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Genetic Variation of Selected *Camellia sinensis* (Cultivated Tea) Varieties in Malaysia Based on Random Amplified Microsatellite (RAMs) Markers

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ABSTRACT

Studies on the genetic variation among *Camellia sinensis* L. varieties (cultivated tea) in Malaysia were conducted by using RAMs markers. Six varieties were selected from Sungai Palas Boh Estate, Cameron Highlands and nine varieties were selected from Bukit Cheeding Boh Estate, Banting. These tea varieties were classified as resistant, intermediate, or susceptible varieties based on the level of infestation by the mosquito bug, *Helopeltis theivora*. DNA was extracted from the leaves of 225 individuals belonging to different varieties from the two populations. Four RAMs primers were used to evaluate the genetic variation in 15 varieties of tea. Distances were calculated based on Nei and Li's (1979) similarity coefficients using the data from the RAMs markers. A cluster analysis employing UPGMA was done and the dendrogram grouped the tea varieties into two clusters with intermediate variety grouping and the resistant (the first cluster) or susceptible (the second cluster) varieties. The first cluster consisted of all the varieties from Cameron Highlands, except for BC223 (resistant) and 63/14 (resistant) from the Banting population, while the other clusters consisted of all the varieties from the Banting population, except for BC196 (resistant) from the Cameron Highlands population. The dendrogram showed that the genetic differences were based on the populations' geographical distributions and partially based on their resistance towards attack by *H. theivora*.

Keywords: Genetic variation, tea, RAMs, resistant, susceptible varieties

INTRODUCTION

Tea, *Camellia sinensis,* is a beverage crop native to South East Asia, and has been introduced into many other countries (Wachira *et al*., 2000). The genus *Camellia* is composed of over 80 taxa (Sealy, 1958), of which only one, *C. sinensis* L. (O. Kuntz), is frequently used commercially as a source of beverage tea (Wachira *et al*., 1997). Two varieties of tea, the *assamica* and the *sinensis*, which differ in their morpho-anatomical, chemical, and genetic points of view are now fully recognized. Despite its agronomic importance, tea is still characterized and selected using environmentally and ontogenetically dependant morpho-anatomical traits (Green, 1971). However, it has been argued that these may not reflect the true level of genetic differentiation as most are subjected to large environmental effects (Wachira *et al*., 1997).

Although the mosquito bug, *Helopeltis theivora*, is recognized as a leaf-destroying

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pest of tea in Malaysia, little is known about the genetic background of this particular insect and its host plant, tea. The lack of information has led to the loss of genetic variability and allelic differences of the tea varieties planted in Malaysia. Moreover, the number of molecular work done on *H. theivora* is still limited; however, some information regarding molecular study on tea is available. Knowledge of genetic diversity among the available tea varieties is important as it will have to be preserved and characterized for future breeding and crop improvement programmes that constitute the fundamental support structure for the tea industry (Balasaravanan *et al*., 2003). Studies have also been carried out to examine the genetic diversity of tea as its diversity has suffered severe erosion over the years because of intensive selection and breeding for desirable agronomic traits. Balasaravanan *et al*. (2003) studied the genetic diversity among South Indian tea germplasm (*C. sinensis, C. assamica, C. assamica* spp. Lasiocalyx) using polymorphic microsatellite markers of *C. sinensis*, but the study focused only on the allelic differences of clones from different regions. RAPD and AFLP markers have intensively been used to develop genetic linkage maps of *C. sinensis* (Christine *et al*., 2000). Meanwhile, studies using Random Amplified Polymorphic DNA (RAPD) and organelle specific polymerase chain reactions were used to establish the affinities for the cultivated tea and its wild relatives (Francis *et al*., 1997). In this study, the RAMs primers were used. These markers are alternatively known as ISSR (Inter Simple Sequence Repeats). It uses simple sequence repeats anchored at the 5' end by a short arbitrary sequence. The RAMs primers are highly polymorphic and dominant in inheritance (Wang *et al*., 2009). The goals of this study were to amplify bands which were used to investigate the level of genetic variation present in the tea variety in Malaysia and the relationships among the resistant and susceptible varieties of tea to the mosquito bug (*H. theivora*) in the highlands and lowlands of Malaysia using random amplified microsatellites (RAMs) as molecular genetic markers.

MATERIALS AND METHODS

Materials

The leaf samples of different tea *C. sinensis* (L) varieties were collected from two populations, namely Bukit Cheeding Boh Estate, Banting, Selangor, Malaysia (lowland tea) and Sungai Palas Boh Estate, Cameron Highlands, Pahang, Malaysia (highland tea). Six varieties were selected from the Cameron Highlands population and there were nine varieties from the Banting population. They were differentiated into being resistant, intermediate, and susceptible varieties of tea (Table 1), based on the infestation values of *H. theivora* on them (unpublished data). The means for all the resistant scores were analyzed using the Duncan LSD. Consequently, fifteen individuals from each of the varieties from the Banting and Cameron Highlands populations were analyzed.

DNA Extraction and Amplification

DNA of individuals belonging to the different varieties of tea was isolated using the protocol of Doyle and Doyle (1987) with some modifications. The PCR was carried out in a 10µl total reaction mixture volume consisting of 20 ng of genomic DNA, 1X PCR buffer (Promega), 250 µM of each dNTPs (Promega), 0.5 µM of primer, 1.5 units of Taq polymerase (Promega), 2.0mM of $MgCl_2$ and topped up with deionized distilled water to 10 µl. The mixture was overlaid with 15 µl mineral oil. The PCR amplifications were carried out in a thermocycler (T3 Biometra) following the method used by Williams *et al*. (1990) with minor modifications done to the thermal cycles, as follows: 96ºC for 3 min; 40 cycles of 96ºC for 10 s, an optimal annealing temperature of each primer for 10 s, 72ºC for 30 s. A final extension step of 72ºC for 5 minutes was included after 40 cycles of amplification. Twenty RAMs primers, designed by Kumar (2003) for mungbeans and Hoh *et al*. (2004) for river catfish, were screened to test for the amplifications using the protocol as stated above. For the primers that produced too many bands or complex banding patterns, the annealing

TABLE 1
Sampling sites, and list of tea varieties and their types of resistance level to mosquito bug *H. theivora's* attack

Sampling sites	Resistant	Intermediate	Susceptible
Cameron Highlands	BC 196	AT 53	BC 1248a
	BC 664	TRI 2024	TV 9
	BC 223	66/3	65/6
Banting	63/14	65/4	BC 1248
	63/12	65/16	63/4

temperature was increased by 5°C. Nonetheless, the primers that did not produce distinct banding patterns were eliminated from the study. With some combinations of primers and genomic DNA template, a non-discrete range of amplification products that appeared as a 'smear' visualized on a gel could be converted to discretely sized bands by reducing the concentration of either the polymerase or the genomic DNA (Williams *et al*., 1990). The amplification products were analyzed by electrophoresis on a 2.0% (w/v) horizontal agarose gel in 1 x TBE buffer at 78 V for 1 to 1½ hours, depending on the size of the amplified fragments from each primer. A 100bp ladder (Promega) was used as a molecular size standard. The PCR products were detected by staining the agarose gel in ethidium bromide (10 μg/μl) and subsequently visualizing the gel over UV light. The gel was photographed and documented using an Alpha®Imager 2200 (Alpha Innotech, USA) system.

Statistical Analysis

The RAMs banding profiles were visually scored for all the DNA samples and for each primer. The recording of the data was according to the presence/absence criterion (1= presence of band; 0 = no band). It is worth highlighting that faintly stained bands that were not clearly resolved were not considered in the data collection. Similarity coefficients were calculated across all the possible pairwise comparisons of individuals, both within and among populations, using the following formula:

$$S_{xy}=2n_{xy} / n_x + n_y$$

where n_{xy} is the number of common bands shown in both individuals x and y, and n_x and n_y are the total numbers of bands observed in individuals x and y respectively (Nei and Li, 1979). The data obtained were used to compile pairwise distance matrices based on the similarity coefficient of Nei and Li (1979), using the RAPDistance version 1.04 software (Armstrong *et al*., 1995). As a means of providing a visual representation of the genetic relationships, a dendrogram was constructed based on the distance values derived from $1\text{-}S_{xy}$ between pairs of individuals within and between populations. Then the dendrogram was constructed using the unweighted pair group method with arithmetic averaging (UPGMA) employing the SAHN (Sequential, agglomerative, hierarchical, and nested clustering) programme from NTSYS-pc version 1.6 (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 1993). The UPGMA method defines the intercluster distance as the average of all the pairwise distances for members of different clusters.

RESULTS

After initially screening 20 RAMs primers, four primers (Table 2) were identified to be informative for the purpose of resolving genetic marker differences, within and among the varieties of tea. An example of the polymorphisms detected among some test samples by primer BP-05 is shown in *Fig. 1*. From the 15 varieties of tea studied, a total of 63 bands or DNA markers were generated using the four primers. The overall data showed that the largest fragment was 1,000bp, while the

TABLE 2
The optimized conditions for each of the RAMs primers

Primer code	Sequence (5' to 3')	$MgCl_2$ concentration	Annealing temperature	DNA volume
BP-05	NNN YYB MBM $B(AG)_6$	3.0mM	51ºC	20ng
BP-08	KKY HYH YHY $(GTT)_5$	2.5mM	51ºC	20ng
BP-11	KKY HYH Y $(CAG)_5$	2.0mM	48ºC	20ng
BP-13	KKB SBS BSB $(CT)_6$	2.0mM	51ºC	20ng

Note: K=G/T; N=A/C/G/T; H=A/C/T; Y=T/C; B=C/G/T; M=A/C; S=C/G

smallest fragment was 200bp in size. Table 3 shows the number of RAMs bands generated by the four primers in each of the 15 varieties of tea studied. The total number of polymorphic bands was 57 out of the 63 reproducible bands. In particular, primers BP-05 and BP-08 showed the highest percentage of polymorphic bands (100.0%), followed by BP-13 and BP-11 with 89.5% and 63.6%, respectively. Meanwhile, BC 196 (85.7% of polymorphic bands) had the highest number of polymorphic bands as compared to the other varieties, variety 65/4 showed the lowest number of polymorphic bands with 68.3% of polymorphic bands.

The matrixes of distances between the 15 varieties of teas are shown in Table 4. The highest distance value between varieties was found to be between variety TRI 2024 (D) and variety 66/3 (J), which were from Cameron Highlands and Banting, respectively, (0.7092) while the lowest distance value was found to be between variety 65/6 (M) and variety BC 1248 (N), which were from the same population in Banting (0.5496). This finding showed that the genetic differences were based on the populations' geographical distributions. In the clustering analysis, two major groups were formed; however, these were not conclusively

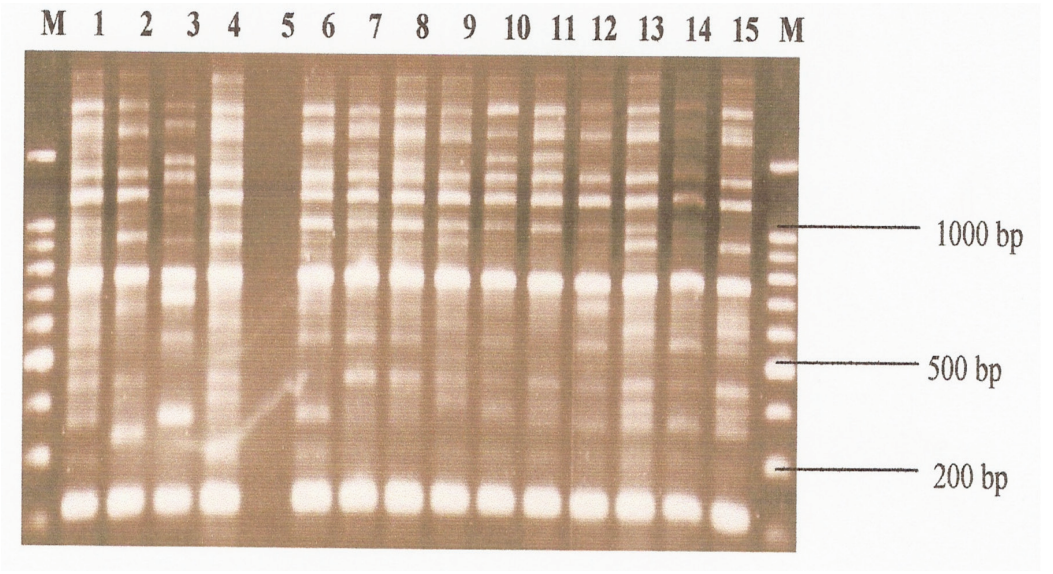

Fig. 1: RAMs profile of C. sinensis *variety AT 53 generated using primer BP-05. PCR product was run on 2% agarose gel using 1X TBE buffer. Lane M: 100bp size marker (Promega); Lane number 1-15: Different individuals of variety AT 53*

TABLE 3
The number of RAMs bands generated by four primers in each of the 15 tea varieties

Primer	Total reproducible bands	Total polymorphic bands	Number of polymorphic bands according to varieties of tea														% of polymorphic bands
			A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
BP-05	18	18	18	18	17	18	18	18	18	18	17	18	18	18	18	18	17	100.0
BP-08	15	15	14	14	14	15	13	15	13	14	14	14	12	15	15	14	14	100.0
BP-11	11	7	5	2	5	3	4	5	6	4	5	3	2	4	7	4	4	63.6
BP-13	19	17	17	15	16	14	13	12	10	11	15	13	11	11	13	14	14	89.5
Total	63	57	54	49	52	50	48	50	47	47	51	48	43	48	53	50	49	88.3
% polymorphism			85.7	77.8	82.5	79.4	76.2	79.4	74.6	74.6	81.0	76.2	68.3	76.2	84.1	79.4	77.8	

Varieties:

A=BC 196
B=BC 664
C=AT 53
D=TRI 2024
E=BC 1248a
F=TV 9
G=BC 223
H=63/14
I=63/12
J=66/3
K=65/4
L=65/16
M=65/6
N=BC 1248
O=63/4

TABLE 4
Distances based on Nei and Li's similarity coefficients among 15 varieties of tea

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A	-														
B	0.5728	-													
C	0.6346	0.6032	-												
D	0.6715	0.6370	0.6244	-											
E	0.6615	0.6322	0.6305	0.6388	-										
F	0.6387	0.6106	0.6169	0.6385	0.6018	-									
G	0.6700	0.6244	0.6246	0.6441	0.6304	0.6086	-								
H	0.6213	0.5763	0.5816	0.6171	0.5975	0.5716	0.5754	-							
I	0.6210	0.5900	0.6196	0.6459	0.6155	0.5968	0.6142	0.5735	-						
J	0.6621	0.6645	0.6932	0.7092	0.6993	0.6565	0.6618	0.6379	0.6207	-					
K	0.6670	0.6620	0.6887	0.7068	0.6667	0.6571	0.6721	0.6604	0.6101	0.6610	-				
L	0.6450	0.6372	0.6613	0.6741	0.6786	0.6595	0.6549	0.6444	0.6212	0.6537	0.6441	-			
M	0.6269	0.6174	0.6557	0.6747	0.6627	0.6570	0.6543	0.6278	0.5967	0.6421	0.6528	0.6125	-		
N	0.6369	0.6390	0.6754	0.6972	0.6755	0.6634	0.6712	0.6510	0.6081	0.6308	0.6283	0.6264	0.5496	-	
O	0.5912	0.5919	0.6125	0.6369	0.6210	0.6116	0.6185	0.5848	0.5569	0.6075	0.6082	0.5712	0.5608	0.5687	-

Cameron Highlands population:

A=BC 196	D=TRI 2024	
B=BC 664	E=BC 1248a	
C=AT 53	F=TV 9	

Banting population:

G=BC 223	J=66/3	M=65/6
H=63/14	K=65/4	N=BC 1248
I=63/12	L=65/16	O=63/4

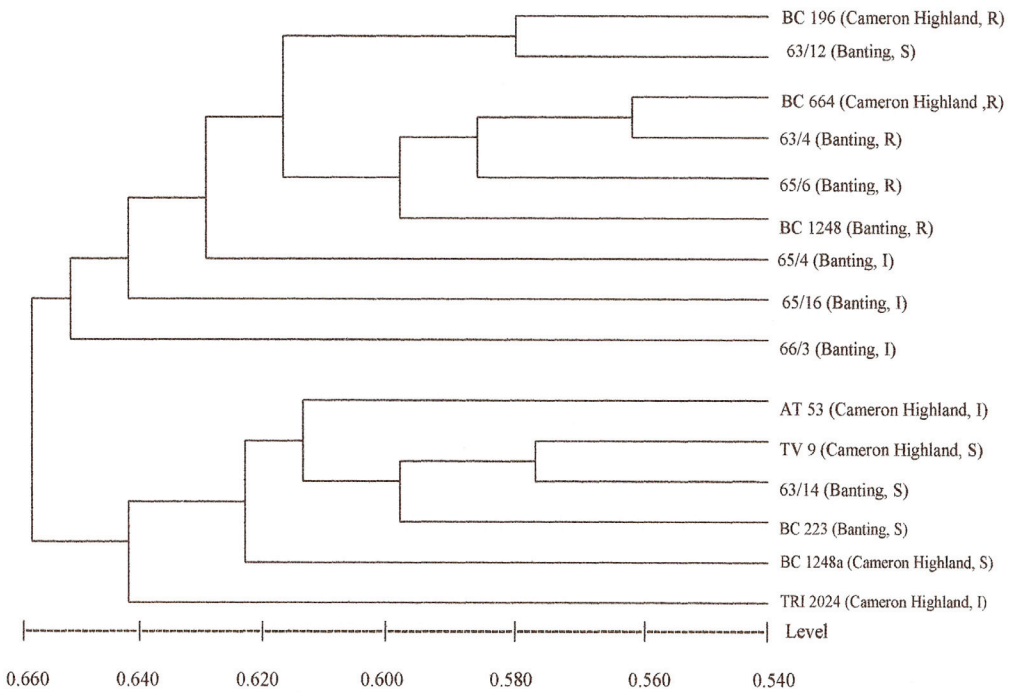

Fig. 2: Dendrogram showing the relationships between the varieties of C. sinensis *derived from UPGMA cluster analysis using distances derived from the Nei and Li (1979) similarity coefficients based on RAMs markers *R=Resistant, I=Intermediate, S=Susceptible*

grouped according to their insect resistance types (i.e. resistant, intermediate, and susceptible varieties of tea), as this was mainly based on their geographical populations (*Fig. 2*). The first cluster consisted of the tea varieties from the Banting population, except for BC 196 (resistant) and BC 664 (resistant) which were from the Cameron Highlands population. The tea varieties found in this cluster were all of the resistant type, except for variety 63/12, which was a susceptible variety and varieties 66/3, 65/4 and 65/16, which were intermediate varieties. The second cluster consisted of mainly the varieties from the Cameron Highlands population, except for BC 223 and 63/14 which were from Banting. The tea varieties in this cluster were mainly of the susceptible type, except for AT 53 and TRI 2024, which were included in the intermediate varieties. The resistant, susceptible, and intermediate varieties were clustered within their own sub-clusters in each of the two major clusters.

DISCUSSION

The dendrogram showed that the differences were mainly based on the populations' geographical distributions and partially based on their resistance towards insect (*H. theivora*) attack. The cluster analysis had mainly grouped the fifteen varieties based on the areas from which the samples were collected (except for BC 196 and BC 664 which were from the Cameron Highlands population that were clustered in the first cluster, and grouped most of the Banting varieties together), while BC 223 and 63/14 (from the Banting population) were clustered in the second cluster that grouped most of the Cameron Highlands varieties together. A possible reason for this could be that some of

the varieties at Cameron Highlands were brought from Banting to be planted there and vice-versa, since both plantations are owned by the same company.

If the genetic differences were fully based on the tea varieties' resistance towards *H. theivora* attack, the dendrogram would have shown three main clusters. The first cluster would consist of the varieties that showed resistance, the second cluster would consist of the intermediate strains, and the third cluster would consist of varieties that showed susceptibility. The reason why they were not clustered could be that the resistance levels of these strains were also dependant on the environment and the developmental stages of the plants (Kaundun and Park, 2002). The visual insect resistance scorings (data not shown), which were done in the present study, might not be as accurate as compared to the DNA markers, especially for the intermediate varieties. The results gathered in this study on the same tea varieties, but based on 153 dominant RAPD markers (data not shown) produced by the ten primers from Kit A of Operon Technology Inc., USA also gave similar clustering patterns as those based on the RAMs markers presented here. This finding suggests that both DNA marker methods could potentially be used for the determination of genetic variations among the tea varieties.

CONCLUSIONS

The results from the RAMs markers complemented the observation that feeding damage on tea leaves could be used to indicate the resistance levels of the plants to insect attacks. The varieties which were of the resistant, susceptible and intermediate types tended to be clustered within their own sub-clusters in each of the two major clusters. In this study, the RAMs markers were found to be capable of detecting high levels of polymorphisms in the various tea varieties that had enabled the researchers to determine their genetic diversity. Further studies could be done using codominant genetic markers such as single locus DNA microsatellites markers and allozymes to obtain a better understanding of their genetic relationships. This will help in preserving and characterizing the existing tea varieties for future breeding and crop improvement programmes that constitute the fundamental support structure for the Malaysian tea industry.

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REFERENCES

Armstrong, A., Gibbs, A., Peakell, R. and Weiler, G. (1995). *RAPDistance Program Version 1.04*. Canberra: Australia National University.

Balasaravanan, T., Pius, P.K., Kumar, R.R., Muraleedharan, N. and Shasany, A.K. (2003). Genetic diversity among south Indian tea germplasm (*Camellia sinensis, C. assamica* and *C. assamica* spp. *lasiocalyx*) using AFLP markers. *Plant Science*, *165*, 365-372.

Christine, A.H., Wachira, F.N., Paul, S., Powell, W. and Waigh, R. (2000). Construction of genetic linkage map for *Camellia sinensis* (tea). *Heredity*, *35*, 346-355.

Doyle, J.J. and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, *19*, 11-15.

Francis, N.W., Wayne, P. and Robbie, W. (1997). An assessment of genetic diversity among *Camellia sinensis* L. (cultivated tea) and its wild relatives based on randomly amplified polymorphic DNA and organelle specific STS. *Heredity*, *78*, 603-611.

Green, M.J. (1971). An evolution of some criteria used in selecting large yielding tea clones. *Journal of Agricultural Science*, *76*, 143-156.

Hoh, B.P., Tan, S.G., Siraj, S., Yusoff, K. and Chew, W.C. (2004). A rapid method of isolating microsatellite markers from the Asian Red-tailed catfish *Mystus nemurus*. *Malaysia Bulletin of the Genetics Society*, June, 16-18.

Kaundun, S.S. and Park, Y.G. (2000). Genetic structure of six Korean tea populations as revealed by RAPD-PCR markers. *Crop Science*, *42*, 594-601.

Kumar, V. (2003). Isolation, characterization and application of DNA microsatellite markers in mungbean (*Vigna radiata* L. W. Iczek) and other selected legumes. Dissertation, Universiti Putra Malaysia.

Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variations in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the United States of America*, *76*, 5269-5273.

Rohlf, F.J. (1993). *NTSYS-pc. Numerical Taxonomy and Multivariate Analysis System, Version 1.60*. Exeter Software, New York.

Sealy, J.R. (1958). *A Revision of the Genus Camellia*. Royal Horticultural Society London.

Wachira, F., Hackett, C.A., Paul, S., Powell, W. and Waugh, R. (2000). Construction of a genetic linkage map for *Camellia sinensis* (tea). *Journal of Heredity*, *85*, 346-355.

Wachira, F.N., Powell, W. and Waugh, R. (1997). An assessment of genetic diversity among *Camellia sinensis* L. (cultivated tea) and it's wild relative based on randomly amplified polymorphisms DNA and organelle-spesific STS. *Journal of Heredity*, *78*, 603-611.

Wang, H.Z., Wu, Z.X., Lu, J.J., Shi, N.N., Zhao, Y., Zhang, Z.T. and Liu, J.J. (2009). Molecular diversity and relationships among *Cymbidium goeringii* cultivars based on inter-simple sequence repeat (ISSR) markers. *Genetica*, *136*, 391–399.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalsky, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research*, *18*, 6531-6535.