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# Assessment of genetic diversity of *Stephania rotunda* Lour. collected in Northern Vietnam using RAPD and ISSR markers

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**Abstract:** *Stephania rotunda* Lour. is a valuable traditional medicinal plant that is in danger of extinction. This research aimed to examine the genetic relationship of 32 *Stephania rotunda* Lour. accessions collected in Vietnam by using RAPD and ISSR markers. 14 RAPD and 14 ISSR primers successfully detected 163 loci of which 133 (80.19%) were polymorphic and a total of 3047 scorable bands were obtained. The 14 RAPD markers produced 1346 scorable bands, 89.63% of which were polymorphic while 14 ISSR primers resulted in 1701 bands, 70.76% of which were polymorphic. The RAPD primers showed a mean PIC of 0.3 and Rp value of 2.28 while the average PIC and Rp indexes for ISSR markers were 0.20 and 1.47, respectively. UPGMA dendrogram obtained from cluster analysis of RAPD and ISSR combined data grouped 32 accessions into five clusters at 76% variation with Jaccard's similarity coefficient varying from 0.586 to 0.951. Moreover, principal component analysis was also used to determine genetic relationships among 32 collected accessions. The results showed high genetic dissimilarity among 32 *Stephania rotunda* Lour. Correlation analysis between the matrices of similarity coefficient was measured using Mantel's test. A moderate correlation value ($r$ = 0.543) between RAPD vs ISSR matrices, but the strong correlation value between RAPD and pooled data ($r$ = 0.930) and between ISSR and combined matrices ($r$ = 0.814) were obtained, suggesting a combination of RAPD and ISSR markers appears to be productive in studying the genetic variance in *Stephania rotunda* Lour. Our results could provide a solid foundation for further conservation management and breeding programs of *Stephania rotunda* Lour.

**Keywords:** ISSR; genetic diversity; *Stephania rotunda* Lour.; RAPD.
**Abbreviations:** ISSR_Inter-Simple Sequence Repeats; PCA_Principal Coordinate Analysis; PIC_Polymorphism Information Content RAPD_Random Amplified Polymorphic DNA; Rp_Resolving Power; UPGMA_Unweighted Pair-Group Method with Arithmetic. Average

## Introduction

*Stephania rotunda* Lour. (*S. rotunda*) is a medicinal creeper plant belonging to the large genus *Stephania* of Menispermaceae with about 60 species, most of which are mainly distributed in Southeast Asia (Nguyen, 2003; Luo et al., 2008; Desgrouas et al., 2014). In this genus, 37 species in China, 15 species in Thailand, and about 16 species in Vietnam have been recorded (Lo, 1978; Nguyen, 2003; Hu et al., 2008; Vu et al., 2019). As a folk traditional medicine, it has been used for the treatment of a wide range of illnesses including headache, asthma, fever, and diarrhea (Semwal and Semwal, 2015). Phytochemical analyses identified at least 40 types of alkaloids in various parts of this plant (Desgrouas et al., 2014).
In Vietnam, *S. rotunda* is commonly found in a wide range of regions, especially in calcareous mountain areas. With its rich biodiversity, Vietnam is a part of South-Central China that is one of 25 biodiversity hotspots listed by Myers et al. (2000). However, the biodiversity of *S. rotunda* plants in these regions has been critically endangered by a chaotic history. Moreover, the indiscriminate overexploitation of *Stephania* plants has resulted in the endangerment of many species. In Vietnam, *S. rotunda* is classified as Class IIA for the species that is threatened with extinction listed in Decree 84/2021/ND-CP (Vietnamese Government, 2021). Thus, it is important to provide an efficient and reliable strategy for the conservation and development of this species. Molecular analysis and assessment of genetic diversity can provide useful information about taxonomic identification, evolution amongst the species as well as the molecular background of different natural phenomena (Csillery et al., 2010). However, the knowledge of this species is still limited in Vietnam.

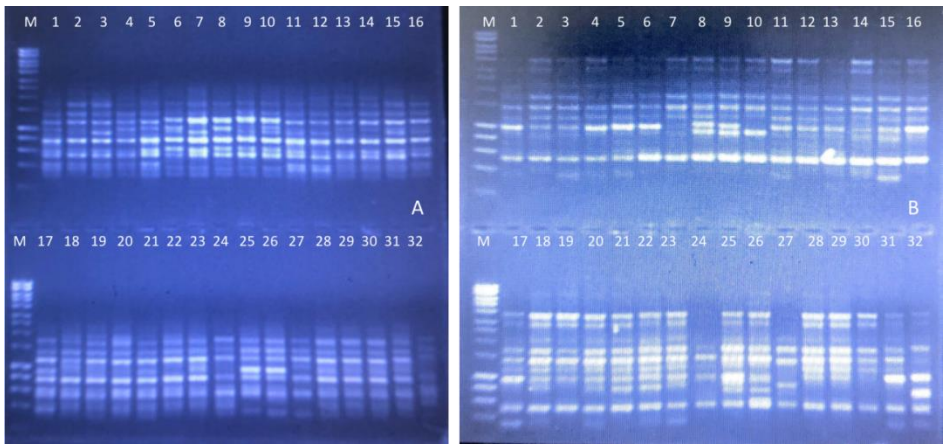


**Fig 1. (A)** RAPD banding profile obtained with primer OPA-01 and (B) ISSR banding profile obtained with primer UBC-811. Lane M: 1kb ladder, lane from 1 - 32 were PCR products from accessions in terms of No. given in Table 1.

**Table 1.** Amplification of 14 RAPD markers in 32 *Stephania rotunda* Lour. accessions.

| Primer | Sequence | Tm (°C) | Total No. of loci | Polymorphic loci | Polymorphism (%) | Total No. of bands | PIC | Rp |
|---|---|---|---|---|---|---|---|---|
| OPA-01 | GAGGCCCTTC | 34 | 8 | 8 | 100.00 | 143 | 0.30 | 3.44 |
| OPA-02 | TGCCGAGCTG | 34 | 8 | 7 | 87.50 | 101 | 0.27 | 1.81 |
| OPB-01 | GTTTCGCTCC | 32 | 8 | 8 | 100.00 | 66 | 0.19 | 1.88 |
| OPB-04 | GGACTGGAGT | 32 | 7 | 5 | 71.43 | 102 | 0.20 | 1.88 |
| OPC-03 | GGGGGTCTTT | 32 | 8 | 7 | 87.50 | 168 | 0.29 | 3.25 |
| OPC-08 | TGGACCGGTG | 34 | 7 | 7 | 100.00 | 116 | 0.32 | 3.25 |
| OPD-01 | ACCGCGAAGG | 34 | 4 | 3 | 75.00 | 55 | 0.25 | 1.44 |
| OPE-04 | GTGACATGCC | 32 | 4 | 3 | 75.00 | 66 | 0.09 | 0.38 |
| OPE-07 | AGATGCAGCC | 32 | 5 | 5 | 100.00 | 135 | 0.25 | 1.56 |
| OPN-03 | GGTACTCCCC | 34 | 6 | 6 | 100.00 | 82 | 0.40 | 3.75 |
| OPO-01 | GGCACGTAAG | 32 | 6 | 5 | 83.33 | 50 | 0.16 | 1.13 |
| OPO-02 | ACGTAGCGTC | 32 | 10 | 10 | 100.00 | 128 | 0.27 | 3.50 |
| OPS-05 | ACAGGTGCGT | 32 | 6 | 6 | 100.00 | 66 | 0.29 | 2.38 |
| OPR-12 | TTTGGGGCCT | 32 | 4 | 3 | 75.00 | 68 | 0.35 | 2.25 |
| **Total** | | | **91** | **83** | **1254.76** | **1346** | **3.63** | **31.88** |
| **Average/ Primer** | | | **6.5** | **5.93** | **89.63** | **96.14** | **0.30** | **2.28** |

The examination of the genetic dissimilarity of medicinal plants plays a vital role in the conservation and utilization of plant genetic materials. Both morphological and DNA markers can be utilized to determine the genetic variance within and among plant individuals or populations. The morphological markers are cheap and simple, but they have demerits since the phenotypes are greatly influenced by environmental conditions and the developmental stages of the plants. In contrast, molecular markers, also known as DNA markers, including random-amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSRs), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNPs), and sequence tag sites (STSs), etc. are stable, detectable in every part of the plant, unaffected by environmental factors, and exhibit greater levels of polymorphism (Semwal and Semwal, 2015). RAPD and ISSR have been successfully effective in revealing the diversity in the DNA of many medicinal plants in particular, such as ginseng (Wei et al., 2014), *Nilgirianthus ciliates* (Ramakrishnan et al., 2019), *Podophyllum hexandrum* (Naik et al., 2010), *Justicia adhatoda* (Kumar et al., 2014), medicinal plants of *Solanaceae* family (Singh et al., 2022).

This study is the first effort to evaluate the genetic diversity among the 32 *S. rotunda* accessions grown in various geographical locations of Vietnam by utilizing RAPD and ISSR markers. The research's findings will facilitate the effective conservation, management, and development of *S. rotunda* and other endangered medicinal plants.

## Results

### *RAPD analysis*

A total of 91 loci were yielded by 14 RAPD primers with a mean of 6.5 loci per primer (Table 1, Fig 1A). The percentage of polymorphism was from 71.43% for primer OPB-04 to 100% for primers OPA-01, OPB-01, OPC-08, OPE-07, OPN-03, OPO-02, and OPS-05 with an average of 89.63% polymorphism per primer. The Polymorphism Information Content (PIC) value of 14 RAPD markers varied from a lowest of 0.09 (OPE-04) to a highest of 0.40 (OPN-03) with a mean of 0.3. The resolving power (Rp) value was lowest for primer OPE-04 (0.38) and highest for primer OPN-03 (3.75) with an average of 2.28 per primer (Table 1).

The Jaccard similarity index resulting from RAPD data ranged from 0.478 between accession 8 and accession 13 to 0.956 between accession 29 and 30, with an average of 0.735 (Supplemental Table 1). At 73.5% similarity, the 32 *S. rotunda*

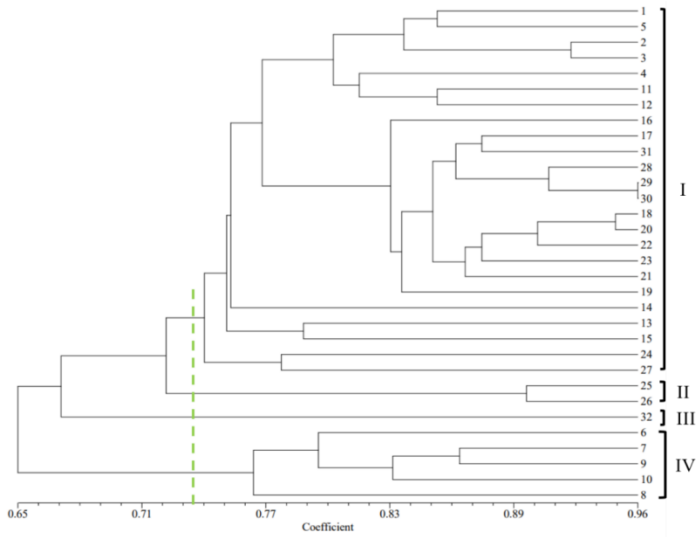


**Fig 2.** Dendrogram obtained with UPGMA from the Jaccard's similarity coefficients of 32 *Stephania rotunda* Lour. accessions based on RAPD data analysis.

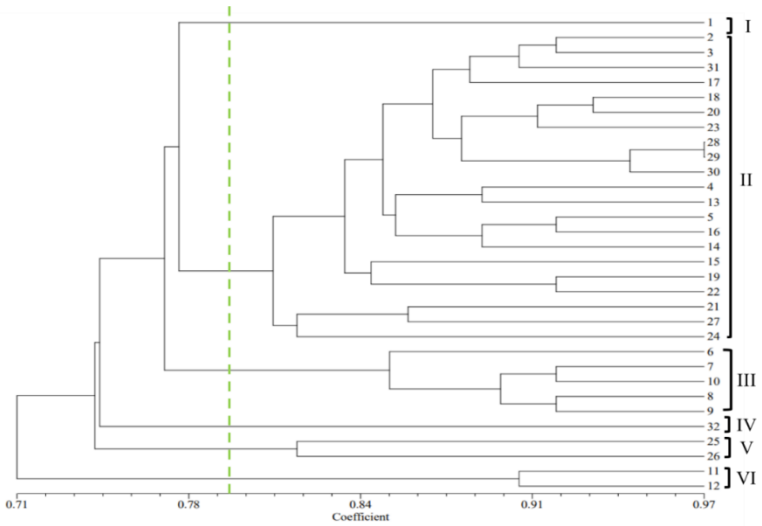


**Fig 3.** Dendrogram obtained with UPGMA from the Jaccard's similarity coefficients of 32 *Stephania rotunda* Lour. accessions based on ISSR data analysis.

**Table 2.** Amplification of 14 ISSR markers in *Stephania rotunda* Lour. accessions.

| Primer | Sequence | Tm ($^{o}$C) | Total No. of loci | Polymorphic loci | Polymorphism (%) | Total No. of bands | PIC | Rp |
|---|---|---|---|---|---|---|---|---|
| UBC-807 | $(AG)_8T$ | 50.0 | 5 | 4 | 80.00 | 95 | 0.06 | 0.31 |
| UBC-808 | $(AG)_8C$ | 50.0 | 7 | 5 | 71.43 | 103 | 0.13 | 1.06 |
| UBC-811 | $(GA)_8C$ | 50.0 | 8 | 7 | 87.50 | 162 | 0.36 | 4.75 |
| UBC-812 | $(GA)_8A$ | 49.0 | 5 | 3 | 60.00 | 102 | 0.33 | 2.5 |
| UBC-813 | $(CT)_8T$ | 50.0 | 5 | 3 | 60.00 | 128 | 0.20 | 1.5 |
| UBC-823 | $(TC)_8C$ | 50.0 | 6 | 4 | 66.67 | 158 | 0.24 | 2.13 |
| UBC-824 | $(TC)_8G$ | 50.0 | 5 | 4 | 80.00 | 140 | 0.19 | 1.25 |
| UBC-827 | $(AC)_8G$ | 52.4 | 4 | 4 | 100.00 | 80 | 0.28 | 1.5 |
| UBC-848 | $(CA)_8RG$ | 53.0 | 4 | 3 | 75.00 | 94 | 0.32 | 2.13 |
| UBC-864 | $(ATG)_6$ | 45.0 | 4 | 4 | 100.00 | 107 | 0.27 | 1.31 |
| UBC-873 | $(GACA)_4$ | 45.0 | 4 | 2 | 50.00 | 118 | 0.13 | 0.63 |
| UBC-888 | $(CA)_7BDB$ | 47.0 | 4 | 2 | 50.00 | 125 | 0.04 | 0.19 |
| UBC-889 | $(AC)_8DB$ | 47.0 | 5 | 2 | 40.00 | 152 | 0.08 | 0.5 |
| UBC-891 | $(TG)_7HVH$ | 47.0 | 6 | 3 | 50.00 | 137 | 0.1 | 0.81 |
| **Total** | | | **72** | **50** | | **1701** | **2.75** | **20.56** |
| **Average/primer** | | | **5.14** | **3.57** | **70.76** | **121.5** | **0.20** | **1.47** |

*R = A/G; B = non-A; D = non-C; H = non-G; V=non-T.

**Table 3.** Relative efficiency of molecular markers for determining polymorphism in *Stephania rotunda* Lour. accessions.

| Parameters for marker efficiency | Molecular marker system | | |
|---|---|---|---|
| | RAPD | ISSR | Combined RAPD and ISSR |
| Number of cultivars | 32 | 32 | 32 |
| Total number of primers | 14 | 14 | 28 |
| Total number of loci | 91 | 72 | 163 |
| Total number of polymorphic loci | 83 | 50 | 133 |
| Polymorphism (%) | 89.63 | 70.76 | 80.19 |
| Total number of scorable bands | 1346 | 1701 | 3047 |
| Polymorphism information content (PIC) | 0.30 | 0.20 | 0.23 |
| Resolving power (Rp) | 2.28 | 1.47 | 1.87 |

**Table 4.** Matrix comparisons of Mantel test between markers.

| Comparison | Matrix correlation (*r*) | p-value (two-tailed) | Alpha |
|---|---|---|---|
| RAPD vs. ISSR | 0.543 | < 0.0001 | 0.05 |
| RAPD vs. combined RAPD and ISSR | 0.930 | < 0.0001 | 0.05 |
| ISSR vs. combined RAPD and ISSR | 0.814 | < 0.0001 | 0.05 |

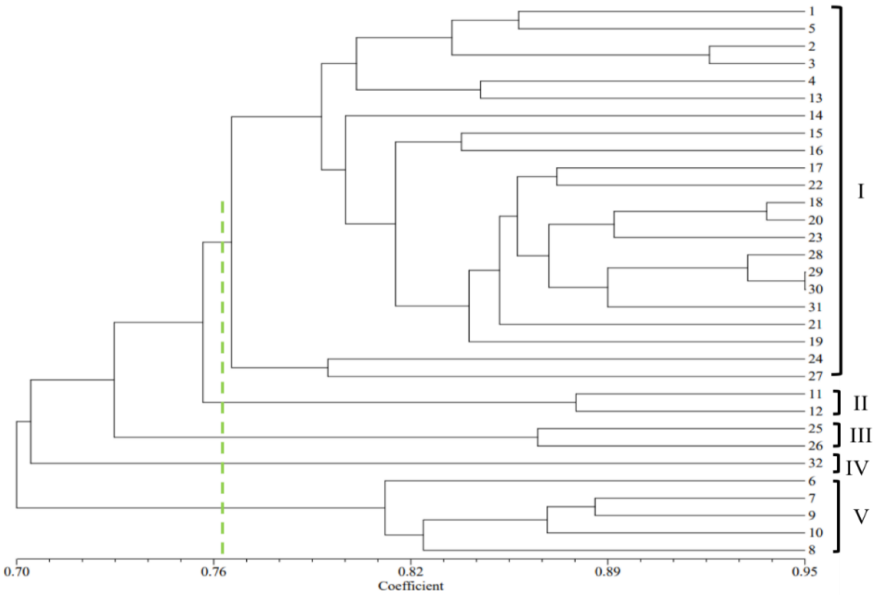


**Fig 4.** Dendrogram obtained with UPGMA from the Jaccard's similarity coefficients of 32 *Stephania rotunda* Lour. accessions based on RAPD and ISSR data analysis.

accessions were separated into four main clusters as shown in Fig 2. Cluster I contained most of the accessions including 1, 5, 2, 3, 4, 11, 12, 16, 17, 31, 28, 29, 30, 18, 20, 22, 23, 21, 19, 14, 13, 15, 24, and 27. Cluster II consisted of accessions 25 and 26. Cluster III had only one accession 32. Cluster IV was composed of five accessions including 6, 7, 9, 10, and 8 (Fig 2).

***ISSR analysis***

The 14 ISSR markers produced a total of 72 amplification loci with an overall mean of 5.14 loci per primer (Table 2, Fig 1B). UBC-811 primer gave the maximum loci (8) while UBC-827, UBC-848, UBC-864, UBC-873, and UBC-888 primers exhibited the smallest number of loci (4.0). The percentage of polymorphism was from 40.00% (UBC-889 primer) to 100% (UBC-827 and UBC-864 primers) with a mean of 70.76% polymorphism per primer. The lowest PIC index of 0.04 was for primer UBC-888 whereas the maximum PIC index of 0.36 was found in UBC-811 primer with a mean PIC value of 0.20 per primer. The mean Rp index of 14 ISSR primers was 1.47 with a maximum index of 4.75 for UBC-811 primer and the smallest index of 0.31 for UBC-807 primer (Table 2).

The Jaccard similarity index based on ISSR data ranged from 0.583 to 0.972 with a mean of 0.794 (Supplemental Table 2). Accessions 28 and 29 exhibited the biggest similarity value (0.972) and accessions 9 and 12 showed the least similarity (0.583). The dendrogram displays the construction of five main clusters of 32 *S. rotunda* accessions at the coefficient value of 79.4% (Fig 3). Cluster II is the largest cluster with 23 accessions including 2, 3, 31, 17, 18, 20, 23, 28, 29, 30, 4, 13, 5, 16, 14, 15, 19, 22, 21, 27, and 24. Cluster III consisted of five accessions 6, 7, 10, 8, and 9. Each cluster I and IV had only accession 1 and 32, respectively. Cluster V contained accessions 25 and 26. Cluster VI comprised two accessions 11 and 12.

***Combined RAPD and ISSR analyses***

A combination of 14 RAPD and 14 ISSR primers generated 80.19% polymorphic bands with the mean PIC and Rp indexes of 0.23 and 1.87, respectively (Table 3). The Jaccard similarity

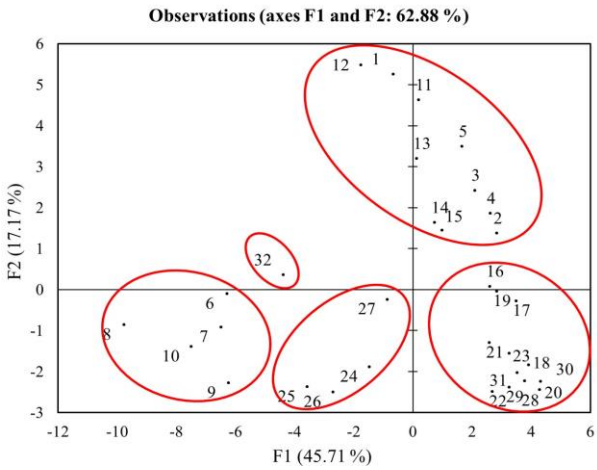


**Fig 5.** PCA analysis of 32 *Stephania rotunda* Lour. accessions using Jaccard's similarity coefficients generated by RAPD and ISSR markers. F1, F2 are the main components of the PCA biplot contributed 62.88% of the total variation in genetic relationship among 32 accessions. Each point represents as accession number.

**Table 5.** Detail of the *Stephania rotunda* Lour. accessions collected from different regions of Vietnam used in this study.

| Accession No. | Collection site | Laditude/longtitude | Altitude (m) |
|---|---|---|---|
| 1 | Vinh Tien, Kim Boi, Hoa Binh | 20°43'00"N, 105°25'13"E | 425 |
| 2 | Vinh Tien, Kim Boi, Hoa Binh | 20°42'53"N, 105°26'02"E | 479 |
| 3 | Vinh Tien, Kim Boi, Hoa Binh | 20°43'35"N, 105°26'40"E | 299 |
| 4 | Vinh Tien, Kim Boi, Hoa Binh | 20°43'36"N, 105°26'47"E | 359 |
| 5 | Vinh Tien, Kim Boi, Hoa Binh | 20°43'49"N, 105°26'22"E | 440 |
| 6 | Dao Duc, Vi Xuyen, Ha Giang | 22°43'28"N, 104°58'16"E | 175 |
| 7 | Dao Duc, Vi Xuyen, Ha Giang | 22°43'15"N, 104°57'22"E | 146 |
| 8 | Dao Duc, Vi Xuyen, Ha Giang | 22°43'14"N, 104°34'16"E | 126 |
| 9 | Cao Bo, Vi Xuyen, Ha Giang | 22°45'00"N, 104°51'56"E | 873 |
| 10 | Cao Bo, Vi Xuyen, Ha Giang | 22°44'28"N, 104°52'46"E | 689 |
| 11 | Cao Son, Luong Son, Hoa Binh | 20°48'44"N, 105°29'27"E | 242 |
| 12 | Cao Son, Luong Son, Hoa Binh | 20°48'32"N, 105°30'25"E | 224 |
| 13 | Cao Son, Luong Son, Hoa Binh | 20°48'32"N, 105°31'28"E | 125 |
| 14 | Cao Son, Luong Son, Hoa Binh | 20°48'33"N, 105°30'45"E | 241 |
| 15 | Cao Son, Luong Son, Hoa Binh | 20°48'41"N, 105°32'11"E | 382 |
| 16 | Tu Son, Kim Boi, Hoa Binh | 20°43'44"N, 105°23'53"E | 348 |
| 17 | Tu Son, Kim Boi, Hoa Binh | 20°43'26"N, 105°23'34"E | 453 |
| 18 | Tu Son, Kim Boi, Hoa Binh | 20°43'12"N, 105°25'19"E | 454 |
| 19 | Tu Son, Kim Boi, Hoa Binh | 20°42'50"N, 105°25'19"E | 614 |
| 20 | Tu Son, Kim Boi, Hoa Binh | 20°44'52"N, 105°23'46"E | 375 |
| 21 | An Binh, Lac Thuy, Hoa Binh | 20°24'02"N, 105°45'40"E | 71 |
| 22 | An Binh, Lac Thuy, Hoa Binh | 20°23'59"N, 105°45'47"E | 142 |
| 23 | An Binh, Lac Thuy, Hoa Binh | 20°23'47"N, 105°45'47"E | 70 |
| 24 | Nam Ke, Muong Nhe, Dien Bien | 22°06'03"N, 102°38'43"E | 526 |
| 25 | Muong Thin, Tuan Giao, Dien Bien | 21°37'05"N, 103°22'00"E | 871 |
| 26 | Muong Thin, Tuan Giao, Dien Bien | 21°37'03"N, 103°21'56"E | 833 |
| 27 | Tan Uyen, Tan Uyen, Lai Chau | 22°04'34"N, 103°39'49"E | 764 |
| 28 | Doan Ket, Da Bac, Hoa Binh | 20°56'00"N 105°02'55"E | 810 |
| 29 | Doan Ket, Da Bac, Hoa Binh | 20°55'28"N 105°01'59"E | 1054 |
| 30 | Doan Ket, Da Bac, Hoa Binh | 20°55'40"N 105°02'35"E | 896 |
| 31 | Doan Ket, Da Bac, Hoa Binh | 20°56'31"N 105°02'36"E | 944 |
| 32 | Quang Thanh, Thanh Hoa | 19°45'45"N, 105°46'56"E | 4 |

value based on pooled RAPD and ISSR data analysis ranged from 0.586 to 0.951 with a mean of 0.762. The highest similarity value was between 29 and 30 accessions (0.951) whereas accessions 9 and 13 exhibited the least similarity of 0.586 (Supplemental Table 3). The UPGMA dendrogram based on combining data grouped 32 *S. rotunda* accessions into five different groups at 76.2% similarity. Cluster I contained 22 accessions including 1, 5, 2, 3, 4, 13, 14, 15, 16, 17, 22, 18, 20, 23, 28, 29, 30, 31, 21, 19, 24, and 27. Cluster II had two accessions 11 and 12. Cluster III comprised accessions 25 and 26. Cluster IV had only one accession 32. Cluster V consisted of five accessions including 6, 7, 9, 10, and 8 (Fig 4).

The Mantel test and Pearson's correlation (*r*) exhibited a moderate coefficient of 0.543 ($p<0.0001$) between ISSR and

RAPD markers whereas genetic matrics of RAPD or ISSR makers and integrated data showed a strong correlation with $r$ = 0.930 and 0.814 ($p<0.0001$), respectively (Table 4). The results indicated that the efficiency of genetic variation analysis of RAPD to 32 *S. rotunda* accessions is higher than that of ISSR.

Principal component analysis (PCA) based on the combined RAPD and ISSR data displayed the genetic relationships of *S. rotunda* accessions in two-dimensional space accounted for 45.71% and 17.17% of the total genetic varia difference. The PCA data separated 32 *S. rotunda* into major clusters that appeared similar to the tree diagram generated from the cluster analysis (Fig 5). Accessions 1, 12, 11, 13, 5, 14, 15, 3, 4, and 2 were grouped into one cluster whereas accessions 16, 19, 17, 21, 23, 18, 30, 31, 22, 29, 28, and 20 were in the same cluster. Accessions 6, 7, 8, 9, and 10 were gathered together in one cluster, and accessions 24, 25, 26, and 27 were in one group. Unsurprisingly, accession 32 was isolated into one group from the remaining accessions (Fig 5).

**Discussion**

DNA markers such as ISSRs, RAPDs, AFLPs, SSRs, and SNPs have been demonstrated to be practical in examining genetic dissimilarity in medicinal plants' germplasm (Lee et al., 2012; Liu et al., 2018; Bi et al., 2021; Singh et al., 2022). In this study, the genetic diversities of 32 *S. rotunda* accessions collected from different geographical zones in Vietnam were analyzed through RAPD and ISSR molecular markers. A large number of studies previously showed that RAPD markers provide a higher capacity for detecting polymorphism and genetic diversity than ISSR markers (Ninh et al., 2022; Gupta et al., 2008; Verma et al., 2017) while other research indicated that ISSR markers have more efficiency than RAPD in identifying polymorphism in many plant species (Zietkiewicz et al., 1994; Pham et al., 2021). In medicinal plant research, Hamouda (2019) has shown that the percentage of polymorphism among 14 collections of *Silybum marianum* populations in Egypt identified by RAPD markers was 73.2% whereas by ISSR markers was 79.3%. However, Baruah et al. (2017) revealed that 90.68% of bands generated by ISSR primers showed polymorphism among *Cymbopogon* species while this number in the case of RAPD primers was lower (88.62%). In our study, the RAPD primers are more efficient, detecting 89.63% polymorphism in 32 accessions of *S. rotunda*, compared to ISSR which detected 70.76% polymorphism. The higher PIC value observed in RAPD markers also added strength to the above observation. Similarly, Bui et al. (2022) also found that the mean PIC index of RAPD (0.72) was higher than that of ISSR primers (0.64) in an important medicinal plant in Vietnam, *Pseuderanthemum latifolium*.

The resolving power (Rp) is a measure of the ability of a molecular marker to differentiate among individuals (Prevost and Wilkinson, 1999). In this study, Rp value of RAPD markers (2.28) was higher than that of ISSR markers (1.47) (Table 3) suggesting a higher power of RAPD for the discrimination of genetic diversity among *S. rotunda* accessions. Supporting these findings, our UPGMA clustering analysis showed that the dendrogram generated by RAPD and pooled RAPD+ISSR data grouped 32 accessions of *S. rotunda* in similar clusters except that accessions 11 and 12 were separated into one group in the combined data-generated dendrogram (Fig 3 and Fig 4). Moreover, the correlation test showed that RAPD gave a stronger correlation coefficient ($r$ = 0.930) than ISSR ($r$ = 0.814) with pooled data (Table 4). According to Ninh et al. (2022) and Chowdhury et al. (2002), the ISSR system produced more complex marker patterns and was more reproducible than the RAPD approach meaning that ISSR is advantageous when discriminating closely related accessions. Nonetheless, based on higher polymorphism, PIC, Rp, and correlation coefficient values, the RAPD method was slightly more effective than ISSR in the evaluation of genetic variation in *S. rotunda*. This is because the target sequences in the genome detected by the two marker systems were different. While RAPD primers are distributed along the genome, ISSRs are found only between microsatellite sequences (Bachmann, 1997; Landergott et al., 2001; Penner, 1996).

The similarity coefficient was from 0.478 - 0.957 in RAPD, 0.583 - 0.972 in ISSR, and 0.586 - 0.951 in joined data which indicated high genetic diversity among 32 *S. rotunda* accessions collected from different geographical areas in Vietnam. Different environmental conditions such as temperature, relative humidity, sunlight, rainfall, wind, etc. may be the main contributors to the high genetic variation among the *S. rotunda* populations. Supporting our observations, previous studies showed similar results in medicinal plants, such as *Caragana microphylla* (Huang et al., 2016) and *Nilgirianthus ciliates* (Ramakrishnan et al., 2019) wherein climate factors affect genetic variability. It was found that *S. rotunda* accession 28 was closely related to accession 29 based on the similarity matrix obtained from pooled data analysis. This finding was not surprising since these two accessions were both collected from Doan Ket, Da Bac, Hoa Binh province. The last similarity value was obtained between accessions 8 and 13, accessions 9 and 12, and 9 and 13 according to RAPD, ISSR, and pooled data analyses (Supplemental Tables 1, 2, and 3). These results can be attributed to the fact that these accessions were collected from different geographical areas such as accession 8 and 9 was collected in Ha Giang province whereas accession 12 and 13 were collected from Hoa Binh province.

The dendrogram of the combined RAPD and ISSR data demarcated 32 *S. rotunda* accessions into five main clusters. Cluster I contained 20 accessions collected from Hoa Binh (1, 5, 2, 3, 4, 13, 14, 15, 16, 17, 22, 18, 20, 23, 28, 29, 30, 31, 21, 19), one accession collected from Muong Nhe, Dien Bien (24) and one accession collected from Tan Uyen, Lai Chau (27). Cluster II comprised two accessions collected from Luong Son, Hoa Binh (11 and 12). Two accessions collected in Tuan Giao commune, Dien Bien province were in cluster III (25 and 26) whereas all five accessions from Vi Xuyen commune, Ha Giang province (6, 7, 9, 8, 10) were grouped in cluster V. Accession 32 collected from Thanh Hoa province singled out from the rest of the accessions (Fig 4). It can be seen that the accessions collected in different communes but belonging to the same provinces were separated into one group. This could be the result of the plants growing in similar climate conditions (Huang et al., 2016). The mixing of accession 24 collected in Muong Nhe commune, Dien Bien province, and accession 27 collected in Tan Uyen commune, Lai Chau province in cluster I of 20 accessions collected from Hoa Binh province could mean that the region-specific diversity does appear when the plants are grown in different geographical zones or the environmental conditions in these locations are moderately similar. The results acquired by cluster analysis also were further supported by the principal component analysis (PCA) that indicates the separation of the 32 accessions based on

their geographical areas. In wild conditions, the genetic diversity of each *Stephania rotunda* Lour. was successfully uncovered by ISSR and RAPD markers.

## Materials and methods

### *Plant materials*

Thirty-two accessions of *S. rotunda* were collected from different locations in central and Northern areas of Viet Nam being maintained at the Faculty of Biotechnology, Vietnam National University of Agriculture. The list of *S. rotunda* accessions is presented in Table 5.

### *DNA extraction*

Genomic DNA was isolated from fresh leaves of *S. rotunda* plants by the standard CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle, 1987). DNA concentration and purity were measured by a Nanodrop Spectrophotometer (Thermo). The DNA was stored at -20°C.

### *RAPD-PCR and ISSR-PCR amplification*

Fourteen RAPD and 14 ISSR primers were used for PCR amplification. The sequences and melting temperature (Tm) of each primer are presented in Table 2 and Table 3. PCR reactions were conducted with 1 µl of primer (10 µM), 1.0 µl DNA (10 ng/µl), 10 µl MyTaq Buffer (Bioline, USA), and the final volume made up of 20 µl with distilled water. The PCR was run in ASTEC Thermal Cyclers (Gene Atlas, ASTEC, Japan) using a program of initial denaturation at $95^0$C for 5 min, 35 cycles of 30 s denaturation at $95^0$C, 30 s annealing at Tm, and 90 s extension at $72^0$C, followed by a 10 min final extension at $72^0$C. PCR products were visualized on 1% agarose gel in 1×TAE buffer by electrophoresis at 100 V for 30 min and photographed by Bio-image System (BioRad, Germany).

### *Data analysis*

Clear and reproducible bands amplified with ISSR and RAPD primers were manually scored as presence (1) or absence (0).

The polymorphism information content (PIC) index for each primer was measured according to Roldán-Ruiz et al. (2000): $\mathrm{PIC} = 2f(1-f)$ where ƒ is the proportion of the present bands and $(1-f)$ is the proportion of the absent bands.

The resolving power (Rp) of each primer was calculated as proposed by Prevos and Wilkinson (1999): $\mathrm{Rp} = \sum \mathrm{BI}$. In which, BI (Band Informativeness) equals $1 - (2 \times |0.5 - p|)$ where *p* is the frequency of accessions carrying a particular band.

Dendrograms were produced using the unweighted pair-group method with arithmetic average (UPGMA) on the basis of Jaccard's similarity coefficient TREE program of NTSYS 2.1 software. Genetic similarity matrices by ISSR, RAPD and combined ISSR and RAPD markers calculated by Jaccard's similarity coefficient in NTSYS 2.1 software were used as input for principal coordinate analysis (PCA). Estimation of correlation value between markers was done using a Mantel test in XLSTAT 2018 package.

## Conclusion

This is the first study on the genetic diversity analysis of the medicinal plant *S. rotunda* Lour. using molecular markers. The findings of the research provide important information for conservation and breeding programs of *S. rotunda* Lour.

## References


Bachmann K (1997) Nuclear DNA markers in plant biosystematics research. Opera Bot. 132:137–148.

Baruah J, Gogoi B, Das K, Ahmed NM, Sarmah DK, Lal M, Bhau BS (2017) Genetic diversity study amongst Cymbopogon species from NE-India using RAPD and ISSR markers. Ind Crops Prod. 95: 235-243.

Bi D, Chen D, Khayatnezhad M, Hashjin ZS, Li Z, Ma Y (2021) Genetic response of growth phases for abiotic environmental stress tolerance in cereal crop plants. Genetika. 53(1): 393-405.

Bui ST, Khong TT, Ho VT (2022) Genetic diversity and characterization of *Pseuderanthemum latifolium* by RAPD and ISR molecular markers. J Anim Plant Sci. 32(1): 78-83.

Chowdhury MA, Vandenberg B, Warkentin T (2002) Cultivar identification and genetic relationship among selected breeding lines and cultivars in chickpea (*Cicer arietinum* L.). Euphytica. 127: 317–325.

Csillery K, Blum MGB, Gaggiotti OE, Francois O (2010) Approximate bayesian computation (ABC) in practice. Trends Ecol. Evol. 25: 410–418.

Desgrouas C, Taudon N, Bun SS, Baghdikian B, Bory S, Parzy D, Ollivier E (2014) Ethnobotany, phytochemistry and pharmacology of *Stephania rotunda* Lour. J Ethnopharmacol. 154(3): 537-563.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Lett. 19: 11-15.

Gupta S, Srivastava M, Mishra GP, Naik PK, Chauhan RS, Tiwari SK, Kumar M, Singh R (2008) Analogy of ISSR and RAPD markers for comparative analysis of genetic diversity among different *Jatropha curcas* genotypes. Afr. J. Biotechnol. 7: 4230-4243.

Hamouda M (2019) Molecular analysis of genetic diversity in population of *Silybum marianum* (L.) Gaertn in Egypt. J. Genet. Eng. Biotechnol. 17: 12.

Hu CM, Lou HS, Chen T, Gilbert MG (2008) Menispermaceae. In: Wu ZY, Revan P, Hong DY. (Ed.) Flora of China. Science Press, MO. 1–31.

Huang W, Zhao X, Zhao X, Li Y, Lian J (2016) Effects of environmental factors on genetic diversity of *Caragana microphylla* in Horqin Sandy Land, northeast China. Ecol. Evol. 321(6): 8256–8266.

Kumar A, Mishra P, Singh SC, Sundaresan C (2014) Efficiency of ISSR and RAPD markers in genetic divergence analysis and conservation management of *Justicia adhatoda* L., a medicinal plant. Plant Syst. Evol. 300: 1409–1420.

Landergott U, Holderegger R, Kozlowski G, Schneller JJ (2001) Historical bottlenecks decrease genetic diversity in natural populations of *Dryopteris cristata*. J. Hered. 87: 344–355.

Lee OR, Kim MK, Yang DC (2012) Authentication of medicinal plants by SNP-based multiplex PCR. Methods Mol. Biol. 862: 135-147.

Liu S, An Y, Li F, Li S, Liu L, Zhou Q, Zhao S, Chaoling W (2018) Genome-wide identification of simple sequence repeats and development of polymorphic SSR markers for genetic studies in tea plant (*Camellia sinensis*). Mol. Breed. 38(5): 59.

Lo HS (1978) A preliminary study on the genus *Stephania* in China. Acta Phytotax. Sin. 16: 10–40.

Luo XR, Chen T, Gilbert MG (2008) *Stephania.* In: Wu ZY, Revan P, Hong DY (Ed.) Flora of China. Science Press, MO. 15–27.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, Kent J (2000) Biodiversity hotspots for conservation priorities. Nature. 403: 853-858.

Naik PK, Alam MA, Singh H, Goyal V, Parida S, Kalia S, Mohapatra T (2010) Assessment of genetic diversity through RAPD, ISSR and AFLP markers in *Podophyllum hexandrum*: a medicinal herb from the Northwestern Himalayan region. Physiol Mol Biol Plants. 16(2): 135-148.

Nguyen TB (2003) Menispermaceae. Checklist of plant species of Vietnam. Angiosperm Agricultural Publishing House, Ha Noi. 140–152.

Ninh TT, Doan HT, Nguyen MT, Tran TT, Dang PDD, Dinh ST, Nguyen HT, Nong H, Nguyen CX (2022) Genetic diversity of avocado (*Persea americana* Mill.) germplasm in Vietnam using RAPD and ISSR molecular markers. Aust. J. Crop Sci. 16(06): 856-862.

Penner GA (1996) RAPD analysis of plant genomes. In: Jauhar PP (ed.) Methods of genome analysis in plants. CRC, Boca Raton. 251–268.

Pham KN, Ninh TP, Pham HT, Nguyen NQ, Do NH, Dinh ST (2021). High genetic diversity of *Dysosma tonkinense* revealed by ISSR and RAPD markers. Asian J. Plant Sci. 20: 637-647.

Prevost A, Wilkinson MJ (1999) A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet. 98: 107-112.

Ramakrishnan R, Subramani P, Periyasamy R, Chinnar Tamil S, Lakkakula S, Shanmugaraj G, David LWM, Manikandan R (2019) Genetic diversity and phylogenetic relationship of *Nilgirianthus ciliatus* populations using ISSR and RAPD markers: Implications for conservation of an endemic and vulnerable medicinal plant. Biocatal. Agric. Biotechnol. 18.

Roldán-Ruiz I, Dendauw J, van Bockstaele E, Depicker A, De Loose M (2000) AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). Mol. Breed. 6: 125-134.

Semwal DK, Semwal RB (2015) Efficacy and safety of *Stephania glabra*: an alkaloid-rich traditional medicinal plant. Nat. Prod. Res. 29(5): 396-410.

Singh R, Mahendra PS, Sadhana C (2022) Genetic diversity analysis in five medicinal plants of Solanaceae family using RAPD markers. Int. J. Pharm. Biol. Sci. 12(1): 120-125.

Verma KS, Ul Haq S, Kachhwaha S, Kothari SL (2017) RAPD and ISSR marker assessment of genetic diversity in *Citrullus colocynthis* (L.) Schrad: a unique source of germplasm highly adapted to drought and high-temperature stress. 3 Biotech. 7: 288.

Vietnamese Government (2021) Decree No. 84/2021/ND-CP dated September 22, 2021 on management of endangered, rare and precious species of forest fauna and flora and observation of Convention on International Trade in Endangered Species of Wild Fauna and Flora.

Vu TC, Nong VD, Tran VT, Xia N (2019) *Stephania polygona* (Menispermaceae), a new species from Southern Vietnam. Phytotaxa. 400 (3): 211–214.

Wei XY, Tian YX, Zhao ZL, Sun FR (2014) RAPD and ISSR analyses of genetic diversity of American ginseng germplasm from different habitats in China. Chin. Tradit. Herb. Drugs. 45: 3153-3158.

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics. 20: 178-183.