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Full Length Article

Diversity of Tea (*Camellia sinensis*) Grown in Vietnam based on Morphological Characteristics and Inter-primer Binding Sites (iPBS) Marker

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Abstract

Six iBPS primers and twenty-one morphological traits were used to assess the genetic diversity and relationships of 15 tea [*Camellia sinensis* (L.) O. Kuntze] accessions grown in Vietnam. The similarity index matrix generated from Dice coefficient (iPBS analysis) and the dissimilarity index matrix generated from Euclidian distance coefficient (morphological analysis) were used to build the respective un-weighted pair group method with arithmetic average (UPGMA) derived-dendrograms revealing the genetic relationships among the tested tea accessions. The iPBS analysis showed a slightly different result as compared to the result of morphological analysis which indicated that the group of Shan and Assam type were separate from the group of China type and the hybrids related to China type. Meanwhile, the iPBS analysis clustered 15 tea genotypes into two main groups; the tea genotypes of small leaved China, Assam-small leaved China hybrid and Shan-large leaved China hybrid type, with small leaf size, came together into one group; the other one was mainly nested by the tea genotypes of Shan, Assam and Shan-small leaved China type, with large leaf size. However, the iPBS analysis also shared the same basic agreements with the morphological analysis. All the tea genotypes, which originated from the same parents or the same type of crossing parental couple, revealed close relatedness. The results of morphological analysis are conformable with the conventional classification of tea taxa and give basic relative information among 15 tea genotypes to demonstrate particularly the result of molecular analysis. It could be concluded that iPBS is a useful DNA based-marker for evaluation of genetic diversity and relationships of tea.

Keywords: *Camellia sinensis*; Diversity; iPBS; Morphology; Tea

Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] is one of the most popular and lowest cost beverages in the world, and consumed by a wide range of age groups in all levels of society with more than three billion cups daily worldwide (Hick, 2009). Tea is not only considered to be a part of the huge beverage market, but also be of interest to functional foods markets (Hick, 2009). In most of the tea producing countries, tea has been a source of revenue and has contributed significantly to the local rural economies (Paul *et al.*, 1997).

The origin of the tea tree is considered as contributing around the Burma regions, from which tea has been spread to China, Indonesia and India (Rajkumar *et al*., 2010). Because of the prolonged dispersal among different geological regions together with the freely interbreed nature of it and crossing activities of humanity, tea becomes multiform on both morphological and molecular features (Tran, 2009). Therefore, taxonomy of tea has been still controversial for many years (Chen *et al.*, 2006). According to Yamamoto *et al.* (1997) subdivided tea into four varieties: small-leaved China tea (*C. sinensis* var. *microphylla*), large-leaved China tea (*C. sinensis* var. *macrophylla*), Shan tea (*C. sinensis* var. *shan*) and Assam tea (*C. sinensis* var. *assamica*). This taxonomic system has been used for tea classification in Vietnam (Tran, 2009). Sealy (1958), based on leaf and growth characteristics, classified tea as two distinct taxa, *C. sinensis* var. *sinensis* (China tea), and *C. sinensis* var. *assamica* (Masters) (Assam tea). This classification was revised by Wight (1962) who assigned specific ranks to China tea and Assam tea, of which he named the nomenclature *Camellia sinensis* (L.) as *C. sinensis* var. *sinensis* (L.), but named *C. sinensis* var. *assamica* (Masters) as *C. assamica* (Masters); in addition, he considered Planchon's *Thea lasiocalyx*, which equated to Cambod race (Barua, 1963), as a sub-species of *C. assamica* and named as *C. assamica* ssp. *lasiocalyx* (Planchon ex Watt). Later, Chang (1984) subdivided *C. sinensis* into three varieties, *C. sinensis* var. *sinensis*, *C. sinensis* var. *assamica*,

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and *C. sinensis* var. *pubilimba*, which equaled to *C. sinensis* var. *shan* mentioned by Cohen-Stuart (Pandolfi *et al*., 2009).

Tea is a cross-pollinated plant (Banerjee, 1992). Long-term allogamy makes it highly heterogeneous and consequently with broad genetic variation (Chen and Chen, 2012). Hybridization among few limited parents may lead to a narrow genetic base of tea clones. For that reason, it is important to select breeding parents with a distant relationship to sustain the genetic diversity of tea cultivars (Yao and Chen, 2012). Studying on genetic diversity and relationship of tea accessions may give useful information for a tea-breeding program.

Numerous studies to evaluate tea diversity have been conducted by using morphological markers (Vo, 2006; Tran, 2009; Rajkumar *et al*., 2010), biochemical markers (Magoma *et al*., 2000; Ramkumar *et al*., 2011), digital marker (Pandolfi *et al*., 2009) and DNA-based markers. Among them, DNA-based markers have been used more popularly. Genetic identity and relationship of tea has been determined using several DNA-based markers such as restriction fragment length polymorphism (RFLP) (Devarumath *et al*., 2002), random amplified polymorphic DNA (RAPD) (Wachira *et al*., 1997; Boonerjee *et al.*, 2013), amplified fragment length polymorphism (AFLP) (Paul *et al*., 1997; Mishra *et al*., 2009), simple sequence repeats (SSR) and inter-simple sequence repeats (ISSR) (Vo, 2006; Tran, 2009; Ji *et al.*, 2011). Nevertheless, as mentioned by many previous reports (not discussed particularly in this report), all of them are old markers, and each system has its own disadvantages.

Recently, Kalendar *et al*. (2010) developed a new universal method for DNA fingerprinting, which is based on the location of specific inter-primer binding sites of the long terminal repeat (LTR) retrotransposons, called as "Inter Primer Binding Sites" (iPBS) method. The iPBS technique has been applied to investigate the genetic relationships of *Saussurea esthonica* (Gailite and Rungis, 2012), *Psidium guajava* (Mehmood *et al*., 2013), *Myrica rubra* (Chen and Liu, 2014), etc. However, it seems new to genetic diversity analysis of tea.

In this study, we employed the morphological characteristics and iPBS marker as a molecular marker for identifying the genetic variation and revealing the genetic relationship among 15 tea accessions grown in Vietnam.

Materials and Methods

Plant Materials

Fifteen tea genotypes collected from Northern Mountainous Agricultural and Forestry Science Institute (NOMAFSI) (located at Phu Tho province) in Vietnam were used in the present study (Table 1). These included widely planted tea cultivars or new tea clones that had released by NOMAFSI. Based on the parental plants and morphological data, these genotypes were categorized into six types of tea, which consisted of 2 accessions of Shan tea, 2 accessions of Assam tea, 5 accessions of China tea and 6 accessions of hybrid tea. Out of them, there were two couples of hybrid genotypes originated from the same parents, of which one couple is PH8 and PH9 and the other one is LDP1 and LDP2.

Morphological Analysis

Twenty-one important quantitative and qualitative characteristics of stem, leaf, shoot, and flower were investigated from the currently harvested tea plants following the guidelines of International Plant Genetic Resources Institute (IPGRI, 1997) with some minor adjustments (Table 2). The selected leaves for characterizing were the 4th leaf of the flush shoot. The selected shoots for analysis were plucked at the position of 2/3 of the internode between the 2nd and the 3rd leaf counted from the 2nd leaf.

Molecular Analysis

DNA of 15 selected tea genotypes was separately extracted from the young leaves using the CTAB procedure of Doyle and Doyle (1990) with some modifications. The leaf tissues were ground in liquid nitrogen then mixed with 700 µL of fresh 2X CTAB buffer (2% CTAB, 1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* Tris HCl, pH 8.0) together with 2 µL of ß-mercaptoethanol and incubated at 65ºC for 120 min. The sample was then extracted with an equal volume of chloroform/isoamylalcohol (ratio of 24:1, respectively) and centrifuged at 21,910 x g for 5 min to get the aqueous phase, which was later added with 2 µL of RNase (10 mg/mL) before incubating at 37ºC for 30 min, after that followed by added with 50 µL of 10% CTAB (in 0.7 *M* NaCl). The procedure was repeated by adding with 700 µL of chloroform/isoamylalcohol (ratio of 24:1, respectively) and again centrifuged as described above. The collected aqueous phase was added to 500 mL of cold isopropanol and incubated at -20ºC for 15 min, and was then centrifuged at 21,910 x g for 20 min to precipitate DNA. The DNA pellet was washed with 1 mL of 70% ethanol and continued to spin at 21,910 x g for 30 min. This step was repeated with 90% ethanol. Afterward, the DNA pellet was dried by incubating at 37ºC for 20 min. Finally, the DNA pellet was dissolved in 50 µL of $T_{10}E_1$ buffer (10 m*M* Tris HCl, 1 m*M* EDTA, pH 8.0), and incubated at 37ºC for 1 h, then stored at -20ºC for the future experiment. The high molecular weight DNA was checked for quality and quantity using agarose gel (1%) electrophoresis and with a Spectrophotometer machine (Eppendorf BioPhotometer).

The iPBS analysis was done using the method of Chen and Liu (2014) with some modifications. The total volume of each PCR mixture was 20 µL, which consisted of 4.0 µL of DNA template (50 ng/µL), 4.0 µL of dNTPs (200 µ*M*), 0.2 µL of Taq polymerase (1 Ū), 2.0 µL of Taq reaction

buffer (1 X), 0.2 µL of $MgCl_2$ (0.5 m*M*), 2.0 µL of iPBS primer (2 pmoL/µL) and deionized water (topped up to 20 µL). The PCR reaction was performed in a Thermal Cycler (Eppendorf Mastercycler EP Gradient S) and consisted of initial denaturation at 95ºC for 3 min with 30 cycles of the following: denaturation at 95ºC for 15 s, annealing at 50ºC or 55ºC (depending on the primers) for 60 s and extension at 68ºC for 60 s, final extension was performed at 72ºC for 5 min and the reaction was ended at 4ºC. Sixteen iPBS primers were screened using an optimized reaction system with 15 selected tea accessions as DNA templates, among these 6 primers (Table 3) with high clarity and repeatability were selected for polymorphic assessment. Amplification products were separated by electrophoresis using 1.5% agarose gel in 1 X TBE buffer at 50 voltage for 60 min, then stained with ethidium bromide before examined. The gel was checked using a Gene Genius Bio-imaging System (SynGene). A GeneRuler 100 bp Plus DNA Ladder was used as a molecular size standard. For each primer, the PCR was repeated twice to confirm band pattern consistency.

Data Analysis

All the data were analyzed using NTSYS-pc v2.1 software (Rohlf, 2000). Morphological data presented in this report were the mean of the observed values with the standard deviations. The data were standardized before used for analysis. The SIMINT module was used to calculate the dissimilarity index matrix based on Euclidian distance coefficient (e_{jk}) (Romesburg, 2004) calculated from 21 morphological traits as below:

$$e_{jk} = \sqrt{\sum_{i=1}^{n} (X_{ij} - X_{jk})^2}$$

Where: e_{jk}: the Euclidian distance coefficient between the j^{th} object and the k^{th} object; X_{ij}: the value of the i^{th} attribute measured on the j^{th} object; X_{ik}: the value of the of the i^{th} attribute measured on the k^{th} object; n: numbers of attributes.

DNA bands were sized and scored by Phoretix 1D Pro software (TotalLab Ltd.), then carefully and manually checked. An electrophoretic band at the same migration location (assumed to represent a single locus) was recorded as 1, whereas no band was as 0, and an original matrix consisting of "0" and "1" recorded was generated. Only the fragments, which had a molecular weight range of 200-3000 bp and were visualized with medium or high intensity, were considered for data analysis; while the faint bands were ignored. Polymorphic Information Content (PIC) was calculated as: $PIC = 1-[f^2 + (1-f)^2]$, where "f" is frequency of the marker in the data set. For each primer, the PIC value was the mean of calculated PIC of all loci (Mehmood *et al*., 2013). The SIMGEND module was used to calculate the similarity index matrix based on Dice genetic similarity coefficient (Nei and Li, 1979) with the formula:

Dice genetic similarity coefficient = $2N_{ab}/(2N_{ab} + N_a + N_b)$; where, N_{ab}: number of common bands shared by the sample a and the sample b; N_a: number of bands only present in the sample a; N_b: number of bands only present in the sample b.

The Unweighted Pair Group Method with Arithmetic Average (UPGMA) in the SHAN module was used to build the dendrogram revealing the genetic relationships among 15 tested tea accessions for both morphology and molecular analysis. The principal component analysis (PCA) was also done based on Dice genetic similarity coefficient to reveal the relationships among 15 tested tea accessions. The cophenetic value matrix was calculated from the tree matrix of the iPBS analysis (produced by the SAHN clustering module) using the COPH module and the goodness fit of the UPGMA derived–dendrogram to the Dice similarity index matrix was checked using the MXCOMP module.

Results

The genetic diversity and relationships among 15 tested tea accessions was revealed in the UPGMA-derived dendrogram (Fig. 1A) generated from their morphological traits. The dissimilarity value ranged from 3.37, between the most similar genotypes, PH8 and PH9 (Shan-small leaved China hybrid type, and also known as offspring of the same parents), to 10.59, between the most distant genotypes, VN1 (small leaved China type) and PH11 (Assam type) (data not showed). At the dissimilarity value of about 7.18, all the tested tea accessions were divided into 2 principal clusters. The cluster C1 (Fig. 1A) mainly consisted of China genotypes and the hybrids related to China type (Assam-China hybrid and Shan-China hybrid), PH1 (Assam type) was also grouped in this cluster, while all the Shan **geno**types (PH12, PH14) and the other Assam genotype (PH11) came together into the cluster C2 (Fig. 1A). At the dissimilarity value of about 5.87, within the cluster C1 (Fig. 1A), two sub-groups were differentiated and two small sub-groups were formed within each sub-group. In the sub-group SuC1 (Fig. 1A), VN1, VN3 and Keoamtich were presented in one small sub-group forming a group of small-leaved China type, and the remaining small sub-group was nested by the crosses LDP1 and LDP2 (Assam-small leaved China type, and also known as offspring of the same parents), and PH10 (small leaved China type). Meanwhile, the sub-group SuC2 (Fig. 1A) included three types of tea genotype comprising Assam type, Shan-China hybrid type and Assam-China hybrid type; among these, two genotypes of Shan-large leaved China hybrid (DS13 and DS15) and one genotype of Assam (PH1) were grouped together into one small sub-group, and the rest small sub-group was clustered by two genotypes of Shan-small leaved China hybrid (PH8 and PH9) and their mother, Kimtuyen (Assam-small leaved China type) which was also identified as Small leaved China type (Vo, 2006).

Out of 16 iPBS primers screened, 6 primers which consistently produced well defined bands, were selected for

Table 1: Source and type of the fifteen tea accessions used in the present study

Name	Origin, acquisition place	Type*	Code
PH8	Crossing, Vietnam	Shan-small leaved China hybrid	Sh-SC.H
PH9	Crossing, Vietnam	Shan-small leaved China hybrid	Sh-SC.H
PH10	Imported, China	Small leaved China	SC
PH11	Imported, India	Assam	As
PH12	Selected, Vietnam	Shan	Sh
PH14	Selected, Vietnam	Shan	Sh
VN1	Imported, China	Small leaved China	SC
VN3	Imported, China	Small leaved China	SC
DS15	Crossing, Vietnam	Shan-large leaved China hybrid	Sh-LC.H
DS13	Crossing, Vietnam	Shan-large leaved China hybrid	Sh-LC.H
LDP1	Crossing, Vietnam	Assam- small leaved China hybrid	As-SC.H
LDP2	Crossing, Vietnam	Assam- small leaved China hybrid	As-SC.H
Keoamtich	Imported, China	Small leaved China	SC
Kimtuyen	Imported, Taiwan	Assam-small leaved China hybrid	As-SC.H
PH1	Imported, India	Assam	As

*The type of the tea accessions were identified following the taxonomic system of Cohen-Stuart (1919)

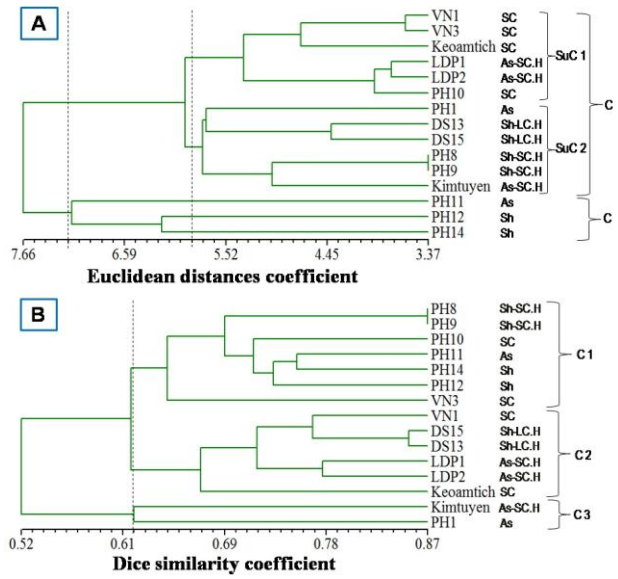

Fig. 1: Phylogenetic dendrogram of 15 tested tea accessions constructed using data from 21 morphological traits (A) and 6 iPBS primer markers (B)

the present study. An example of the polymorphisms detected among 15 tested tea accessions by primer 2224 is presented in Fig. 2. The number of bands, number of polymorphic bands, percentage of polymorphism and the mean of PIC value from these primers are shown in Table 4. From 15 tested tea accessions, total of 101 bands whose fragment size ranged from 230 to 3000 bp were observed, among which 96 were polymorphic bands (95.05%). Primer 2224 and 2238 produced the highest number of bands (22 bands for each) with 100% of polymorphic percentage, while primer 2389 produced the lowest number of bands (13 bands) with the lowest polymorphic percentage (84.62%). The mean of PIC value generated by 6 primers was 0.3; out of these, primer 2224 gave the highest PIC value (0.35),

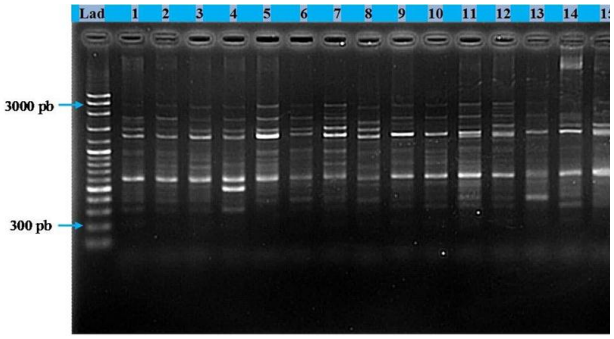

Fig. 2: DNA fingerprinting of 15 tested tea accessions based on iPBS marker using primer 2224. Lad = 100 pb plus ladder; lane 1-15: PH8, PH9, PH10, PH11, PH12, PH14, VN1, VN3, DS15, DS13, LDP1, LDP2, Keoamtich, Kimtuyen, PH1, respectively

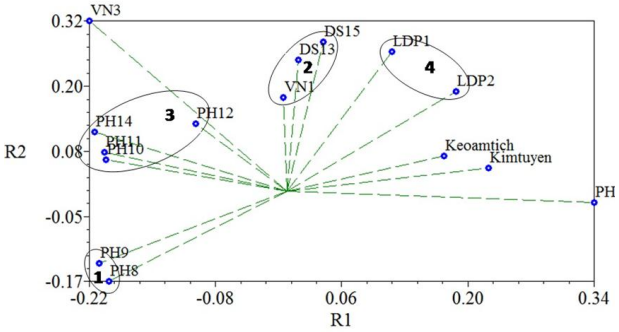

Fig. 3: Principal component analysis (PCA) of 15 tested tea accessions constructed using data from 6 iPBS primer markers

followed by primer 2238 and 2398 with 0.34 for each, and giving the lowest PIC value was primer 2389 (0.2).

The similarity index matrix (Table 5) generated from Dice similarity coefficient was used for UPGMA cluster analysis. The dendrogram for revealing the genetic relationships among 15 tea accessions based on Dice similarity coefficient is shown in Fig. 1B. The values of Dice similarity coefficient between 15 tested tea genotypes ranged from 0.394, the lowest value was found to be between PH14 (Shan type) and Kimtuyen (small leaved China type), to 0.868, the highest value was found to be between PH8 and PH9 (Shan-small leaved China hybrid type).

The UPGMA derived-dendrogram (Fig. 1B) generated from Dice similarity coefficient was seen not to separate clearly 15 tested tea accessions into main tea varieties. At the similarity coefficient value of about 0.618, all the tested tea genotypes were divided into 3 clusters of 7, 6 and 2, respectively, of which the small leaved Chine type appeared under all the three clusters. The cluster C1 (Fig. 1B) mainly contained the tea genotypes with large leaf size, wherein the tea genotypes of PH12, PH14 and PH11, with the leaf size

Table 2: Morphological characteristics of the fifteen tested tea genotypes

Morphological traits	PH8	PH9	PH10	PH11	PH12	PH14	VN1	VN3	DS15	DS13	LDP1	LDP2	Keoamtich	Kim tuyen	PH1
Tree type	3	3	1	2	2	2	1	1	3	3	1	1	1	1	2
Leaf length** (cm)	8.48 (0.89)*	8.43 (0.84)	6.56 (0.70)	11.28 (1.05)	11.88 (0.66)	8.27 (1.29)	5.16 (0.73)	5.82 (0.48)	8.48 (1.23)	6.73 (0.74)	7.04 (0.86)	7.08 (0.87)	6.82 (0.61)	6.29 (0.55)	7.07 (0.83)
Leaf breadth** (cm)	3.44 (0.36)	4.08 (0.32)	2.48 (0.27)	4.55 (0.51)	5.03 (0.28)	3.39 (0.64)	2.37 (0.31)	2.97 (0.32)	3.07 (0.44)	3.02 (0.36)	3.07 (0.46)	3.05 (0.35)	2.98 (0.21)	3.13 (0.21)	3.42 (0.42)
Leaf length/breadth ratio**	2.46 (0.13)	2.06 (0.12)	2.65 (0.20)	2.48 (0.20)	2.36 (0.09)	2.44 (0.52)	2.18 (0.19)	1.96 (0.14)	2.76 (0.22)	2.23 (0.14)	2.29 (0.21)	2.32 (0.15)	2.29 (0.11)	2.01 (0.13)	2.07 (0.20)
Leaf area** (cm^2)	29.17 (5.86)	34.39 (5.75)	16.27 (3.35)	51.32 (9.47)	59.76 (6.23)	28.04 (8.45)	12.23 (3.26)	17.29 (3.16)	26.03 (7.59)	20.32 (4.51)	21.61 (5.72)	21.59 (4.82)	20.32 (3.20)	19.69 (2.84)	24.18 (5.30)
Leaf shape	4	4	2	4	2	3	2	2	2	2	4	3	4	4	1
Leaf color	1	1	3	4	3	5	2	1	3	3	3	3	1	1	1
Length of the leaf petiole** (cm)	0.23 (0.06)	0.26 (0.05)	0.21 (0.04)	0.45 (0.07)	0.34 (0.05)	0.23 (0.06)	0.24 (0.05)	0.37 (0.05)	0.33 (0.04)	0.31 (0.05)	0.23 (0.05)	0.24 (0.07)	0.41 (0.06)	0.29 (0.05)	0.33 (0.06)
Length from leaf petiole to the first serration** (cm)	2.13 (0.33)	1.80 (0.19)	0.84 (0.20)	1.87 (0.28)	1.49 (0.33)	1.72 (0.24)	1.09 (0.11)	1.00 (0.07)	1.47 (0.19)	1.08 (0.10)	1.01 (0.26)	1.06 (0.21)	1.65 (0.19)	1.06 (0.11)	1.62 (0.23)
Serrula form of the leaf	2	2	2	1	3	1	2	2	1	3	1	3	3	2	2
Number of pair of main vein on the leaf surface**	8.87 (1.48)	8.73 (1.05)	6.93 (0.96)	10.41 (1.47)	11.73 (1.28)	9.00 (1.18)	5.44 (1.06)	5.87 (0.73)	9.71 (1.00)	8.82 (1.19)	7.15 (1.10)	6.96 (1.04)	5.73 (0.98)	8.07 (0.66)	8.22 (0.98)
Leaf base shape	1	1	2	2	1	2	1	1	1	1	2	2	1	1	1
Leaf apex shape	1	1	1	2	2	2	1	1	1	1	1	2	1	1	1
Color of the shoot	5	5	3	1	3	5	5	1	1	1	4	5	5	1	1
Pubescence density on bud	3	2	3	1	3	2	3	3	3	3	2	2	3	3	1
Pubescence density on lower surface of the 1st leaf	2	2	3	1	3	3	3	3	2	2	2	2	4	2	1
Length of the shoot** (cm)	6.54 (0.89)	6.21 (1.03)	5.11 (1.02)	5.93 (1.81)	5.95 (0.79)	6.72 (1.31)	4.00 (0.76)	5.06 (0.82)	4.70 (1.06)	5.20 (0.76)	5.93 (0.58)	5.98 (0.80)	4.22 (0.58)	6.47 (0.73)	5.94 (1.08)
Mean of fresh shoot weight (g/shoot)	0.62	0.55	0.56	1.24	1.16	1.08	0.54	0.54	0.48	0.53	0.61	0.66	0.53	0.60	1.07
Color of the flower	2	4	2	3	1	1	2	1	2	2	3	2	2	4	2
Position of style splitting	1	1	3	2	1	1	3	3	3	1	3	3	1	2	3
Position of stigma in relation to stamen	1	1	1	3	1	1	1	1	2	2	1	1	1	1	1

*The deviation of each morphological trait was shown in the bracket

**The quality traits were code as below:

Leaf shape
Ovate 1
Oblong 2
Lanceolate 3
Elliptic 4

Leaf base
Rounded 1
Acute 2

Tree type
Shrub 1
Arbor 2
Semi-arbor 3

Splitting of style
Ascending 1
Geniculate 2
Terminal 3

Leaf serrula form
Serrurate 1
Blunt serrulate 2
Biserrurate 3

Pubescence density on the bud
Glabrous to rare 1
Light pubescent 2 indumentums
Dense pubescent layer 3

Color of the flower
White 1
Bluish white 2
Yellowish whit 3
Milky 4

Pubescence density on lower surface of the 1st leaf
Glabrous leaves with only hair on the mid-rib 1
Pubescence extends about half-way to the margin 2
Leaves with entire under surface of lamina pubescent 3
Dense pubescent indumentums 4

Leaf apex shape
Acute 1
Acuminate 2

Position of stigma in relation to stamen
Extrose 1
Co-planar 2
Introse 3

Color of the leaf and the shoot
Green 1
Dark green 2
Light green 3
Yellowish green 4
Purplish green 5

of 8.27–11.88 × 3.39–5.03 cm (Table 2), were grouped in one single sub-group, while PH8 and PH9, with the leaf size of 8.43–8.44 × 3.44–4.08 cm (Table 2), came together in the other single sub-group. Surprisingly, VN3 and PH10, with the small leaf size of 5.16–6.56 × 2.48–2.97 cm (Table 2), were also presented in this cluster, but they distantly placed from each other and outliers from the other sub-group. The cluster C2 (Fig. 1B) included all the tea genotypes of small leaf size, which consisted of four genotypes of the hybrid tea and two genotypes of the small leaved China tea. Among them, only Keoamtich was appeared alone, while the same parental crosses LDP1 and LDP2 were presented in one sub-group, and the single cluster consisted of DS13 and DS15 together with VN1 were presented in the other sub-group. It was known that only Keoamtich and VN1 are original small leaved China type, with the leaf size of 5.16–6.82 × 2.37–2.98 cm, meanwhile LDP1, LDP2, DS13 and DS15 are all hybrid type with their leaf size of 6.73–8.48 × 3.02–3.07 cm (Table 2). Those interprets why the genotypes of LDP1, LDP2, DS13 and DS15 were grouped together with Keoamtich and VN1 in the same cluster (C2-Fig. 1B), and

Table 3: Six iPBS primers for the present study

Primers Code	Primer sequences	Annealing temperature (ºC)
2389	5'-ACATCCTTCCCA-3'	50
2373	5'-GAACTTGCTCCGATGCCA-3'	
2398	5'-GAACCCTTGCCGATACCA-3'	
2238	5'-ACCTAGCTCATGATGCCA-3'	55
2077	5'-CTCACGATGCCA-3'	
2224	5'-ATCCTGGCAATGGAACCA-3'	

Table 4: Analysis of polymorphisms obtained with iPBS primers in 15 tested tea accessions

Primers	Number of bands	Polymorphic bands	Percentage of polymorphism (%)	Mean PIC value	Fragment size (bp)
2398	18	17	94.44	0.34	362-3000
2389	13	11	84.62	0.20	284-1674
2373	18	16	88.89	0.27	230-2231
2238	19	19	100.00	0.34	283-2895
2224	19	19	100.00	0.35	314-2956
2077	14	14	100.00	0.30	256-2061
Total: 6	Total: 101	Total: 96	Average: 95.05	Average: 0.3	

Table 5: Similarity index matrix of Dice similarity coefficient

	PH8	PH9	PH10	PH11	PH12	PH14	VN1	VN3	DS15	DS13	LDP1	LDP2	Keoamtich	Kimtuyen	PH1
PH8	1.000														
PH9	0.868	1.000													
PH10	0.709	0.718	1.000												
PH11	0.736	0.744	0.732	1.000											
PH12	0.636	0.690	0.723	0.725	1.000										
PH14	0.658	0.667	0.703	0.756	0.747	1.000									
VN1	0.588	0.643	0.700	0.705	0.742	0.650	1.000								
VN3	0.562	0.591	0.691	0.674	0.667	0.691	0.622	1.000							
DS15	0.575	0.602	0.607	0.701	0.694	0.629	0.800	0.626	1.000						
DS13	0.615	0.622	0.605	0.723	0.758	0.651	0.739	0.646	0.852	1.000					
LDP1	0.557	0.542	0.609	0.620	0.673	0.609	0.694	0.608	0.710	0.731	1.000				
LDP2	0.568	0.553	0.578	0.633	0.626	0.511	0.708	0.560	0.743	0.745	0.778	1.000			
Keoamtich	0.554	0.561	0.539	0.581	0.598	0.513	0.691	0.523	0.624	0.644	0.667	0.745	1.000		
Kimtuyen	0.421	0.453	0.423	0.456	0.550	0.394	0.546	0.444	0.558	0.627	0.562	0.621	0.640	1.000	
PH1	0.472	0.455	0.429	0.457	0.559	0.405	0.578	0.426	0.586	0.542	0.628	0.660	0.659	0.617	1.000

from that they revealed a close relationship to the small leaved China type. In the cluster C3 (Fig. 1B), PH1 and Kimtuyen were grouped in the same cluster showing an opposite result to the morphological analysis (PH1 and Kimtuyen were out grouped).

To confirm the results of UPGMA analysis, Principal Component Analysis (PCA) (Fig. 3) was performed based on 101 principal components of the iPBS analysis with the sum of eigenvalue was 96% (data not showed). The goodness of fit of the UPGMA derived-dendrogram to the Dice similarity coefficient matrix was also checked. The matrix correlation (*r*) equaled to 0.7912, which showed poor fit of the dendrogram to the Dice similarity index matrix.

Discussion

The classification of tea was initially proposed by Sealy (1958) based on leaf characteristics. Later, Wight (1962) revised this classification on the basic morphological characters such as leaf size, leaf shape, length of the pistil and flower sizes. The morphological analysis, which was performed basing on 21 morphological traits, grouped 15 tested tea accessions into two principal clusters, of which the China type and the hybrids related to the China type were separate from the Shan and Assam type. This result is in accordance with the conventional classification of tea taxa, and similar to the study of genetic diversity of teas grown in Vietnam based on leaf characters of Pandolfi *et al.* (2009).

The iPBS marker is developed basing on the location of specific inter-primer binding sites of the long terminal repeat (LTR) retrotransposons (Kalendar *et al*., 2010). Retrotransposons that are considered as a class of repetitive and mobile sequences are ubiquitous and abundant components of virtually all known eukaryotic genomes (Voytas *et al*., 1992). They can constitute more than half of the repetitive DNA in higher plants (Schnable *et al*., 2009), and to be dynamic genome components with their ability to integrate new copies and facilitate intra-chromosomal recombination (Kalendar *et al.*, 2000). Because of dispersed and ubiquitous transposable elements, the "copy and paste"

life cycle of replicative transposition of the LTR retrotransposons leads to new genome insertions without excision of the original element, so that they are considered well suited as molecular markers (Kalendar *et al.* 2010). From 15 tested tea accessions, 6 iPBS primers produced total of 101 bands, among which 96 were polymorphic bands occupying 95.05% of total number of produced bands. These indicated that iPBS marker is capable for detecting substantial numbers of polymorphic loci on tea with a relatively small number of primers (6 primers). This result is similar to the report of Mehmood *et al.* (2013), which showed that 6 iPBS primers generated 113 scorable bands on Guava (*Psidium guajava* L.), out of these 95 were polymorphic bands (84.07%).

The molecular analysis showed that the values of Dice similarity coefficient between 15 tested tea genotypes ranged from 0.394 to 0.868. This result is different from the study of genetic diversity of teas grown in Vietnam using ISSR marker (Vo, 2006) and SSR markers (Tran, 2009), which showed a large variation of the similarity values among the tested tea samples, ranging from 0.09 to 1.00 and 0.00 to 0.83, respectively. Using very large number of tea samples (77 tea genotypes for Vo and 96 tea genotypes for Tran) might be a reason for this difference. However, it is moderately similar to the study of Wachira *et al.* (1997) (using RAPD marker), in which the similarity values among the tested tea samples was 0.33 – 0.74.

The UPGMA derived-dendrogram (Fig. 1B) generated from Dice similarity coefficient was seen not to separate clearly 15 tested tea accessions into main tea varieties. This result is consistent with the study of Vo (2006) and Tran (2009). The appearance of the small leaved China genotypes under all the three clusters indicated that gene introgression occurred between China, Shan and Assam type because of the crosspollination during long-term cultivation and improvement. Vo (2006) suggested that it is difficult to find the true archetypal China (small- and large-leaved), Shan and Assam varieties. However, the dendrogram also showed that 15 tested tea genotypes were clustered into two main groups; in which, small leaved China genotypes and the group of Assam-small leaved China hybrid and Shan-large leaved China hybrid type, which had small leaf size, came together into one group (C2-Fig. 1B), and the genotypes of Shan and Assam tea together with Shan-small leaved China type, which had large leaf size, grouped into the other one (C1-Fig. 1B). The leaf size of the tea genotypes was characterized following Sharma and Venkataramani (1974), and it significantly contributed to explain this clustering. These result moderately met with the originative classification of the tested tea genotypes based on morphological characteristics. The judgment which of relationship and the isolation of cultivar types can be correlated with leaf size characters (Rajkumar *et al.*, 2010) could be used to explain for the appearance of all tea genotypes in the cluster C1 and C2 (Fig. 1B). Furthermore, one small group C3 (Fig. 1B) was also seen in the dendrogram with the appearance of PH1 and Kimtuyen. The reason why PH1 and Kimtuyen, which are different type of tea from each other (PH1: Assam tea, Kimtuyen: small leaved China tea), were grouped in the same cluster in the molecular analysis might be explained by that Kimtuyen was crossed between Assam type and small leaved China type, therefore its genetic pattern may contain genetic type of Assam tea, hence the molecular analysis showed this genotype has close relationship to PH1. The study of genetic diversity of teas grown in Vietnam of Vo (2006) and Pandolfi *et al.* (2009) also showed Kimtuyen and PH1 were nested in the same cluster.

The result of PCA from the molecular analysis showed poor fit of the UPGMA derived-dendrogram to the Dice similarity index matrix. There were several disagreements between the PCA and the dendrogram, for example, Kimtuyen was grouped together with PH1 in one group in the cluster analysis (C3) (Fig. 1B), but in the PCA (Fig. 3) this genotype disposed more closely to Keoamtich than to PH1 showing more conformable with the reputed taxonomy of Kimtuyen and Keoamtich; or PH10 distantly placed to the tea genotypes of PH11, PH12 and PH14 in the cluster analysis (C1) (Fig. 1B), but this tea genotype distributed more closely to PH11 and PH14 than PH12 did in the PCA (Group 3) (Fig. 3). However, the PCA also showed many similarities to the cluster analysis, e.g., all the Shan tea (PH12, PH14) and Assam tea (PH11) were distributed to one group (Group 3) (Fig. 3); or the two couples of tea genotype (PH8 and PH9, and LDP1 and LDP2), which were originated from the same parents for each, were also seen separately gathering in one group (Group 1) for PH8 and PH9 (Fig. 3), and (Group 4) for LDP1 and LDP2 (Fig. 3); another group showed the same to the UPGMA analysis was Group 2 (Fig. 3) with the appearance of DS13, DS15 and VN1.

Conclusion

The iPBS analysis showed a slight different result as compared to the result of morphological analysis. However, they also shared many basic agreements. Most of the morphological characters showed continuous variation and high plasticity as a result of aging and environmental effects (Lai *et al.*, 2001). Therefore, the result of molecular analysis, which is free from the environmental influence (Vo, 2006), is more consistent than that of the morphological analysis. It could be pointed out that the result of morphological analysis gave basic relative information among 15 tested tea genotypes to demonstrate particularly the result of molecular analysis. Both the morphology and molecular analysis confirmed the closeness of many tested tea accessions. It could conclude that iPBS is an useful DNA based-marker for evaluation of genetic diversity and relationships of tea.

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