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Wild *Piper* species from Vietnam and Laos with genetic diversity, *Phytophthora capsici* resistance, and eugenol-rich chemotypes

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Abstract. *Ho NTH, Ho HN, Truong HTH. 2026. Wild* Piper *species from Vietnam and Laos with genetic diversity,* Phytophthora capsici *resistance, and eugenol-rich chemotypes. Biodiversitas 27 (4): d270427. https://doi.org/10.13057/biodiv/d270427.* Black pepper (*Piper nigrum*) is an economically important spice, but its productivity is severely constrained by *Phytophthora capsici*. Wild *Piper* species represent a valuable genetic resource for improving disease resistance and exploring bioactive compounds; however, the knowledge about them remains insufficiently characterized. This study aimed to evaluate the genetic diversity of wild *Piper* accessions collected from Vietnam and Laos, assess their resistance to *P. capsici*, and analyze leaf chemical composition associated with resistance. Genetic diversity was examined using the ITS_{u1-4} region and RAPD markers, while resistance was evaluated through in vitro detached-leaf assays. Chemical profiling of selected accessions was performed using GC-MS analysis. The *ITS* analysis identified four species, including *Piper betle*, *Piper pendulispicum*, *Piper thomsonii,* and *Piper tricolor*. The inclusion of wild accessions increased overall genetic diversity within the studied population, and RAPD analysis revealed high polymorphism, separating the accessions into three distinct clusters. Chemical analysis indicated that accessions resistant to *P. capsici* contained high proportions of eugenol and methyl eugenol, suggesting a relationship between resistance and eugenol-rich chemotypes. Although limited by sample size and the use of detached-leaf assays, the findings highlighted the potential of wild *Piper* germplasm as a genetic resource for breeding programs and as a source of natural compounds for biological control of *P. capsici*.

Keywords: Biological control, black pepper, eugenol, *ITS* region, *Piper*, RAPD markers

INTRODUCTION

Piper nigrum L. (black pepper) is known as "the king of spices" and brings a great economic value in many countries, such as Vietnam, Brazil, Indonesia, India, and Malaysia (Sarma et al. 2014). Remarkably, *Phytophthora capsici* is a common pathogen severely affecting *P. nigrum* cultivation (Obieze et al. 2023; Truong et al. 2023). It can infect all parts of the *Piper* plant, causing sudden death, wilting, and rapid plant mortality, or create black spots, leading to leaf fall (Suriany et al. 2021). Each year, this pathogen can cause a loss of about 2% in pepper-growing areas and 15-20% production (Nguyen et al. 2022). Many methods are provided to manage *P. capsici*, such as the development of resistant varieties, cultural practices, chemical fungicides, and biological control agents (Santos et al. 2023; Sukamto et al. 2025). However, fungicides have high costs, cause environmental pollution, and are harmful to humans and other organisms (Ngo et al. 2020). Agricultural management activities are time-consuming and labor-intensive (Van Loocke et al. 2025). Therefore, selecting resistant species is necessary for sustainable, long-term management. Current breeding programs favor the employment of wild varieties because they are resistant to diseases and pests, and provide genetic diversity (Nas et al. 2023).

Instead of selecting varieties using traditional methods with much time and effort, molecular markers can facilitate selection by providing genome-wide polymorphisms that are independent of the environment (Meena et al. 2023). DNA barcoding is a suitable method for molecular identification due to its high reproducibility, standardization, and ability to use short, specific gene sequences. The Internal Transcribed Spacer (*ITS*) region of nuclear ribosomal DNA is commonly employed because it exhibits relatively high variation and strong discriminatory power at the species level (Jaramillo 2006; Jaramillo et al. 2008; Jayarathna et al. 2016; Letsiou et al. 2024). In addition, Random Amplified Polymorphic DNA (RAPD) markers are frequently used to analyze genetic diversity and relationships among *Piper* species and accessions (Jiang et al. 2009; Sen et al. 2010; Jiang and Liu 2011; Chowdhury et al. 2014; Meilawati et al. 2020, 2022). RAPD markers are simple and cost-effective; however, they also have certain limitations, including lower reproducibility, dominance that prevents the detection of heterozygous loci, and potential band co-migration (Kumari and Thakur 2014). Therefore, combining these two techniques (*ITS* and RAPD markers) can help address the shortcomings of RAPD and analyze many aspects of the population.

Beyond genetic diversity, the chemical composition of *Piper* species attracted considerable attention due to its potential role in plant defense. Several studies reported that plant extracts rich in eugenol and methyl eugenol exhibit strong antifungal activity against plant pathogens (Da Silva et al. 2014; Miri et al. 2023; Minh et al. 2024). In particular, some *Piper* species, including *Piper colubrinum* Link, *Piper argyrophyllum* Miq., and *Piper flaviflorum*

C.DC., showed resistance to *P. capsici* infection (Chinnapappa et al. 2019; Hao et al. 2025). These findings suggested that eugenol-rich chemotypes may be associated with antifungal properties and disease resistance. Previous work also reported several wild *Piper* accessions, such as *Piper hancei* Maxim. and *Piper divaricatum* G.Mey., exhibiting resistance to *P. capsici* and showing genetic differentiation from cultivated accessions (Rasphone et al. 2022a; Truong et al. 2023). Nevertheless, knowledge about wild *Piper* germplasm remains limited, particularly regarding the relationship between genetic diversity, disease resistance, and chemical composition.

Therefore, further investigation of wild *Piper* accessions is necessary to better understand their genetic diversity, resistance potential, and chemical characteristics. This study aimed to identify wild *Piper* accessions from Vietnam and Laos using ITS DNA barcoding, assess their genetic diversity using RAPD markers, evaluate their resistance to *P. capsici*, and characterize leaf chemical composition, particularly eugenol-related compounds, using GC-MS. In this way, this research seeks to identify promising wild *Piper* resources that could support pepper breeding programs and the development of sustainable disease management strategies.

MATERIALS AND METHODS

Plant materials

Nine *Piper* accessions (from seven species) from wild populations were utilized in this study (Table 1). Among them, five accessions included HUIB_PB1 (*Piper betle*), HUIB_PB2 (*P. betle*), HUIB_PP3 (*Piper pendulispicum*), HUIB_PTh4 (*Piper thomsonii*), and HUIB_PTr1 (*Piper tricolor*) were collected in Vietnam and Laos in 2024; the remaining four accessions (HUIB_PN27 (*P. nigrum*), HUIB_PH30 (*Piper hancei*), HUIB_PD36 (*Piper divaricatum*), and HUIB_PH46 (*P. hancei*) were previously published by Rasphone et al. (2022a).

Procedures

DNA extraction, amplification, and ITS_{u1-4} *sequencing*

Genomic DNA was extracted from fresh leaves by the CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle and Doyle 1990) and purified to achieve a ratio A260:A280 from 1.8 to 2.0. Next, they were amplified with ITS_{u1-4} primers in a PCR system (Applied Biosystems, USA) (Rasphone et al. 2022a). The pair of ITS_{u1-4} primers included ITS_{u1}: `GGAAGKARAAGTCGTAACAAGG` and ITS_{u4}: `RGTTTCTTTTCCTCCGCTTA` (Cheng et al. 2016). Each 30 µL PCR mixture contained 90 ng DNA, 15 µL of 2X MyTaq Mix (Meridian Bioscience, USA), and 20 µM of primer. The thermocycling program was conducted as follows: predenaturation at 95°C for 5 minutes; repeated 35 cycles including: denaturing at 95°C for 15 seconds, annealing at 56°C for 15 seconds, and extension at 72°C for 30 seconds, and 72°C for 5 minutes to complete reactions. PCR products were then sequenced using the Sanger sequencing method in 1st BASE (Apical Scientific Sdn. Bhd., Malaysia) and analyzed with BLASTn available on the NCBI.

DNA profiling by RAPD markers

The genomic DNA of three accessions (HUIB_PD36, HUIB_PP3, and HUIB_PTr4) was screened with 100 RAPD primers (Bioneer company, Korea) to identify primers that produced at least three clear and polymorphic bands. These primers and nine primers from the previously studied by Rasphone et al. (2022a) were then used to amplify four accessions (HUIB_PD36, HUIB_PB1, HUIB_PP3, and HUIB_PTh4). As a result, twenty-one primers that could be replicated in two independent runs were selected to amplify all nine accessions (Table 2). At least two independent RAPD runs were performed on each primer. Each PCR mixture (10 µL) contained 50 ng DNA, 5 µL of 2X MyTaq Mix (Meridian Bioscience, USA), 1.675 mM $MgCl_2$, 0.67 µM of RAPD primer, and 167.5 µM of each dNTP. Besides, PCR amplification followed the thermal cycle of Rasphone et al. (2022b). It began with predenaturation at 94°C for 3 minutes, followed by 40 cycles consisting of denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute, and elongation at 72°C for 2 minutes, and a final extension of 1 cycle at 72°C for 7 min. Next, PCR products were separated on a 1.5% agarose gel and observed under ultraviolet light.

Identification of Phytophthora capsici*-resistant accessions*

To screen for *P. capsici* resistance in five accessions (HUIB_PB1, HUIB_PB2, HUIB_PP3, HUIB_PTh4, and HUIB_PTr1) collected in 2024, the in vitro method of Truong et al. (2023) was applied with some changes. Specifically, three adult leaves of each accession were put in Petri dishes. Next, a wound was created at the midrib of the lower surface leaf. A 0.5 × 0.5 cm layer on Potato Dextrose Agar (PDA) plates that cultured *P. capsici* was then placed in this wound. The inoculated leaves were incubated for 10 days at a temperature of 25°C-26°C with regular wetting (humidity of 80%-90%). After 4, 6, and 10 days of inoculation, the leaf lesion diameter was measured and scored on a 0-4 scale as 0: no lesion, 1: 1-5 mm lesion, 2: 6-10 mm lesion, 3: 11-15 mm lesion, and 4: >15 mm lesion, based on the study of Prakash et al. (2019).

Gas Chromatography-Mass Spectrometry (GC/MS) analysis

Four accessions from the previous study (HUIB_PN27, HUIB_PH30, HUIB_PD36, and HUIB_PH46), one new sample from Laos (HUIB_PB1), and one new sample from Vietnam (HUIB_PTr1) were analyzed. These accessions had different resistance levels and geographic origins. Approximately 100 g of adult leaves from each plant was harvested and finely ground. Next, 20.0 g of this homogenized sample was transferred into a 250 mL Erlenmeyer flask containing 100 mL of n-hexane and sonicated for 15 minutes. This mixture was then macerated for 24 hours at room temperature. The final extract was collected through two filtration processes, using a coarse filter paper and a 0.45 µm membrane filter, respectively.

Table 1. *Piper* accessions used in this study

Accession code	Species name	Coordinates (N, E)	Place of collection
HUIB_PB1	*Piper betle* L.	19°44'58.5", 101°59'35.6"	Kuang Si waterfall, Luang Prabang District, Laos
HUIB_PB2	*Piper betle* L.	19°44'58.5", 101°59'35.6"	Kuang Si waterfall, Luang Prabang District, Laos
HUIB_PP3	*Piper pendulispicum* C.DC.	19°44'60.0", 101°59'35.0"	Kuang Si waterfall, Luang Prabang District, Laos
HUIB_PTh4	*Piper thomsonii* (C.DC.) Hook.f.	19°44'60.0", 101°59'35.7"	Kuang Si waterfall, Luang Prabang District, Laos
HUIB_PTr1	*Piper tricolor* Y.C.Tseng	16°29'32.5", 107°36'10.5"	My Thuong Ward, Hue City, Vietnam
HUIB_PH30	*Piper hancei* Maxim.	16°44'52.3", 106°57'56.0"	Quang Tri District, Vietnam
HUIB_PD36	*Piper divaricatum* G.Mey.	13°57'34.0", 108°00'43.5"	Gia Lai District, Vietnam
HUIB_PH46	*Piper hancei* Maxim.	16°44'52.3", 106°57'56.0"	Quang Tri District, Vietnam
HUIB_PN27	*Piper nigrum* L.	17°01'54.0", 107°04'06.0"	Quang Tri District, Vietnam

Table 2. List of RAPD primers used in the study

Primer name	DNA sequence (5'-3')
UBC#303	GCGGGAGACC
UBC#333	GAATGCGACG
UBC#347	TTGCTTGGCG
UBC#352	CACAACGGGT
UBC#359	AGGCAGACCT
UBC#363	ATGACGTTGA
UBC#377	GACGGAAGAG
UBC#382	ATACACCAGC
UBC#392	CCTGGTGGTT
UBC#450	CGGAGAGCCC
UBC#453	AGTACAAGGG
UBC#457	CGACGCCCTG
UBC#458	CTCACATGCC
UBC#459	GCGTCGAGGG
UBC#460	ACTGACCGGC
UBC#462	CATAGCGGCA
UBC#478	CGAGCTGGTC
UBC#485	AGAATAGGGC
UBC#492	GTGACTGCTC
UBC#493	CCGAATCACT
UBC#494	TGATGCTGTC

After extraction, components of the *Piper* extract were analyzed using a GC-MS/MS TQ8040 system (Shimadzu Corporation, Tokyo, Japan) in the Center for Testing Drugs, Cosmetics and Food of Hue City. This system was run according to the procedure of Ji et al. (2023) with some modifications: DB-5 ms (60 m 0.25 mm; 0.25 µm film thickness) fused-silica capillary column; programmed temperature: 40°C-250°C (5°C/min); injector temperature: 250°C; carrier gas: helium; injection type: 1 µL of solution and a flow rate of 3.45 mL/min. The mass spectrometer operated over a scan range of 40-500 m/z. The chemical composition of *Piper* leaf extract was determined by comparing the mass spectra obtained from the GC-MS test with those obtained from the computer library. Additionally, the relative amounts of each component were expressed as the percentage of the peak area relative to the total defined area. Major components with >0.2% peak area were presented. This analysis had three repeats, with one plant in each repeat.

Data analysis

In ITS_{u1-4} region analysis, BLASTn parameters were used, including >97% identity, 0.0 e-value, >1250 max score, and >90% coverage, to reference sequencing results on the NCBI. Then, multiple sequence alignment was performed by MEGA 12 software to construct a phylogenetic tree (Kumar et al. 2024). The Neighbor-Joining method was based on a Tamura 3-parameter model, and 1000 bootstrap replicates were used. Additionally, DnaSP v6 software was used to evaluate parameters of sequences in the new population (nine accessions), including the number of variable (polymorphic) sites, the number of mutations, nucleotide diversity, average number of nucleotide differences, minimum number of recombination events, number of haplotypes, and haplotype diversity (Rozas et al. 2017). This result was then compared with previously published results in the old population of Rasphone et al. (2022a).

Only RAPD bands in both runs were scored to develop a binary matrix, where presence was recorded as 1 and absence as 0, and faint bands were not scored. This matrix was used to calculate evaluation indicators, Jaccard similarity coefficients, and to develop a UPGMA clustering tree based on NTSYS software v2.10m (Ho et al. 2025). The closer this coefficient is to 1, the closer the species are genetically and vice versa, the greater the difference between accessions (Zhang et al. 2024). These indicators included the total number of bands, polymorphic band rate, Polymorphism Information Content (PIC), Marker Index (MI), and Resolving power (Rp). Additionally, this matrix was analyzed using R v4.5.2 and RStudio v2025.09.2+418 to generate a heatmap of loci with the ComplexHeatmap package and perform Principal Coordinates Analysis (PCoA) with ggplot2, ggfortify, and cluster packages.

The Disease Severity Index (DSI) was calculated using the formula in the study of Prakash et al. (2019): DSI = (Sum of ratings of each leaf) ×100/(Maximum score × Number of leaves). These results were evaluated by the Kruskal-Wallis test in IBM SPSS Statistics (version 27). The DSI value showed that <30% = resistant, 31%-40% as moderately resistant, and >40% as susceptible (Prakash et al. 2019). This classification was based on the time trajectory. Results presented averages of three repeats, with three randomly selected leaves from a plant in each repeat. Additionally, the *Phytophthora* strain in this test was identified and provided by Truong et al. (2023). It was cultured for 5 days, and plugs were taken from the actively growing margin of the same plate.

GC-MS results were analyzed by SPSS software. Their mean values were compared and evaluated using the Kruskal-Wallis test. This analysis had three repeats, with one plant in each repeat. Finally, results regarding eugenol and methyl-eugenol content, resistance level (DSI after 10 days of inoculation), and genetic clustering in RAPD data of accessions were compiled. This data was then analyzed using PAST v4.03 software to generate the UPGMA clustering tree. Additionally, multivariate relationships were explored using Principal Component Analysis (PCA) based on the Euclidean Similarity matrix.

RESULTS AND DISCUSSION

ITS$_{u1-4}$ sequence analysis

Jaramillo (2006), Jaramillo et al. (2008), and Jayarathna et al. (2016) demonstrated that the *ITS* region was effective in assessing the genetic relationships of *Piper* varieties. *ITS$_{u1}$* and *ITS$_{u4}$* are universal and plant-specific PCR primers for amplifying the whole *ITS* sequence and a part of it (*ITS1* or *ITS2*) of plants (Cheng et al. 2016). They were used effectively to identify the *Piper* species in our previous study (Rasphone et al. 2022a). In this work, based on BLASTn searches with the *ITS$_{u1-4}$* region, three *Piper* species (*P. betle*, *P. pendulispicum,* and *P. thomsonii*) in Laos and *P. tricolor* in Vietnam were identified successfully (Tables 1 and 3). *ITS$_{u1-4}$* regions had a sequence length of 719-725 bp with 58.9-59.5% GC content, which was highly similar to GenBank sequences (99.31-99.86%) (Table 3). Notably, the addition of five collected accessions to the previous population increased all evaluation indices of sequences. Specifically, the number of variable sites and the number of mutations rose by 15 sites and 22 mutations, respectively. This resulted in large changes to nucleotide diversity (increasing from 30.930×10^{-3} to 37.840×10^{-3}) and the average number of nucleotide differences (increasing from 20.352 to 24.711). It showed greater sequence variation, supporting a broader breeding base. Besides, the increase in Recombination events (Rm) was consistent with higher genetic diversity in the expanded population (Salgotra and Chauhan 2023). Four new haplotypes were created in the new population. This slightly increased the haplotype diversity to 0.964 (Table 4). Bhat et al. (2021) indicated that any individual has two haplotypes in a stretch of chromosomal DNA, while many haplotypes can be found in a population for the same stretch.

ITS$_{u1-4}$ sequence data of five new accessions (HUIB_PB1, HUIB_PB2, HUIB_PP3, HUIB_PTh4, and HUIB_PTr1) were selected to compare with sequences of four accessions from a previous study conducted by Rasphone et al. (2022a), including HUIB_PN27, HUIB_PH30, HUIB_PD36, and HUIB_PH46. HUIB_PD36 had the largest pairwise distances (0.096-0.161), followed by HUIB_PTh4 (0.070-0.127) (Table 5). In the Neighbor-Joining tree, HUIB_PD36 and HUIB_PTh4 formed two distinct branches separated from the other accessions. Meanwhile, the remaining accessions were clustered together. This showed *ITS* divergence among nine accessions and the large sequence difference of HUIB_PD36 and HUIB_PTh4. In addition, HUIB_PB1 and HUIB_PB2 were homologous to HUIB_PTr1 with 91-100 bootstraps, while HUIB_PP3 was homologous to HUIB_PN27 (99 bootstraps) (Figure 1). Accessions of the same species, such as HUIB_PB1 and HUIB_PB2, HUIB_PH30 and HUIB_PH46, had completely identical *ITS$_{u1-4}$* sequences. Overall, the *ITS* region of five new accessions differed from each other and from *P. hancei*, *P. divaricatum,* and *P. nigrum*. Based on these findings, *ITS* can be a very good marker for identifying *Piper* species.

Furthermore, when considering collecting locations of accessions, it can be seen that there was no correlation between clade separation and geographical distribution. This was easily observed in the four accessions (HUIB_PB1, HUIB_PB2, HUIB_PP3, and HUIB_PTh4) originating from Kuang Si waterfall, Luang Prabang District, Laos. They were located on three different branches, indicating significant differences in the *ITS* sequence. This study was similar to Bouda et al. (2019), which confirmed that variation observed among populations using *ITS* markers did not follow a geographical pattern. Furthermore, the principal factor affecting genetic differentiation was the gene flow.

Table 4. Evaluation indices of *Piper* population based on the *ITS$_{u1-4}$* region

Indices	**Old population (Rasphone et al. 2022a)**	**Current study**
No. of variable sites (S)	150	165
No. of mutations (Eta)	169	191
Nucleotide diversity (per site) (Pi) ($\times 10^{-3}$)	30.930	37.840
Average number of nucleotide differences (k)	20.352	24.711
Number of haplotypes (h)	23	27
Haplotype diversity (Hd)	0.947	0.964
Minimum number of recombination events (Rm)	18	22

Table 3. Results obtained from referencing the *ITS$_{u1-4}$* sequences of 5 newly collected accessions with GenBank data

Accessions	**GenBank accession**	**Size (bp)**	**GC (%)**	**Percent identities (%)**	**e-value**	**Reference sequence**
HUIB_PB1	PX393519	721	59.3	99.31	0	MG208083 (*Piper betle*)
HUIB_PB2	PX393520	719	59.2	99.58	0	MG208083 (*Piper betle*)
HUIB_PP3	PX393521	720	59.4	99.72	0	LC033836 (*Piper pendulispicum*)
HUIB_PTh4	PX393522	724	59.4	99.58	0	MG208068 (*Piper thomsonii*)
HUIB_PTr1	PX393523	725	58.9	99.86	0	DQ868750 (*Piper tricolor*)

Table 5. Pairwise distance in the ITS_{u1-4} region among accessions

	HUIB_PB1	HUIB_PB2	HUIB_PP3	HUIB_PTh4	HUIB_PTr1	HUIB_PH30	HUIB_PD36	HUIB_PH46	HUIB_PN27
HUIB_PB1	0.000								
HUIB_PB2	0.000	0.000							
HUIB_PP3	0.041	0.041	0.000						
HUIB_PTh4	0.070	0.070	0.074	0.000					
HUIB_PTr1	0.003	0.003	0.041	0.070	0.000				
HUIB_PH30	0.075	0.075	0.094	0.083	0.073	0.000			
HUIB_PD36	0.128	0.129	0.136	0.127	0.131	**0.161**	0.000		
HUIB_PH46	0.075	0.075	0.094	0.083	0.073	0.000	0.161	0.000	
HUIB_PN27	0.048	0.048	0.037	0.096	0.050	0.096	0.152	0.096	0.000

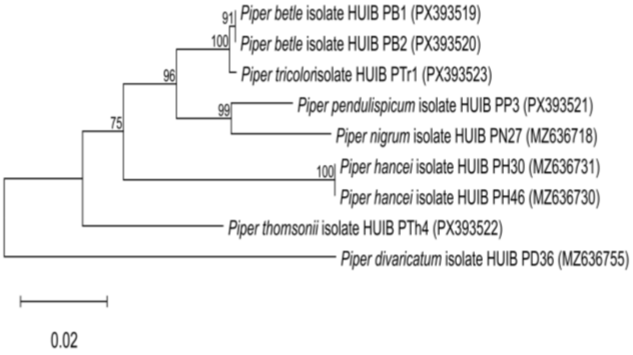

Figure 1. The phylogenetic tree of the ITS_{u1-4} region using the Neighbor-Joining method

RAPD analysis

Results of RAPD analysis showed the presence of multiple *Piper* species, thus high polymorphism was expected. The RAPD data showed that 532 DNA bands were generated using 21 selected RAPD primers, with a polymorphic rate of 100%. The band size ranged from 200 bp to 3500 bp. The primer possessing the largest polymorphic bands was UBC#457 with 31 bands, while the fewest number was UBC#333 (19 bands) (Figure 2; Table 6). Besides, a very high allelic richness was shown via the large mean number of amplified RAPD loci/ polymorphic loci (25.33). This number was much higher than studies conducted by Sen et al. (2010) and Jiang and Liu (2011), but it was lower than Chowdhury et al. (2014), because they proceeded with different populations of *Piper* species.

Among evaluation indicators of primers, Polymorphism Information Content (PIC) was the most noteworthy because it determined the ability of markers to establish polymorphism in samples (Roy et al. 2023). The results showed that UBC#363, UBC#485, and UBC#492 primers had the highest PIC values. All primers brought large information with high PIC indices ($0.25<PIC<0.5$) (Botstein et al. 1980), ranging from 0.333 to 0.414 (Table 5). MI suggested that applying primers with a large number of bands was more effective than primers with only their polymorphic bands (Ho et al. 2025). The average values of this index were 9.47. This index was most prominent in primers with the largest PIC values. Meanwhile, Rp reached its highest values at UBC#363, UBC#457 and UBC#492 primers (Table 5). The higher the Rp of a primer, the more successful it was in classifying genotypes, and vice versa (Ho et al. 2025). Overall, values of PIC, MI and Rp in this study were higher than the results of Kulkarni et al. (2024) in the *P. nigrum* population. This showed that although RAPD primers had dominant inheritance and potential reproducibility issues, they were effective in assessing genetic diversity in the *Piper* population. Besides, UBC#363, UBC#485, and UBC#492 were the most superior primers.

The values of similarity coefficients were quite low, ranging from 0.477 to 0.793 (Table 7). This was equivalent to a genetic distance of 0.207-0.523. Furthermore, Jaccard's similarity matrix was used to generate the UPGMA dendrogram. *Piper* accessions were located on three different branches of the dendrogram at a coefficient of 0.58. *Piper hancei*, including HUIB_PH30 and HUIB_PH46, was branched first. Meanwhile, the third branch contained two *P. betle* accessions (HUIB_PB1 and HUIB_PB2), and the remaining accessions were in the second branch. The two species, *P. hancei* and *P. betle*, occupied the farthest positions among all the accessions (Figure 3). Similarly, the PCoA plot also clearly divided the accessions into 3 groups based on the 2 principal coordinates (PC1 and PC2) with a total variance of 38.11% (Figure 4.A). These clusters revealed the presence of genetic variation among accessions. However, they did not link to geographical diversity despite geographical differences that may influence genetic variation within populations (Zhang et al. 2017; Xu et al. 2024). *Piper hancei* and *P. betle* were clearly separated genetically and geographically, while the remaining accessions did not exhibit this separation. Besides, a heatmap was constructed to cluster all the loci generated from the RAPD markers (Figure 4.B). No clear accession- or species-specific loci were observed among the 532 RAPD loci, which may reflect high polymorphism and the limited sample size. Therefore, future studies with more accessions, reproducibility checks and additional marker systems (e.g. SSR, SNP) are needed to identify species-diagnostic markers and reassess the association of the geographical factor for population polymorphism.

Phytophthora capsici-resistant analysis

The results reported that the newly collected accessions were highly divergent from the rest in terms of ITS_{u1-4} sequences, and RAPD data showed resistance to *P. capsici*.

In contrast, genetic differences in HUIB_PN27 (*P. nigrum*), HUIB_PH30 (*P. hancei*), and HUIB_PH46 (*P. hancei*) suggested a higher susceptibility compared to other accessions. At 4 days post-inoculation, HUIB_PN27 was the most susceptible accession to *P. capsici* with 50.00% DSI, while leaves of HUIB_PH30 and HUIB_PH46 began to show signs of mild infection (DSI = 8.33%). After 6 days, leaves of HUIB_PN27 completely died, whereas HUIB_PH30 and HUIB_PH46 expressed moderate resistance with *P. capsici* (DSI = 25.00-33.33%). However, this resistance of HUIB_PH30 and HUIB_PH46 was relative and time-dependent. The resistance level of HUIB_PH30 and HUIB_PH46 after 10 days decreased (susceptible). Their leaves showed signs of a more serious infection (yellowing and partial death of leaves). Meanwhile, the remaining accessions, including *P. divaricatum* (HUIB_PD36), *P. betle* (HUIB_PB1 and HUIB_PB2), *P. pendulispicum* (HUIB_PP3), *P. thomsonii* (HUIB_PTh4), and *P. tricolor* (HUIB_PTr1), showed no signs of infection at 4 to 10 days post-inoculation. These accessions were classified into the resistant group to *P. capsici* (Figure 5; Table 8).

There was ample evidence that *P. nigrum* was susceptible to *P. capsici* (Truong et al. 2010; Nguyen 2015; Truong et al. 2023). Meanwhile, bioactive compounds of different *Piper* species had potential therapeutic effects against various diseases. Namely, eugenol, methyl eugenol, and isoeugenol in *P. divaricatum*, *P. tricolor,* and *P. betle* enabled these species to resist pathogenic fungi (Sanubol et al. 2014; Erisléia-Meireles et al. 2016; Salehi et al. 2019). Furthermore, extracts from *P. hancei* exhibited potential insecticidal and certain toxicity (Lan et al. 2025). Stems of woody pepper (*P. pendulispicum*) and *P. thomsonii* had high piperine content (Ajit et al. 2024; Huong et al. 2025), which was known for its pharmacological actions, such as antifungal, antibacterial, larvicidal, and insecticidal.

Table 6. Statistical characteristics of RAPD markers used

Primer	Band size (bp)	Total number of bands	Polymorphic bands	Polymorphic band rate (%)	Polymorphism Information Content (PIC)	Resolving power (Rp)	Marker Index (MI)
UBC#303	450-2500	22	22	100	0.364	11.333	8.000
UBC#333	280-2250	**19**	**19**	100	0.377	10.444	7.160
UBC#347	400-2250	20	20	100	0.356	9.778	7.111
UBC#352	300-3000	21	21	100	0.390	12.889	8.198
UBC#359	250-2400	25	25	100	0.397	14.889	9.926
UBC#363	240-2300	29	29	100	**0.398**	**17.556**	**11.556**
UBC#377	320-2750	23	23	100	0.391	13.111	8.988
UBC#382	200-2000	30	30	100	0.357	16.000	10.716
UBC#392	250-1900	24	24	100	0.377	13.556	9.037
UBC#450	200-2250	27	27	100	0.351	13.556	9.481
UBC#453	300-3500	30	30	100	0.356	14.889	10.667
UBC#457	200-3000	**31**	**31**	100	0.363	16.444	**11.259**
UBC#458	480-2300	26	26	100	0.368	14.000	9.580
UBC#459	400-2400	25	25	100	0.391	14.667	9.778
UBC#460	350-2000	25	25	100	0.383	14.667	9.580
UBC#462	500-2050	26	26	100	0.357	13.333	9.284
UBC#478	250-1850	23	23	100	0.382	13.111	8.790
UBC#485	200-2000	26	26	100	**0.412**	**16.889**	10.716
UBC#492	330-2500	28	28	100	**0.414**	**18.222**	**11.605**
UBC#493	250-2100	29	29	100	0.337	14.000	9.778
UBC#494	350-2250	23	23	100	0.333	10.444	7.654
Mean	200-3500	25.33	25.33	100	0.374	13.989	9.470

Table 7. Genetic similarity coefficients among these accessions based on RAPD data

	HUIB_PH30	HUIB_PD36	HUIB_PH46	HUIB_PN27	HUIB_PB1	HUIB_PB2	HUIB_PP3	HUIB_PTh4	HUIB_PTr1
HUIB_PH30	1.000								
HUIB_PD36	0.566	1.000							
HUIB_PH46	**0.793**	0.547	1.000						
HUIB_PN27	0.555	0.586	0.551	1.000					
HUIB_PB1	0.504	0.577	0.515	0.521	1.000				
HUIB_PB2	0.481	0.573	**0.477**	0.532	0.602	1.000			
HUIB_PP3	0.568	0.618	0.545	0.611	0.590	0.564	1.000		
HUIB_PTh4	0.551	0.624	0.573	0.617	0.581	0.562	0.667	1.000	
HUIB_PTr1	0.556	0.637	0.538	0.603	0.594	0.575	0.665	0.641	1.000

Table 8. Reaction of 9 *Piper* accessions to *Phytophthora* infection

Accessions	Disease Severity Index (DSI) (%) after inoculation			Classification
	4 days	6 days	10 days	
HUIB_PH30	8.33a±0.00	25.00a±8.33	50.00a±0.00	From moderately resistant to susceptible
HUIB_PD36	0.00b±0.00	0.00b±0.00	0.00b±0.00	Resistant
HUIB_PH46	8.33a±0.00	33.33a±0.00	41.67ab±0.00	From moderately resistant to susceptible
HUIB_PN27	50.00a±8.33	100.00a±8.33	100.00a±0.00	Susceptible
HUIB_PB1	0.00b±0.00	0.00b±0.00	0.00b±0.00	Resistant
HUIB_PB2	0.00b±0.00	0.00b±0.00	0.00b±0.00	Resistant
HUIB_PP3	0.00b±0.00	0.00b±0.00	0.00b±0.00	Resistant
HUIB_PTh4	0.00b±0.00	0.00b±0.00	0.00b±0.00	Resistant
HUIB_PTr1	0.00b±0.00	0.00b±0.00	0.00b±0.00	Resistant

Note: Within a column, mean values having a letter in common are not significantly different at p = 0.002 by the Kruskal-Wallis test (mean±SD)

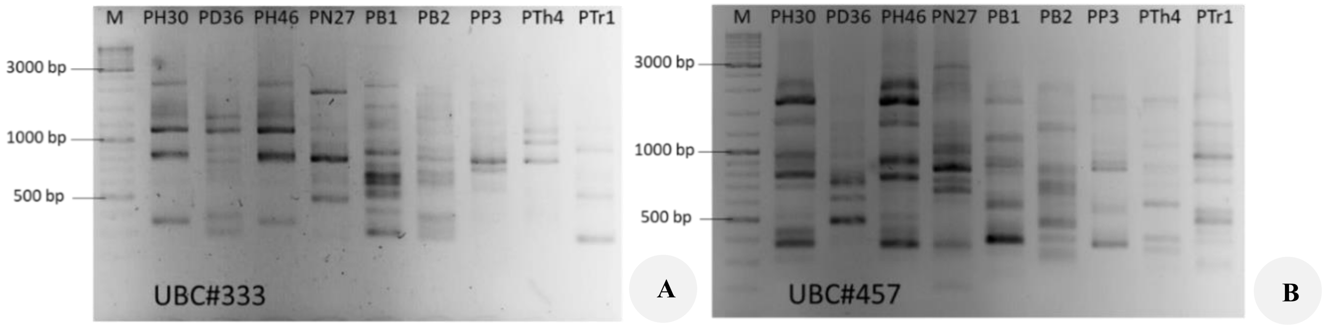

Figure 2. RAPD banding patterns of *Piper* spp. from Vietnam and Laos generated by: A. UBC#333, and B. UBC#457 primers. M: GeneRuler 1 kb DNA Ladder, PH30: HUIB_PH30, PD36: HUIB_PD36, PH46: HUIB_PH46, PN27: HUIB_PN27, PB1: HUIB_PB1, PB2: HUIB_PB2, PP3: HUIB_PP3, PTh4: HUIB_PTh4, PTr1: HUIB_PTr1

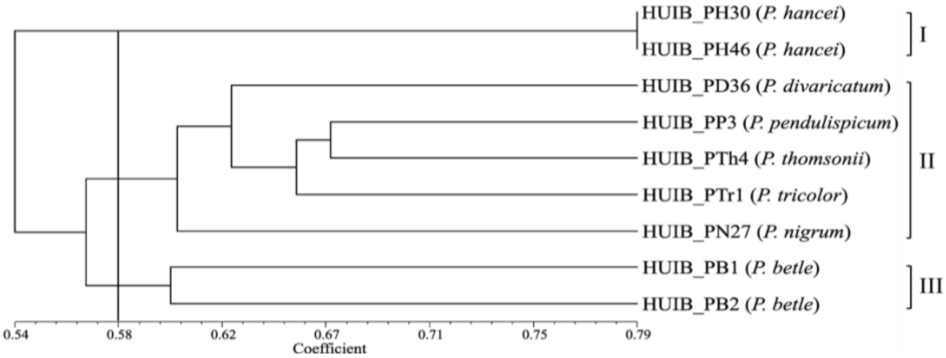

Figure 3. A dendrogram among 7 accessions of *Piper* from Vietnam and Laos was generated by the UPGMA cluster analysis

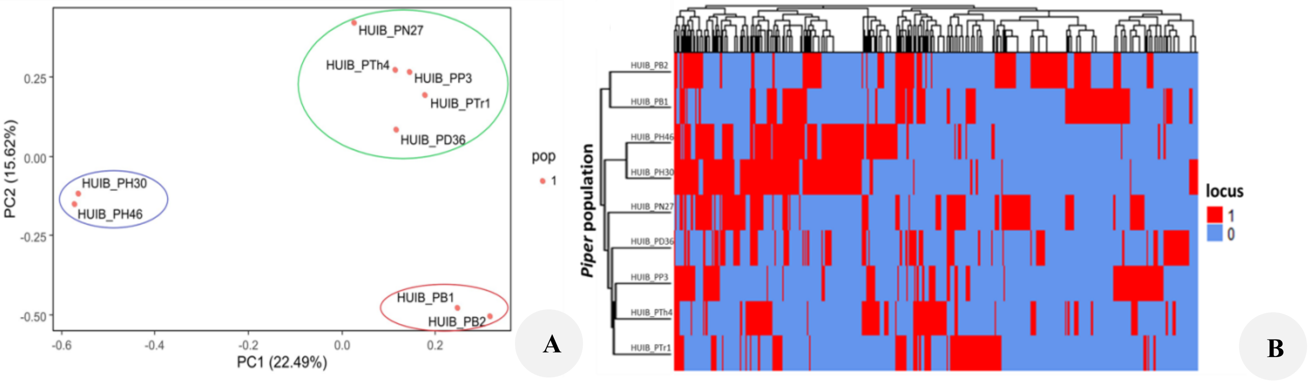

Figure 4. A. The PCoA plot separating nine *Piper* accessions, and B. Heatmap showing clustering of loci generated from RAPD markers

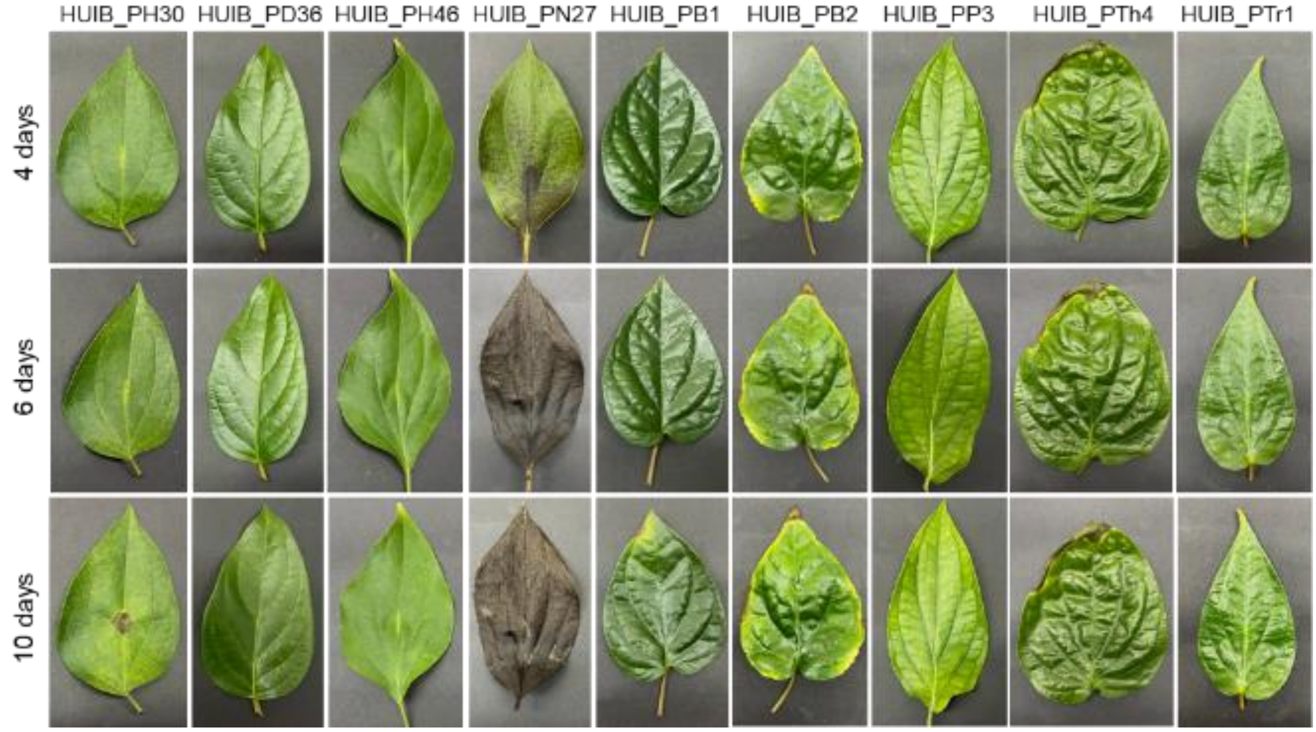

Figure 5. Leaves of accessions after inoculation with *Phytophthora capsici* for 4 days (row 1), 6 days (row 2), and 10 days (row 3)

Chemical characterization of *Piper* extract with potential activity against *Phytophthora capsici*

The GC-MS analysis revealed the detection of 29 bioactive compounds in six *Piper* accessions. The proportions of compounds in each accession varied. HUIB_PH30 contained the highest concentrations of (-)-alpha-pinene, 2-beta-pinene, 1,8-cineole, trans-caryophyllene, germacrene-D, beta-cedrene, elemol, and beta-eudesmol. Camphene and endo-1-bourbonanol were only present in HUIB_PTr1. Beta-ocimene Y and gamma-elemene were only present in *P. divaricatum* (HUIB_PD36). Sabinene was only discovered in *P. hancei* (HUIB_PH46). Similarly, only *P. nigrum* (HUIB_PN27) had delta-elemene, alpha-cubebene, alpha-copaene, and (Z,Z)-farnesal. In addition, compounds such as beta-phellandrene, beta-myrcene, alpha-humulene, beta-selinene, and alpha-selinene had high concentrations in HUIB_PH46. Linalool L, nerolidol, and phytol-isomer were abundant in HUIB_PN27. HUIB_PB1 was the only accession lacking beta-elemene (Table 9).

Eugenol and methyl eugenol, two phenylpropanoid chemicals, are naturally found in numerous plant essential oils. Tan and Nishida (2012) and Iwayemi et al. (2024) reported antifungal actions of these two compounds against phytopathogenic species such as *Aspergillus*, *Penicillium*, *Emericella*, and *Fusarium*. In particular, eugenol and methyl-eugenol in *P. divaricatum* showed activity against fungi such as *Cladosporium cladosporioides*, *Cladosporium sphareospermum*, and *Fusarium solani* f. sp. *piperis* (Da Silva et al. 2010, 2014; Erisléia-Meireles et al. 2016). In this analysis, the majority of the extract content of HUIB_PB1 was eugenol, accounting for 93.71%. Meanwhile, methyl-eugenol accounted for the majority of HUIB_PD36 (56.17%). Only three accessions (HUIB_PD36, HUIB_PB1, and HUIB_PTr1) showed the presence of eugenol at very high concentrations (29.67-93.71%), associated with *P. capsici* resistance. In addition, methyl-eugenol was only present in HUIB_PD36 (56.17%) and HUIB_PB1 (0.73%) (Table 7). The high eugenol and methyl-eugenol contents in HUIB_PB1, HUIB_PTr1, and HUIB_PD36 were consistent with previous reports of antifungal activity of these compounds and putatively associated with their *P. capsici* resistance (Minh et al. 2024). This was probably because eugenol caused disruption of the cytoplasmic membrane and destruction of cellular proteins; in this way, it can affect active transport, electron flow, proton motive force, and cell coagulation (Davidson et al. 2012; Cai et al. 2019). Meanwhile, methyl eugenol delayed mold mycelial initiation and spore development, and inhibited aflatoxin synthesis (Tan and Nishida 2012). Moreover, the presence of bicyclogermacrene, elemol, germacrene-D, caryophyllene, alpha-pinene, and beta-pinene in the extracts may be responsible for the significant antifungal and cytotoxic effects. Of these, beta-caryophyllene (inactive) could be a synergist and did not possess antifungal activity (Ruiz-Vásquez et al. 2022). This study performed in vitro detached-leaf assays and GC-MS with leaf extracts, but the functions of potential compounds (eugenol and methyl-eugenol) need to be confirmed in further work.

Table 9. Major components (% area) identified by GC-MS in extracts from *Piper* species

Accessions	Alpha-Pinene, (-)-	Camphene	Beta-Phellandrene	2-Beta-Pinene	1,8-Cineole	Beta-Ocimene Y
HUIB_PH30	7.13a±0.02	0.00b±0.00	1.25b±0.03	10.59a±0.11	1.30a±0.04	0.00b±0.00
HUIB_PD36	0.28cd±0.00	0.00b±0.00	0.00c±0.00	0.20b±0.01	0.00b±0.00	1.24a±0.05
HUIB_PH46	3.26ab±0.07	0.00b±0.00	20.17a±0.56	4.80ab±0.06	0.00b±0.00	0.00b±0.00
HUIB_PN27	0.00d±0.00	0.00b±0.00	0.00c±0.00	0.00c±0.00	0.00b±0.00	0.00b±0.00
HUIB_PB1	0.00d±0.00	0.00b±0.00	0.00c±0.00	0.00c±0.00	0.00b±0.00	0.00b±0.00
HUIB_PTr1	0.96bc±0.02	0.44a±0.02	10.56ab±0.11	0.00c±0.00	0.00b±0.00	0.00b±0.00

Accessions	Linalool L	Gamma-Elemene	Beta-Myrcene	Eugenol	Sabinene	Beta-Elemene
HUIB_PH30	0.00b±0.00	0.00b±0.00	0.00b±0.00	0.00c±0.00	0.00b±0.00	4.90bc±0.07
HUIB_PD36	0.00b±0.00	0.40a±0.01	0.00b±0.00	29.67b±0.39	0.00b±0.00	4.11cd±0.06
HUIB_PH46	0.00b±0.00	0.00b±0.00	2.20a±0.04	0.00c±0.00	5.29a±0.08	5.80ab±0.14
HUIB_PN27	0.67a±0.01	0.00b±0.00	0.00b±0.00	0.00c±0.00	0.00b±0.00	11.1a±0.06
HUIB_PB1	0.24a±0.02	0.00b±0.00	0.00b±0.00	93.71a±0.29	0.00b±0.00	0.00e±0.00
HUIB_PTr1	0.00b±0.00	0.00b±0.00	0.61a±0.01	64.82ab±0.35	0.00b±0.00	1.48de±0.02

Accessions	Delta-Elemene	Alpha-Cubebene	Alpha-Copaene	Methyl-Eugenol	Trans-Caryophyllene	Alpha-Humulene
HUIB_PH30	0.00b±0.00	0.00b±0.00	0.00b±0.00	0.00c±0.00	7.04a±0.12	4.65ab±0.21
HUIB_PD36	0.00b±0.00	0.00b±0.00	0.00b±0.00	56.17a±0.55	1.35cd±0.02	0.00c±0.00
HUIB_PH46	0.00b±0.00	0.00b±0.00	0.00b±0.00	0.00c±0.00	3.88bc±0.03	5.22a±0.10
HUIB_PN27	6.96a±0.04	1.66a±0.04	2.93a±0.03	0.00c±0.00	6.07ab±0.03	3.48b±0.05
HUIB_PB1	0.00b±0.00	0.00b±0.00	0.00b±0.00	0.73a±0.05	0.81de±0.02	0.00c±0.00
HUIB_PTr1	0.00b±0.00	0.00b±0.00	0.00b±0.00	0.00c±0.00	0.57e±0.02	0.00c±0.00

Accessions	Germacrene-D	Bicyclogermacrene	Beta-Selinene	Alpha-Selinene	Beta-Cedrene	Elemol
HUIB_PH30	9.70a±0.02	0.00b±0.00	13.53b±0.14	11.72b±0.03	3.11a±0.14	3.37a±0.13
HUIB_PD36	4.57ab±0.05	2.01a±0.01	0.00d±0.00	0.00c±0.00	0.00b±0.00	0.00b±0.00
HUIB_PH46	0.00d±0.00	0.00b±0.00	19.08a±0.15	17.15a±0.06	0.00b±0.00	0.00b±0.00
HUIB_PN27	0.00d±0.00	0.00b±0.00	18.35ab±0.07	16.02ab±0.05	0.00b±0.00	0.00b±0.00
HUIB_PB1	2.99bc±0.14	0.47a±0.03	0.00d±0.00	0.00c±0.00	0.00b±0.00	0.00b±0.00
HUIB_PTr1	2.97c±0.08	0.00b±0.00	0.00d±0.00	0.00c±0.00	0.00b±0.00	0.00b±0.00

Accessions	Nerolidol	(Z,Z)-Farnesal	Endo-1-Bourbonanol	Beta-Eudesmol	Phytol-Isomer
HUIB_PH30	3.39b±0.08	0.00b±0.00	0.00b±0.00	15.69a±0.21	2.63ab±0.12
HUIB_PD36	0.00c±0.00	0.00b±0.00	0.00b±0.00	0.00b±0.00	0.00d±0.00
HUIB_PH46	11.20ab±0.30	0.00b±0.00	0.00b±0.00	0.00b±0.00	1.97bc±0.09
HUIB_PN27	26.66a±0.22	2.39a±0.09	0.00b±0.00	0.00b±0.00	3.73a±0.02
HUIB_PB1	0.00c±0.00	0.00b±0.00	0.00b±0.00	0.00b±0.00	1.05cd±0.09
HUIB_PTr1	0.00c±0.00	0.00b±0.00	17.60a±0.35	0.00b±0.00	0.00d±0.00

Note: within a column, mean values having a letter in common are not significantly different at $p < 0.001$ by the Kruskal-Wallis test (mean±SD)

The relationship among accessions based on genetic diversity, resistance, and eugenol and methyl-eugenol content

Accessions were separated based on four components, including genetic diversity, resistance, eugenol, and methyl-eugenol rate. While resistance to *P. capsici*, eugenol content and methyl-eugenol content were the three main factors differentiating accessions, cluster in RAPD data was less important (Figure 6). *P. betle* (HUIB_PB1) and *P. tricolor* (HUIB_PT1) tended to be strongly associated with high eugenol content, while *P. divaricatum* (HUIB_PD36) was characterized by methyl-eugenol content. Conversely, *P. hancei* (HUIB_PH30, HUIB_PH46) and *P. nigrum* (HUIB_PN27) were more related to the post-infectious disease index. Overall, accessions resistant to *P. capsici* (HUIB_PD36, HUIB_PB1, and HUIB_PTr1) were grouped into one subgroup (Figure 7). They had a 0% DSI and contained eugenol and methyl-eugenol. Meanwhile, accessions susceptible to *P. capsici* (HUIB_PH30, HUIB_PH46 and HUIB_PN27) were in a different subgroup (Figure 7), had 41.67-100.00% DSI, and lacked eugenol and methyl-eugenol in their extracts. These findings, based on a limited number of accessions and qualitative resistance testing, suggest that eugenol- and methyl-eugenol-rich chemotypes are promising sources of *P. capsici* resistance, but further functional validation and field trials are required before deployment in breeding programs or producing biological control compounds for *P. capsici*.

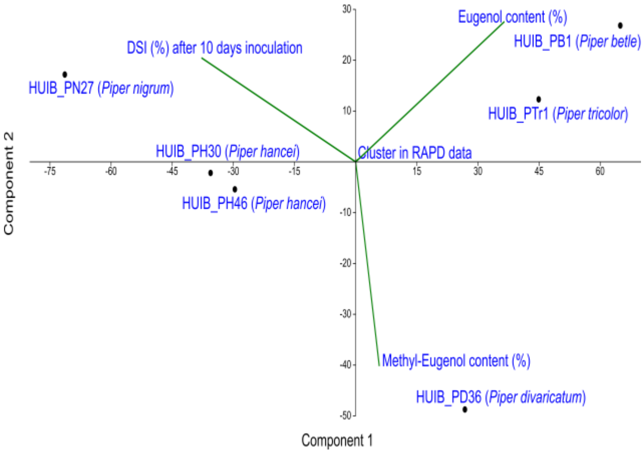

Figure 6. PCA plot showing correlation among accessions based on four components

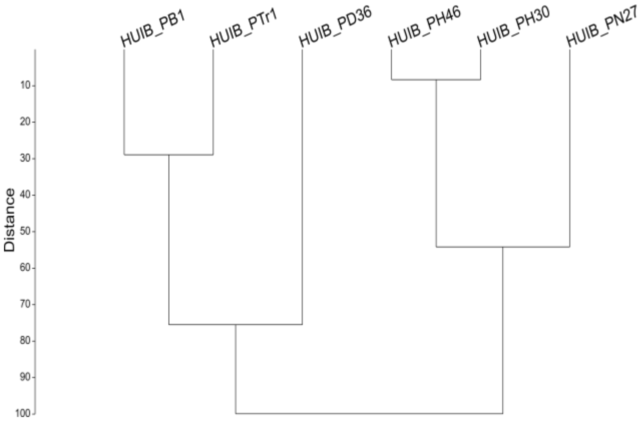

Figure 7. UPGMA clustering tree of *Piper* accessions based on genetic diversity, resistance, and eugenol and methyl-eugenol content

In conclusion, four *Piper* species collected in Vietnam and Laos were successfully identified based on the *ITS*$_{u1\text{-}4}$ regions. Furthermore, the addition of these accessions increased the population's polymorphism. Genetic diversity among accessions was reported based on RAPD data. In particular, new accessions were associated with the high proportions of eugenol and methyl-eugenol in the GC-MS analysis and expressed resistance to *P. capsici* in in vitro testing. Although this work had some limitations, including a small sample size, limited replication, and a detached-leaf assay only, it was likely to provide potential materials for breeding and producing biological fungicides that control *P. capsici*. In further research, field trials, SSR/SNP markers, and functional assays of eugenol/methyl-eugenol should be conducted to overcome the mentioned limitations.

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