Australian Journal of Crop Science AJCS
AJCS 8(1):9-17 (2014) ISSN:1835-2707

Genetic diversity of lemba (*Curculigo latifolia*) populations in Peninsular Malaysia using ISSR molecular markers

Ali Ranjbarfard, Ghizan Saleh*, Nur Ashikin Psyquay Abdullah and Pedram Kashiani

Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

***Corresponding author: ghizan@agri.upm.edu.my**

Abstract

Genetic diversity of 45 populations of lemba (*Curculigo latifolia*) collected from various ecotypes of Peninsular Malaysia was analyzed using ISSR markers. Initially, 12 ISSR primers were selected and applied on populations. Only seven primers were found to produce polymorphic and reproducible bands. The seven ISSR primers generated a total of 162 amplification products, of which the percentage of polymorphic bands for populations ranged from 22.22 % to 72.22 %. Mean Nei's gene diversity value (h) and mean Shannon's Information Index (I) estimated from the 45 populations were 0.1915 and 0.2861, respectively. Furthermore, when all 45 populations pooled, h and I were 0.3697 and 0.5504, respectively. The coefficient of genetic differentiation among populations (G_{ST}) was 0.48. The results of AMOVA showed highly significant genetic differences among and within populations. Of the total genetic variation among 225 *C. latifolia* samples collected from 45 populations of 11 states, 62% was due to genetic differences within populations, while only 38% was due to variations among populations. Result of the Mantel test showed that there was no significant relationship between genetic distance among populations and geographical distance among the collection sites (r = 0.22). This pattern was further confirmed by the UPGMA tree constructed based on Jaccard's genetic similarity coefficients. The populations could be generally grouped into eight major clusters, each mostly presents populations from the same state. The average genetic similarity between populations equaled 0.606. The principal coordinate analysis (PCoA) revealed similar grouping of the populations. In conclusion, a wide range of genetic diversity was revealed among and within the *C. latifolia* populations studied. These variations could be utilized for further breeding purposes to produce new *C. latifolia* varieties.

Keywords: Lemba; *Curculigo latifolia*; Genetic diversity; ISSR; Peninsular Malaysia.
Abbreviations: ISSR_Inter Simple Sequence Repeats; h_Nei's gene diversity value; I_mean Shannon's Information Index; AMOVA_Analysis of molecular variance.

Introduction

Curculigo latifolia, locally known as *lemba*, is a member of the family Hypoxidaceae. The genus *Curculigo* comprises of about 20 species distributed in the tropical regions of Asia and Africa (Kocyan, 2007). The most common species found in Peninsular Malaysia and Borneo Island are *C. capitulata* and *C. latifolia* (Shaari, 2005). Curculin, which is extracted from *C. latifolia* fruits, has been found to have both a sweet taste and sweetness-modifying characteristics of natural sweeteners and has been shown to be a potentially good low-calorie sweetener (Yamashita et al., 1990; Koizumi et al., 2007). Neoculin, a taste-modifying protein has been shown to be present in the fruits of *C. latifolia* (Shirasuka et al., 2004). Leaves, stem-tips and roots of *C. latifolia* have all been used domestically as traditional medicine against fever (Brink and Escobin, 2003). Decoctions of the flowers and roots are used as a stomachic and diuretic, whereas rhizome decoctions are used to treat menorrhagia and applied in traditional medicine as a lotion against ophthalmia (Brink and Escobin, 2003). The rhizomes have also been found useful to treat eye diseases in north-eastern India (Brink and Escobin, 2003). In Borneo, the leaves of *C. latifolia* were used in traditional healing ceremonies (Brink and Escobin, 2003). The rhizomes of the plant have also been used as a traditional cure for jaundice and the rhizome extract of *C. latifolia* inhibits hepatitis B virus *in-vitro*, which further confirmed its use as traditional medicine (Wiart, 2000). Thus, *C. latifolia* has great potential for the pharmaceutical industry.

Despite considerable medicinal significance and economic potential of this plant as a sweetness modifier, no attempt has been made to conserve and cultivate the plant as a commercial crop. Except for the limited information on *C. latifolia* as a wild plant, little is known about its genetics, habitat, biotic and abiotic factors influencing the growth, and other characteristics.

In the past two decades, molecular markers have numerously been used to reveal genetic structure and variability in many plant species (Varshney et al., 2006; Barcaccia, 2010; Xu, 2010; Kashiani et al., 2012a, b). However, the genetic structure of *C. latifolia* has not been characterized utilizing any kind of DNA markers. Characterization of the available genetic variation in this species is necessary for further breeding works towards enhancing its productivity. Inter-simple sequence repeats (ISSRs) are microsatellite-derived genetic fingerprints based on amplification of DNA segments occurring in the genome in regions, where a particular short sequence repeat (SSR) motif occurs on opposing strands within a short and amplifiable distance (Zietkiewicz et al., 1994). They are similar to RAPD, as they require no prior knowledge of the genome, cloning or specific primer design, yet have higher

Table 1. List of ISSR primers, their sequences and the number and size range of bands amplified on 45 populations of *C. latifolia*.

Primer	Sequence (5'-3')	NAB	NPB	Size range (bp)	Annealing temperature (°C)
UBC815	CTC TCT CTC TCT CTC TG	20	20	300 - 1840	57
UBC823	TCT CTC TCT CTC TCT CC	26	26	350 - 1760	57
UBC825	ACA CAC ACA CAC ACA CT	25	25	240 - 1500	54
UBC835	AGA GAG AGA GAG AGA GYC	27	27	200 - 1780	58
UBC848	CAC ACA CAC ACA CAC ARG	27	27	230 - 1800	58
UBC868	GAA GAA GAA GAA GAA GAA	19	19	270 - 1840	50
UBC891	HVH GTG TGT GTG TGT GT	18	18	260 - 1240	55

R: (A, G), Y: (C, T), H: (A, C, T) (i.e. not G), V: (A, C, G) (i.e. not T), NAB: number of bands amplified; NPB: number of polymorphic bands amplified.

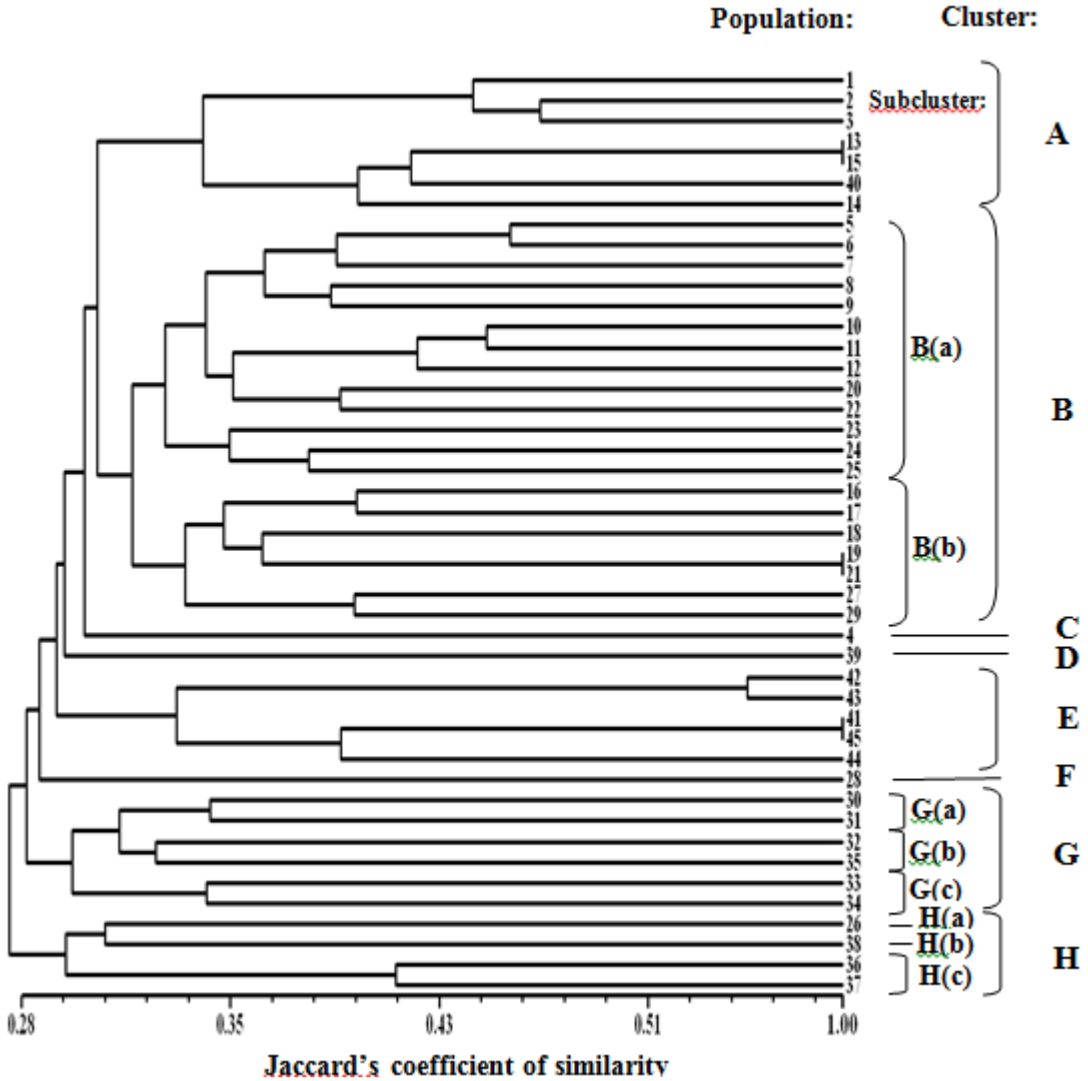

Fig 1. Dendrogram indicating relationships among populations of *C. latifolia* based on Jaccard's similarity coefficients derived from seven ISSR primers

reproducibility than RAPD because of high annealing temperatures. The cost of these analyses is lower than that of AFLPs (Zietkiewicz et al., 1994; Qian et al., 2001; Reddy et al., 2002). ISSRs have been broadly and successfully used in studies of genetic diversity, phylogenetics, genetic mapping and evolutionary biology in a wide range of plant species (Salimath et al., 1995; Fang et al., 1998; Ajibade et al., 2000; Chapman et al., 2000; Martin and Sánchez-Yélamo, 2000; Camacho and Liston, 2001; Sankar and Moore, 2001; Cheghamirza et al., 2004; Lu et al., 2011).

The objective of this study was to estimate genetic diversity among 45 populations of *C. latifolia* which were collected from 11 states of Peninsular Malaysia, using ISSR DNA markers.

Results

Genetic diversity among populations

The seven polymorphic primers out of 12 generated a sum of 162 unambiguous and reproducible bands that could be scored, with the size ranging from 200 bp to 1840 bp. The highest numbers of polymorphic bands revealed among the

Table 2. Genetic diversity estimates among 45 populations of *C. latifolia* collected from 11 states of Peninsular Malaysia.

Population	N	PPB (%)	n_a	n_e	H	I
1	5	50.6	1.5062±0.5015	1.3175±0.3747	0.1848±0.2014	0.2753±0.2893
2	5	46.9	1.4691±0.5006	1.3195±0.3942	0.1806±0.2091	0.2656±0.2982
3	5	51.2	1.5123±0.5014	1.3062±0.3653	0.1803±0.1973	0.2706±0.2839
4	5	61.7	1.6173±0.4876	1.3570±0.3588	0.2133±0.1925	0.3221±0.2762
5	5	42.6	1.4259±0.4960	1.2771±0.3754	0.1589±0.2010	0.2354±0.2885
6	5	55.6	1.5556±0.4984	1.3646±0.3999	0.2071±0.2087	0.3063±0.2957
7	5	72.2	1.7222±0.4493	1.4319±0.3666	0.2543±0.1892	0.3820±0.2660
8	5	22.2	1.2222±0.4170	1.1447±0.2936	0.0846±0.1651	0.1253±0.2408
9	5	56.2	1.5617±0.4977	1.3526±0.3798	0.2050±0.2023	0.3054±0.2894
10	5	53.7	1.5370±0.5002	1.3376±0.3851	0.1949±0.2035	0.2903±0.2904
11	5	48.1	1.4815±0.5012	1.2858±0.3692	0.1670±0.1963	0.2511±0.2818
12	5	51.8	1.5185±0.5012	1.2734±0.3355	0.1672±0.1843	0.2564±0.2683
13	5	50.6	1.5062±0.5015	1.3583±0.4193	0.1981±0.2163	0.2894±0.3051
14	5	51.8	1.5185±0.5012	1.3129±0.3718	0.1832±0.1986	0.2747±0.2851
15	5	50.6	1.5062±0.5015	1.3583±0.4193	0.1981±0.2163	0.2894±0.3051
16	5	49.4	1.4938±0.5015	1.3228±0.3846	0.1853±0.2056	0.2742±0.2943
17	5	47.5	1.4753±0.5009	1.2911±0.3677	0.1700±0.1981	0.2544±0.2852
18	5	59.9	1.5988±0.4917	1.3835±0.3763	0.2233±0.2020	0.3315±0.2891
19	5	69.7	1.6975±0.4608	1.4719±0.3907	0.2681±0.2016	0.3946±0.2831
20	5	57.4	1.5741±0.4960	1.3432±0.3723	0.2017±0.1981	0.3030±0.2834
21	5	69.7	1.6975±0.4608	1.4719±0.3907	0.2681±0.2016	0.3946±0.2831
22	5	59.3	1.5926±0.4929	1.3516±0.3663	0.2081±0.1961	0.3129±0.2812
23	5	67.3	1.6728±0.4706	1.3970±0.3762	0.2330±0.1943	0.3508±0.2740
24	5	67.9	1.6790±0.4683	1.4069±0.3678	0.2399±0.1930	0.3601±0.2735
25	5	55.6	1.5556±0.4984	1.3234±0.3686	0.1910±0.1957	0.2881±0.2806
26	5	66.7	1.6667±0.4729	1.4006±0.3749	0.2348±0.1953	0.3524±0.2763
27	5	43.2	1.4321±0.4969	1.2713±0.3572	0.1591±0.1966	0.2370±0.2848
28	5	53.7	1.5370±0.5002	1.3272±0.3793	0.1903±0.2006	0.2850±0.2866
29	5	54.3	1.5432±0.4997	1.3111±0.3672	0.1839±0.1948	0.2781±0.2791
30	5	46.3	1.4630±0.5002	1.3035±0.3845	0.1735±0.2047	0.2566±0.2930
31	5	59.3	1.5926±0.4929	1.3717±0.3838	0.2155±0.2024	0.3210±0.2881
32	5	52.5	1.5247±0.5009	1.2759±0.3367	0.1688±0.1840	0.2591±0.2680
33	5	56.2	1.5617±0.4977	1.3197±0.3574	0.1912±0.1925	0.2896±0.2775
34	5	50.6	1.5062±0.5015	1.3066±0.3761	0.1783±0.1995	0.2672±0.2855
35	5	54.3	1.5432±0.4997	1.3205±0.3678	0.1890±0.1969	0.2844±0.2825
36	5	58.0	1.5802±0.4950	1.3694±0.3920	0.2121±0.2050	0.3153±0.2910
37	5	56.8	1.5679±0.4969	1.3288±0.3744	0.1932±0.1966	0.2917±0.2803
38	5	47.5	1.4753±0.5009	1.2437±0.3209	0.1507±0.1793	0.2322±0.2632
39	5	22.2	1.2222±0.4170	1.1480±0.2880	0.0876±0.1665	0.1293±0.2443
40	5	41.4	1.4136±0.4940	1.2412±0.3421	0.1435±0.1880	0.2164±0.2734
41	5	57.4	1.5741±0.4960	1.3632±0.3791	0.2111±0.2026	0.3141±0.2898
42	5	61.7	1.6173±0.4876	1.3705±0.3706	0.2182±0.1968	0.3275±0.2800
43	5	48.1	1.4815±0.5012	1.3206±0.4020	0.1801±0.2092	0.2657±0.2965
44	5	41.4	1.4136±0.4940	1.2941±0.4201	0.1592±0.2124	0.2325±0.2981
45	5	57.4	1.5741±0.4960	1.3632±0.3791	0.2111±0.2026	0.3141±0.2898
Mean	5	53.3	1.5331±0.4898	1.3291±0.3791	0.1915±0.1976	0.2861±0.2898
Total	225	100.0	2.0000	1.6294±0.2587	0.3697±0.1063	0.5504±0.1230

N: Number of samples in population; PPB: percent of polymorphic bands; n_a: observed number of alleles; n_e: effective number of alleles; h: Nei's (1973) gene diversity; I: Shannon's information index.

45 populations (27 bands) were obtained from UBC835 (AG microsatellite repeats) and UBC848 (CA microsatellite repeats), while the lowest number was amplified by UBC891 (18 bands) (Table 1). This indicates that AG and CA microsatellite repeats were able to reveal high variation among the *C. latifolia* populations. The total number of DNA fragments amplified from each primer in each population varied from 1 (UBC815 in Population 8, UBC825 in Population 43 and UBC868 in Population 39) to 25 (UBC835 in Population 26). This also indicates the ability of AG microsatellite repeats in revealing the variation among the *C. latifolia* populations.

The percentage of polymorphic bands revealed among the populations ranged from 22.2% (Populations 8 and 39) to 72.2% (Population 7), with an average of 53.3% (Table 2). Among populations, the average number of alleles per locus (n_a) ranged from 1.2222 (Populations 8 and 39) to 1.7222 (Population 7), with a mean of 1.533.

The effective number of alleles per locus (n_e) ranged from 1.1447 (Population 8) to 1.4719 (Populations19 and 21), with a mean of 1.329. Assuming Hardy-Weinberg equilibrium, the mean Nei's gene diversity value (h) was 0.1915, ranging from 0.0846 (Population 8) to 0.2681 (Populations19 and 21), and the mean Shannon's Information index (I) was 0.2861, ranging from 0.1253 (Population 8) to 0.3946 (Populations19 and 21). The Nei's gene diversity and Shannon's information indices at all population level were 0.3697 and 0.5504, respectively, and the n_a and n_e values

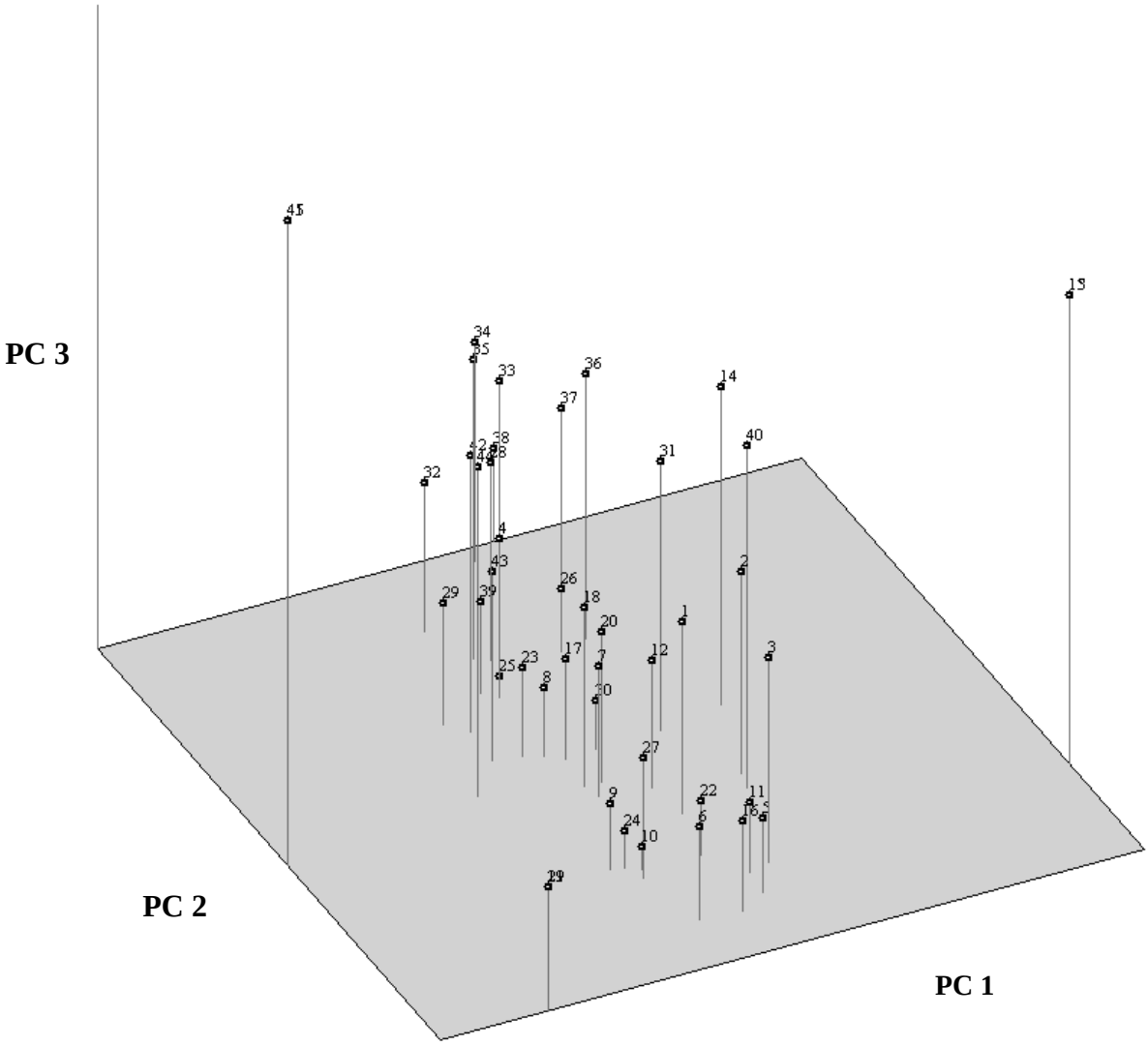

Fig 2. Three-dimensional plot of the principle components from results of ISSR marker data obtained from the 45 populations of *C. latifolia* (PC 1, PC 2, and PC 3: The three axes represent the first three principle components. Numbers in the graph show the 45 populations of *C.latifolia* collected in Peninsular Malaysia).

equaled 2.000 and 1.629, respectively. Among the 45 populations, Population 21 had the highest genetic diversity level, while Population 8 had the lowest (Table 2). The distribution of genetic variation among and within populations was illustrated by Nei's (Nei, 1973) gene diversity statistics. The Nei's total genetic diversity (H_T), within population genetic diversity (H_S), and coefficients of genetic differentiation (G_{ST}) were found to be 0.3697, 0.1915 and 0.482, respectively.

Genetic differentiation and results of AMOVA

The coefficient of genetic differentiation among populations (G_{ST}) was 0.482, indicating the presence of intermediate genetic diversity among the 45 populations of *C. latifolia*. Results of AMOVA showed that there were significant genetic differences among and within *C. latifolia* populations collected from the 11 states. Of the total genetic differences, 62% was due to genetic variation within populations, while the remaining 38% was due to variation among populations. Based on AMOVA results, of the 38% genetic variation among populations, 28% was due to genetic differences among populations within states and only 10% was due to differences among the states (Table 3).

Relationship among populations

Based on Jaccard's similarity coefficient, a dendrogram was generated to represent the genetic relationships among the 45 *C. latifolia* populations (Fig1). The populations were generally grouped into eight major clusters (A–H). In addition, Clusters B, G and H were further divided into different sub-clusters (Fig1). Populations from the same and neighboring states or regions were found to cluster together. All populations from Terengganu were grouped into Cluster E, and all populations from Kelantan state were grouped into Cluster G with Population 35 from Pahang. All Populations from Negeri Sembilan, Melaka and Kedah were grouped into Cluster B(a) with Population 12 from Johor, Population 20 from Penang and Population 25 from Perlis. Strong pairing was observed between Populations 15 and 13 from Johor, Populations 21 and 19 from Penang, and Populations 45 and

Table 3. Molecular analysis of variance (AMOVA) among and within *C. latifolia* populations collected from 11 states of Peninsular Malaysia.

Source of variation	d.f.	SS	MS	Variance component	Variation percentage (%)	P value
Among states	10	1423.1	142.3	3.6	10	<0.001
Among populations	34	2366.9	69.6	9.6	28	<0.001
Within populations	180	3870.4	21.5	21.5	62	<0.001
Total	224	7660.4		34.7	100	

d.f: the degrees of freedom; SS: sum of squares; MS: mean squares.

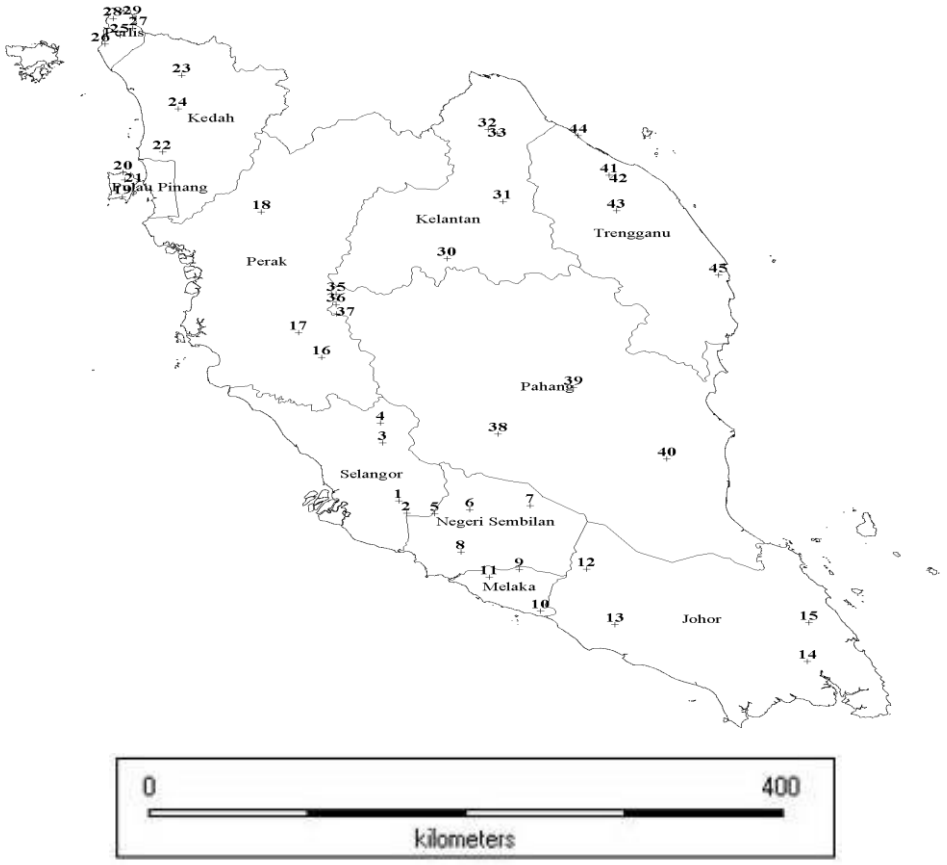

Fig 3. Map showing the geographical collection sites of *C. latifolia* in Peninsular Malaysia (Numbers on the map refer to each of 45 populations collected as described in Table 4).

41 from Terengganu. Their high genetic similarity was also indicated after 1000 simulations of the data using bootstrapping technique. Results of the Mantel test showed that there was no significant relationship between genetic distance among populations and geographical distance among the collection sites (r = 0.22). The highest Jaccard's similarity coefficient (1.000) was found for three pairs of genotypes, between Populations 15 and 13, between Populations 21 and 19, and between Populations 45 and 41.

The lowest similarity coefficient (0.195) was found between populations 38 and 32, which indicates that they were relatively remote in relationship. Result of the principal coordinate analysis (Fig 2) revealed a similar grouping of populations as that shown by the cluster analysis. The first three principal components (PCs) extracted a cumulative of 38.8% of the variance in the 45 populations of *C. latifolia*. The first three components (PC 1, PC 2 and PC 3) had variances of 31.9, 3.5 and 3.4%, respectively.

Discussion

In the present study, microsatellite-based ISSR markers were used to detect genetic variation in *C. latifolia*. A number of ISSR primers with AG, CA, CT, TC, AC, GT and GAA repeats were assayed in order to select suitable polymorphic markers. The investigation showed that the AG and CA microsatellite repeats and their adjacent regions were highly polymorphic in the genome of *C. latifolia*. These microsatellite repeats can be utilized for designing new SSR DNA markers for further genetic mapping and QTL studies of this species.

In this study, the coefficient of differentiation among populations (G_{ST}) was found to be 0.48. This indicates that 48% of variation was due to among population differences, while 52% was due to within population differences. Hamrick et al. (1990) reported that cross-pollinating species are characterized by a relatively high total genetic diversity,

Table 4. Geographical distribution of *C. latifolia* populations collected from 11 states in Peninsular Malaysia.

Population	Collection site	State	Latitude	Longitude	Altitude
1	Universiti Putra Malaysia(Serdang)	Selangor	2˚59.210	101˚42.454	48
2	Kampung Sungai Buah (Dengkil)	Selangor	2˚53.559	101˚45.030	29
3	Batang Kali	Selangor	3˚25.428	101˚37.430	30
4	Kerling (Hulu Selangor)	Selangor	3˚34.615	101˚36.750	54
5	Kampung Daching (Lenggeng)	Negeri Sembilan	2˚53.351	101˚53.857	41
6	Sungai Jelebu (Jelebu)	Negeri Sembilan	2˚55.174	102˚04.926	148
7	Kampung Baru Batu Tempurung (Bahau)	Negeri Sembilan	2˚57.018	102˚24.040	91
8	Rantau (Rembau)	Negeri Sembilan	2˚35.897	102˚02.120	60
9	Kampung Tebong (Selandar)	Melaka	2˚27.882	102˚20.539	51
10	Batu Gajah (Merlimau)	Melaka	2˚08.829	102˚27.257	29
11	Ayer Pak Abas (Alor Gajah)	Melaka	2˚24.370	102˚10.990	32
12	Kampung Sungai Siput (Segamat)	Johor	2˚28.094	102˚41.727	13
13	Yong Peng	Johor	2˚02.792	102˚50.764	31
14	Kota Tinggi	Johor	1˚46.018	103˚51.568	11
15	Jemaluang	Johor	2˚03.619	103˚51.988	27
16	Kampung Jalan Paku (Bidor)	Perak	4˚04.511	101˚18.090	52
17	Kampar	Perak	4˚15.734	101˚10.783	65
18	Lenggong (Hulu Perak)	Perak	5˚10.621	100˚59.101	91
19	Barat Daya	Penang	5˚17.640	100˚15.220	11
20	Batu Feringgi	Penang	5˚28.729	100˚15.373	28
21	Bukit Bendera	Penang	5˚25.229	100˚16.002	689
22	Sungai Petani (Kuala Muda)	Kedah	5˚38.011	100˚27.890	11
23	Pokok Sena	Kedah	6˚12.950	100˚33.973	29
24	Bukit Jenun (Pendang)	Kedah	5˚57.641	100˚32.749	23
25	Beseri	Perlis	6˚31.062	100˚14.402	18
26	Sungai Batu Pahat (Kangar)	Perlis	6˚27.131	100˚09.810	44
27	Bukit Kedak (Guar Jentik)	Perlis	6˚34.355	100˚18.380	43
28	Gua Kelam	Perlis	6˚38.667	100˚12.208	135
29	Padang Besar	Perlis	6˚39.285	100˚18.380	40
30	Felda Aring (Gua Musang)	Kelantan	4˚49.400	101˚57.777	82
31	Kampung Derdap (Kuala Krai)	Kelantan	5˚15.320	102˚15.381	69
32	Kampung Paloh Rawa (Tanah Merah)	Kelantan	5˚48.032	102˚10.647	33
33	Kampung Paloh Rawa (Tanah Merah)	Kelantan	5˚48.032	102˚10.647	33
34	Bukit Belah (Temangan)	Kelantan	5˚46.376	102˚13.643	16
35	Trinkap (Cameron Highlands)	Pahang	4˚33.295	101˚22.749	1435
36	Tanah Rata	Pahang	4˚28.530	101˚22.756	1465
37	Ringlet (Cameron Highlands)	Pahang	4˚24.279	101˚22.890	1135
38	Lancang	Pahang	3˚29.557	102˚13.802	18
39	Bandar Pusat Jengka	Pahang	3˚50.588	102˚37.643	99
40	Muadzam Shah	Pahang	3˚18.298	103˚07.165	12
41	Setiu	Terengganu	5˚27.300	102˚48.939	41
42	Setiu	Terengganu	5˚27.300	102˚48.939	41
43	Kampung Basung (Kuala Berang)	Terengganu	5˚11.191	102˚51.222	43
44	Kampung Apal (Jertih)	Terengganu	5˚45.379	102˚39.175	26
45	Bukit Bauk	Terengganu	4˚41.855	103˚23.461	44

high genetic diversity within populations, and low coefficient of gene differentiation. They reported that coefficient of genetic differentiation (G_{ST}) for outbreeding species was about 0.2, while coefficient of genetic differentiation (G_{ST}) for inbreeding species was 0.5 and above (Hamrick et al., 1990).

Although *C. latifolia* is reported to be a cross-pollinating species (Kocyan, 2007), the current study revealed that G_{ST} obtained for 45 populations (0.48) was near to the value obtained from inbreeding populations (0.5). This could be due to low rate of seed set and high reproduction by rhizome, which were observed in *C. latifolia* populations at collection sites (Ren et al., 2005). The rhizome propagation theoretically has similar effects for population genetic structure as strict inbreeding that has been observed in this study. This has been reported in some cases, e.g. Kashiani et al. (2012b) reported very high G_{ST} among corn populations (0.931), indicating high rate of self-pollination; however, corn is well-known to be a cross-pollination species. They reported that this was due to eight generations of selfing applied on those corn lines. In our study, rhizome propagation of *C. latifolia* populations can be considered as strict selfing. On the other hand, it might also be possible to conclude that *C. latifolia* implies the mixture of simultaneous self-pollination (through rhizome propagation) and cross-pollination reproduction since the G_{ST} obtained from the 45 populations was a slightly lower than those for self-pollinating species but higher than those for cross-pollinating species.

The coefficient of genetic differentiation (G_{ST}) reported for a self-pollinating species (*Elymus fibrosis*) was 0.65 with allozymes and 0.63 with RAPD (Díaz et al., 2000).

The results of AMOVA showed highly significant genetic differences among and within populations. Of the total genetic variation among the 225 *C. latifolia* samples from 45 populations collected from 11 states, 62% was due to genetic

differences within populations, while only 38% was due to variations among the populations. The magnitude of variation among populations obtained from AMOVA (0.38) was close to the coefficient of genetic differentiation (G_{ST} = 0.48) mentioned earlier. This indicates that there were high genetic dissimilarities among plants sampled within a population.

Materials and Methods

Plant materials

Two hundred and twenty-five samples from 45 different populations of *C. latifolia* were collected from 11 states of Peninsular Malaysia from July 2007 to September 2008 (Table 4, Fig 3). The collection area covered latitudes of 1°46.018 N to 6°39.285 N, longitudes of 100° 09.81 E to 103°51.988 E, and altitudes of 11 m to 1465 m. The sites of collection include primary and undisturbed secondary forests, plantations (especially rubber (*Hevea brasiliensis*) plantation) and along road sides. Sampling was carried out according to the method of Hawkes (1980), where samples were collected every 30 km along state roads in different directions (Hawkes, 1980). Plant samples were planted in polyethylene bags filled with soil mixture (clay, sand and organic matter in a 1:1:2 ratio) and maintained under shelter in Field 2, Universiti Putra Malaysia for further experiment.

DNA extraction

Fresh leaf tissue of *C. latifolia* samples were ground to a fine powder using a mortar and pestle under liquid nitrogen. DNA was extracted from 100 mg of ground leaf tissue using the GENE ALL™ Plant SV Mini Kit (from General Biosystem, Seoul, Korea) following the manufacturer's instruction with minor modification to the washing steps. The DNA samples were dissolved in 100µl TE and kept at 4 °C. The purity of genomic DNA extracted was verified using MassRuler™ High Range DNA Ladder from Fermentas Company on a 0.8 % ethidium agarose electrophoresis gel. The concentration of DNA extracted was determined by measuring the absorbance ratio at wavelengths of 260 over 280 nm using the spectrophotometer (The NanoPhotometer™).

ISSR primers

Twelve ISSR primers (UBC809, UBC810, UBC815, UBC823, UBC825, UBC835, UBC848, UBC866, UBC868, UBC886, UBC889 and UBC891) designed by the Biotechnology Laboratory, University of British Columbia (UBC), Vancouver, Canada, were screened for polymorphism and reproducibility of bands. However, only seven primers produced polymorphic and reproducible bands, and were further utilized to reveal genetic difference among the populations (Table 1).

PCR Amplification

PCR amplifications were performed using volumes of 20 µl PCR reaction containing 5 µl (25–30 ng) of genomic DNA, 1.5 µl PCR 10x buffer, 1.5 µl of 25 mM MgCl2, 0.3 µl dNTP Mix (10 mM each of dATP, dGTP, dCTP, dTTP), 0.2 unit of *Taq* polymerase (all from QIAGEN®, *Taq* DNA Polymerase Kit) 1.8 µl (4 pmol/µl) of each primer, and 9.7 µl distilled water. The amplification was performed on BIO-RAD Mycyceler thermal cycler (Bio-Rad Laboratories, USA) with the touchdown thermal cycling protocol starting at 5 °C above optimum annealing temperature and then reduced by 1 °C per cycle until 5 °C below optimum annealing temperature. Eight minutes denaturing step at 94 °C was followed by the remaining thermal cycling protocol which was set to 94 °C for 45 sec, optimum annealing temperature for 50 sec, and extension at 72 °C for 70 sec, for a total of 38 cycles with a final ten minutes extension at 72 °C.

Ten µl of each PCR product were subjected to electrophoresis on 2% agarose gels, stained with ethidium bromide (0.5 µg/ ml) and documented in a gel documentation imaging system (ChemilImager™ Gel Doc., Alpha Innotech Corporation, California, USA). The fragment sizes were estimated by comparing to 50 bp and 100 bp DNA ladders (GeneRuler™, Fermentas). Positive and negative controls were included in each set of PCR amplification reactions to account for possible contamination.

Data analysis

A binary data matrix was obtained by scoring the ISSRs on 2% agarose gels in which the presence or absence of a band was scored as 1 or 0, respectively. The data matrix was constructed and used to reveal differences among the 45 populations studied. The percentage of polymorphic bands (PPB), observed number of alleles (n_a) effective allele number (n_e), Nei's gene diversity value (h) and Shannon's information index (I) were calculated to estimate genetic variation among the populations studied. Population differentiation was analyzed for polymorphism between populations by G_{ST}. Gene flow (N_m) was estimated from $N_m = (1/4)(1- G_{ST})/G_{ST}$ (Nei, 1987). The total genetic diversity (H_T), genetic diversity within the population (H_S), genetic diversity among populations (D_{ST}) and coefficient of genetic differentiation (G_{ST}) was related by the equations $H_T = H_S + D_{ST}$, and $G_{ST} = D_{ST}/H_T$. The estimate of overall population differentiation was obtained from the G_{ST} value (Nei, 1973). All the analyses were carried out withthe software POPGENE version 1.3.1 (Yeh et al., 1999).

Analysis of molecular variance (AMOVA) was performed to describe genetic structure and variability among the populations. The effect of spatial separation on genetic structure was tested by Mantel test (Mantel and Valand, 1970) on genetic matrices (Nei, 1978) and geographic distances among populations. Mantel test was performed with 1000 random permutations using the software GenAlEx version 6.2 (Peakall and Smouse, 2006).

For revealing genetic relationships among the populations, Jaccard's coefficient of genetic similarity (Sneath and Sokal, 1973) was estimated to construct a dendrogram using the method of Unweighted Pair Group with Arithmetic Averages (UPGMA). Principal Coordinate Analysis (PCoA) was also carried out using NTSYS-PC software version 2.1 (Rohlf, 1998). The co-phenetic correlation coefficients were calculated and the Mantel test (Mantel and Valand, 1970) was performed to check the goodness of fit of cluster analysis.

Conclusion

Significant genetic variation was revealed among and within *C. latifolia* populations collected throughout Peninsular Malaysia, as indicated by ISSR markers utilizing Nei's gene diversity coefficients, Shannon's information indices and AMOVA. Variation within populations seemed to be greater than that among populations. The investigation showed that the AG and CA repeats from UBC835 and UBC848 primers produced a great number of polymorphic bands, and thus suggests that the AG and CA microsatellite repeats and their

adjacent regions can be utilized for detecting wide range of genetic diversity through the genome of *C. latifolia*. In addition, these microsatellite repeats can be utilized for designing new SSR markers for further marker-trait associations and QTL investigations. The genetic variation obtained could be utilized for improvement of populations with specific characteristics in future breeding programs for the identification of commercial value properties in this plant using these markers. It is interesting to note that the present study is the first such investigation on revealing genetic diversity in *C. latifolia* using molecular markers.

Acknowledgements

The authors would like to extend their gratitude to Ministry of Higher Education (MOHE) for funding this project under Fundamental Research Grant Scheme (Grant No: 01-10-07-293FR). Special thanks go to the Plant Breeding Laboratory Staff of the Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, for their assistance.

References

Ajibade S, Weeden N, Chite S (2000) Inter simple sequence repeat analysis of genetic relationships in the genus *Vigna*. Euphytica. 111(1): 47-55

Barcaccia G (2010) Molecular markers for characterizing and conserving crop plant germplasm. In: Mohan Jain S, Brar DS (ed) Molecular techniques in crop improvement, 2nd edn. Springer, New York, NY, 772

Brink M, Escobin RP (2003) Plant Resources of South-East Asia. In: Brink M, Escobin RP(ed) Fibre plants. Backhuys Publishers, Leiden

Camacho FJ, Liston A (2001) Population structure and genetic diversity of *Botrychium pumicola* (Ophioglossaceae) based on inter-simple sequence repeats (ISSR). Am J Bot. 88(6): 1065-1070

Chapman HM, Parh D, Oraguzie N (2000) Genetic structure and colonizing success of a clonal, weedy species, *Pilosella officinarum* (Asteraceae). Heredity. 84(4): 401-409

Cheghamirza K, Koveza O, Konovalov F, Gostimsky S (2004) Identification and mapping of chi115 gene and DNA markers linked to it in pea (*Pisum sativum* L.). Russ J Genet. 40(7): 737-742

Diaz O, Sun GL, Salomon B, von Bothmer R (2000) Levels and distribution of allozyme and RAPD variation in populations of *Elymus fibrosus* (Schrenk) Tzvel.(Poaceae). Genet Resour Crop Ev. 47(1): 11-24

Fang D, Krueger RR, Roose ML (1998) Phylogenetic relationships among selected *Citrus* germplasm accessions revealed by inter-simple sequence repeat (ISSR) markers. J Am Soc Hortic Sci. 123(4): 612-617

Hamrick JL, Godt MJW (1990) Allozyme diversity in plant species. 43-63. In: Brown AHD, Clegg MT, Kahler AL, Weir BS (ed) Plant population genetics, breeding, and genetic resources.Sinauer Associates Inc, Sunderland, Massachusetts

Hawkes JG (1980) Crop Genetic Resources Field Collection Manual: For Seed Crops, Root and Tuber Crops, Tree Fruit Crops and Related Wild Species. International Board for Plant Genetic Resources, Rome

Kashiani P, Saleh G, Jothi MP, Abdullah NAP, Ahmad S (2012a) Demarcation of informative chromosomes on tropical sweet corn inbred lines using microsatellite DNA markers. Gent Mol Biol 35(3): 614-621

Kashiani P, Saleh G, Jothi MP, Abdullah NAP, Ahmad S (2012b) Molecular Characterization of Tropical Sweet Corn Inbred Lines Using Microsatellite Markers. Maydica 57(2): 154-163

Kocyan A (2007) The discovery of polyandry in *Curculigo* (Hypoxidaceae): implications for androecium evolution of asparagoid monocotyledons. Ann Bot -London. 100(2): 241-248

Koizumi A, Nakajima K, Asakura T, Morita Y, Ito K, Shmizu-Ibuka A, Misaka T, Abe K (2007) Taste-modifying sweet protein, neoculin, is received at human T1R3 amino terminal domain. Biochem Bioph Res Co. 358(2): 585-589

Mantel N, Valand RS (1970) A technique of nonparametric multivariate analysis. Biometrics. 547-558.

Martin J, Sanchez-Yelamo M (2000) Genetic relationships among species of the genus *Diplotaxis* (Brassicaceae) using inter-simple sequence repeat markers. Theor Appl Genet. 101(8): 1234-1241

Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA. 70(12): 3321-3323

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89(3): 583-590

Nei M (1987) Molecular evolutionary genetics. Columbia Univ Pr, New York

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes. 6(1): 288-295

Qian W, Ge S, Hong DY (2001) Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers. Theor Appl Genet. 102(2): 440-449

Reddy MP, Sarla N, Siddiq E (2002) Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128(1): 9-17.

Ren MX, Zhang QG, Zhang DY (2005) Random amplified polymorphic DNA markers reveal low genetic variation and a single dominant genotype in Eichhornia crassipes populations throughout China. Weed Res 45(3): 236-244

Rohlf FJ (1998) On applications of geometric morphometrics to studies of ontogeny and phylogeny. Syst Biol. 47(1): 147-158

Salimath SS, de Oliveira AC, Bennetzen JL, Godwin ID (1995) Assessment of genome origins and genetic diversity in the genus *Eleusine* with DNA markers. Genome. 38(4): 757-763

Sankar AA, Moore G (2001) Evaluation of inter-simple sequence repeat analysis for mapping in *Citrus* and extension of the genetic linkage map. Theor Appl Genet. 102(2): 206-214

Shaari N (2005) Lemba (*Curculigo latifolia*) Leaf as a New Materials for Textiles. Paper presented at the 4th International Symposium on Environmentally Conscious Design and Inverse Manufacturing, Tokyo, 12-14 December 2005

Shirasuka Y, Nakajima K, Asakura T, Yamashita H, Yamamoto A, Hata S, Nagata S, Abo M, Sorimachi H, Abe K (2004) Neoculin as a new taste-modifying protein occurring in the fruit of *Curculigo latifolia*. Biosci Biotech Bioch. 68(6): 1403-1407

Sneath PHA, Sokal RR (1973) Numerical taxonomy. The principles and practice of numerical classification. W. H. Freeman & Co, San Francisco, USA

Wiart C (2000) Medicinal Plants of Southeast Asia. Pelanduk Pubns Sdn Bhd, Kuala Lumpur, Malaysia

Varshney RK, Hoisington DA, Tyagi AK (2006) Advances in cereal genomics and applications in crop breeding. Trends Biotechnol 24(11): 490-499

Xu Y (2010) Molecular plant breeding. CAB International, Oxfordshire, UK

Yamashita H, Theerasilp S, Aiuchi T, Nakaya K, Nakamura Y, Kurihara Y (1990) Purification and complete amino acid sequence of a new type of sweet protein taste-modifying activity, curculin. J Biol Chem. 265(26): 15770-15775

Yeh F, Yang C, Boyle T (1999) POPGENE version 1.31 Microsoft window-based freeware for population genetic analysis, University of Alberta. Edmonton, AB, Canada

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20(2): 176-183.