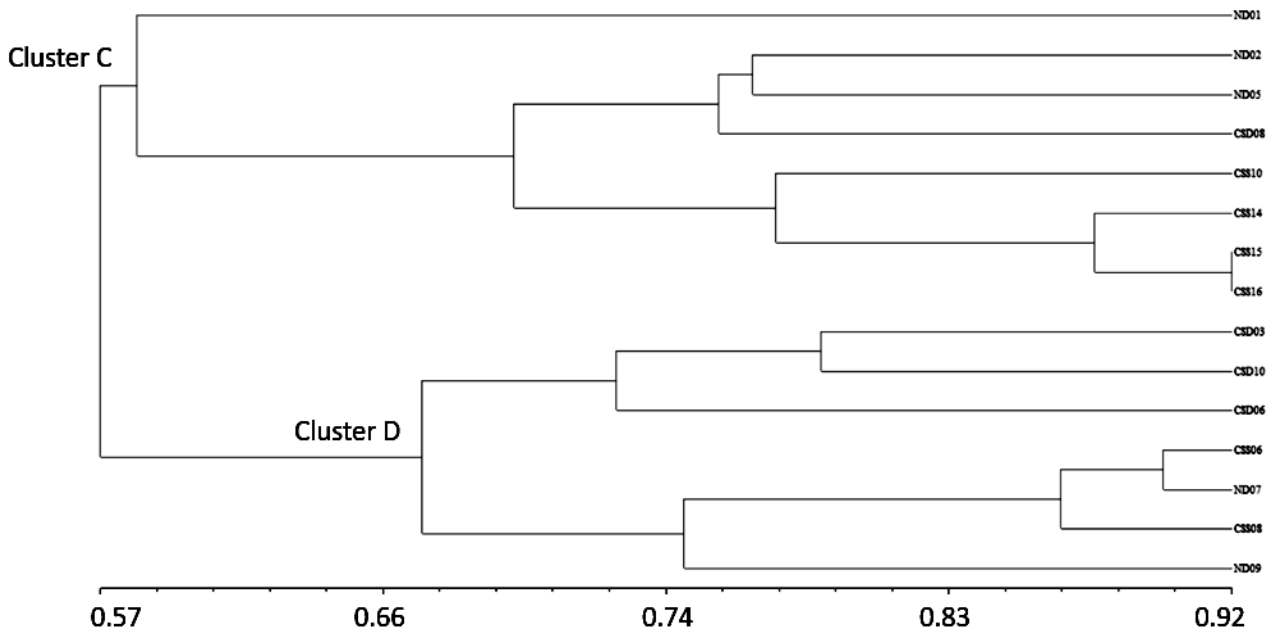

Figure 7. The dendrogram of 5 RAPD markers expressed the similarity coefficients for markers among 15 *Nanhaia speciosa* genotypes

The RAPD method

The dendrogram illustrates two main clusters, C and D, with genetic similarity ranging from 0.57 to 0.92 (Figure 7). Cluster C includes 3 individuals of the ND breed (ND1, ND2, ND5) and 4 individuals of the CSS breed (CSS10, CSS14, CSS15 and CSS16) and 1 individual of CSD08. The similarity of this group is estimated to be about 0.58. Branch D is divided into branch D1 including 3 individuals of the CSD genotypes (CSD03, CSD10, CSD09), branch D2 includes 4 samples CSS06, ND07, CSS08 and ND09.

In our study, from both clustering output by ISSR and RAPD, the grouping result was partially correlated with the population geography. Furthermore, based on the broad range of genetic similarity from these two molecular markers, three *N. speciosa* populations showed wide genetic diversity. Our study is partly similar to that of Aminah et al. (2017) when RAPD markers were used to estimate the genetic diversity between *Pongamia pinnata* (L.) Pierre species on Java Island, Indonesia. According to this paper, the cluster analysis of genetic similarity in five Malapari populations resulted in the classification into two main clusters correlated with their geographic area. In detail, the first cluster consisted of four populations: Carita, Batukaras, Kebumen, and Alas Purwo, while the second cluster was only a population from Baluran.

In conclusion, the study revealed the anatomy and morphology of the foliar and flower parts of *Nanhaia speciosa* in detail, which showed that the characteristics of the flower are stable between genotypes from different regions and more reliable than the characters of the leaves. Interestingly, the number of leaflets of the ND individuals collected in Tan Yen District, Bac Giang Province, is less, usually 5 (rarely 3) leaflets per leaf, compared to the number of leaflets of samples collected at CSD and CSS. No significant differences were noted in leaf and flower anatomy. Moreover, the genetic diversity of *N. speciosa* genotypes was investigated, and 15 genotypes were evaluated using ISSR and RAPD markers. Based on two molecular markers, ISSR and RAPD, it was shown that ND individuals have different genetic characteristics from CSS and CSD genotypes. Specifically, 80% of ND individuals with the ISSR indicator and 60% with the RAPD indicator are classified into a separate branch compared to the remaining genotypes. These ND individuals may be used as inherited materials to improve traits concerned with pharmaceutical value and for breeding programs. The results of this study provide initial basic data on the genetic diversity of *N. speciosa* in Northern Vietnam. It is necessary to carry out further analyses to clarify the genetics of *N. speciosa* species at the genetic level, especially the ND group while expanding the sample size and scope of research to develop this medicinal material effectively without major ecological impacts.

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REFERENCES

Aminah AAM, Supriyanto S, Suryani A, Siregar IZ. 2017. Genetic diversity of *Pongamia pinnata* (*Millettia pinnata*, aka. malapari) populations in Java Island, Indonesia. Biodiversitas 18 (2): 677-681. DOI: 10.13057/biodiv/d180234.

Amiryousefi A, Hyvönen J, Poczai P. 2018. iMEC: Online marker efficiency calculator. Appl Plant Sci 6 (6): e01159. DOI: 10.1002/aps3.1159.

Ammar MH, Alghamdi SS, Migdadi HM, Khan MA, El-Harty EH, Al-Faifi SA. 2015. Assessment of genetic diversity among faba bean genotypes using agro-morphological and molecular markers. Saudi J Biol Sci 22 (3): 340-350. DOI: 10.1016/j.sjbs.2015.02.005.

Bussotti F, Pollastrini M. 2015. Evaluation of leaf features in forest trees: Methods, techniques, obtainable information and limits. Ecol Indic 52: 219-230. DOI: 10.1016/j.ecolind.2014.12.010.

Chen D, Yi X, Yang H, Zhou H, Yu Y, Tian Y, Lu X. 2015. Genetic diversity evaluation of winged bean (*Psophocarpus tetragonolobus* (L.) DC.) using Inter-Simple Sequence Repeat (ISSR). Genet Resour Crop Evol 62: 823-828. DOI: 10.1007/s10722-015-0261-3.

Chen Q, Wei T, Li M, Liu S, Wu J, Xu G, Zou Z, Xie S. 2024. Effect of aqueous extract of *Millettia speciosa* Champ on intestinal health maintenance and immune enhancement of *Cyprinus carpio*. Fish Shellfish Immunol 144: 109227. DOI: 10.1016/j.fsi.2023.109227.

Chesnokov YV, Artemyeva AM. 2015. Evaluation of the measure of polymorphism information of genetic diversity. Agric Biol 50 (5): 571-578. DOI: 10.15389/agrobiology.2015.5.571eng.

Compton JA, Schrire BD, Könyves K, Forest F, Malakasi P, Mattapha S, Sirichamorn Y. 2019. The *Callerya* group redefined and Tribe Wisterieae (Fabaceae) emended based on morphology and data from nuclear and chloroplast DNA sequences. PhytoKeys 125: 1-112. DOI: 10.3897/phytokeys.125.34877.

da Silva Júnior AL, Cabral RLR, Sartori L, de Souza LC, de Miranda FD, Caldeira MVW, Moreira SO, de Oliveira Godinho T. 2020. Evaluation of diversity and genetic structure as strategies for conservation of natural populations of *Dalbergia nigra* (vell.) Allemão ex Benth. Cerne 26 (4): 435-443. DOI: 10.1590/01047760202026042754.

Dao DT, Le QT, Nguyen TT, Ma F. 2021. Nitrogen-containing heterocyclic compounds from the roots of *Callerya speciosa*. Vietnam J Sci Technol Eng 64 (3): 49-52. DOI: 10.31276/vjste.64(3).49-52.

Desmond SC, Garner M, Flannery S, Whittemore AT, Hipp AL. 2021. Leaf shape and size variation in bur oaks: An empirical study and simulation of sampling strategies. Am J Bot 108 (8): 1540-1554. DOI: 10.1002/ajb2.1705.

Fu M-Q, Xiao G-S, Xu Y-J, Wu J-J, Chen Y-L, Qiu S-X. 2016. Chemical constituents from roots of *Millettia speciosa*. Chin Herb Med 8 (4): 385-389. DOI: 10.1016/S1674-6384(16)60068-0.

Gogoi B, Wann SB, Saikia SP. 2020. Comparative assessment of ISSR, RAPD, and SCoT markers for genetic diversity in *Clerodendrum* species of North East India. Mol Biol Rep 47: 7365-7377. DOI: 10.1007/s11033-020-05792-x.

Hovenden MJ, Vander Schoor JK. 2006. The response of leaf morphology to irradiance depends on altitude of origin in *Nothofagus cunninghamii*. New Phytol 169: 291-297. DOI: 10.1111/j.1469-8137.2005.01585.x.

Huang B, Xu L, Li K, Fu Y, Li Z. 2017. Embryo induction and plant regeneration of *Callerya speciosa* (Fabaceae) through anther culture. Aust J Bot 65 (1): 80-84. DOI: 10.1071/bt16112.

Huang Z, Zeng Y-J, Chen X, Luo S-Y, Pu L, Li F-Z, Zong M-H, Lou W-Y. 2020. A novel polysaccharide from the roots of *Millettia speciosa* Champ.: Preparation, structural characterization and immunomodulatory activity. Intl J Biol Macromol 145: 547-557. DOI: 10.1016/j.ijbiomac.2019.12.166.

Jahdi R, Arabi M, Bussotti F. 2020. Effect of environmental gradients on leaf morphological traits in the Fandoghlo forest region (NW Iran). iForest Biogeosci For 13 (6): 523-530. DOI: 10.3832/ifor3391-013.

Kamble VV, Tamboli AS, Umdale SD, Rather SA, Liu H, Wani SH, Gaikwad NB. 2023. Evaluating genetic diversity of geographically diverse populations of *Embelia ribes* Burm f., a highly medicinal woody liana from the Western Ghats of India, using Random Amplified Polymorphic DNA (RAPD) and Intersimple Sequence Repeats (ISSR) markers. Mol Biol Rep 50: 1603-1615. DOI: 10.1007/s11033-022-08099-1.

Lam VQ, Anh LH, Quan NV, Xuan TD, Hanamura I, Uchino K, Karnan S, Takami A. 2022. Cytotoxicity of *Callerya speciosa* fractions against myeloma and lymphoma cell lines. Molecules 27 (7): 2322. DOI: 10.3390/molecules27072322.

Li D, Xu Z, Li Y, Gan L, Wu P, Wu R, Jin J, Zheng X, Zhang K, Ma H, Li L. 2022. Polysaccharides from *Callerya speciosa* alleviate metabolic disorders and gut microbiota dysbiosis in diet-induced obese C57BL/6 mice. Food Funct 13: 8662-8675. DOI: 10.1039/d2fo00337f.

Maraş-Vanlioğlu FG, Yaman H, Kayaçetin F. 2020. Genetic diversity analysis of some species in Brassicaceae family with ISSR markers. Biotech Stud 29 (1): 38-46. DOI: 10.38042/biost.2020.29.01.05.

Moghaieb REA, Abdelhadi AA, El-Sadawy HA, Allam NAT, Baiome BA, Soliman MH. 2017. Molecular identification and genetic diversity among Photorhabdus and Xenorhabdus isolates. 3 Biotech 7 (1): 6. DOI: 10.1007/s13205-016-0594-4.

Nasiruddin, Ji M, Yu Z, Chen G, Masood T, Ma F. 2020. Chemical constituents and biological functions of different extracts of *Millettia speciosa* leaves. J Food Nutr Res 8: 506-515. DOI: 10.12691/jfnr-8-9-7.

Nong Y, Zhang C, Guo Y, Qin Y, Zhong X, Feng L, Pan Z, Deng L, Guo H, Su Z. 2023. Quality control for a traditional Chinese medicine, *Millettia speciosa* Champ, using ultra-high-performance liquid chromatography fingerprint, serum pharmacochemistry and network pharmacology. Anal Methods 15: 5166-5180. DOI: 10.1039/d3ay01051a.

Osman SA, Ali HBM. 2020. Genetic diversity of five *Lathyrus* species using RAPD, ISSR and SCoT markers. Asian J Plant Sci 19 (2): 152-165. DOI: 10.3923/ajps.2020.152.165.

Pharmawati M, Wrasiati LP, Yowani SC. 2021. ISSR and RAPD primers selection for assessing genetic diversity of *Enhalusacoroides* (L.f.) Royle. IOP Conf Ser: Earth Environ Sci 709: 012054. DOI: 10.1088/1755-1315/709/1/012054.

Phong DT, Hien VTT, Thanh TTV, Tang DV. 2011. Comparison of RAPD and ISSR markers for assessment of genetic diversity among endangered rare *Dalbergia oliveri* (Fabaceae) genotypes in Vietnam. Genet Mol Res 10 (4): 2382-2393. DOI: 10.4238/2011.October.6.3.

Rogers SO, Bendich AJ. 1988. Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA, Verma DPS (eds). Plant Molecular Biology Manual. Springer, Dordrecht. DOI: 10.1007/978-94-009-0951-9_6.

Sirichamorn Y, Balslev H, Mattapha S. 2016. Two new species of *Callerya* Endl. (Leguminosae: Papilionoideae) from Thailand. Phytotaxa 263 (1): 42-50. DOI: 10.11646/ phytotaxa.263.1.4.

Strock CF, Schneider HM, Lynch JP. 2022. Anatomics: High-throughput phenotyping of plant anatomy. Trends Plant Sci 27 (6): 520-523. DOI: 10.1016/j.tplants.2022.02.009.

Vu HTT, Dinh PT. 2012. Genetic diversity among endangered rare *Dalbergia cochinchinensis* (Fabaceae) genotypes in Vietnam revealed by Random Amplified Polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers. Afr J Biotechnol 11: 8632-8644. DOI: 10.5897/ajb11.3598.

Wang MY, Ma WY, Wang QL, Yang Q, Yan XX, Tang H, Li ZY, Li YY, Feng SX, Wang ZN. 2022a. Flavonoid-enriched extract from *Millettia speciosa* Champ prevents obesity by regulating thermogenesis and lipid metabolism in high-fat diet-induced obese C57BL/6 mice. Food Sci Nutr 10 (2): 445-459. DOI: 10.1002/fsn3.2664.

Wang H, Wang R, Harrison SP, Prentice IC. 2022b. Leaf morphological traits as adaptations to multiple climate gradients. J Ecol 110 (6): 1344-1355. DOI: 10.1111/1365-2745.13873.

Wei Z, Pedley L. 2010. *Callerya*. In: Wu Z-Y, Raven PH, Hong DY (eds). Flora of China 10. Science Press, Beijing; Missouri Botanical Garden Press, St Louis.

Xiao Y, Li M, Zhong L, Qin Y, Zhang X, Wei Q, Qin Z, Zhang Y, Chen B. 2022. The complete chloroplast genome sequence and phylogenetic analysis of *Nanhaia speciosa* (Fabaceae). Mitochondrial DNA B Resour 7 (1): 266-268. DOI: 10.1080/23802359.2021.2008828.

Yao S, Lan Z, Huang R, Tan Y, Huang D, Gu J, Pan C. 2021. Hormonal and transcriptional analyses provides new insights into the molecular mechanisms underlying root thickening and isoflavonoid biosynthesis in *Callerya speciosa* (Champ. ex Benth.) Schot. Sci Rep 11: 9. DOI: 10.1038/s41598-020-76633-x.

Yao S-C, Bai L-H, Lan Z-S, Tang M-Q, Zhai Y-J, Huang H, Wei RC. 2016. Hairy root induction and polysaccharide production of medicinal plant *Callerya speciosa* Champ. Plant Cell Tiss Organ Cult 126: 177-186. DOI: 10.1007/s11240-016-0988-3.

Yu D, Liang X. 2019. Characterization and identification of isoflavonoids in the roots of *Millettia speciosa* Champ. by UPLC-Q-TOF-MS/MS. Curr Pharm Anal 15: 580-591. DOI: 10.2174/1573412914666180608095922.

Zhao XN, Liang JL, Chen HB, Liang YE, Guo HZ, Su ZR, Li YC, Zeng HF, Zhang XJ. 2015a. Anti-fatigue and antioxidant activity of the polysaccharides isolated from *Millettiae speciosae* Champ. Leguminosae. Nutrients 7 (10): 8657-8669. DOI: 10.3390/nu7105422.

Zhao XN, Wang XF, Liao JB, Guo HZ, Yu XD, Liang JL, Zhang X, Su ZR, Zhang XJ, Zeng HF. 2015b. Antifatigue effect of *Millettiae speciosae* Champ. (Leguminosae) extract in mice. Trop J Pharm Res 14 (3): 479-485. DOI: 10.4314/tjpr.v14i3.17.

Zhao Z, Liu P, Ma S, Wang S, Li A, Liu J, Wang M. 2017. Botanical characteristics, chemical and nutritional composition and pharmacological and toxicological effects of medicinal and edible plant *Millettia speciosa* Champ. Food Sci 38 (9): 293-306. DOI: 10.7506/spkx1002-6630-201709046.