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RESEARCH ARTICLE

Evaluation of genetic diversity of *Clinacanthus nutans* (Acanthaceaea) using RAPD, ISSR and RAMP markers

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Abstract Three polymerase chain reaction (PCR) techniques were compared to analyse the genetic diversity of *Clinacanthus nutans* eight populations in the northern region of Peninsular Malaysia. The PCR techniques were random amplified polymorphic deoxyribonucleic acids (RAPD), inter-simple sequence repeats (ISSR) and random amplified microsatellite polymorphisms (RAMP). Leaf genomic DNA was PCR amplified using 17 RAPD, 8 ISSR and 136 RAMP primers . However, only 10 RAPD primers, 5 ISSR primers and 37 RAMP primers produced reproducible bands. The results were evaluated for polymorphic information content (PIC), marker index (MI) and resolving power (RP). The RAMP marker was the most useful marker compared to RAPD and ISSR markers because it showed the highest average value of PIC (0.25), MI (11.36) and RP (2.86). The genetic diversity showed a high percentage of polymorphism at the species level compared to the population level. Furthermore, analysis of molecular variance revealed that the genetic diversity was higher within populations, as compared to among populations of *C. nutans*. From the results, the RAMP technique was recommended for the analysis of genetic diversity of *C. nutans*.

Keywords *Clinacanthus nutans* · Genetic diversity · RAPD · ISSR · RAMP

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Introduction

Sabah Snake Grass, which is also known as *Daun Belalai Gajah* is a popular medicinal plant in South-East Asia. Its scientific name is *Clinacanthus nutans* Lindau and it belongs to the Acanthaceae family. It has been used in many local remedies and its extracts had been used to treat skin rashes, snake bites, insect stings, inflammation, cancer and as antivirals against herpes simplex virus (HSV) and varicella zoster virus (VZV) (Arullappan et al. 2014; Sakdarat et al. 2006; Wanikiat et al. 2008). *C. nutans* is well distributed in Malaysia, Thailand, Indonesia, Vietnam and China (Aslam et al. 2015). Local communities, and farmers have cultivated *C. nutans* through stem cuttings and this has resulted in informal exchanges of *C. nutans*. The most common method of propagation of *C. nutans* is via stem cutting (vegetative propagation) rather than sexual reproduction, as sexual reproduction results in poor multiplication rate and is time consuming (Fong et al. 2014). Therefore, there is a great need for wholesalers, retailers and consumers to know the genetic relationship of *C.*

Table 1 Locations of *C. nutans* sampling areas in northern Peninsular Malaysia

Population code	Accession number	Coordinates		Environmental Conditions	
		Latitude	Longitude	Elevation (m)	Temperature (°C)
PBF	PBF1 to PBF10	5.46913	100.24655	14	33
PTG	PTG1 to PTG10	5.48783	100.50535	16	30
SBP	SBP1 to SBP10	6.53414	100.16906	47	30
KSP	KSP1 to KSP10	5.70244	100.51216	13	34
KKK	KKK1 to KKK10	5.60518	100.65115	21	33
PPS	PPS1 to PPS10	5.49663	100.44532	10	33
KJN	KJN1 to KJN10	5.81501	100.62629	34	33
PBM	PBM1 to PBM10	5.28496	100.28013	7	32

nutans in different populations. Besides that, information on genetic diversity of *C. nutans* can be useful in developing agricultural practices that can avoid genetic erosion and can offer conservation strategies for the long-term vigour of the species (Brake et al. 2014).

With respect to variation between species, DNA fingerprinting remains the most effective and easiest tool for genetic analyses (Ganie et al. 2015; Ntuli et al. 2015). Hence, three different kinds of markers namely RAPD, ISSR and RAMP markers were employed to determine the phylogenetic relationship in *C. nutans* species. RAPD has been successfully applied in genetic variation investigations of *Mucuna pruriens* due to its usefulness as an informative and cheap tool for plant breeding programs (Patil et al. 2016). There are also many researchers using these markers as one of their techniques to study the genetic diversity of their desired plant species (Desai et al. 2015; Kumar et al. 2009, 2010; Mei et al. 2015; Patel et al. 2015; Patil et al. 2016; Pu et al. 2009; Saleh 2015; Zhao et al. 2013; Zhao et al. 2015). RAPD and ISSR are dominant markers that function well in DNA fingerprinting studies of plant species. The combination of RAPD and ISSR can produce another type of marker, which is called RAMP. RAMP has been shown to be useful in detecting and mapping co-dominant microsatellite polymorphisms without tedious cloning and sequencing (Wu et al. 1994). Although the RAMP molecular marker has not been widely tested in other plant species, it is a useful technique because it is suitable for genetic analyses of plants whose genetic background is unclear (Zhao et al. 2013). The information on genetic diversity is useful as it serves as a guide on proper growing practice, whether by stem-cutting or sexual reproduction. Genetic diversity also offers insight as to whether a plant can survive over the long-term and its adaptability in its particular environment (Booy et al. 2000).

Currently, there is no report on the genetic variation of *C. nutans* using RAPD, ISSR and RAMP markers. Therefore, the objectives of this study are to provide information on the genetic diversity of *C. nutans* by using three different markers and, the relationship among accessions, which can be valuable for the conservation of *C. nutans* in herbal medicines industry.

Materials and methods

Plant collection

Leaf samples were collected from 80 *C. nutans* accessions collected from the northern region of Peninsular Malaysia (Table 1). The samples were from 8 different populations, with each population contributing 10 sample accessions. The plant identification was compared to the voucher collection (No: 11536) in the herbarium of the School of Biological Sciences, Universiti Sains Malaysia. The various sampling sites of *C. nutans* are shown in the map in Fig. 1.

Genomic DNA extraction

The genomic DNA of *C. nutans* were extracted from the leaves as described by Sunar et al. (2009). The quantity and quality of DNA were measured using a UV spectrophotometer at the wavelengths of 260/280 nm and the quality of extraction was observed using 0.8% agarose gel electrophoresis.

RAPD, ISSR and RAMP fingerprinting

RAPD, ISSR and RAMP amplifications were performed using 17 RAPD primers, 8 ISSR primers and 136 RAMP primers (a combination of RAPD and ISSR markers). The information is in Table S1. An aliquot of 25 μL master mix was made up of 2.5 μL of 10X Taq buffer, 0.75 μL of 50 mM $MgCl_2$, 0.5 μL of 10 mM dNTP mix, 1.0 μL of 0.4 μM primers, 0.125 μL of 5U of Taq DNA polymerase and 1.0 μL of genomic DNA and 19.125 μL of sterile deionized water.

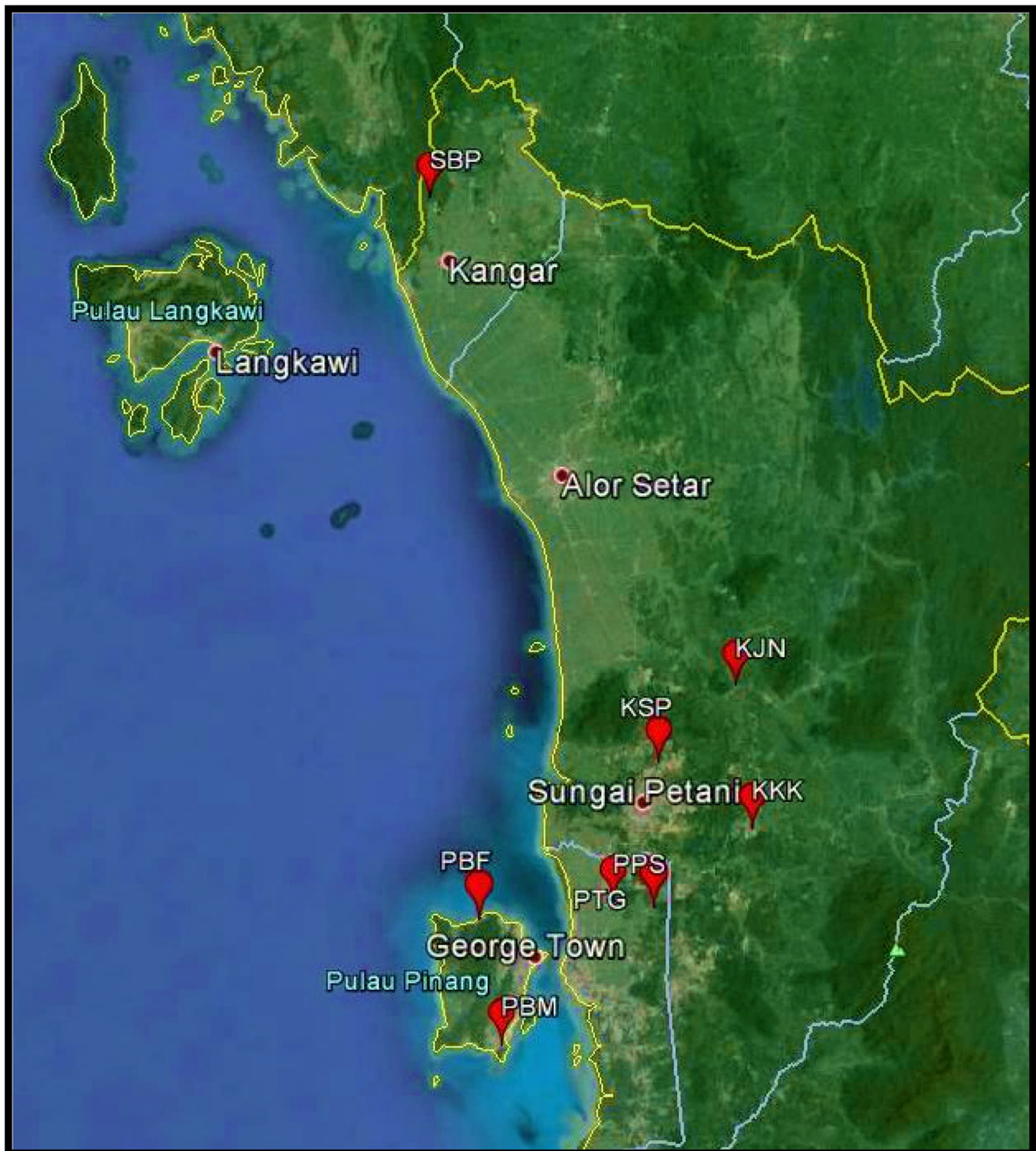

Fig. 1 Distribution of *C. nutans* populations in northern region of Peninsular Malaysia. The code names for populations above were shown in Table 1

Amplification was performed in a MyCycler^{TM} Thermal Cycler (Bio-Rad, USA) programmed for an initial 1 min denaturation at 94 °C, 45 cycles of 1 min denaturation at 94 °C, 1 min annealing (annealing temperature dependent on primers used) and 2 min extension at 72 °C followed by a final extension for 5 min at 72 °C and hold at 4 °C. Amplified PCR products were analysed using 1.5% agarose gel electrophoresis at 90 V for 30 min.

Table 2 Details of the banding pattern revealed through RAPD and ISSR primers

Primer code		T (°C)	BP	TB	PB	MP	PPB (%)	PIC	EMR	MI	RP
RAPD											
OPA-11	CAATCGCCGT	42	600–2500	5	4	1	80.00	0.19	56.64	10.76	1.15
OPA-18	AGGTGACCGT	37	800–1500	6	4	2	66.67	0.20	42.44	8.49	1.80
OPA-09	GGGTAACGCC	37	200–2500	6	5	1	83.33	0.37	45.14	16.70	3.48
OPAS-06	GGCGCGTTAG	38	600–15,000	4	3	1	75.00	0.13	44.81	5.83	0.58
OPAA-16	GGAACCCACA	34	400–1000	4	4	0	100.00	0.23	66.00	15.18	1.40
OPAP-07	ACCACCCGCT	42	200–900	6	6	0	100.00	0.19	68.33	12.98	1.65
OPAO-03	AGTCGGCCCA	42	300–800	5	4	1	80.00	0.20	53.92	10.78	1.53
OPAK-03	GGTCCTACCA	32	600–3500	5	5	0	100.00	0.18	71.20	12.82	1.10
OPAM-07	AACCGCGGCA	45	200–600	3	1	2	33.33	0.06	25.78	1.55	0.20
OPAH-03	GGTTACTGCC	32	300–1000	6	5	1	83.33	0.18	55.97	10.08	1.43
Total				50	41	9					
Average				5.00	4.10	0.90	80.17	0.19	53.02	10.52	1.43
ISSR											
(GTG)5	GTGGTGGTGGTGGTG	53	400–1000	5	4	1	80.00	0.27	43.36	11.71	2.14
(GACA)4	GACAGACAGACAGACA	47	300–1500	9	6	3	66.67	0.27	40.59	10.96	3.56
(GCA)4	GCAGCAGCAGCA	49	400–5000	8	6	2	75.00	0.16	49.22	7.88	1.43
(AGG)6	AGGAGGAGGAGGAGGAGG	57	250–1000	8	7	1	87.50	0.21	59.72	12.54	2.35
(CAA)5	CAACAACAACAACAA	41	300–3500	7	7	0	100.00	0.15	73.29	10.99	1.33
Total				37	30	7					
Average				7.4	6.0	1.4	81.83	0.21	53.24	10.82	2.16

T (°C) annealing temperature, *BP* base pairs, *TB* total band, *PB* polymorphic band, *MB* monomorphic band, *PPB (%)* percentage polymorphic band (%), *PIC* polymorphic information content, *EMR* effective multiplex ratio, *MI* marker index, *RP* resolving power of primer

Data collection and analysis

The DNA analyses were scored by the presence (1) and absence (0) of bands for each primer genotype for RAPD, ISSR and RAMP analysis. Clear and reproducible amplified bands were chosen for the analyses. The PIC value for each locus was calculated using the formula (Roldan-Ruiz et al. 2000);

$$PICi = 2fi(1 - fi)$$

PICi—Polymorphic information content of the locus i; fi—Frequency of the amplified fragments; 1 − fi—Frequency of non-amplified fragments.

For each primer, PIC was calculated using an average PIC value from all loci of each primer. It was used to describe the linkage analysis of polymorphisms for marker locus. The MI was calculated by using the formula (Varshney et al. 2007);

$$MI = EMR \times PIC$$

EMR = n × b; n = Average number of fragments amplified by accession to a specific system marker; b = PB/(PB + MB); PB = Number of polymorphic loci; MB = Number of non-polymorphic loci.

MI was used to determine the usefulness of the system marker used in each primer. EMR analysis depends on the fraction of polymorphic fragments. The RP of each primer was calculated by using the formula (Prevost and Wilkinson 1999);

$$RP = \Sigma Ib$$

Ib = 1 − (2 × |0.5 − pi|); Ib— represents the informative fragments that can be represented on a scale of 0/1 by the following formula; pi—Proportion of accessions containing the ith band.

Data matrices of the three different types of markers were analyzed using POPGENE version 1.32 (Yeh et al. 1997) and Unweighted pair group method arithmetic average (UPGMA) cluster analysis was performed to explore the relationship among populations based on Nei's genetic distance. Estimated gene flow (N_m) was calculated as;

$$N_m = 0.5(1 - G_{ST})/G_{ST},$$

where G_{ST} is the gene differentiation index.

The AMOVA and Mantel test with distance permutation (999) were performed using GenAlEx 6.5 (Peakall and Smouse 2012). The AMOVA was used to calculate the

Table 3 Details of the banding pattern revealed through RAMP markers

Primer code	T (°C)	BP	TF	PB	MB	PPB (%)	PIC	EMR	MI	RP
RAMP										
(GACA)4/CAGGCCCTTC	32.2	400–2500	10	8	2	80.00	0.27	52.00	14.04	3.75
(GCA)4/CAGGCCCTTC	32.2	300–2500	12	8	4	66.67	0.22	45.50	10.01	3.53
(AGG)6/CAGGCCCTTC	32.2	250–2500	8	6	2	75.00	0.30	46.22	13.87	3.68
(GTG)5/GGGTAACGCC	33.4	200–2500	7	5	2	71.43	0.26	47.35	12.31	2.40
(GACA)4/GGGTAACGCC	33.4	200–2500	10	6	4	60.00	0.16	42.78	6.84	2.18
(GCA)4/GGGTAACGCC	33.4	200–2500	9	7	2	77.77	0.25	50.99	12.75	3.25
(AGG)6/GGGTAACGCC	33.4	200–2500	9	6	3	66.66	0.21	45.41	9.54	2.68
(GTG)5/AGGTGACCGT	31.9	400–1500	6	3	3	50.00	0.21	34.00	7.14	1.80
(GACA)4/AGGTGACCGT	32.0	300–1500	9	5	4	55.55	0.21	37.65	7.91	2.75
(GCA)4/AGGTGACCGT	31.9	400–5000	8	6	2	75.00	0.27	47.81	12.91	3.25
(GTG)5/GGAACCCACA	29.7	400–1000	9	6	3	66.67	0.25	49.92	12.48	3.03
(GACA)4/GGAACCCACA	30.0	300–1500	8	4	4	50.00	0.20	33.81	6.76	2.48
(GCA)4/GGAACCCACA	29.7	400–5000	8	5	3	62.50	0.21	40.08	8.42	3.18
(AGG)6/GGAACCCACA	29.7	250–1500	8	5	3	62.50	0.24	41.56	9.97	2.70
(CAA)5/GGAACCCACA	29.7	200–1000	6	4	2	66.67	0.27	42.89	11.58	2.35
(GCA)4/ACCACCCGCT	38.3	200–1500	9	6	3	66.67	0.29	42.52	12.33	3.65
(AGG)6/ACCACCCGCT	38.3	200–5000	8	6	2	75.00	0.30	46.50	13.95	3.60
(CAA)5/ACCACCCGCT	35.4	200–1000	8	5	3	62.50	0.21	42.97	9.02	2.25
(GTG)5/AGTCGGCCCA	38.2	300–1000	6	3	3	50.00	0.21	42.19	8.86	1.88
(GACA)4/AGTCGGCCCA	38.2	300–1500	8	5	3	62.50	0.22	42.27	9.30	2.48
(GCA)4/AGTCGGCCCA	38.2	300–5000	7	5	2	71.43	0.24	42.05	10.09	2.23
(AGG)6/AGTCGGCCCA	38.2	250–1000	7	6	1	85.71	0.31	53.63	16.63	3.05
(CAA)5/AGTCGGCCCA	35.4	300–3500	9	6	3	66.67	0.22	58.48	12.87	2.65
(GTG)5/GGTCCTACCA	27.4	400–3500	6	3	3	50.00	0.18	34.92	6.29	1.53
(GACA)4/GGTCCTACCA	27.4	300–3500	9	7	2	77.77	0.25	52.02	13.01	2.95
(GCA)4/GGTCCTACCA	27.4	400–3500	8	4	4	50.00	0.19	53.67	10.20	2.20
(AGG)6/GGTCCTACCA	27.4	250–3500	8	6	2	75.00	0.40	51.28	20.51	2.33
(GTG)5/AACCGCGGCA	41.2	200–1000	6	4	2	66.67	0.23	44.78	10.30	1.93
(GACA)4/AACCGCGGCA	41.2	200–1500	9	7	2	77.78	0.32	40.44	12.94	4.25
(CAA)5/AACCGCGGCA	35.4	200–5000	7	5	2	71.43	0.30	48.22	14.47	3.15
(GTG)5/GGTTACTGCC	30.2	300–1000	7	4	3	57.15	0.27	44.69	12.07	3.05
(GACA)4/GGTTACTGCC	30.2	300–1500	9	6	3	66.67	0.24	38.10	9.14	3.00
(GCA)4/GGTTACTGCC	30.2	300–1500	9	5	4	55.56	0.23	42.74	9.83	2.98
RAMP										
(AGG)6/GGTTACTGCC	30.2	300–1500	8	6	2	75.00	0.27	48.94	13.21	2.95
(CAA)5/GGTTACTGCC	27.2	300–1500	8	6	2	75.00	0.30	45.94	13.78	3.65
(GTG)5/GGCGCGTTAG	34.8	400–15,000	8	7	1	77.78	0.33	54.47	17.98	3.55
(GACA)4/GGCGCGTTAG	34.8	300–15000	10	5	5	50.00	0.21	33.75	7.09	3.13
Total			301	201	100					
Average			8.14	5.43	2.70	66.29	0.25	44.93	11.36	2.85

T (°C) annealing temperature, *BP* base pairs, *TF* total fragment loci, *PB* polymorphic band, *MB* monomorphic band, *PPB (%)* percentage polymorphic band (%), *PIC* polymorphic information content, *EMR* effective multiplex ratio, *MI* marker index, *RP* resolving power of primer

variance components and their significance levels for variation among populations and within populations.

Results and discussions

Analysis of amplified band

The uses of three different markers (RAPD, ISSR and RAMP) in this study allow us to compare the effectiveness of each genetic marker in the DNA fingerprinting of *C. nutans*. Based on Tables 2 and 3, only 10 primers of RAPD, 5 primers of ISSR and 37 primers of RAMP produced reproducible bands. The highest percentage of polymorphisms were from primers OPAA-16, OPAP-07 and OPAK-03 for RAPD, (CAA)5 for ISSR and (AGG)6/AGTCGGCCCA for RAMP markers. Both RAPD and ISSR markers showed 100% polymorphisms whereas RAMP markers detected 86% polymorphism. The RAMP primers on average, returned a PIC value of 0.25, which is considered as an informative marker. According to Botstein et al. (1980), primers that show a PIC value of $0.5 > \text{PIC} > 0.25$ is considered informative marker. The discriminatory power and the usefulness of each marker can be evaluated by comparing its PIC, MI and RP values. Among the three different markers, the highest linkage analysis of polymorphism was from RAMP markers, which had the highest value of PIC, MI and RP compared to RAPD and ISSR (Table 4). In Table 5, the RAMP result obtained here is compared to the RAMP average value for *Opuntia* sp., *Phoenix dactylifera* L. and *Arthrocnemum macrostachyum*. From Table 5, *C. nutans* had highest MI value compared to other species, which indicated a high degree of polymorphism had been detected (Adhikari et al. 2015). Thus, RAMP markers proved to be a valuable molecular marker compared to ISSR and RAPD (Saleh 2015). This finding concurs with the report by Linh et al. (2007), who observed that the success of RAMP marker is due to the natural dominant and co-dominant characteristics of RAPD and ISSR respectively.

Analysis of genetic diversity

Table 6 shows RAPD, ISSR and RAMP results that had been analysed using POPGENE version 1.32 (Yeh et al. 1997). The highest percentage of polymorphism loci (PPB) was from the SBP population (RAPD = 80%, ISSR = 81% and RAMP = 63%) with the observed number of alleles (Na) (RAPD = 1.80 ± 0.40, ISSR = 1.81 ± 0.39 and RAMP = 1.63 ± 0.48). The effective number of alleles (Ne) of RAPD, ISSR and RAMP showed less variability than Na with an average value of 1.24 ± 0.28, 1.24 ± 0.25 and 1.38 ± 0.39 respectively among the eight populations. The Nei's gene diversity index (H) and Shannon's Index (I) were also highest in population SBP [RAPD: $H = 0.21 \pm 0.15$ and $I = 0.34 \pm 0.21$, ISSR: $H = 0.20 \pm 0.14$ and $I = 0.33 \pm 0.20$ and RAMP: $H = 0.26 \pm 0.22$ and $I = 0.37 \pm 0.31$)].

Table 4 Comparison of highest PIC, MI and RP values of *C. nutans* between three markers

Type of markers	PIC	MI	RP
RAPD	0.19	10.52	1.43
ISSR	0.21	10.82	2.16
RAMP	0.25	11.36	2.86

PIC Polymorphic information content, *MI* marker index, *RP* resolving power of primer

Table 5 Comparison of *C. nutans* and other plants based on RAMP markers average value

Type of plants	PIC	MI	RP	References
C. nutans	0.25	11.36	2.86	This study
Opuntia sp.	0.80	4.64	2.77	Bendhifi et al. (2014)
P. dactylifera	0.58	5.98	4.06	Soumaya et al. (2013)
A. macrostachyum	0.43	2.84	–	Saleh (2015)

PPB percentage polymorphic band, *PIC* polymorphic information content, *MI* marker index, *RP* resolving power of primer

Based on Nei's genetic diversity index, the SBP population showed the highest genetic diversity compared to other populations. SBP population is located in Taman Herba Perlis, a commercial herb garden in northern part of the west coast of Peninsular Malaysia. This finding is in line with Gao et al. (2012), who reported that populations in well-established gardens that had been managed over a long period of time showed the highest genetic diversity. This is because many gardens have maintained multiple collections from diverse wild population over the years, facilitated by the existence of a village and human society in that region. Population PBM showed low genetic diversity as it was situated in a cultivated farm that practised intensive harvesting practices preventing *C. nutans* to reach maturity and thus preventing production of flowers which is essential for cross-pollination. This is in contrast to other species in the Acanthaceae family, especially the herbaceous species that can be spread from seeds easily such as *Ruellia nudiflora* (Ramos-Zapata et al. 2010) that had higher genetic variation but cultivated in disturbed open sites and in diverse agricultural areas.

Table 6 Summary of genetic diversity as revealed through RAPD, ISSR and RAMP among 8 populations of *C. nutans*

Population code	Na (mean ± SD)	Ne (mean ± SD)	H (mean ± SD)	I (mean ± SD)	Polymorphic loci	PPB (%)
RAPD						
PBF	1.62 ± 0.49	1.26 ± 0.31	0.17 ± 0.16	0.27 ± 0.24	31	62.00
PTG	1.64 ± 0.49	1.22 ± 0.25	0.15 ± 0.14	0.25 ± 0.21	32	64.00
SBP	1.80 ± 0.40	1.32 ± 0.28	0.21 ± 0.15	0.34 ± 0.21	40	80.00
KSP	1.6 ± 0.50	1.22 ± 0.26	0.15 ± 0.15	0.24 ± 0.23	29	58.00
KKK	1.68 ± 0.47	1.28 ± 0.30	0.18 ± 0.16	0.29 ± 0.23	34	68.00
PPS	1.58 ± 0.50	1.22 ± 0.27	0.15 ± 0.15	0.24 ± 0.23	29	58.00
KJN	1.58 ± 0.50	1.25 ± 0.31	0.16 ± 0.17	0.26 ± 0.25	29	58.00
PBM	1.60 ± 0.50	1.22 ± 0.25	0.15 ± 0.15	0.25 ± 0.23	30	60.00
Average	1.64 ± 0.48	1.24 ± 0.28	0.17 ± 0.15	0.27 ± 0.23		63.50
Species level	1.82 ± 0.39	1.32 ± 0.33	0.20 ± 0.17	0.32 ± 0.22	41	82.00
ISSR						
PBF	1.62 ± 0.49	1.30 ± 0.30	0.19 ± 0.17	0.30 ± 0.26	23	62.16
PTG	1.68 ± 0.4	1.26 ± 0.28	0.18 ± 0.16	0.28 ± 0.23	25	67.57
SBP	1.81 ± 0.39	1.29 ± 0.26	0.20 ± 0.14	0.33 ± 0.20	30	81.08
KSP	1.59 ± 0.50	1.24 ± 0.30	0.16 ± 0.16	0.25 ± 0.24	22	59.46
KKK	1.70 ± 0.46	1.21 ± 0.21	0.15 ± 0.12	0.26 ± 0.19	26	70.27
PPS	1.65 ± 0.48	1.19 ± 0.18	0.14 ± 0.12	0.24 ± 0.19	24	64.86
KJN	1.73 ± 0.45	1.24 ± 0.23	0.17 ± 0.13	0.28 ± 0.20	27	72.97
PBM	1.54 ± 0.51	1.19 ± 0.25	0.13 ± 0.14	0.21 ± 0.22	20	54.05
Average	1.67 ± 0.47	1.24 ± 0.25	0.16 ± 0.14	0.27 ± 0.22		66.55
Species level	1.84 ± 0.37	1.32 ± 0.30	0.21 ± 0.15	0.34 ± 0.21	31	83.78
RAMP						
PBF	1.51 ± 0.50	1.40 ± 0.41	0.22 ± 0.22	0.32 ± 0.31	154	51.16
PTG	1.58 ± 0.49	1.46 ± 0.41	0.25 ± 0.22	0.36 ± 0.31	176	58.47
SBP	1.63 ± 0.48	1.47 ± 0.40	0.26 ± 0.22	0.37 ± 0.31	191	63.46
KSP	1.40 ± 0.49	1.29 ± 0.39	0.16 ± 0.21	0.24 ± 0.30	120	39.87
KKK	1.60 ± 0.49	1.41 ± 0.36	0.24 ± 0.20	0.35 ± 0.28	180	59.80
PPS	1.49 ± 0.50	1.38 ± 0.41	0.21 ± 0.22	0.30 ± 0.31	147	48.84
KJN	1.57 ± 0.50	1.36 ± 0.36	0.21 ± 0.20	0.32 ± 0.29	173	57.48
PBM	1.35 ± 0.48	1.26 ± 0.38	0.15 ± 0.21	0.21 ± 0.29	106	35.22
Average	1.52 ± 0.49	1.38 ± 0.39	0.21 ± 0.21	0.31 ± 0.30		51.79
Species level	1.67 ± 0.47	1.41 ± 0.34	0.24 ± 0.19	0.37 ± 0.27	201	66.78

Na observed number of alleles, *Ne* effective number of alleles, *H* Nei's genetic diversity, *I* Shannon's information index, *PPB* percentage of polymorphic loci

Genetic relationships of *C. nutans* at species level and population level

At the species level, the percentage of polymorphic loci were high compared to the average percentage at population level (RAPD: species level = 82% and population level = 63.50%, ISSR: species level = 83.78% and population level = 66.55% and RAMP: species level = 66.78% and population level = 51.79%). As shown in Table 7, AMOVA analysis from the three markers showed that variance high within populations (85–95%) than among populations (11–15%). Hence, the result of the analysis of genetic diversity by POPGENE corroborates the result of GenA1EX 6.5. Other plants that had been propagated vegetatively such as *Sinopodophyllum hexandrum* also have low genetic diversity at the population level (H = 0.06) but higher at the species level (H = 0.14) (Liu et al. 2014). Studies on *Calanthe tsoongiana*, *Leersia hexandra* and *Jatropha curcas* also showed high genetic diversity at the species level (H = 0.40, 0.26 and 0.11) than at the population level (H = 0.18, 0.15 and 0.10) respectively (Biabani et al. 2013; Song et al. 2006).

Table 7 The AMOVA results based on RAPD, ISSR and RAMP markers

Markers	DF	SS	MS	Est. Var	Value (%)	*P* value
RAPD						
Among populations	7	71.83	10.26	0.57	11.00	
Within populations	72	330.40	4.59	4.59	89.00	0.001
Total	79	402.23		5.16	100.00	
ISSR						
Among populations	7	66.86	9.55	0.62	15.00	
Within populations	72	243.50	3.38	3.38	85.00	0.001
Total	79	310.36		4.00	100.00	
RAMP						
Among populations	7	375.61	53.66	1.80	5.00	
Within populations	72	2566.90	35.66	35.65	95.00	0.001
Total	79	2942.51		37.45	100.00	

df degree of freedom, *SS* sum of squared deviation, *MS* mean squared deviation, *Est. Var* estimated variance, *p value* the probability of obtaining a more extreme component estimate by chance alone

The high genetic diversity at the species levels is due to many factors such as a recent reduction of population size with inadequate time for isolation to spread and regular N_m (Maguire and Sedgley 1997). Geographical distribution is one of the factors that gives impact on genetic diversity of a species. The Acanthaceae family is consist of mainly herbs and shrubs, which have been largely distributed. In this case, *C. nutans* has been widely distributed in South China, Thailand, Vietnam, Malaysia and Indonesia (Chelyn et al. 2014) occurring in wild and cultivated habitat, including grasslands, hillsides, shrubs, valley, coastal regions and dense and open forest. *C. nutans* has a wide range of geographical distribution and is characterised by its high genetic diversity at the species level but only moderate at the population level, which will further deteriorate as commercial plantings of *C. nutans* practised vegetative propagation through stem-cutting. In the longer term, the moderate diversity at the population level, frequent harvesting before maturity and vegetative propagation will further reduce the genetic diversity at population level and species level due to the loss of rare alleles (Schoettle et al. (2011).

Populations relationship among *C. nutans*

UPGMA cluster analysis was performed to explore the relationship among populations based on Nei's genetic distance. As shown in Fig. 2, the common nodes of similarity for RAPD, ISSR and RAMP are different, occurring at 6.4, 8.0 and 6.5% respectively. The same finding was obtained by Kumar el al. (2009), where the clusters did not show clear correlation between populations. However, the results explained a degree of commonness in the genotypes of *C. nutans* accessions. Among the eight populations of *C. nutans,* population PTG, KSP, KKK and PPS showed high similarity and low genetic distance based on the similarity matrix in Table 8. These populations are distributed in lowland areas and undergo intensive agricultural practices. On the other hand, populations PBF and PBM present low similarity and high genetic distance most probably attributed to the different growing conditions and agricultural practises. Population PBF is located on hilly coastal area in the northern coast, while PBM is situated in agricultural lowlands in the southern coast of Penang island.

The analysis on gene differentiation and N_m is summarised in Table 9. We followed the conventions proposed by Zhao et al. (2016) who defined gene differentiation (G_{ST}) values of $0 \leq 0.05$ as low, $0.05 \leq 0.15$ as medium and $0.15 < 0.25$ as high level of genetic differentiation (G_{ST}). Similarly, we followed Kumar et al. (2014), who had classified N_m values <1 as low, $N_m > 1$ as moderate and $N_m > 4$ as extensive N_m. In this study, the average G_{ST} value of *C. nutans* is 0.17, indicating a high level of G_{ST}, while the average N_m value of 2.51 was only moderate. This result is similar to a study on a related species *Justicia adhatoda*, (a member of the family Acanthaceae), which reported comparable values; ($G_{ST} = 0.30$ for RAPD; $Nm = 1.28$ for RAPD and $G_{ST} = 0.31$ for ISSR; $Nm = 1.30$ for ISSR), which mirrored our findings of moderate N_m but high level of G_{ST} values. High G_{ST} values result in variations within populations, while moderate N_m indicated that one species in every generation can link the gene pools (set of genetic information) among populations (Kumar et al. 2014). Therefore, we can conclude that, *C. nutans* populations had been moderately dispersed among populations but showed high genetic diversity within a population.

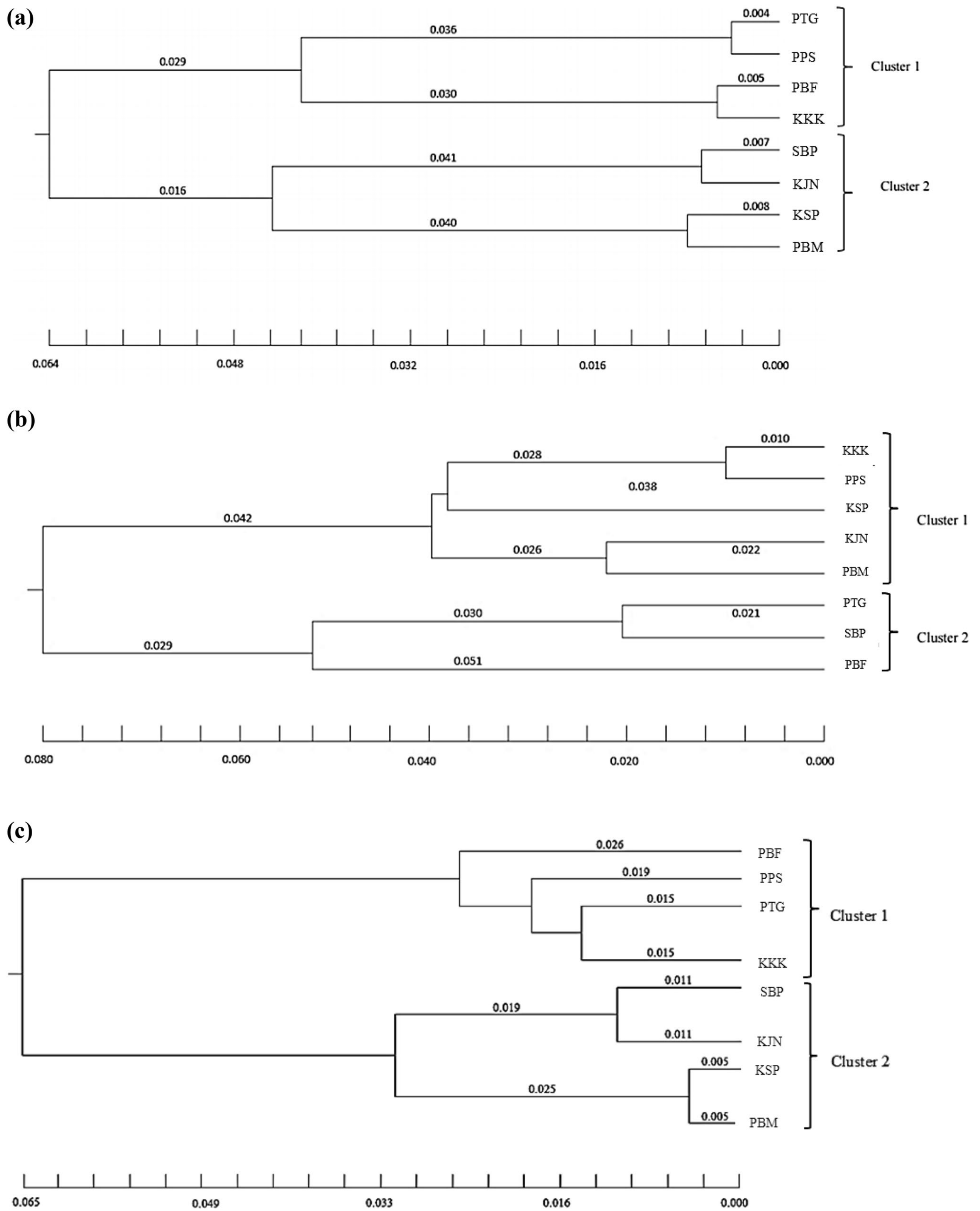

Fig. 2 The relationship of C. nutans among populations according to UPGMA cluster analysis: **a** RAPD analysis, **b** ISSR analysis, and **c** RAMP analysis

Table 8 Similarity matrix of eight populations of *C. nutans* in (a) RAPD analysis and (b) ISSR analysis and (c) RAMP analysis

Population Code	PBF	PTG	SBP	KSP	KKK	PPS	KJN	PBM
(a)								
PBF	****	0.9594	0.9547	0.9212	0.9947	0.9601	0.9512	**0.9206**
PTG	0.0414	****	0.9274	0.9381	0.9592	**0.9955**	0.9283	0.9348
SBP	0.0463	0.0754	****	0.9541	0.9646	0.9335	0.9934	0.9540
KSP	0.0821	0.0639	0.0470	****	0.9312	0.9365	0.9503	0.9925
KKK	0.0053	0.0417	0.0360	0.0713	****	0.9608	0.9610	0.9316
PPS	0.0407	**0.0045**	0.0688	0.0656	0.0399	****	0.9346	0.9331
KJN	0.0500	0.0744	0.0067	0.0510	0.0398	0.0676	****	0.9536
PBM	**0.0827**	0.0674	0.0471	0.0076	0.0709	0.0692	0.0475	****
(b)								
PBF	****	0.9567	0.9439	0.9360	0.9256	0.9034	0.9126	**0.8868**
PTG	0.0443	****	0.9801	0.9524	0.9268	0.9417	0.8974	0.9002
SBP	0.0577	0.0201	****	0.9651	0.9280	0.9484	0.9166	0.9336
KSP	0.0661	0.0488	0.0355	****	0.9578	0.9673	0.9557	0.9468
KKK	0.0773	0.0761	0.0747	0.0431	****	**0.9902**	0.9327	0.9149
PPS	0.1016	0.0601	0.0530	0.0333	**0.0099**	****	0.9386	0.9362
KJN	0.0914	0.1083	0.0870	0.0453	0.0696	0.0633	****	0.9780
PBM	**0.1202**	0.1051	0.0687	0.0547	0.0890	0.0659	0.0222	****
(c)								
PBF	****	0.9787	0.9440	0.9280	0.9722	0.9739	0.9405	**0.9261**
PTG	0.0215	****	0.9574	0.9355	0.9856	0.9846	0.9490	0.9323
SBP	0.0577	0.0436	****	0.9693	0.9525	0.9489	0.9890	0.9653
KSP	0.0747	0.0666	0.0312	****	0.9319	0.9291	0.9748	**0.9947**
KKK	0.0282	0.0146	0.0487	0.0706	****	0.9784	0.9438	0.9251
PPS	0.0264	0.0155	0.0525	0.0736	0.0219	****	0.9444	0.9266
KJN	0.0613	0.0523	0.0111	0.0256	0.0579	0.0572	****	0.9706
PBM	**0.0768**	0.0701	0.0353	**0.0053**	0.0778	0.0763	0.0298	****

Genetic similarity is listed above diagonal and genetic distance is listed below diagonal. The bold value showed genetic similarity and genetic distance between *C. nutans* populations

Table 9 The G_{ST} and N_m value within and among populations of *C. nutans*

	H_s	H_t	H_s/H_t	G_{ST}	N_m
RAPD					
Nei's gene diversity	0.17	0.20	0.82	0.18	2.30
ISSR					
Nei's gene diversity	0.16	0.21	0.78	0.22	1.82
RAMP					
Nei's gene diversity	0.21	0.24	0.87	0.13	3.42
Total	0.54	0.65	2.48	0.52	7.54
Average	0.18	0.22	0.83	0.17	2.51

H_s within-population gene diversity, H_t total gene diversity, H_s/H_t ratio of gene diversity within population, G_{ST} genetic differentiation coefficient, N_m gene flow estimated from G_{ST}

Conclusions

We have identified 10 RAPD, 5 ISSR and 37 RAMP markers that can produced reproducible bands and can be used to study the genetic diversity of 80 accessions of *C. nutans*. We have shown that out of the three markers, the RAMP markers are recommended for evaluating the genetic diversity of *C. nutans*. Among the eight populations, SBP showed the highest genetic diversity. For future studies, we would like to compare the phytochemical profiling among populations of *C. nutans*. This is essential in order to understand the relationship of genetic diversity and the phytochemical profile in *C. nutans,* which is essential for its development for medicinal purposes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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