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Assessing Genetic Diversity and Potential Disease Resistance of some Sen Peanut (*Arachis hypogaea* L.) Samples by Molecular Markers

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Abstract

Peanut (Arachis hypogae L.) is considered one of the main foods and oil crops in Vietnam. The improvement of local Sen peanut varieties is still limited because the identification of the varieties is mainly based on morphological characteristics. In order to identify genomic features and conserve the genetic resources of this variety in Nam Dinh province, an analysis of the genetic diversity of 20 Sen peanut variety samples was conducted using 32 SSR markers. These markers were selected based on their association with important traits of peanuts. The marker TC9H09 is effective for selecting lines with high resistance to rust disease, as indicated by a DNA band size of 220 bp. The markers TC3A12 and TC3E02, with DNA band sizes of 220 bp and 250 bp, respectively, were found to be effective in selecting lines with high oil content. Meanwhile, the marker TC9F10 could be used to select lines tolerant to rust disease and late leaf spot disease, with resulting DNA band sizes of 170 bp, 240 bp, 290 bp, and 350 bp. There were four line groups with high genetic similarity, namely (1,2); (3, 4, 5, 6, 7, 8, 9, 10); (11, 12, 15, 16, 18); and (13, 14, 17, 19, 20). Combining data from phenotypic and genotypic evaluation, it was found that lines 3, 4, 5, 6, 7, 8, 9, and 10 exhibited high resistance to rust disease and had genetic similarity. Similarly, lines 13, 14, 16, and 17 demonstrated high lipid content. These lines, which possess valuable traits for quality and productivity, will be multiplied to effectively improve the genetic resources of the Sen peanut variety in Nam Dinh province.

Keywords: genetic diversity, genetic resource, Sen peanut variety, DNA markers, SSR

Introduction

Peanut (*Arachis hypogaea* L.) (2n = 4x = 40) is a highly important oilseed crop, cultivated extensively in various parts of the world, including the Americas, Africa, and Asia. The global peanut growing areas reached 32.72 million hectares, and the total peanut production

was to 53.93 million tons in 2021 (FAO, 2023). In 2021, Vietnam produced peanuts across an area of 65.2 thousand hectares, with a total production of 430.4 thousand tons and an average yield of 2.6 tons per hectare. Vietnam has ranked fifth area in the total of 25 Asian countries planting peanuts, and the yield is significantly higher than the global average. In Vietnam, the focus of peanut breeding has been on developing local varieties that boast high adaptability, short growth duration, drought tolerance, and strong resistance to pests and diseases (Cuc et al., 2021a). These pests and diseases include leaf diseases such as rust, black spot, and brown spot, as well as bacterial wilt disease. Additionally, the breeding efforts aim to produce peanuts suitable for oil extraction, exportation, and domestic consumption while maintaining optimal quality.

The current peanut breeding combines traditional methods with molecular marker methods, which are effective for studying genetic diversity, evolution, genetic conservation, and mapping. The use of DNA markers linked to genes associated with disease resistance, tolerance, and quality in genetic maps can greatly contribute to improving local crop varieties and identifying valuable traits in selected lines. In Vietnam, molecular markers have been applied in research on the genetic diversity of numerous important foods and vegetable crops for breeding purposes, for example, the selection of brown plant hopper resistance genes in rice (Huyen, 2005), aromatic and color rice landraces (Trung et al., 2017; Hue et al., 2018), mapping genes of black spot in peanut (Cuc et al., 2009), soybean rust resistance (Khanh et al., 2013). Various markers, including RAPD, RFLP, AFLP, and SSR, can be used to assess the genetic diversity of peanuts, with SSR markers being considered more suitable for genetic studies of cultivated peanuts than other markers (Tang et al., 2007; Cuc et al., 2021b; Kassie et al., 2023). Genetic mapping for major disease resistance in peanuts has also been conducted, paving the way for the recent application of molecular marker methods in peanut breeding worldwide.

Sen peanut variety is a local variety in Nam Dinh province with great potential for commercial production due to its valuable traits, which include good quality and high resistance to major pests and diseases. In order to enhance and develop local genetic resources, it is necessary to utilize molecular markers to evaluate the genetic diversity in peanut lines and identify lines with desirable traits. Therefore, the objective of this study was to assess the genetic diversity of 20 lines of the Sen peanut variety by employing 32 SSR markers that are associated with the genes responsible for disease resistance (such as rust disease, black spot disease, brown disease, and bacterial wilt disease) as well as high lipid content, as identified in published genetic maps. The data collected from this study will be utilized to categorize, conserve, improve, and develop local peanut genetic resources.

Materials and Methods

Materials

In this study, a total of 20 lines of peanut varieties (*Arachis hypogaea* L.) were collected from Hai Hau and Giao Thuy districts of Nam Dinh province. Thirty-two SSR markers linked to genes associated with disease resistance (rust disease, black spot disease, brown disease, bacterial wilt disease) and high lipid content were selected from published genetic maps for peanuts. These markers are described on the website

http://peanutbase.org. Additional materials and chemicals were used for molecular biology purposes.

Methods

Total DNA extraction and genetic evaluation using SSR markers

The study was conducted at Labatory of GMO, Department of Agricultural Genetics Institute from February to April 2022. The collected peanut line samples were extracted and purified using the method (Mace et al., 2003). The primer PCR reaction and volume per reaction are presented in Table 1.

Table 1. *The formula in PCR reaction*

No.	Content	Vol per reaction
1	10x PCR buffer	2 µl
2	dNTPs (5 mM)	0,5 µl
3	Primer F (100 pM/µl)	1,2 µl
4	Primer R (100 pM/µl)	1,2 µl
5	Taq polymerase 1 U/µl	0,2 µl
6	DNA (30 ng/µl)	2 µl
7	H_2O	12,9 µl
	Total	20µl/reaction

The PCR program consisted of an initial denaturation step at 94℃ for 2 minutes, repeating 30 cycles at 94℃ for 45 seconds. The temperature for annealing primers varied depending on the markers used with options of 65℃, 60℃, and 55℃ for 1 min. The extension step was performed at 72 ℃ for 1 min, and there was a final extension at 72℃ for 10 min, respectively.

Electrophoresis and data analysis

The products of the PCR reaction were electrophoresed, and the results were analyzed using a 3.5% agarose gel in 1X TAE buffer for a duration of 2.5 to 3 hours. Visualization was done using the RED SAFE staining solution. The data from analyzing the PCR products were recorded in binary format in Excel version 2016. For each line on each marker, the presence or absence of bands at each allele position was recorded as 1 or 0, respectively. The standard DNA scale used to determine allele sizes was a 100 bp ladder, consisting of bands ranging from 100 bp to 1000 bp, divided into 10 bars. The DNA data were analyzed using the NTSYS 2.1 program version 2.1. The similarity coefficient was calculated using the formula of Nei and Li (1979) within the NTSYS 2.1 program, in which:

$$Sij = \frac{2a}{(2a+b+c)}$$

Where:

Sij: The similarity coefficient between sample i and sample j

aij is the number of bands present in both samples i and j

bij is the number of bands present in sample i but not in sample j

cij is the number of bands present in sample j but not in sample i.

The similarity matrix was calculated using the UPGMA method. The gene diversity coefficient or polymorphism information content (PCI) between markers was calculated according to Weir (1990):

$$PIC = 1-\Sigma P_{ij}^2$$

where Pij is the frequency of the i^{th} allele for the j^{th} marker.

Results and Discussion

DNA extraction and purification

The first step in the analysis process was extracting and purifying the DNA of 20 studied peanut lines. Purification of extracted DNA products is an initial requirement because peanuts contain high polysaccharides and polyphenols. The total DNA of the studied peanut lines was extracted and purified according to the method of Mace et al (2003).

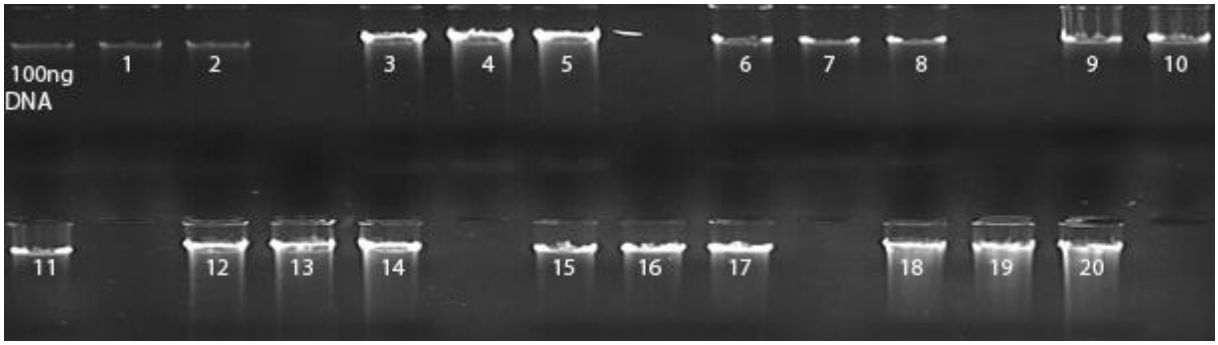

Figure 1. *DNA extraction and purification result of peanut samples. Lanes: 1-20 are the numbers of samples collection*

DNA extracted from peanut leaves had high quality and quantity, with the concentration from 200 to 1500 ng/µl, and $OD_{(260/280)}$ between 1.7 to 2.0, respectively. Thus, these samples' quality and DNA concentration met the standards for the next steps in the analysis process.

Genetic diversity evaluation of peanut samples using the molecular marker SSR

Based on the genetic map of genes/QTLs that control traits of resistance to the rust disease, black spot, brown spot, bacterial wilt, and high oil content in peanuts published around the world, there were 32 SSR markers associated with resistance genes/QTLs that were selected for the analysis of 20 studied peanut lines (Cuc et al., 2009).

Table 2*. PIC values and polymorphism of 20 lines of Sen peanut samples*

No.	Marker	PIC	PS	HS	Total	RP	STS
1	TC9H09	0.091	2	1	3	66.67	40
2	TC3B04	0.095	1	1	2	50.00	24
3	TC11A04	0.175	2	1	3	66.67	40
4	TC7H11	0.295	2	0	2	100	34
5	TC5A06	0.252	2	1	3	66.67	45
6	LEC 1	0.073	3	0	3	100	22
7	Seq4F7	0.235	2	0	2	100	20
8	Seq3B5	0.235	2	0	2	100	24
9	Seq8D9	0.200	2	0	2	100	22
10	TC3A12	0.365	4	0	4	100	34
11	TC11H06	0.195	2	0	2	100	20
12	TC6E04	0.263	3	1	4	75.00	45
13	IPAHM524	0.120	2	1	3	66.67	42
14	TC3E02	0.275	2	1	3	66.67	42
15	TC4G02	0.236	2	1	3	66.67	50
16	TC9F10	0.345	6	0	6	100	38

17	IPAHM0509	0.225	3	1	4	75.00	41
18	IPAHM108	0.090	3	0	3	100	40
19	Seq19H3	0.125	2	0	2	50.00	20
20	GM1878	0	0	3	3	0	60
21	GMLQ976	0	0	2	2	0	40
22	GA161	0	0	1	1	0	20
23	TC6G09	0	0	1	1	0	20
24	TC4E12	0	0	1	1	0	20
25	PM375	0	0	1	1	0	20
26	TC3E05	0	0	1	1	0	20
27	TC1E01	0	0	1	1	0	20
28	TC4F12	0	0	1	1	0	20
29	Seq2B10	0	0	1	1	0	20
30	GM1760	0	0	1	1	0	20
31	GMRQ843	0	0	1	1	0	20
32	IPAHM0569	0	0	1	1	0	20
	Total		**46**	**26**	**72**		**963**

PS: Polymorphic segments; Homomorphic segments; RP: Ratio of polymorphism; STS: Segment total of all samples

The purified DNA samples underwent PCR reactions for analysis. Electrophoresis on a 3.5% agarose gel was used to analyze the PCR products. A total of 32 SSR molecular markers were used to analyze 20 peanut lines. This analysis resulted in 963 alleles, which were divided into 72 DNA bands. Of these bands, 46 were found to be polymorphic segments. Out of the 32 markers used, 11 resulted in only one homomorphic DNA segment. Two markers resulted in 2-3 isomorphic segments, while the remaining 19 markers resulted in 1 to 6 polymorphic segments. The PIC values of the polymorphic segments ranged from 0 to 0.365 and were associated with specific molecular markers.

Molecular marker TC9F10:

The electrophoresis analysis of PCR products on agarose gel with TC9F10 marker showed that there were 5 alleles found in 20 peanut lines with sizes of 170bp. 240bp. 260bp, 270bp, 290bp, and 350bp. respectively. The allele 1 at the position of 170 bp only appeared in line 11 to line 20. The allele 2 at 180 bp appeared in line 1 to line 10. and the allele 3 at 200 bp appeared in line 11 to line 18. and line 20.

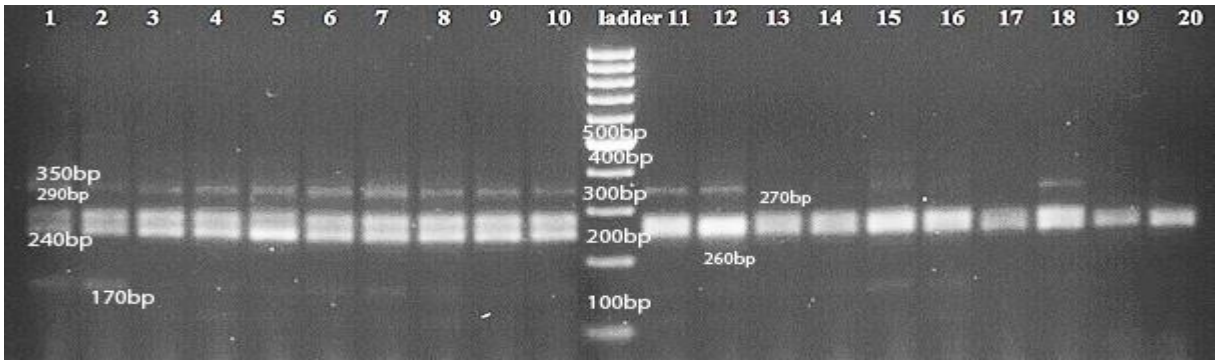

Figure 2. *Electrophoresis analysis of PCR products using TC9F10 marker on agarose gel for 20 peanut lines numbered from 1 to 20*

Molecular m*arker* LEC 1:

The results of electrophoresis analysis of agarose gel PCR products with Lec1 marker of 20 peanut lines/varieties obtained 3 alleles with sizes 170bp, 180bp, and 200bp. In which allele 1 at position about 170 bp only appears in lines 11 to 20; the 2nd allele equivalent to position 180 bp appeared in lines 1 to 10; The 3rd allele at position 200 bp appeared in lines 11-18 and 20.

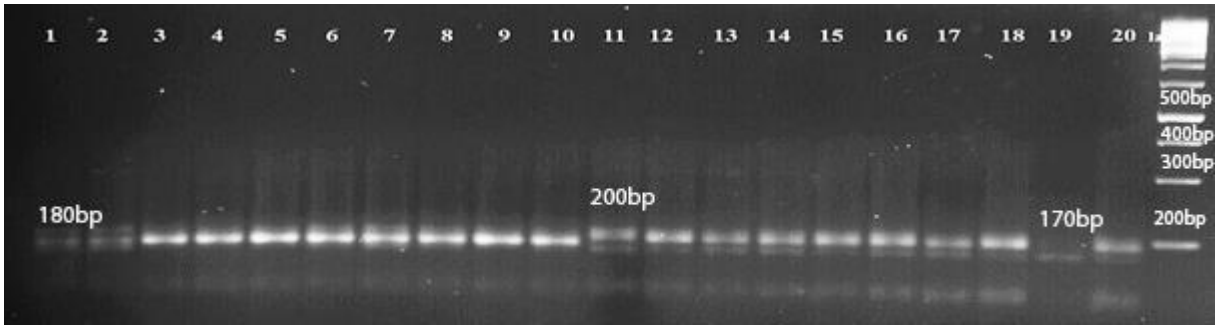

Figure 3. *Electrophoresis analysis of PCR products using Lec1 marker on agarose gel for 20 peanut lines numbered from 1 to 20*

Molecular m*arker TC9H9*:

The results of electrophoresis analysis of PCR products agarose gel with TC9H9 marker of 20 peanut lines/varieties shown in Figure 4. obtained 3 alleles with sizes 150 bp, 220 bp, and 250 bp. In which, allele 1 is at position, position 150 bp occurs in all lines 1-20; the 2nd allele at position 220 bp only appeared in lines 11-20; The 3rd allele at position about 250 bp only appeared in lines 1-10.

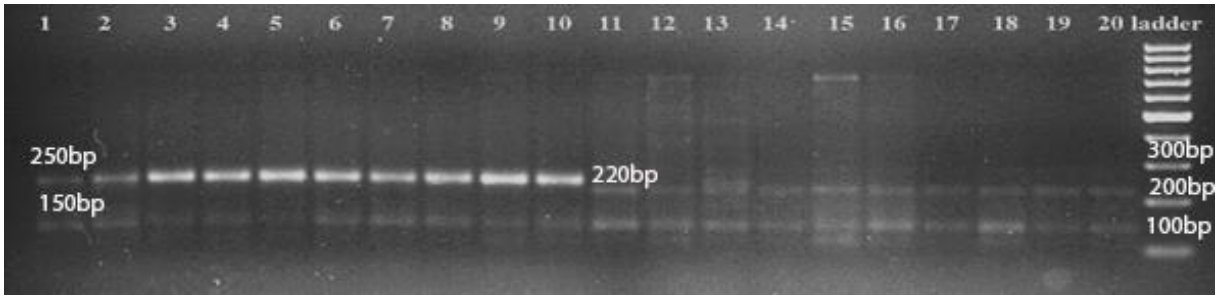

Figure 4. *Electrophoresis analysis of PCR products using TC9H9 marker on agarose gel for 20 peanut lines numbered from 1 to 20*

The table of genetic similarity coefficients for 20 peanut lines showed that the coefficients ranged from 0.5 to 1. Line 2 and line 19 had the lowest coefficients, both with a value of 0.5. The software NTSYSpc 2.1 was used to analyze the relationship of genetic diversity among the peanut lines. The results included a Jaccard similarity index between the 20 peanut lines and a tree diagram constructed using the UPGMA method (Figure 5). The degree of difference between peanut lines was expressed using difference coefficients. Peanut lines with high genetic similarity were grouped together. Based on a genetic similarity threshold of 0.7, the 20 peanut lines were divided into 2 main groups: Group 1 consisted of lines numbered from 1 to 10. Within this group, a sub-group with a genetic similarity of 0.93 was further divided into 2 sub-groups: Group 1.1 included line 1 and line 2, while Group 1.2 included lines 3 to 10 (with lines 3, 4, 5, 6, 7, 8, and 9 showing no genetic difference when analysed using 32 molecular markers).

Group 2 consisted of lines numbered from 11 to 20. Within this group, a sub-group with a genetic similarity of 0.82 was further divided into 2 sub-groups: Group 2.1 included lines 11, 12, 15, 16, and 18, while Group 2.2 included lines 13, 14, 17, 19, and 20. The studied peanut lines exhibited high genetic similarity, ranging from 82% to 100%. This indicates that the collected peanut lines have relatively similar genetic bases.

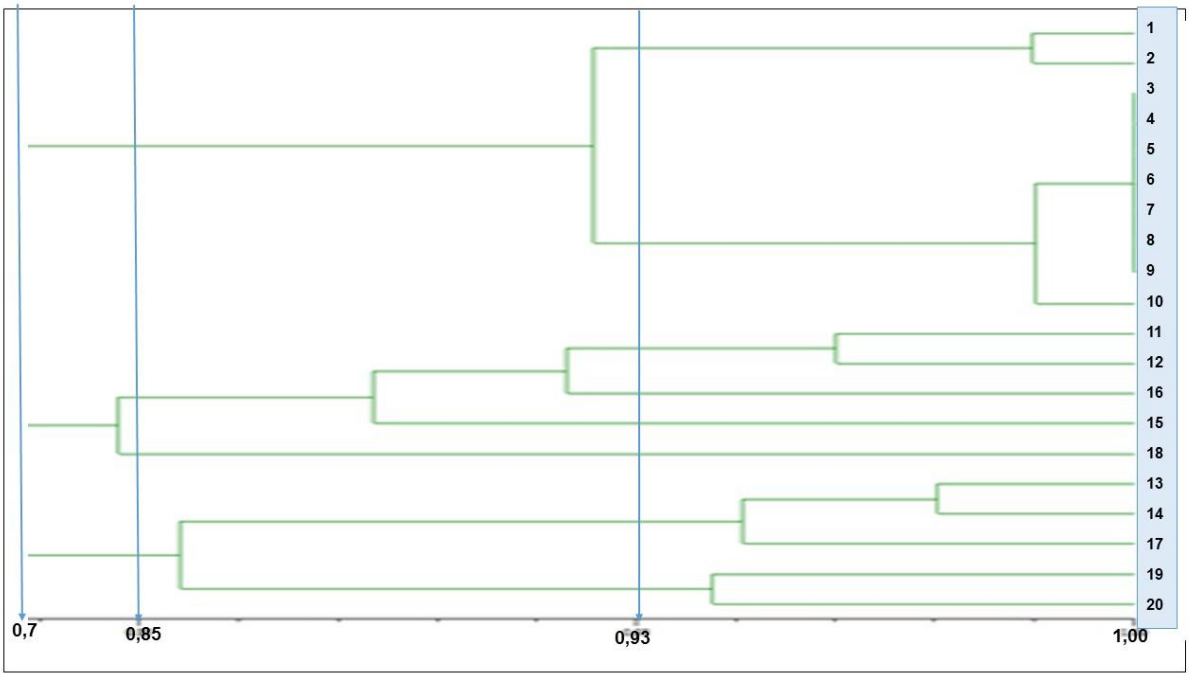

Figure 5. *Tree diagram of genetic clustering of 20 peanut lines based on data analysis using NTYSYS 2.1 software*

However, analysis using 32 SSR markers revealed differences between these lines. Therefore, evaluating the genetic diversity among lines within a peanut variety is valuable for identifying lines with high genetic similarity. This can aid in improving peanut genetic resources. Additionally, selecting markers associated with target genes that confer resistance to diseases such as rust disease, early leaf spot disease, late leaf spot disease, and bacterial wilt can accurately distinguish between peanut lines in the same Sen peanut variety, grouping them into 1 and 2. Hence, as mentioned by other authors, this method can be employed to assess lines of crops with a limited genetic base, including peanuts, which are difficult to differentiate. Thus, the analysis of genetic diversity in 20 peanut lines using 32 SSR molecular markers demonstrated that the following line groups exhibit similar genetic similarity: (1, 2); (3, 4, 5, 6, 7, 8, 9, and 10); (11, 12, 15, 16, and 18); and (13, 14, 17, 19, and 20).

Selection of molecular markers associated with genes controlling quality and disease resistance of peanut lines

Identification of molecular associated with genes controlling disease resistance

When selecting markers for this study, we consulted documents and genetic maps of traits published by other authors. Varshney et al. (2009) conducted a genetic analysis and mapping of QTL linkages for rust resistance in peanuts and identified markers that bind QTL for rust resistance. Out of these markers, 10 SSR markers were selected to effectively screen for rust resistance traits in peanut breeding. These markers were used

to evaluate rust resistance in 20 studied peanut lines. Based on the results of the phenotypic evaluation of rust resistance in the field, it was found that line 14 was infected with a severity score of 5. In contrast, line 1 was resistant with a severity score of 1. When evaluating the genotype of the lines using molecular markers associated with the rust-resistant QTLs, in combination with the results of the phenotypic evaluation, lines with DNA bands similar to line 14 were temporarily considered as infected lines, while lines with DNA bands similar to line 1 were temporarily considered as resistant lines.

Molecular m*arker TC11A04*:

The molecular marker TC11A04 was a specific marker that amplified regions of the rust-resistant genes. Therefore, if the peanut lines express the DNA band at the position of 220bp, They had rust-resistant genes (the size of the DNA band was similar to line 1). The peanut lines that expressed DNA bands at the position of 200 bp were determined not to have rust-resistant genes (the DNA band size was similar to line 14). 11 lines had rust-resistant genes, including 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 15.

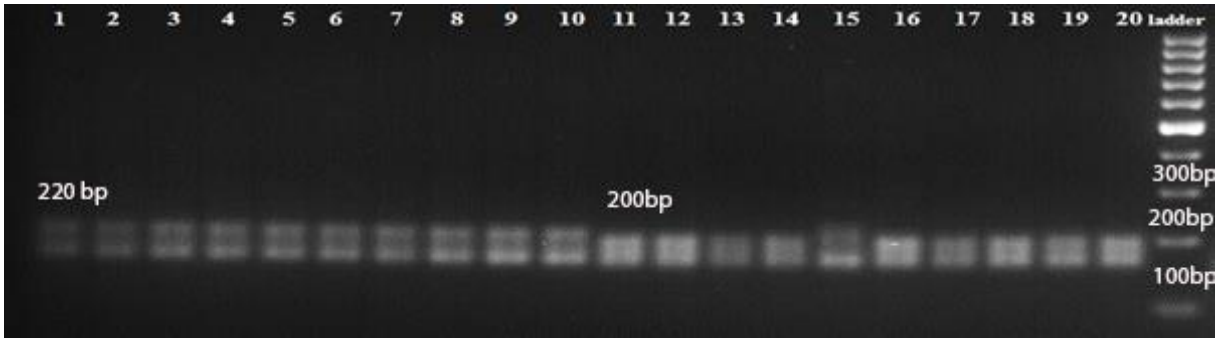

Figure 6. *Electrophoresis analysis of PCR products using TC11A04 marker on agarose gel for 20 peanut lines numbered from 1 to 20*

The remaining lines expressing a DNA band at 200 bp were rust-infected lines, including 11, 12, 13, 14, 16, 17, 18, 19, and 20. Comparing the results of DNA genotype assessment with phenotypic assessment, the above analysis was consistent with assessing rust resistance in the Sen peanut lines in Nam Dinh in the autumn-winter seasons of 2021. Thus, the TC11A404 markers could be used for early screening for rust resistance in selected lines to improve peanut varieties.

Molecular m*arker IPAHM 524*:

The molecular marker IPAHM524 amplifies the regions of rust-resistant genes and is considered a specific marker. However, when using this marker for both infected and resistant controls, it was found that the expressed DNA bands appeared at the same position of 300 bp. Consequently, the marker IPAHM524 cannot be used to differentiate between rust-resistant and susceptible peanut lines. In simpler terms, there is no variation in DNA size among the peanut lines when using the IPAHM524 marker to screen for rust-resistant genes.

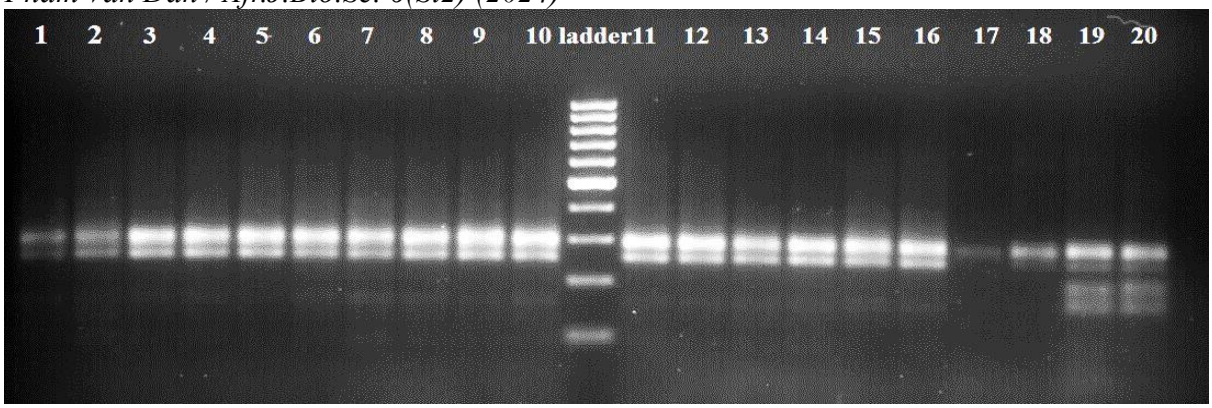

Figure 7. *Electrophoresis analysis of PCR products using IPAHM524 marker on agarose gel for 20 peanut lines numbered from 1 to 20*

Using the same analytical method, 10 molecular markers associated with rust-resistant QTL could be used to screen rust-resistant genes in 20 peanut lines, as presented in Table 3.

Table 3. *Molecular markers for screening rust-resistant genes of 20 studied peanut lines*

No.	Molecular marker	Rust-resistant lines	Rust-infected lines
1	**TC9H09**	1,2,3,4,5,6,7,8,9,10	11,12,13,14,15,16,17,18,19, 20
2	**TC11A04**	1,2,3,4,5,6,7,8,9,10,15	11,12,13,14,16,17,18,19,20
3	TC7H11	Not applicable	Not applicable
4	TC5A06	Not applicable	Not applicable
5	Lec1	Not applicable	Not applicable
6	**TC3A12**	1,2,3,4,5,6,7,8,9,10,11,12, 15,16,17,18,19,20	13,14
7	TC6E01	Not applicable	Not applicable
8	IPAHM524	Not applicable	Not applicable
9	**TC4G02**	1,2,3,4,5,6,7,8,9,10,11,12 15,16, 18,	13,14,17,19,20,
		Rust-and late-leaf spot-resistant lines	Rust-and late leaf spot-infected lines
10	**TC9F10**	1,2,3,4,5,6,7,8,9,10,11,12,18	13,14,15,16,17,19,20

There were 5 molecular markers identified as involved in rust-resistant QTLs on the reference genetic maps: TC9H09, TC11A04, TC3A12, TC4G02, and TC9F10. These markers could be used to identify rust-resistant lines by analysing DNA bands similar to the DNA band of line 1. Combined with the results of phenotypic assessment of rust resistance in the field, two markers, TC9H09 and TC11A04, showed greater effectiveness than the other markers in screening for rust-resistant genes in peanuts. Additionally, the marker TC9F10 is associated with both rust and late-leaf spot-resistant QTLs, making it a useful tool for screening lines with genes controlling resistance to both diseases.

Identification of molecular associated with genes controlling lipid content

The lipid content is important for evaluating peanut quality. The quality of peanut oil depends on the composition of fatty acids, specifically oleic (O) and linoleic (L) acids, which comprise 80% of the peanut oil. Most popular peanut varieties today contain 55% oleic acid and 25% linoleic acid. Linoleic acid is less saturated and less stable compared to oleic acid. The O/L ratio affects peanut products' oxidative stability and shelf life, making peanut varieties with high oleic acid content beneficial for consumers' health. Selecting peanut varieties for high oil yield per planted area improves peanut lines. In this study, we screened 20 lines of the Sen peanut variety for molecular markers associated with quality traits, specifically lipid content. These markers are described on the website http://peanutbase.org . Three molecular markers, TC3A12, TC11A04, and TC3E02, were found to be associated with the genes controlling lipid content. These markers have the effect of increasing lipid content in peanuts. We have previously presented the marker TC11A04. Electrophoresis analysis of PCR products amplified by the marker TC3A12 revealed a specific DNA band of 220 bp in lines 13 and 14, which differed from other lines. Additionally, a DNA band of 450 bp indicated differences in lines 1 to 11, 15, and 16.

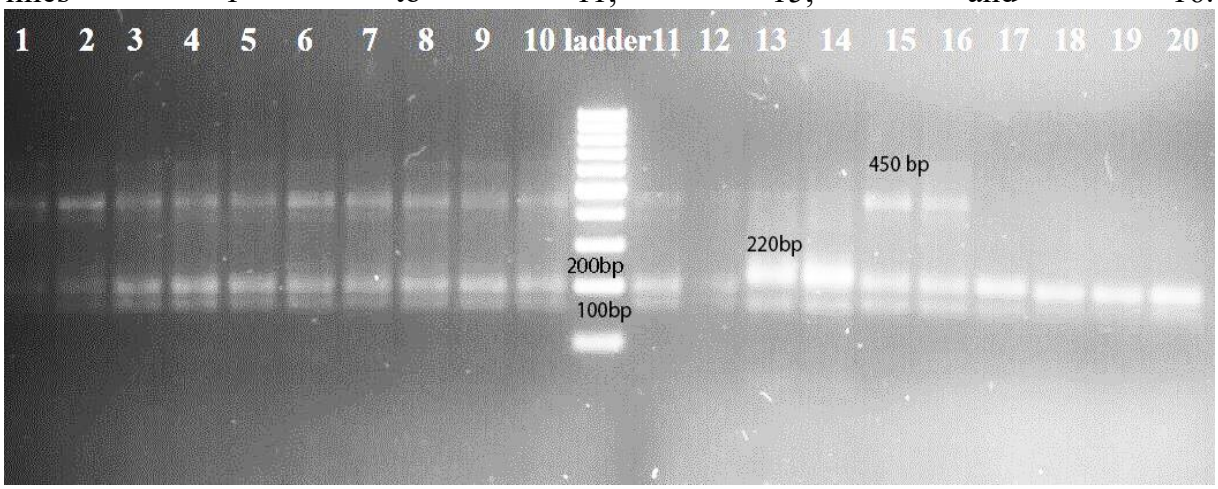

Figure 8. *Electrophoresis analysis of PCR products using TC3A12 marker on agarose gel for 20 peanut lines numbered from 1 to 20*

Molecular marker TC3E02

Figure 5 shows the results of the electrophoresis analysis of PCR products of 20 peanut lines on agarose gel using the marker TC3E02. Alleles with sizes of 250 bp, 310 bp, and 320 bp, respectively, appeared in lines 1 to 20. Allele 1 at the position of 250 bp only appeared in lines 1 to 20; allele 2 at the position of 310 bp appeared in lines 1 to 10, and allele 3 at the position of 320 bp appeared in lines 11,12,14 and 15.

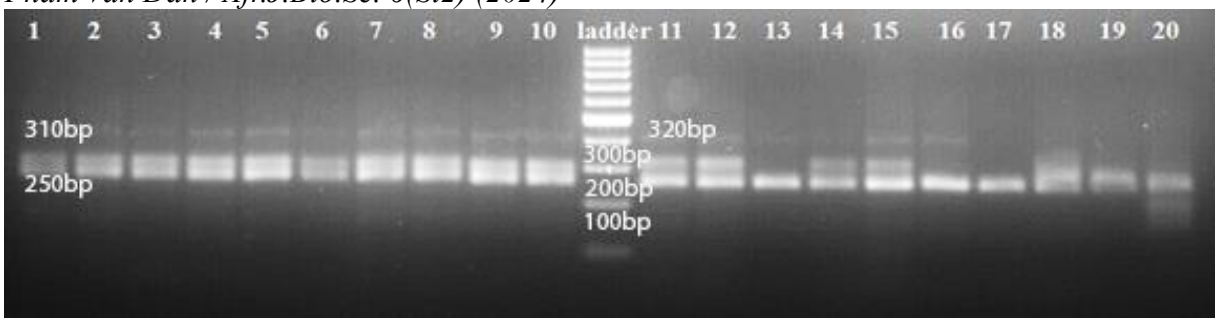

Figure 9. *Electrophoresis analysis of PCR products using TC3E02 marker on agarose gel for 20 peanut lines numbered from 1 to 20*

Combined with the results of lipid content analysis, the marker TC3A12, used in lines 13 and 14, had a DNA band of 220 bp. The marker TC3E02, used in lines 13, 16, and 17, had a DNA band of 260 bp. These markers could be associated with QTL of high lipid content through the combination of genotypic analysis and actual lipid content analysis in line 13. Thus, the markers TC3A12 and TC3E02 can be used to screen peanut lines with high lipid content. The evaluation of 20 Sen peanut lines using molecular markers associated with genes controlling disease resistance, including rust disease and late leaf spot, as well as high lipid content, was summarized in Table 4, along with the phenotypic and trait analysis of the actual oil content of the peanut lines. Our previous studies successfully applied SSR markers to select some elite peanut lines resistant to late leaf spot disease (Cuc et al., 2021a, Cuc et al., 2021b)

Table 4. Molecular markers used for selecting peanut lines with disease-resistant and high lipid content

No.	Molecular marker	Disease resistance / High lipid content	DNA size	Peanut lines
1	TC9H09	Rust resistance	220bp	Line 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10
2	TC3A12	High lipid content	220bp	Line 13, line 14
3	TC3E02	High lipid content	250bp	Line 13, line 16, line 17
4	TC9F10	Rust and late leaf spot resistance	170bp, 240bp, 290bp, 350 bp	Line 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 18

Conclusions

In conclusion, we analyzed the genetic diversity of 20 peanut lines using 32 SSR molecular markers. We identified line groups that exhibited similar genetic similarity, namely: (1,2); (3, 4, 5, 6, 7, 8, 9, and 10); (11, 12, 15, 16, and 18); and (13, 14, 17, 19, and 20). Our analysis determined that two molecular markers, TC9H09 and TC9F10, can effectively screen peanut lines for resistance to rust and late leaf spot diseases. Similarly, the molecular markers TC3A12 and TC3E02 can be utilized to identify peanut lines with high lipid content. Specifically, lines 3, 4, 5, 6, 7, 8, 9, and 10 exhibited genetic similarity and resistance to rust disease, while lines 13, 14, 16, and 17 showed high lipid content. These findings indicate that these specific peanut lines are suitable candidates for selecting peanut lines that possess both disease resistance and high lipid content.

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