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Evaluation of genetic diversity by molecular markers in Indian gooseberry (*Phyllanthus emblica*)

Poeaim, S.[1*], Sangsoy, V.[2] and Tangthirasunun, N.[1]

[1]Department of Biology, School of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Ladkrabang, Bangkok 10520 Thailand; [2]Phrae Agricultural Research and Development Centre, Department of Agriculture, Phrae, 54000 Thailand.

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Abstract The genetic diversity and relationships among 16 cultivars of Indian gooseberry (*Phyllanthus emblica*) using Sequence-Related Amplified Polymorphism (SRAP) and Random Amplified Polymorphic DNA (RAPD) markers were investigated. The cultivars were collected from the Phrae Horticultural Research Center, Thailand. For SRAP analysis, 30 primer combinations were initially tested, comprising five forward and six reverse primers. Seven primer combinations consistently generated well-defined bands and were selected for genetic profiling. These SRAP primers amplified 172 fragments, with 145 (84.30%) polymorphic. For RAPD analysis, 50 primers were evaluated, and eight primers consistently produced clear bands of genetic profiling. These RAPD primers amplified 215 fragments, with 188 (87.44%) polymorphic. The genetic relationships among the cultivars were assessed using a dendrogram constructed with NTSYS-pc (version 2.1X) based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The genetic similarity coefficients ranged from 0.66 to 0.90 for SRAP and from 0.58 to 0.89 for RAPD markers, indicating a high level of genetic diversity among the cultivars.

Keywords: Indian gooseberry, SRAP, RAPD

Introduction

Phyllanthus emblica Linn, the Indian gooseberry, Amla, or Makham-pom in Thailand, is categorized as part of the Euphorbiaceae family. The Indian gooseberry is commonly found across Southeast Asia and is prevalent in tropical forests (Chaturvedi *et al.,* 2022). The plant is commonly applied in traditional medicinal treatments. Its fruit, bark, and leaves are valued for their therapeutic properties, including reducing fever and asthma, promoting wound healing, relieving cough, treating sore throats, and boosting the immune system. It is abundant in essential nutrients, including minerals, amino acids, polysaccharides, unsaturated fatty acids, and vitamins, making it a highly nutritious plant with

[*]**Corresponding Author:** Poeaim, S.; **Email:** supattra.poe@kmitl.ac.th

significant health benefits. Studies demonstrating the well-established pharmacological activities highlight its potential as an antioxidant, anticancer agent, anti-inflammatory, anti-diabetic, and anti-hyperlipidemic compound. This aligns with the presence of a wide array of critical phytochemical compounds, such as gallic acid, quercetin, ellagic acid, and kaempferol, which are found in the fruit, bark, and leaves of Indian gooseberry (Saini *et al*., 2022; Prananda *et al*., 2023; Ma *et al*., 2024). So, Indian gooseberry finds broad application across various fields, particularly in food-related industries such as beverages, preserved fruits, and fermented foods. Additionally, it is a key ingredient in medicines and cosmetics.

In Thailand, most Indian gooseberry fruits are harvested from natural forests or traditional cultivation, but production is limited and inconsistent in quantity and quality. Information on its varieties and quality remains scarce, highlighting the need for genetic diversity studies to support future utilization and breeding efforts. Genetic diversity in Indian gooseberries can be assessed using morphological, biochemical, and molecular markers. Molecular markers are handy as they reveal precise genetic differences unaffected by environmental factors.

Various molecular techniques have been employed to examine Indian gooseberry and investigate genetic diversity. A marker using RAPD-SCAR (random amplified polymorphic DNA-sequence characterized amplified region) was developed by Dnyaneshwar *et al*. (2006) to detect the genus *Phyllanthus*, which has since been utilized for authenticating commercial samples of Indian gooseberry fruit powders used in traditional Chinese medicine. Additionally, RAPD markers were employed to assess the genetic diversity of seven Indian gooseberry varieties, and cluster analysis indicated three groups corresponding to their regions of origin (Chaurasia *et al*., 2009). Besides some molecular techniques such as ISSR: inter simple sequence repeat (Senapati *et al*., 2011), nrITS-RFLP: nuclear ribosomal internal transcribed spacer-restriction fragment length polymorphism (Bandyopadhyay and SenRaychaudhuri, 2014) and SSR: microsatellites (Liu *et al*., 2020). Including the use of multiple markers, such as RAPD and ISSR (Rout and Aparajita, 2010), RAPD and nuclear rDNA-SNPs: single nucleotide polymorphisms (Singh *et al*., 2015) for phylogenetic studies and conservation of *Phyllanthus* species.

Within Thailand, genetic diversity assessments of certain *Phyllanthus* species and Indian gooseberry cultivars and germplasm have employed various techniques, including RAPD and SCAR (Theerakulpisut *et al*., 2008), morphology and AFLP: amplified fragment length polymorphism (Luangprasert *et al*., 2011) and SSR or microsatellites (Pandey and Changtragoon, 2012).

Sequence-related amplified polymorphism: SRAP and RAPD are polymerase chain reaction (PCR)-based techniques extensively used to identify polymorphisms. SRAP is designed to amplify open-reading frames (ORFs) in genomic DNA (Li and Quiros, 2001). In contrast, RAPD is a technique that uses a single decamer primer to amplify random segments of genomic DNA. Both SRAP and RAPD are potent techniques that do not require prior knowledge of the DNA sequence and simplicity. So, the objective was to showcase the application of SRAP and RAPD markers for assessing the genetic diversity among Indian gooseberries gathered from the germplasm collection at the Phrae Agricultural Research and Development Centre, Department of Agriculture, Phrae, Thailand.

Material and method

Plant materials and DNA extraction

The sixteen cultivars of Indian gooseberries were collected from the field crops research center at the Phrae Agricultural Research and Development Centre, Department of Agriculture, Ministry of Agriculture and Cooperatives, Phrae province, Thailand, where they were maintained of Indian gooseberries germplasm. The variety samples name are Pakkang (PE01), India Hang Chat (PE02), Numka (PE03), Si Cafe (PE04), Maelukdok (PE05), Wang Hong (PE06), Nakuha (PE07), Pan Siam (PE08), Pangkho (PE09), Luktor (PE10), Huai Luek (PE11), Nong Ha (PE12), Bo Kaew (PE13), Dongyen (PE14), Yokmanee (PE15), and Napoon (PE16). Young, healthy, uninfected leaves were collected, rinsed with water, air-dried, and stored at -80°C before DNA extraction using a modified CTAB method. The genomic DNA was purified using the GF-1 AmbiClean Kit (Vivantis). Finally, DNA yield and concentration were measured with spectrophotometry and 1% agarose gel electrophoresis in 1X TBE buffer. Purified DNA was diluted in TE buffer to a final 50 ng/µl concentration.

Random Amplified Polymorphic DNA (RAPD) marker

Two Indian gooseberry varieties with two different leaf morphologies, small leaf (Pan Siam, PE08) and big leaf (Pangkho, PE09), were selected for

primer screening in a preliminary study. Thirty decamers of RAPD primers (UBC-University of British Columbia: OPA01- OPA17, OPC02, OPC04, OPC05, OPC06, OPC08, OPC14, OPC16, OPAM03, OPAM12, OPAM16, OPT01, OPT10, and OPT20) were initially screened for genetic profile analysis that described by William *et al*. (1990). The PCR reactions were set up in a 25 µl volume containing 100 ng of high-quality genomic DNA, 1.6 pmol of RAPD primer, 0.25 mM dNTP mix, 2.50 mM $MgCl_2$, 1 U of Taq DNA polymerase, and 1× PCR buffer. Amplification started with an initial denaturation at 94°C for 3 minutes, followed by 36 cycles. Each cycle included denaturation at 94°C for 40 seconds, annealing at 50-55°C for 40 seconds, and elongation at 72°C for 2 minutes. After 36 cycles, a final extension was performed at 72°C for 7 minutes. The PCR products were then analyzed by separating them on a 2% agarose gel in 1X TBE buffer with a 100 bp DNA marker (Vivantis). They were visualized after staining with ethidium bromide to obtain the RAPD profile.

Sequence-related Amplified Polymorphism (SRAP) markers

In a preliminary study, a set of thirty SRAP primer combinations, consisting of five forward primers (ME1-ME5) and six reverse primers (EM1-EM6), as outlined in Table 1 (Li and Quiros, 2001), underwent screening in the Numka (PE03), Nakuha (PE07), and Nong Ha (PE12) cultivars. The experimental procedures follow previously established protocols by Sabpayakom *et al*. (2016) and Poeaim *et al*. (2023). The PCR program used two-step amplification involving the first five cycles at 35°C annealing temperature followed by 35 cycles increasing to 50°C, an initial denaturation at 94°C for 3 minutes, followed by the first five cycles of denaturation at 94°C for 1 minute, annealing at 35°C for 1 minute, and elongation at 72°C for 1 minute. Subsequently, the second step for 35 cycles was performed with denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and elongation at 72°C for 1 minute. The final step was at 72°C for 10 minutes. The SRAP fragments were separated and stained, and the SRAP profile was like the protocol of the RAPD profile.

Data scoring and analysis

RAPD and SRAP profiles were analyzed by scoring DNA fragments at the same locus as present (1) or absent (0) for each primer. Similarity coefficients were calculated, and a dendrogram was generated using UPGMA in NTSYSpc version 2.11X.

Table 1. Sequences of five forward and six reverse primers, as described by Li and Quiros (2001)

Primer		Sequences (5' to 3')	Primer		Sequences (5' to 3')
Forward primer	Me1	TGAGTCCAAACCGGATA	Reverse primer	Em1	GACTGCGTACGAATTAAT
	Me2	TGAGTCCAAACCGGAGC		Em2	GACTGCGTACGAATTTGC
	Me3	TGAGTCCAAACCGGAAT		Em3	GACTGCGTACGAATTGAC
	Me4	TGAGTCCAAACCGGACC		Em4	GACTGCGTACGAATTTGA
	Me5	TGAGTCCAAACCGGAAG		Em5	GACTGCGTACGAATTAAC
				Em6	GACTGCGTACGAATTGCA

Results

RAPD profile analysis

Screening for effective primers, 30 RAPD primers were evaluated for their ability to amplify DNA. The study used two samples, Paen Siam (PE08) and Pang Kha (PE09), which differ significantly in leaf morphology. Primers from the OPA and OPC sets successfully amplified DNA, but eight (OPA04, OPA11, OPA12, OPA16, OPC02, OPC06, OPC08, and OPC18) produced distinct, well-defined bands, making them suitable for analyzing genetic diversity. Reproducibility was assessed by performing two independent trials per primer, confirming the consistency of the RAPD technique. Amplified DNA fragments ranged between 300 and 1300 base pairs. OPAM3, OPAM12, and OPAM16 failed to amplify, while OPT1, OPT10, and OPT20 yielded faint, smeared bands.

DNA amplification was performed on 16 samples using the eight selected primers. Among the primers, OPC02 produced the highest number of DNA bands. For instance, the RAPD profiles of DNA fragments generated using primers OPA16 (Figure 1A) and OPC08 (Figure 1B) from 16 Indian gooseberry samples were analyzed, with a 100 bp plus DNA ladder as the marker. RAPD bands per primer ranged from 21 (OPA12) to 32 (OPC02), averaging 26.88 bands per primer. The eight primers collectively amplified 215 bands, including 188 polymorphic bands, resulting in a polymorphism percentage of 87.44% (Table 2). The highest polymorphic fragment percentage was obtained with OPA11 and OPA12 primers (100.00%), whereas OPC18 produced the fewest polymorphic fragments (76.00%).

Genetic relationships were analyzed using RAPD data by calculating genetic similarity with the simple matching coefficient and constructing a dendrogram using the UPGMA method in NTSys version 2.0e. The similarity coefficients ranged from 0.58 to 0.89. At a coefficient of 0.58, the 16 samples were grouped into two major clusters. The first cluster separated India Hang Chat (PE02) from the other samples. These 15 samples were divided into two

subgroups: subgroup 1 included Pakkang (PE01), Numka (PE03), Si Cafe (PE04), Huai Luek (PE11), Nong Ha (PE12), Bo Kaew (PE13), Dongyen (PE14), Yokmanee (PE15), and Napoon (PE16), while subgroup 2 comprised Maelukdok (PE05), Wang Hong (PE06), Nakuha (PE07), Pan Siam (PE08), Pangkho (PE09), and Luktor (PE10) appeared to have close genetic similarities. The highest genetic similarity of 0.89 was observed between Pakkang (PE01) and Numka (PE03), indicating a closer relationship, similar to Wang Hong (PE06) and Pangkho (PE09), which are closely related. Figure 2 illustrates the dendrogram depicting the genetic relationships among the sixteen Indian gooseberry cultivars.

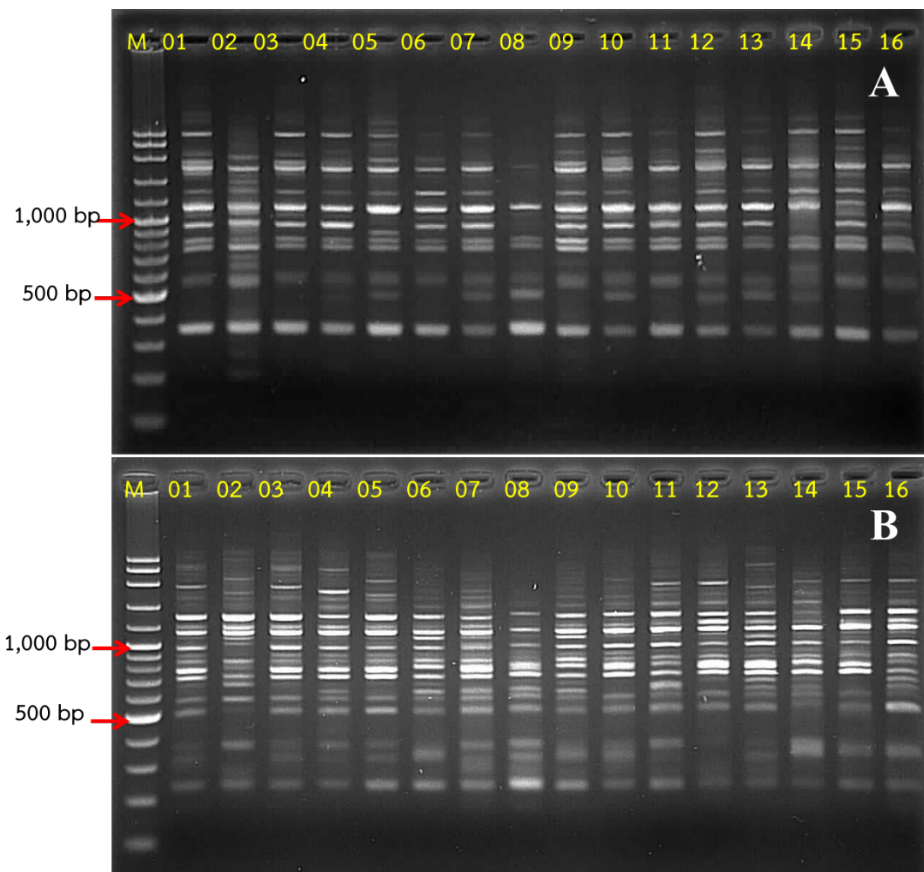

Figure 1. Example of the RAPD profiles from the sixteen cultivars of Indian gooseberry revealed by (A) OPA16 (B) OPC08 primer; VC 100 bp plus DNA ladder used as a marker

Table 2. RAPD primer codes, the total and polymorphic DNA bands, along with the percentage of polymorphism detected in the sixteen cultivars of Indian gooseberry

RAPD primer codes	No. of total DNA bands	No. of polymorphic bands	% of polymorphism
OPA04	27	21	77.78
OPA11	24	24	100.00
OPA12	21	21	100.00
OPA16	27	21	77.78
OPC02	32	30	93.75
OPC06	31	29	93.55
OPC08	28	23	82.14
OPC18	25	19	76.00
Total	**215**	**188**	**-**
Average	**26.88**	**23.50**	**87.44**

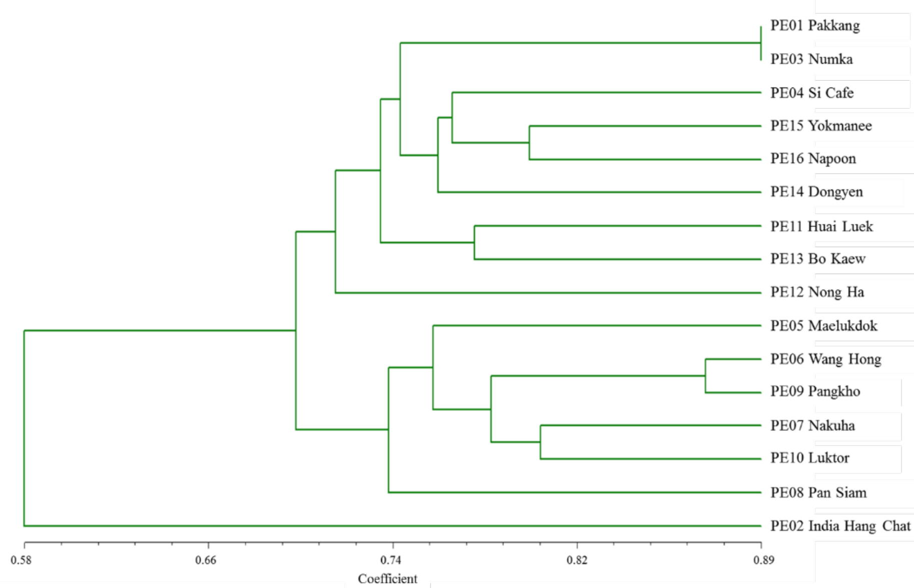

Figure 2. The dendrogram of the sixteen cultivars of Indian gooseberry was analyzed based on the RAPD marker using NTsyspc version 2.11X program and UPGMA method

SRAP profile analysis

Initially, three Indian gooseberry cultivars, Numka (PE03), Nakuha (PE07), and Nong Ha (PE12), each with distinct morphological traits, were chosen to evaluate 30 SRAP primer combinations. Seven combinations (Me2/Em1, Me3/Em5, Me4/Em2, Me4/Em3, Me4/Em5, Me5/Em2, and Me5/Em5) successfully produced prominent, well-defined bands and displayed polymorphism. Those primer combinations made SRAP profiles of 16 Indian gooseberry cultivars. The SRAP profile from the combination of the ME02/EM01 and ME03/EM05 primers is shown in Figure 3A and 3B, respectively. A total of 172 DNA bands were observed from seven primer combinations. The number of SRAP bands per primer combination ranged from 18 (Me4/Em5) to 30 (Me2/Em1), averaging 11.43 bands. The highest number of polymorphic fragments (93.33%) was observed with Me2/Em1 primers. One hundred forty-five polymorphic bands were scored, averaging 7.43, leading to a polymorphism percentage of 84.30% (Table 3).

The sixteen Indian gooseberry cultivars based on the SRAP bands were used to construct a dendrogram. Indian gooseberry DNA samples were analyzed with a simple matching coefficient from selected seven primer pairs by NTsyspc version 2.11X. The genetic similarity coefficient ranges between 0.64-0.90. When a dendrogram was constructed using the UPGMA method, a genetic similarity coefficient of 0.64 resulted in the classification of the 16 samples, which were grouped into two major clusters, as illustrated in Figure 4. The first cluster consisted of only one sample, India Hang Chat (PE02), distinctly separated from all other samples. The other cluster, consisting of only one sample, Pan Siam (PE08), was distinctly separated from the remaining 14 samples. These 14 samples were divided into two subgroups: subgroup 1 included Pakkang (PE01), Numka (PE03), Si Cafe (PE04), Huai Luek (PE11), Nong Ha (PE12), Bo Kaew (PE13), Dongyen (PE14), Yokmanee (PE15), and Napoon (PE16), while subgroup 2 of Maelukdok (PE05), Wang Hong (PE06), Nakuha (PE07), Pangkho (PE09), and Luktor (PE10). The highest genetic similarity of 0.90 was observed between Pakkang (PE01) and Numka (PE03), indicating a closer relationship, comparable to Wang Hong (PE06) and Pangkho (PE09), which exhibit a close genetic relationship. Figure 4 illustrates the dendrogram depicting the genetic relationships among the sixteen Indian gooseberry cultivars.

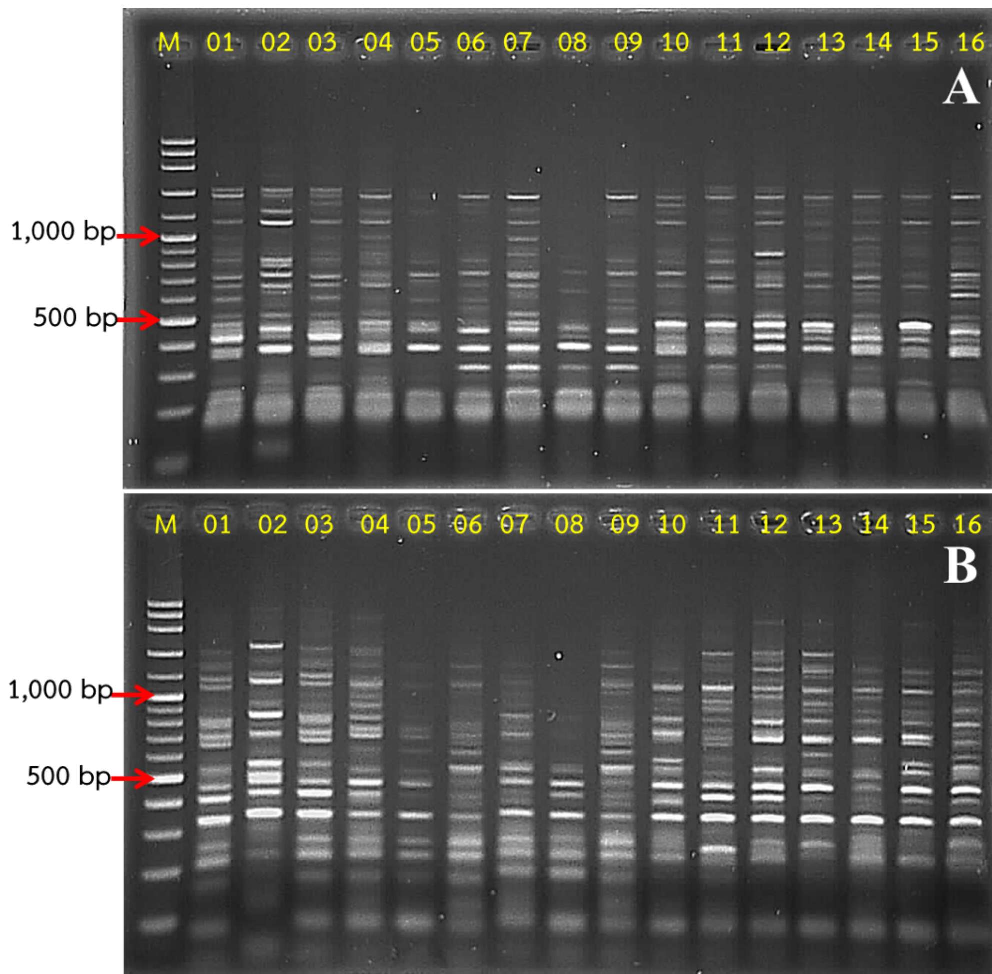

Figure 3. Example of the SRAP profiles from 16 Indian gooseberry samples revealed by (A) ME02/EM01 (B) ME03/EM05 primer combinations; VC 100 bp plus DNA ladder used as a marker

Table 3. Primer code combinations, total DNA bands, polymorphic bands, and the percentage of polymorphism detected by the SRAP marker in the sixteen Indian gooseberry cultivars

Primer codes combination	No. of total DNA bands	No. of polymorphic bands	% of polymorphism
Me2/Em1	30	28	93.33
Me3/Em5	28	23	82.14
Me4/Em2	26	22	84.62
Me4/Em3	19	16	84.21
Me4/Em5	18	14	77.78
Me5/Em2	23	18	78.26
Me5/Em5	28	24	85.71
Total	172	145	-
Average	**11.43**	**7.43**	**84.30**

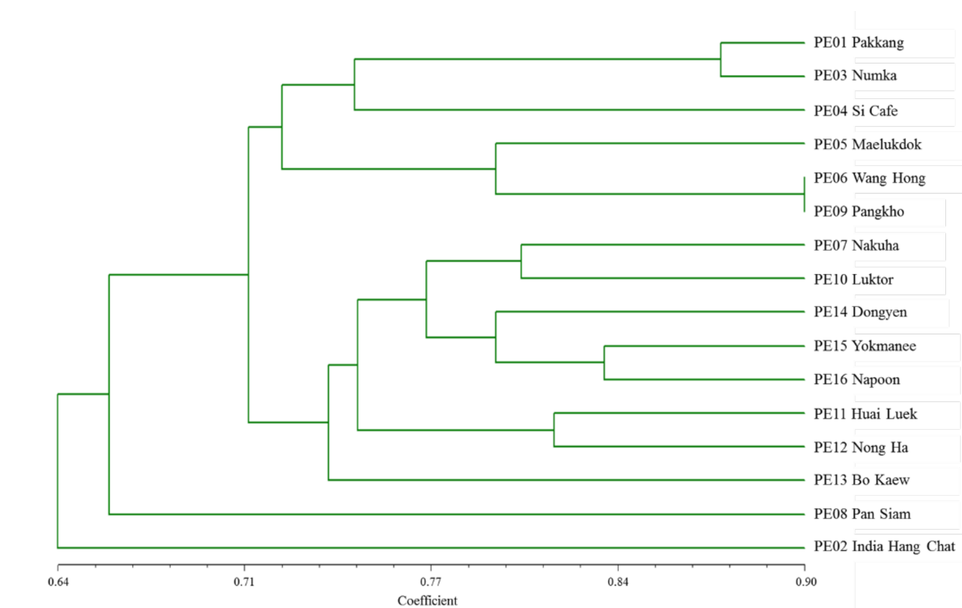

Figure 4. The dendrogram of the sixteen cultivars of Indian gooseberry was analyzed based on SRAP marker using NTsyspc version 2.11X program and UPGMA method

Discussion

This study utilized RAPD and SRAP profiling to analyze the genetic diversity and relationships among 16 Indian gooseberry (*P. emblica*) cultivars grown at the Phrae Agricultural Research and Development Centre, Department

of Agriculture, Phrae Province, as part of the Indian gooseberry germplasm collection of Thailand. For RAPD, the RAPD analysis identified eight effective primers (OPA04, OPA11, OPA12, OPA16, OPC02, OPC06, OPC08, and OPC18), which produced reproducible and polymorphic DNA bands. OPA16 was identified as a practical RAPD primer for clear band generation. It was used to develop a RAPD-SCAR marker for precise Indian gooseberry genotype identification (Dnyaneshwar *et al*., 2006), while Singh *et al*. (2015) used RAPD and rDNA SNPs to reveal genetic diversity and relationships among commercial varieties. OPC02 demonstrated the highest band yield, confirming its utility for detecting genetic variation. The polymorphism percentage of 87.44% indicates a high level of genetic variability among the studied samples, with OPA11 and OPA12 primers yielding the highest number of polymorphic bands (100%). The genetic similarity coefficients ranged from 0.58 to 0.89, highlighting significant genetic diversity among the cultivars. The clustering pattern observed in the dendrogram classified the 16 cultivars into two major clusters, reflecting distinct genetic backgrounds. Interestingly, the first cluster distinctly separated India Hang Chat (PE02) from other samples, suggesting unique genetic traits. The second cluster formed two subgroups, where cultivars such as Pakkang (PE01) and Numka (PE03), similar to Wang Hong (PE06) and Pangkho (PE09), which are closely related, likely due to shared genetic lineage or similar breeding histories. Conversely, lower similarity coefficients among other pairs highlight their genetic distinctiveness. The findings are consistent with previous studies emphasizing the efficacy of RAPD markers in assessing genetic diversity in plants, particularly in identifying polymorphic loci and constructing genetic relationships. The findings support the hypothesis that cultivars with distinct morphological traits, like leaf shape and fruit size, often show genetic divergence, aligning with Chaurasia *et al*. (2009), who linked genetic diversity to geographical origins.

For SRAP, the initial screening of 30 SRAP primer combinations, seven combinations (Me2/Em1, Me3/Em5, Me4/Em2, Me4/Em3, Me4/Em5, Me5/Em2, and Me5/Em5) were selected based on their ability to generate well-defined and polymorphic DNA bands. The primers Me2/Em1 produced the highest number of polymorphic fragments (93.33%), showcasing their effectiveness in detecting genetic variability. The 84.30% polymorphism observed across the study demonstrates the high genetic diversity among the cultivars, consistent with previous findings that SRAP markers are particularly suitable for identifying genetic variation in plant populations. A total of 172 DNA bands were produced, with the average number of polymorphic bands per primer combination being 7.43. This highlights the robustness of the SRAP technique in revealing polymorphic loci, even among closely related samples. The genetic

similarity coefficient, ranging from 0.64 to 0.90, provides further evidence of the genetic heterogeneity within the population. The dendrogram constructed using the UPGMA method grouped the 16 cultivars into two major clusters. India Hang Chat (PE02) and Pan Siam (PE08) were distinctly separated from the remaining samples, emphasizing their genetic uniqueness. Two subgroups were formed among the remaining 14 samples, reflecting closer genetic relationships. For example, the high similarity (0.90) between Pakkang (PE01) and Numka (PE03) suggests they may share a common genetic background, possibly from similar breeding programs or geographic areas, similar to Wang Hong (PE06) and Pangkho (PE09), which are also closely related. Although the SRAP technique offers high reproducibility, simplicity, and the ability to detect genetic variations in non-model organisms without prior sequence information, it has not yet been studied in Indian gooseberry. However, it has shown similar effectiveness in studies on the diversity of durian (Thinhuatoey *et al*., 2016) and Yellow Star tree (Poeaim *et al*., 2023).

Applying RAPD and SRAP techniques in this study provided valuable insights into the genetic diversity and relationships among 16 Indian gooseberry cultivars. The polymorphism percentage was slightly higher in the RAPD analysis (87.44%) than in SRAP (84.30%). However, on average, SRAP markers produced more polymorphic fragments per primer combination, suggesting their ability to identify specific loci more consistently. For example, Me2/Em1 in SRAP showed 93.33% polymorphism, comparable to the highest polymorphism rates from RAPD primers such as OPA11 and OPA12 (100%). The dendrograms based on RAPD and SRAP revealed similar trends in genetic similarity and clustering. The consistent separation of India Hang Chat (PE02) and Pan Siam (PE08) highlights their distinct genetic profiles, making them promising candidates for breeding programs focused on introducing novel traits or enhancing genetic diversity, such as higher vitamin C content, improved yield, stress tolerance, or disease resistance. Including the remaining 14 samples, which show closely related genetic relationships, particularly the high similarity (0.89 for RAPD and 0.90 for SRAP) between Pakkang (PE01) and Numka (PE03), suggesting a shared genetic background, possibly from similar geographical origins. Similarly, Wang Hong (PE06) and Pangkho (PE09) also exhibit close genetic relationships. Therefore, developing cultivars with such close genetic relationships is not advisable, as this could result in a loss of genetic diversity.

Future studies could combine RAPD or SRAP with SSR or AFLP to improve the resolution of genetic relationships. Integrating genetic data with phenotypic traits such as higher vitamin C content, greater yield, larger fruit size, stress tolerance, and disease resistance would enhance targeted breeding and conservation efforts.

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