Original Research Article

Genetic diversity study of *Cinnamomum cassia* Blume using random amplified polymorphic DN A markers (RAPD)

ABSTRACT

Aims: To investigate Genetic diversity study of *Cinnamomum cassia* Blume in Vietnam.

Methodology: Fresh leaves of Cinnamomum cassia Blume sample were grinded in liquid nitrogen and total genomic DNA was extracted and purified by CTAB method. The primer was amplified via the polymerase chain reaction on a Gene Amp PCR system 9700. The binary data set was utilized to compute the pairwise Jaccard similarity index and to construct the related similarity matrix. All analyses were performed by using the NTSYS-PC software application.

Results: Twenty-four accessions of Cinnamomum cassia Blume belongs to Cinnamomum species collected from some mountainous areas of the Yenbai province, Vietnam were examined to assess genetic variation using the RAPD method. The accessions included 4 in Lucyen, 5 in Tranyen, 5 in Vanchan, 6 in Vanyen and 4 in Yenbinh. Eighty-one out of 99 bands detected by 15 selected RAPD primers were polymorphic. The average number of band/primer was 6.6, in which the polymorphic bands/primer was 5.4, corresponding to 82 %. The similarity indexes among accessions fluctuate between 0.67 to 0.91 The coefficient of estimated PIC/primer range from 0.371 to 0.883, with an average of 0.756. The hightpolymophylic and similarity indexes showed large modification of genome. Grouping accessions in phylogenetic tree reflected the fact that the different areas may used the same or different cultivars of Cinnamomum cassia Blume for cultivation.

Conclusion:*Cinnamomum cassia* Blume belongs to *Cinnamomum* species was introduced in Yenbai from multiple sources and possesses hight genetic diversity and variation and suggests that necessary to establish conservation plan, evaluation and selection of the best gene resources for commercial purposes

Keywords: *Cinnamomum cassia; Genetic diversity, RAPD, Vietnam.*

INTRODUCTION

Cinnamomum cassia Blumebelongs to*Cinnamomum*species,a genus with a significant number of more than 200 species. These are widely distributed in East Africa including Seychelles, Madagascar and Asian as the West Coast of India, Indonesia, China, Sri Lanka, and Vietnam[1].In Vietnam, there are three main species of Cinnamomum currently existing, including Cinnamomum zeylanicum Ness, Cinnamomum cassia Blume,

Cinnamomum obtusifolium Ness var Loureirii. Perot Ebernh[2]. The genus *Cinnamomum* are considerable economically significant, common farmed for its bark, whichis condiment made from the inner bark andcommonused in perfumery[3]. These are also a traditional folk medicine and werewidely grown with plantations covering more than 160,000 hectares[4]. The herb bark has a potent aromatic and a flavor that is sweet with a hint of bitterness and spice[5]. Recently, various literature has reported the therapeutic use of this herb in a variety of disease states, including malignancies, peptic ulcer illnesses, and diabetes mellitus[6].The twigs and cortex*Cinnamomum cassia* Blume have been utilized for the treatment of blood circulation problems, gastritis, inflammatory illnesses and diabetes[7]. Currently pharmacological and chemical report focused primarily on the essential containment within twigs and cortex consisted of average 1-2% volatile oil known as cassia oil. The essential oil's principal ingredients are 65-80% cinnamaldehyde, a lesser proportion of eugenol,starch, mucilage, and tannins[8]. So that exhibited in wide range of effects, including antiulcer, anticancer, antibacterial, anti-inflammatory, antidiabetic activities and antioxidant[9].

In the past decades, a various of DNA markerincluding SSR, ISSR, RFLP, RAPD have been quickly included into the molecular techniques available for genome analysis[10]. DNA bands are regarded as unit characteristics, and their existence or absence in an amplicon can be utilized to investigate genetic relationships as well as intra- and inter-specific genetic variants[11]. Given the extensive distribution and distinct morphological variation, including plant development habits within species, that is critical to examine genetic diversity through molecular methods. PCR-based markers have been widely employed to examine genetic variation within species and to determine genetic diversity[12].In this study, RAPD primers were utilized to analyze genetic diversity within *Cinnamomum cassia* Blumepopulations collected from various geographical areas in Yen Bai, Vietnam using molecular methods. This research aim was to access genetic diversity among *Cinnamomum cassia* Blume accessions by RAPD markers in order to offer data on genetics and a theoretical basis for species protection.

Yen Bai is one of the mountainous provinces with the largest cinnamon growing area in Vietnam, about over 12,000 hectares. Cinnamon products are the main source of income for many people living in the province. Along with maintaining the current cinnamon area, the province also always has a policy of improving the quality of cinnamon products, from variety selection, development to sustainable exploitation and use of cinnamon trees. (QĐ Số: 1481/QĐ-UBND)

Picture 1 : Cinnamon Vanyen district, Yenbai Province

MATERIALS AND METHOD

Cinnamomum cassia Blume samples

Cinnamomum cassia Blume samples were gathered from four different Yen Bai, Vietnam areas. Collection locations were shown in the table 1.

Table 1:Signalof population and position of *Cinnamomum cassia* Blume populations in Yen Bai, Vietnam

Number	Sample	Sample information	
		Species	Collection region
1	LY1	*Cinnamomum cassia* Blume	Lucyen, Yen Bai
2	LY2	*Cinnamomum cassia* Blume	Lucyen, Yen Bai
3	LY3	*Cinnamomum cassia* Blume	Lucyen, Yen Bai
4	LY4	*Cinnamomum cassia* Blume	Lucyen, Yen Bai
5	TY1	*Cinnamomum cassia* Blume	Tranyen, Yen Bai
6	TY2	*Cinnamomum cassia* Blume	Tranyen, Yen Bai
7	TY3	*Cinnamomum cassia* Blume	Tranyen, Yen Bai
8	TY4	*Cinnamomum cassia* Blume	Tranyen, Yen Bai
9	TY5	*Cinnamomum cassia* Blume	Tranyen, Yen Bai
10	VC1	*Cinnamomum cassia* Blume	Vanchan, Yen Bai
11	VC2	*Cinnamomum cassia* Blume	Vanchan, Yen Bai
12	VC3	*Cinnamomum cassia* Blume	Vanchan, Yen Bai
13	VC4	*Cinnamomum cassia* Blume	Vanchan, Yen Bai
14	VC5	*Cinnamomum cassia* Blume	Vanchan, Yen Bai
15	VY1	*Cinnamomum cassia* Blume	Vanyen, Yen Bai
16	VY2	*Cinnamomum cassia* Blume	Van Yen, Yen Bai
17	VY3	*Cinnamomum cassia* Blume	Vanyen, Yen Bai
18	VY4	*Cinnamomum cassia* Blume	Vanyen, Yen Bai
19	VY5	*Cinnamomum cassia* Blume	Vanyen, Yen Bai
20	VY6	*Cinnamomum cassia* Blume	Vanyen, Yen Bai
21	YB1	*Cinnamomum cassia* Blume	Yenbinh, Yen Bai
22	YB2	*Cinnamomum cassia* Blume	Yenbinh, Yen Bai
23	YB3	*Cinnamomum cassia* Blume	Yenbinh, Yen Bai
24	YB4	*Cinnamomum cassia* Blume	Yenbinh, Yen Bai

DNA extraction

Fresh leaves of *Cinnamomum cassia* Blume sample were grinded in liquid nitrogen and total genomic DNA was extracted and purifiedby CTAB method described by Doyle and Doyle[13] with some modifications, then stored at – 20^{o}C. The results of extraction were measured using a NanoDrop Spectrophotometer to detect sample absorbance at 260 nm and observed by ethidium bromide (Etbr) staining on 1.0% agarose gel electrophoresis.

RAPD Amplification

The polymerase chain reaction was carried out on a Gene Amp PCR system 9700. The reaction mixtures con¬tained 10X PCR buffer, 2 mM dNTP, 0.5 unit of taq DNA polymerase (Sigma, St. Louis, MO, USA), 5pmol of primer and 5 ng of template DNA in a total volume of 12.5 pl. The amplification program for RAPD was as follows: one first cycle of 95°c for 2 minutes followed by 40 cycles of 94°c for 1 minute, 40°C for 1 minute, and 72°c for 2 minutes, and holding at 72°c for 5 minutes. The amplification products were electrophoresed on 2% agarose gels with 1 xTAE (0.04 M Tris-acetate and 1 mM EDTA, pH 7.6). The gels were stained with ethidium bromide and photographed under uv light.

Data analysis

The primers that yielded polymorphic bands were checked for stability in repeated reactions and only those that showed reproducible bands were selected for analysis. Here, the bands which showed a different status (presence or absence) at least in one individual in total samples were considered to be polymorphic. A 0-1 matrix was built by scoring 1 for the presence and 0 for the absence of bands. The binary data set was utilized to compute the pairwise Jaccard similarity index and to construct the related similarity matrix. According to the UPGMA method, the resultant matrix was utilized to build a dendrogram. The dendrogram distances were compared to the genetic distances between genotype pairings to calculate the cophenetic correlation. All analyses were carried out using the NTSYS-PC software application.

The calculation of the pairwise Jaccard similarity index is based on the formula:

$$J=\frac{A}{N-D}$$

Where A is the occurrence frequency of the polymorphismband, D is theoccurrence frequency of themonomorphic band and N is the total bands.

The Polymorphism Information Content (PIC) value was measured according to Saa and Wricke's formula:

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

Where $P_{ij}$is the frequency of jth allele for ith marker. PIC value ranges from 0 (non- polymorphism) to 1 (completepolymorphism).

RESULTSAND DISSCUSSION

Extraction and purification of DNA

The isolation and purification of high-quality DNA from *Cinnamomum* species are difficult in general due to the possession of a lot of essential oils, polysaccharides, and polyphenols that not only prevent DNA precipitation but also affect the quantity and quality of isolated DNA[14]. Total DNA was isolated through the modified CTAB method to be able to obtain DNA with high quality [13]. The OD values were measured using a NanoDrop spectrophotometer to detect samples absorbance at 260 nm between 1.7 to 1.9. Total DNA was then stained with Etbr for 30 minutes and observed under UV light (Figure 1).

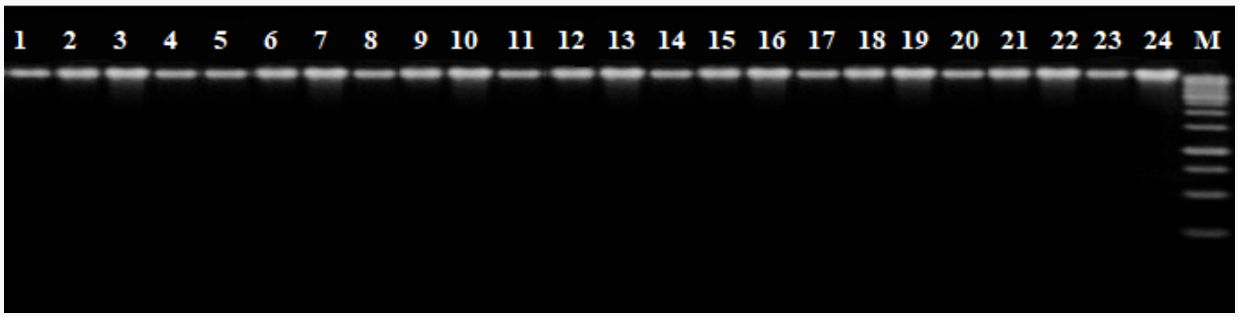


Figure 1: The total DNA isolated from leaves of *Cinnamomum cassia* Blume through the modified CTAB method. *1-24: Electrophoresis results of total DNA parts corresponding to the ordinal numbers in Table 1. M: Marker 1kb (Thermo Scientist, USA).*

Figure 1 demonstrates that the total DNA is high quality, these are characterized by the existence of the smear band beneath the main band. These smear bands contain impurities including RNA, proteins, phenolic substances, and polysaccharides. Although each sample had impurities, the proportion of DNA from the main fragment was still enormous, therefore the DNA was rated as pure enough to be able to perform RAPD-PCR. The concentration of DNA samples was then quantified using a Nanodrop spectrophotometer and diluted to a suitable concentration for PCR reaction of 25 ng/µl.

Polymorphism of RAPD primers

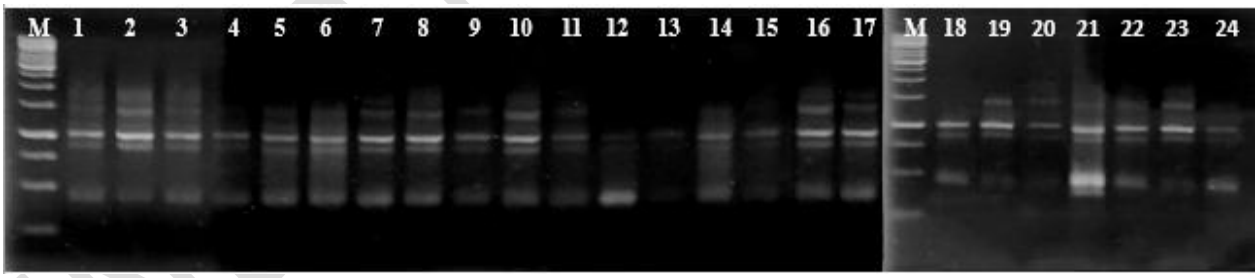
The current research aims to investigate genetic variability and to identify genotypes of cinnamon utilizing PCR-based techniques. A total of 30 primers, including 10 of OPA, 10 of OPB and 10 of OPC, produced a total of 293 bands for 24 individuals in this study. The number of bands detected from each primer ranged from one to 16 bands, with an average of 9.8 bands/primer. The sizes of the bands generated from 230bp to 3.5kb. 26 primers (86.7%) of total 30 gave polymorphic bands,15 of them that produced clearreproducible polymorphic bands in repeated reactions were selected for analysis (Table 2). The photographs of DNA fragments amplified from 24 accessions with OPC 02 and OPC 12 were presented in figure 2. The selected primers amplified a total of 99 bands, 81(82%) of which were polymorphic, while the number of bands ranged from three (OPB 04) to eleven (OPA 09). Primer PIC values were extremely high, ranging from 0.708 to 0.883 with an average of 0.758, except the PIC value of OPB 04 was measured significantly lower at 0.371.

Table 2: Primer; PIC value; number of amplified and polymorphic bands

Primer	Sequence (5'-3')	Length (bp)	Polymorphic information	Number of amplified	Number of polymorph	Percentage of

			content. (PIC)	bands	ic bands	Polymorphism band (%)
OPA 04	AATCGGGCTG	350 – 900	0.722	4	3	75
OPA 05	AGGGGTCTTG	600 – 1800	0.755	8	6	75
OPA 09	GGGTAACGCC	650 – 3500	0.812	11	9	81.9
OPA12	TCGGCGATAG	450 – 1900	0.782	7	4	57.1
OPB 04	GGACTGGAGT	950 – 1500	0.371	3	3	100
OPB 06	TGCTCTGCCC	650 – 3500	0.853	9	7	77.8
OPB 11	GTAGACCCGT	450 – 2800	0.805	8	7	87.5
OPB-13	TTCCCCCGCT	350 – 3200	0.758	6	5	83.3
OPB 20	GGACCCTTAC	250 – 750	0.714	4	3	75
OPC 02	GTGAGGCGTC	450 – 1400	0.747	4	4	100
OPC 05	GATGACCGCC	230 – 980	0.770	5	5	100
OPC 09	CTCACCGTCC	520 – 1150	0.708	4	2	50
OPC 12	TGTCATCCCC	450 – 3000	0.813	6	6	100
OPC 13	AAGCCTCGTC	380 – 1800	0.883	10	7	70
OPC 14	TGCGTGCTTG	300 - 1900	0.871	10	10	100
Overall				99	81	
Average			0.758	6.6	5.4	82.2

A.



B.

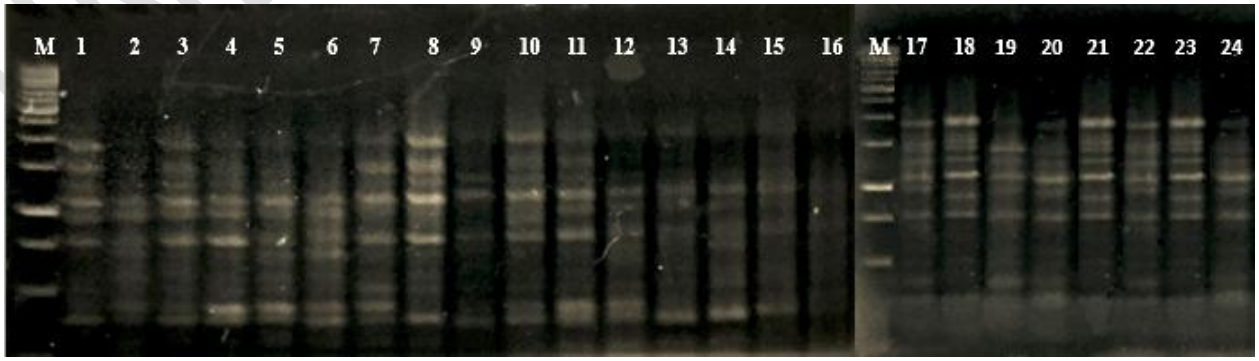


Figure 2: RAPD-PCR profile from *Cinnamomum cassia* Blume individuals, 1-24

Electrophoresis results of RAPD-PCR products corresponding to the ordinal numbers in Table 1. M: Marker 1kb (Thermo Scientist, USA). (A) Primer C02. (B) Primer C09.

Molecular Genetic Diversity of *Cinnamomum cassia* Blume species

The binary data set was used to calculate the pairwise Jaccard similarity index and build the related similarity matrix. The resulting matrix was used to create a dendrogram using the UPGMA method (Figure 3) in which the similarity coefficient value fluctuated between 0.67 to 0.91 and the phenotypes were separated into three main clusters with a 0.68 similarity level. Group I contained nine isolates of *Cinnamomum cassia* Blume species including TY5, VC5, VC2, VC4, VC3, VY5, VY1, VY2, and VY3 with the value of similarity coefficient in the range 0.726 to 0.855. The samples of group I were virtually collected in Van Chan and Van Yen, hence, the Cinnamon individuals belonging to these two regions have a high genetic similarity. Group II had only one isolates LY2 which proves that this cinnamon sample has the furthest genetic relationship compared to the other 23 cinnamon samples in this study. Group III contained fourteen isolates in which the similarity coefficient value was in the range of 0,715 – 0,91 and this group had the highest similarity coefficient. In addition, at the value in the similarity coefficient of 0.715, main group III was separated into three subgroups (III.1, III.2, III.3).

The genetical differencemay be due to effect of abiotic factors such as hydrographic connections, different climate, … or biotic factors such as seed dispersal [15-16].For cultivars, genetical difference also relates to genus sources and the purity of genus. The substantial degree of variance (similarity coefficient of 0.67) between LY1 and VY3 demonstrates they were introduced from different sources geographically isolated and with different environment in Yenbai. The accessions VY6 and YB1 shared a close genetic similarity of 0.91 must be the same source of the genetic material. The similarity coefficient indexes between accession fluctuated between 0.67 to 0.91 shown that the cinnamon samples collected in five regions of Luc Yen, Van Yen, Yen Binh, Van Chan and Tran Yen of Yen Bai province were quite diverse in genetic. Our results have several similarities with the results of Trung's research[2], the *Cinnamomum obtusifolium* Ness samples were collected in Thanh Hoa, Vietnam and detected the similarity coefficient through RAPD marker. Their results shown that all samples had high similarity coefficient between 0.67 to 0.97 and *Cinnamomum* populations in Vietnam were quite genetically diverse. The results attained are useful for categorizing and precisely identifying particular genetic resources that will be offered for conservation and breeding.

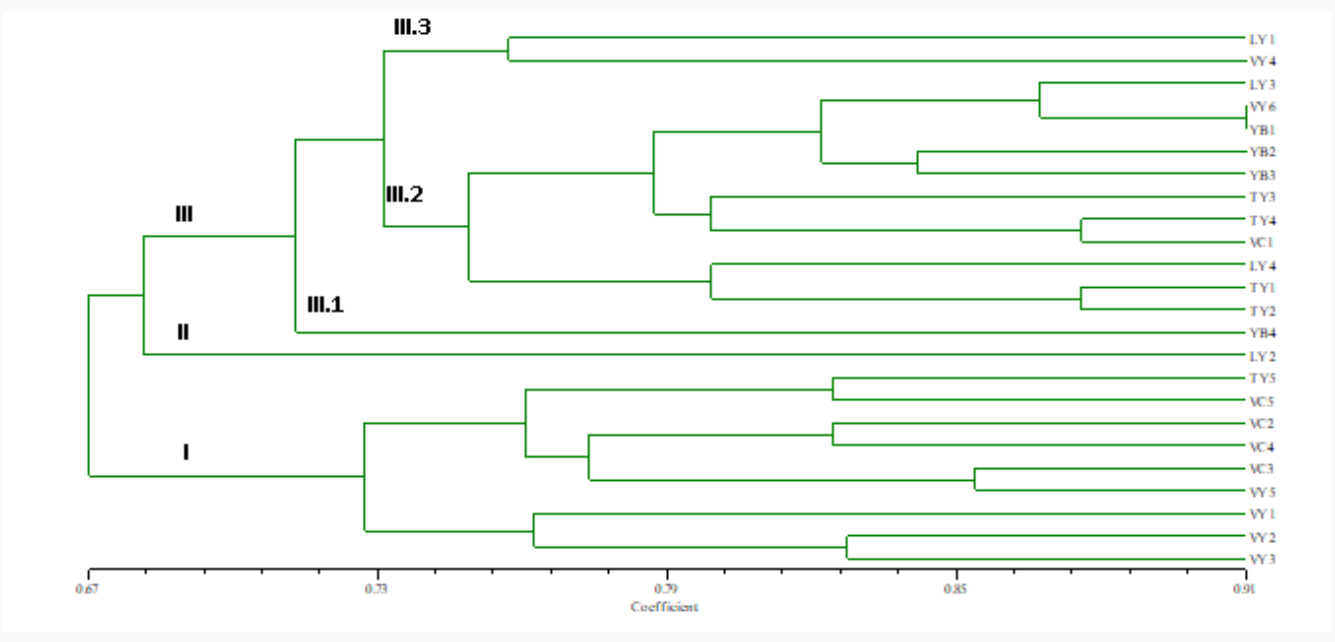


Figure 3. Phylogenetic tree constructedbased ongenetic similarity indexes from pairwise comparision of RAPD bands between accessions

CONCLUSION

This study was undertaken to evaluate the extent and range of genetic diversity ofCinnamomum cassia Blume belongs to Cinnamomum species in some mountainous areas in the Yenbai province, Vietnam. Accurate estimates of diversity are a prerequisite for growing and conserving tree genetic resources. Cinnamomum cassia Blume belongs to Cinnamomum species was introduced in Yenbai from multiple sources and possesses hight genetic diversity and variation and suggests that necessary to establish conservation plan, evaluation and selection of the best gene resources for commercial purposes

References

1. Nair KP, Nair KP 2021, Cassia and Cinnamon. *Minor Spices and Condiments: Global Economic Potential* 99-105.
2. Trung KH, Dung KT, Trung LC, Hoang NH, Luong HT 2017,Evaluation of genetic diversity of Thanh Hoa cinnamon species using RAPD marker. *Journal of Vietnam Science and Technology* **59**: (2).
3. Ravindran P, Nirmal-Babu K, Shylaja M (2003) *Cinnamon and cassia: the genus Cinnamomum*. CRC press.
4. Nguyen KT, Nguyen TQ,2019, Quantitative study of trans-cinnamic acid in Cinnamon by high performance liquid chromatography *TNU Journal of Science and Technology*194(01): 97 – 102.
5. Suriyagoda L, Mohotti AJ, Vidanarachchi JK, Kodithuwakku SP, Chathurika M, Bandaranayake PC, Hetherington AM, Beneragama CK 2021, “Ceylon cinnamon”: Much more than just a spice. *Plants, People, Planet***3**: (4) 319-336.
6. Zaidi SF, Aziz M, Muhammad JS, Kadowaki M 2015, Diverse pharmacological properties of Cinnamomum cassia: A review. *Pakistan journal of pharmaceutical sciences***28**: (4).

7. Ooi LS, Li Y, Kam S-L, Wang H, Wong EY, Ooi VE 2006, Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb Cinnamomum cassia Blume. *The American journal of Chinese medicine***34**: (03) 511-522.
8. Evans WC 2009,*Trease and Evans' pharmacognosy*. Elsevier Health Sciences.
9. Bansode VJ 2012, A review on pharmacological activities of Cinnamomum cassia Blume. *International Journal of Green Pharmacy (IJGP)***6**: (2).
10. Madhumati B 2014, Potential and application of molecular markers techniques for plant genome analysis. *Int J Pure App Biosci***2**: (1) 169-188.
11. Sudupak MA 2004, Inter and intra-species Inter Simple Sequence Repeat (ISSR) variations in the genus Cicer. *Euphytica***135**: 229-238.
12. Sandigawad A, Patil C 2011, Genetic diversity in Cinnamomum zeylanicum Blume.(Lauraceae) using random amplified polymorphic DNA (RAPD) markers. *African Journal of Biotechnology***10**: (19) 3682-3688.
13. Doyle J 1991, DNA protocols for plants. *Molecular techniques in taxonomy* 283-293.
14. Bhau B, Gogoi G, Baruah D, Ahmed R, Hazarika G, Borah B, Gogoi B, Sarmah D, Nath S, Wann S 2015, Development of an effective and efficient DNA isolation method for Cinnamomum species. *Food chemistry***188**: 264-270.
15. Linhart YB, Grant MC 1996, Evolutionary significance of local genetic differentiation in plants. *Annual review of ecology and systematics***27**: (1) 237-277.
16. Agler MT, Ruhe J, Kroll S, Morhenn C, Kim S-T, Weigel D, Kemen EM 2016, Microbial hub taxa link host and abiotic factors to plant microbiome variation. *PLoS biology***14**: (1) e1002352.