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Genetic homogeneity of vegetatively propagated *Clinacanthus nutans* (Acanthaceae)

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Clinacanthus nutans **is a medicinal Asian plant often propagated by stem cuttings but little is known about the genetic relationships between existing accessions and the extent of homogeneity. In this study, we examined the genetic homogeneity in 12** ***C. nutans*** **samples from Malaysia, Thailand and Vietnam reproduced by vegetative propagation from different regions between and within countries, and compared it to sexually propagated** ***Andrographis paniculata*** **(same family), related** ***Clinacanthus siamensis*** **(same family) and an out-group (different family) using restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and microsatellite markers. There was a high genetic similarity between** ***C. nutans*** **accessions from all countries, with identical genetic profiles even though they were geographically distant.** ***C. nutans*** **clustered closely with** ***C. siamensis*** **and was distant from** ***A. paniculata*** **and the out-group. Genetic similarity for** ***C. nutans*** **was almost double that of** ***A. paniculata*****, but the combined clustering analysis revealed higher diversity in** ***C. nutans*****. These results provide fundamental knowledge in future planting decisions and options, and also facilitate further germplasm conservation of** ***C. nutans*** **and other vegetatively propagated medicinal species.**

Key words: *Clinacanthus nutans*, *Andrographis paniculata*, *Clinacanthus siamensis*, Acanthaceae, genetic variation, vegetative propagation, sexual reproduction.

INTRODUCTION

Clinacanthus nutans (Burm. f.) Lindau belongs to the family Acanthaceae and is a highly sought-after medicinal plant with potent bioactivity (Table 1). Growers in agriculture reproduce plants using vegetative propagation by stem cuttings to meet consumer demand. However, little is known on the genetic diversity of existing accessions and whether propagation method influences their homogeneity. *C. nutans* is marketed under the trade name “Sabah Snake Grass” and is generally sold as powdered or whole dried leaves in Malaysian herbal markets. The plant is often misidentified with the closely related *Clinacanthus siemensis* and confused with another locally available medicinal plant in the same family, *Andrographis paniculata* (Burm. f.) Nees, which has

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Table 1. Bioactivity of *C. nutans*, *C. siamensis* and *A. paniculata*.

Taxon	Treatment/Property	Reference
C. nutans	Skin rashes	Sakdarat et al. (2009)
	Snake bites	Daduang et al. (2005)
	Insect stings	Uawonggul et al. (2006)
	Anti-inflammatory	Wanikiat et al. (2008)
	Anti-oxidant	Pannangpetch et al. (2007); Yong et al. (2013)
	Anti-viral against HSV	Jayavasu et al. (1992); Kongkaew and Chaiyakunapruk (2011); Kunsorn et al. (2013)
	Anti-viral against VZV	Thawaranantha et al. (1992); Charuwichitaratana et al. (1996)
	Anti-cancer	Yong et al. (2013)
C. siamensis	Anti-arthritic, anti-inflammatory, traumatic oedema, poison bites/stings	Sreena et al. (2012)
	Anti-viral against HSV	Kunsorn et al. (2013)
	Anti-viral against influenza virus	Wirotesangthong et al. (2009)
A. paniculata	Jaundice and liver conditions	Kapil et al. (1993); Premila (2006)
	Common cold, fever, non-infectious diarrhoea	Caceres et al. (1999)
	Immune-stimulatory	Puri et al. (1993)
	Anti-viral against HIV	Otake et al. (1995); Calabrese et al. (2000)
	Anti-malarial	Najib et al. (1999)

the vernacular name “Indian Snake Grass”. All have similar growth habits and leaf appearance (Figure 1a, b, d and e) but are distinguished by the flowers (Figure 1c and f), stems (Hu and Daniel, 2011) and bitter taste (Chandrasekaran et al., 2009) with different medicinal purposes (Table 1).

Morphological and taste characteristics can be subjective and do not differentiate between closely related individuals, particularly for populations of clonal plants consisting of genets and ramets that often occur with vegetative propagations. Traditional markers based on morphological or allozyme variation have limited abilities to differentiate between genetically similar individuals and have been resolved using DNA-based markers that are efficient in detecting genotypic distribution in clonal populations (Esselman et al., 1999; Keller, 2000). Polymerase chain reaction (PCR)-based DNA markers have been used to assess genetic homogeneity in sexually propagated Acanthaceae representatives (Chua, 2007; Mori et al., 2010; Behera et al., 2011; Suwanchaikasem et al., 2011; Wee et al., 2013), but not on *C. nutans* that is commonly vegetatively propagated.

Little information is available not only on the genetic homogeneity of *C. nutans*, but also on other species of Acanthaceae that are reproduced vegetatively; and the genetic differences between *C. nutans* and *A. paniculata*. Therefore, the current study aimed to: (1) examine the genetic homogeneity of *C. nutans* propagated by stem cuttings as a representative of Acanthaceae from different regions between and within countries, (2) confirm genetic separation of *C. nutans, C. siemensis* and *A. paniculata* as an alternative identification method, and (3) distinguish and compare it to sexually propagated *A. paniculata*.

MATERIALS AND METHODS

Plant

The fresh leaves of 12 accessions of *C. nutans* growing in different conditions/environments (Table 2) were randomly collected from Peninsular Malaysia (CP), East Malaysia (CE), Thailand (CT), and Vietnam (CV) (Figure 2). Leaves of two accessions of *A. paniculata* were collected from Seremban, Negeri Sembilan, Malaysia (AP1 and AP2). *C. siamensis* from Thailand and *Momordica cochinchinensis* from Australia were used as out-groups to compare *C.*

Table 2. The taxon, sample codes, collection sites, environmental and growth conditions of collected samples.

Taxon	Sample	Country	State/Province	Region	Environmental conditions				Growth conditions	
					Elv	Tm		Rn	Potted	Shaded
						H	L			
C. nutans	CP1	Malaysia	Negeri Sembilan	Seremban	66.6	31.3	23.0	2124.0	√	√
	CP2	Malaysia	Negeri Sembilan	Seremban	83.4	31.2	22.4	2010.0	√	√
	CE1	Malaysia	Sabah	Sandakan	158.7	30.8	22.9	2973.0	√	√
	CE2	Malaysia	Sabah	Sandakan	74.9	30.8	22.9	2973.0	X	X
	CE3	Malaysia	Sabah	Tawau	6.8	30.7	23.2	1975.0	√	√
	CE4	Malaysia	Sabah	Kota Kinabalu	9.7	30.7	23.3	2818.0	√	X
	CT1	Thailand	Nakhon Pathom	Map Khae	8.1	32.7	22.7	1237.0	√	√
	CT2	Thailand	Nakhon Pathom	Map Khae	8.1	32.7	22.7	1237.0	X	√
	CT3	Thailand	Nakhon Pathom	Salaya	4.3	32.5	23.4	1334.0	X	X
	CT4	Thailand	Chiang Mai	San Sai	309.5	31.5	19.6	1191.0	X	X
	CT5	Thailand	Chiang Mai	Chiang Dao	439.4	30.6	18.7	1261.0	X	X
	CV1	Vietnam	Ho Chi Minh	Ho Chi Minh	2.2	31.9	23.1	1873.0	X	X
C. siamensis	CS	Thailand	Nakhon Pathom	Salaya	4.3	32.5	23.4	1334.0	X	X
A. paniculata	AP1	Malaysia	Negeri Sembilan	Seremban	83.8	31.2	22.4	2010.0	√	√
	AP2	Malaysia	Negeri Sembilan	Seremban	83.8	31.2	22.4	2010.0	√	√
M. cochinchinensis	MC	Australia	New South Wales	Newcastle	79.0	25.0*	25.0*	NA	√	√

Elv = elevation (m); Tm = mean annual temperature (°C); H = high; L= low; Rn = mean annual rainfall (mm); * = greenhouse conditions.

nutans genotypes and *C. nutans* and *A. paniculata*, respectively. Prior to DNA extraction, all leaf pieces were thoroughly examined and washed using cold tap water. All samples, except samples CP1 and AP2 were air dried for at least 7 days.

DNA extraction

Total DNA from samples CP1 and AP2 was extracted in fresh form, using the GF-1 Plant DNA Extraction Kit (Vivantis Technologies, Malaysia) according to the manufacturer's protocol. The FavorPrep Plant Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan) was used to extract the total DNA from all the other dried samples with slight modifications of the extraction protocol provided by the manufacturer to improve the DNA yield. Twenty milligrams of dried sample was used instead of 100 mg and then DNA extraction was carried out as outlined in the protocol. Finally, 50 µl of preheated elution buffer was added to the column matrix and centrifuged at 6,600 ×g for 2 min to elute the purified DNA.

PCR-RFLP

Primer pair rbcL1F-724R (Applied Biosystems, Australia) was used to partially amplify the ribulose-1,5-biphosphate carboxylase/oxygenase large subunit (rbcL) region (ca. 700 bp) (Olmstead et al., 1992). A total volume of 25 µl PCR mixture contained the following: 12.5 µl 2X GoTaq Green Master Mix (Promega Corporation, Australia), 1.0 µl 0.4 mM forward primer, 1.0 µl 0.4 mM reverse primer, 1.0 µl undiluted DNA, and 9.5 µl nuclease-free water. The control included all of the PCR reagents except the DNA template. Amplification was performed in a G-Storm GS1 thermal cycler (Gene Technologies, England), where the reaction consisted of an initial denaturation step at 95°C for 2 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 7 min and held at 4°C. Amplicons were analysed by gel electrophoresis with a1.4% (w/v) agarose gel in 1XTBE buffer and stained with ethidium bromide (0.5 µg/ml) for visualisation using the Discovery Series Quantity One 1-D Analysis Software

Table 3. Restriction endonuclease digestions of the rbcL 1F and 724R PCR products.

Restriction enzyme	Buffer (10X)	Sequence 5' – 3'	Reaction conditions
*Taq*I (Fermentas)	*Taq*I Buffer with BSA	T-CGA AGC-T	65°C for 3 h
*Msp*I (Fermentas)	Buffer Tango with BSA	C-CGG GGC-C	37°C for 3 h
*Bam*HI (Promega)	Buffer E	G-GATCC CCTAG-G	37°C for 3 h
*Hae*III (Promega)	Buffer C	GG-CC CC-GG	37°C for 3 h
*Hind*III (Promega)	Buffer E	A-AGCTT TTCGA-A	37°C for 3 h
XbaI (Biotech)	*XbaI* Reaction Buffer	T-CTAGA AGATC-T	37°C for 3 h
*Xho*I (Promega)	Buffer D	C-TCGAG GAGCT-C	37°C for 3 hr

Table 4. The five primers and the number of corresponding fragments used in the RAPD and microsatellite analyses.

Primer	Sequence (5' – 3')	No. of polymorphic fragments	Polymorphic fragments (%)
OPA-09	GGGTAACGCC	11	100
OPA-11	CAATCGCCGT	14	100
OPA-18	AGGTGACCGT	11	100
GTG5	GTGGTGGTGGTGGTG	25	100
GACA4	GACAGACAGACAGACA	25	100

(Bio-Rad Laboratories, USA). Aliquots (5 µl) of the PCR products from the rbcL gene were digested with seven restriction enzymes (Table 3). The restriction fragments were separated by 2.0% (w/v) agarose gel electrophoresis in 1X TBE buffer, and stained with ethidium bromide (EtBr) (0.5 µg/ml) for visualisation. PCR amplification and restriction of PCR products were repeated twice for all samples for reproducibility.

RAPD amplification

Of the 22 RAPD primers (Operon Technologies, Australia) screened, three (OPA-09, 11 and 18) that produced polymorphic banding patterns were selected (Table 4). RAPD reactions were performed in a total volume of 25 µl containing 12.5 µl 2X GoTaq Green Master Mix (Promega Corporation), 1.0 µl 0.4 µM primers, 1.0 µl 5 to10 ng of genomic DNA, and 10.5 µl nuclease-free water. RAPD amplifications were performed in a G-Storm GS1 thermal cycler (Gene Technologies) programmed for initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min, elongation at 72°C for 2 min, followed by a final elongation step at 72°C for 8 min and held at 4°C. The amplicons were then subjected to electrophoresis in 1.5% (w/v) agarose gel in 1XTBE buffer and stained with EtBr for visualisation.

Microsatellite amplification

Two microsatellite primers (GTG5 and GACA4) (Operon Technologies) were used to evaluate the genetic relationships in this study (Table 4). Microsatellite reactions were performed in a total volume of 25 µl containing 12.5 µl 2X GoTaq Green Master Mix (Promega Corporation), 1.0 µl 0.4 µM primers, 1.0 µl 5 to 10 ng of genomic DNA, and 10.5 µl nuclease-free water. Microsatellite amplifications were performed in a G-Storm GS1 thermal cycler (Gene Technologies) programmed for initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, elongation at 72°C for 1 min, followed by a final elongation step at 72°C for 7 min and held at 4°C. The amplicons were then separated by electrophoresis in 1.5% (w/v) agarose gel in 1X TBE buffer and stained with EtBr for visualisation.

Data collection and analysis

Each gel was analysed by manually scoring the presence (1) or absence (0) of bands in individual lanes, generating a binary data matrix. The molecular weights of the bands were estimated based on the DNA marker GeneRuler DNA Ladder Mix (Fermentas, Australia). Bands of equal molecular weight generated by similar

Table 5. Restriction endonuclease digestions of rbcL 1F-724R PCR products.

Restriction enzyme	Fragment size (bp)		
	C. nutans	***C. siamensis***	***A. paniculata***
Taq I	150 250 300	150 250 300	250 450 -
Msp I	200 500	200 500	150 550
BamH I	No restriction	No restriction	No restriction
Hae III	No restriction	No restriction	No restriction
Hind III	No restriction	No restriction	No restriction
Xba I	No restriction	No restriction	No restriction
Xho I	No restriction	No restriction	No restriction

Figure 1. Macromorphology of plants investigated in this study. *C. nutans* growth habit (a), leaves (b) and flower (c); *A. paniculata* growth habit (d), leaf (e) and flower (f); and *C. siamensis* leaves (g). Comparisons between *C. nutans* (indicated with black asterisk) leaves with *A. paniculata* (indicated with red asterisk) (h) and *C. siamensis (*indicated with blue asterisk*)* (i).

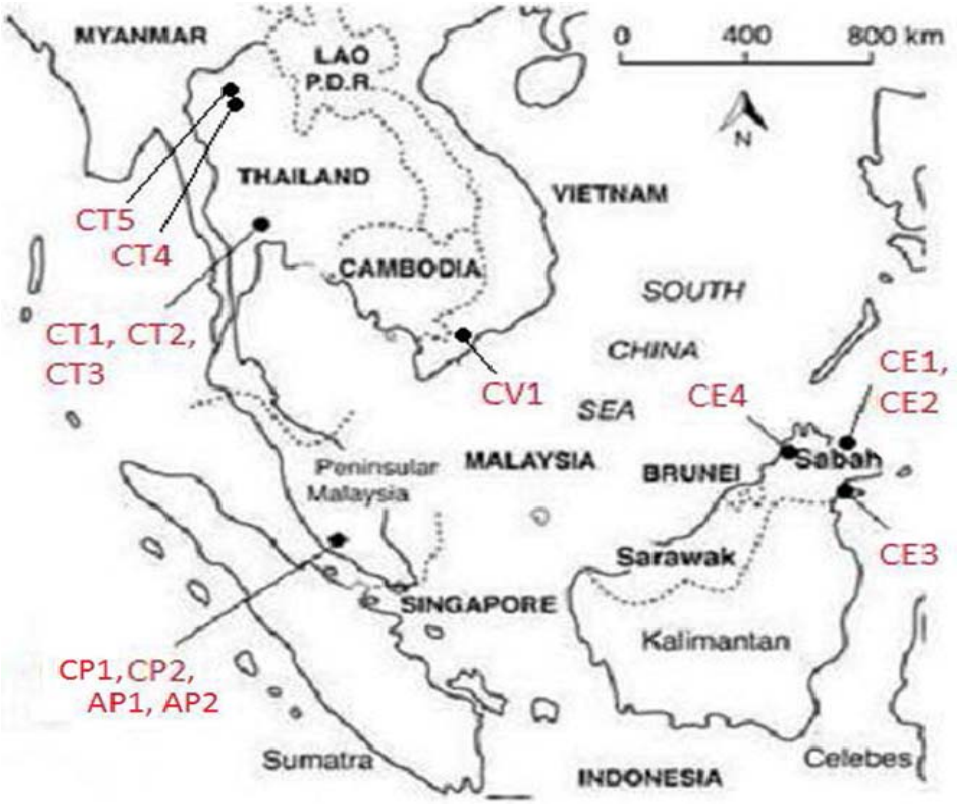

Figure 2. Sampling sites of *C. nutans* and *A. paniculata* in Malaysia, Thailand and Vietnam.

primers were considered to be identical locus. Similarity indices were calculated using the similarity for qualitative data (SimQual) computer algorithm in NTSYS-pc ver. 2.10e (Rohlf, 2000) using Dice's similarity coefficient equation (Dice, 1945): $S_{ij} = 2a/(2a+b+c)$, where S_{ij} = the similarity between two samples, i and j; a = the number of bands present in both i and j; b = the number of bands present in i and absent in j; c = the number of bands present in j and absent in i. From these similarity indices, the sequential, agglomerative, hierarchic and non-overlapping (SAHN) clustering method was performed using the unweighted pair group method with average mean (UPGMA) to generate the dendrograms in the same software. The genetic similarity coefficient value between samples ranged from 0 to 1, where 0 indicated no similarity and 1 indicated that the two samples were genetically identical. Binary data from RAPD and microsatellites were combined for principal component analysis (PCA) using the Minitab statistical software (Minitab, 2010). Score plots were generated and genetically similar accessions were clustered together.

RESULTS

PCR-RFLP analysis

PCR amplification using primer pair rbcL 1F-724R produced a ca. 700 bp amplicon. Of the seven restriction endonucleases used, only two (*Taq*I and *Msp*I) cleaved the 700 bp amplicon (Table 5). Both *Taq*I and *Msp*I digestions revealed that the restriction fragment patterns were different between *C. nutans* and *A. paniculata* but were identical between *C. nutans* and *C. siamenesis. Taq*I endonuclease cut the amplified rbcL gene of *C. nutans* and *C. siamemsis* at two restriction sites, producing three fragments, but only one site for *A. paniculata*, producing two fragments (Table 5; Figure 3a).

All fragments when combined totalled the original template size, that is, ~700 bp. *Msp*I endonuclease cut the amplified rbcL gene of *C. nutans*, *C. siamensis* and *A. paniculata* at only one restriction site and produced the same number of fragments in each species, but at different sites and sizes (results not shown). Restriction digestion of the amplified rbcL gene of *C. nutans* and *C. siamensis* generated 200 and 500 bp, whereas that of *A. paniculata* generated 150 and 550 bp fragments. RFLP marker revealed an identical banding pattern among all samples of *C. nutans* as well as *A. paniculata*.

RAPD analysis

Three primers, OPA-09, OPA-11, and OPA-18, produced amplification patterns in all *C. nutans*, *A. paniculata* and *M. cochinchinensis* samples, except *C. siamensis* (OPA-18 only). Pooled-data revealed that *C. nutans* was genetically different from *A. paniculata* and the out-groups (*C. siamensis* and *M. cochinchinensis*). Within species, *C. nutans* samples were genetically different between

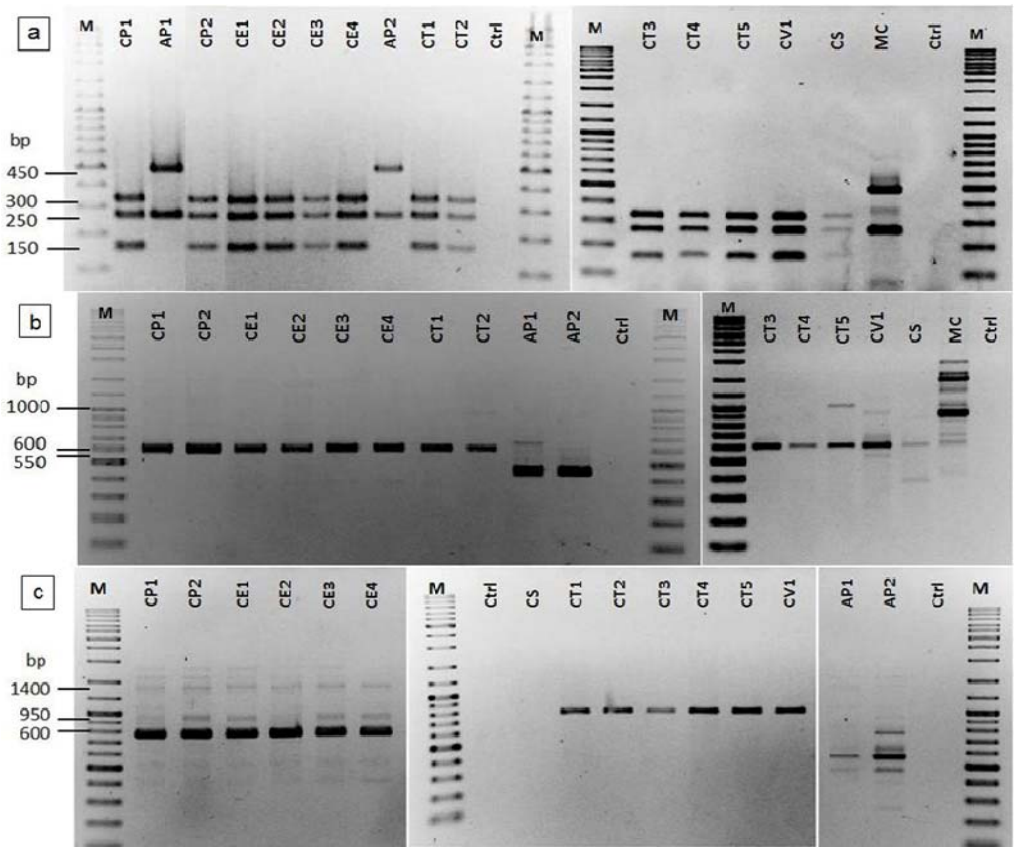

Figure 3. PCR-RFLP patterns of *Taq I* digested rbcL gene (a), RAPD amplification products of OPA-11 (b) and OPA-18 (c). Lane M=100 bp DNA ladder; lanes CP1, CP2, CE1, CE2, CE3, CE4, CT1, CT2, CT3, CT4, CT5, and CV1=*C. nutans*; lanes AP1, AP2=*A. paniculata;* lane CS=*C. siamensis*; lane MC=*M. cochinchinensis*; lane Ctrl=no template control.

countries. All Malaysian *C. nutans* samples were identical while four Thai samples were identical and one was not. Vietnamese sample was separated from other *C. nutans*. Overall, of the 29 loci scored, 100.0% were polymorphic. Eight loci were scored for *C. nutans* and four loci (50.0%), which were produced from OPA-11 (Figure 3b) and -18 (Figure 3c) primers amplifications were polymorphic. All Thai samples revealed an absence of bands at 950 and 1400 bp. All samples showed an absence of bands at 550 and 1000 bp, except CV1 (Vietnam) and CT5 (Thailand), respectively. In contrast, *A. paniculata* samples obtained from the same farm but different sampling sites showed genetic variation. A higher number of polymorphic loci was revealed in *A. paniculata*, where of the 11 loci scored, seven (63.6%) were polymorphic. Fragments produced from RAPD amplification ranged in size from 250 to 2600 bp.

The genetic similarity calculated using Dice's coefficient in NTSYS software showed no to low genetic similarity between *C. nutans* and *A. paniculata* samples, where coefficient values ranged from 0.0000 to 0.2857. Meanwhile, RAPD analysis revealed high genetic similarities between *C. nutans* samples. Malaysian samples although from different regions were identical (1.0000). All Thai samples were also identical except for CT5 with a coefficient value of 0.8889. The genetic similarity coefficient values between Malaysian and Thai samples ranged from 0.7273 to 0.8000. The Vietnamese sample showed a higher similarity to Malaysian *C. nutans* (0.7692) compared to four Thai samples (0.7273). Based on the UPGMA clustering analysis using Dice's coefficient on pooled-RAPD data, the 14 samples (12 *C. nutans* and two *A. paniculata*) were divided into four major divisions at a 0.52 coefficient level (Figure 5a). Division I comprised of all Malaysian samples, division II comprised of all Thai samples, which further separated into two sub-divisions (a:CT1-4; b:CT5), and cluster III comprised of the Vietnamese sample. Cluster IV comprised of the two *A. paniculata* samples, which were genetically different from each other, with a low similarity coefficient of 0.5333.

Microsatellite analysis

Two microsatellite primers, GACA4 (Figure 4a) and GTG5 (Figure 4b), were selected and pooled-microsatellite data was used for genetic analysis. The outcome of the microsatellite analysis was the same as

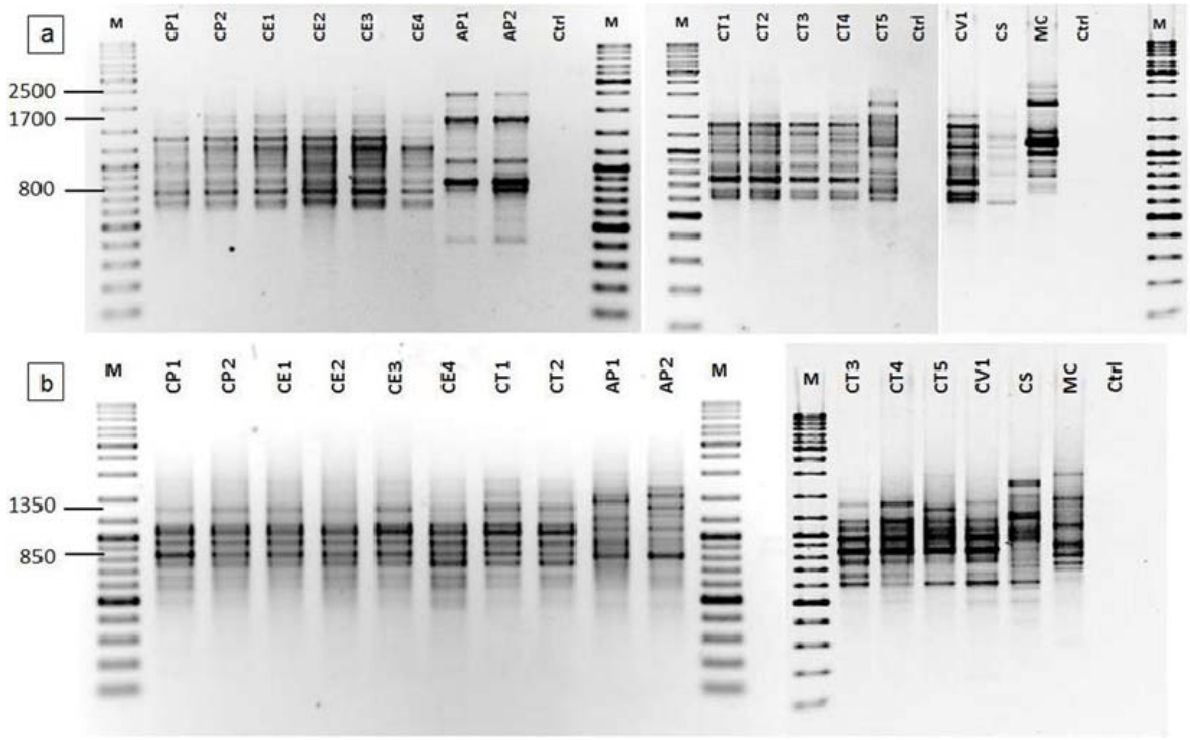

Figure 4. Microsatellite amplification products of GACA4. (a) and GTG5 (b). Lane M=100 bp DNA ladder; lanes CP1, CP2, CE1, CE2, CE3, CE4, CT1, CT2, CT3, CT4, CT5, and CV1=*C. nutans*; lanes AP1, AP2=*A. paniculata;* lane CS=*C. siamensis*; lane MC=*M. cochinchinensis*; lane Ctrl=no template control.

the RAPD analysis in which, (a) *C. nutans* was genetically different from *A. paniculata, C. siamensis* and the out-group; (b) *C. nutans* samples were genetically diverse and different between countries; and (c) some genetic homogeneity existed for Malaysian and Thai *C. nutans* samples, but not all. Overall, of the 36 loci scored, 100.0% were polymorphic. From these samples, 17 loci were scored for *C. nutans* and five loci (29.4%), which were produced from both primer amplifications, were polymorphic. Bands 800 and 1350 were absent only in Malaysian and CT5 samples, respectively. Band 1300 was absent in both Malaysian and Vietnamese samples, but present in all Thai samples. Band 1700 was absent in all samples except Malaysian samples and band 1800 was absent in the Vietnamese sample as well as in all Thai samples, except CT5. For *A. paniculata*, of the 13 loci scored, three (23.1%) were polymorphic. Fragments produced from microsatellite amplification ranged in size from 450 to 2500 bp.

The Dice's coefficient of similarity showed that there was a low genetic similarity between *C. nutans* and *A. paniculata* samples, where the coefficient values ranged from 0.2308 to 0.3077. Meanwhile, microsatellite analysis revealed a high genetic similarity between *C. nutans* samples. All Malaysian samples were identical (1.0000) but were different from Thai and Vietnamese samples. Thai samples were also identical, except for CT5 with a coefficient value of 0.9333. The genetic similarity co-efficient value between Malaysia and Thailand was high at 0.8667. The Vietnamese sample showed a higher similarity to Thai samples (except CT5, 0.8966), with a coefficient value of 0.9655 compared to Malaysian samples (0.8966).

The dendrogram based on UPGMA cluster analysis using Dice's coefficient on pooled-microsatellite data showed a different topology from the dendrogram constructed using RAPD data. The 14 samples (twelve *C. nutans* and two *A. paniculata*) were divided into three major divisions at a 0.86 coefficient level (Figure 5b). Division I comprised all Malaysian samples and division II comprised all Thai and Vietnamese samples, which was further divided into three sub-divisions (a:CT1-4; b:CV1; c:CT5). Cluster III comprised of the two *A. paniculata* samples, which were genetically different from each other, with a similarity coefficient of 0.8696. Combined banding profiles from PCA clustered all *C. nutans* samples together with low genetic diversity between countries and homogeneity from Malaysia (6 samples) and Thailand (4 samples) (Figure 5c). Interestingly, *A. paniculata* samples were also clustered closely together but were not identical and well separated from *C. nutans, C. siamensis* and the outgroup.

All DNA markers were able to detect genetic difference between genera, but only RAPD and microsatellite markers were able to detect genetic variation within and between species (Figure 5c). RAPD and microsatellite

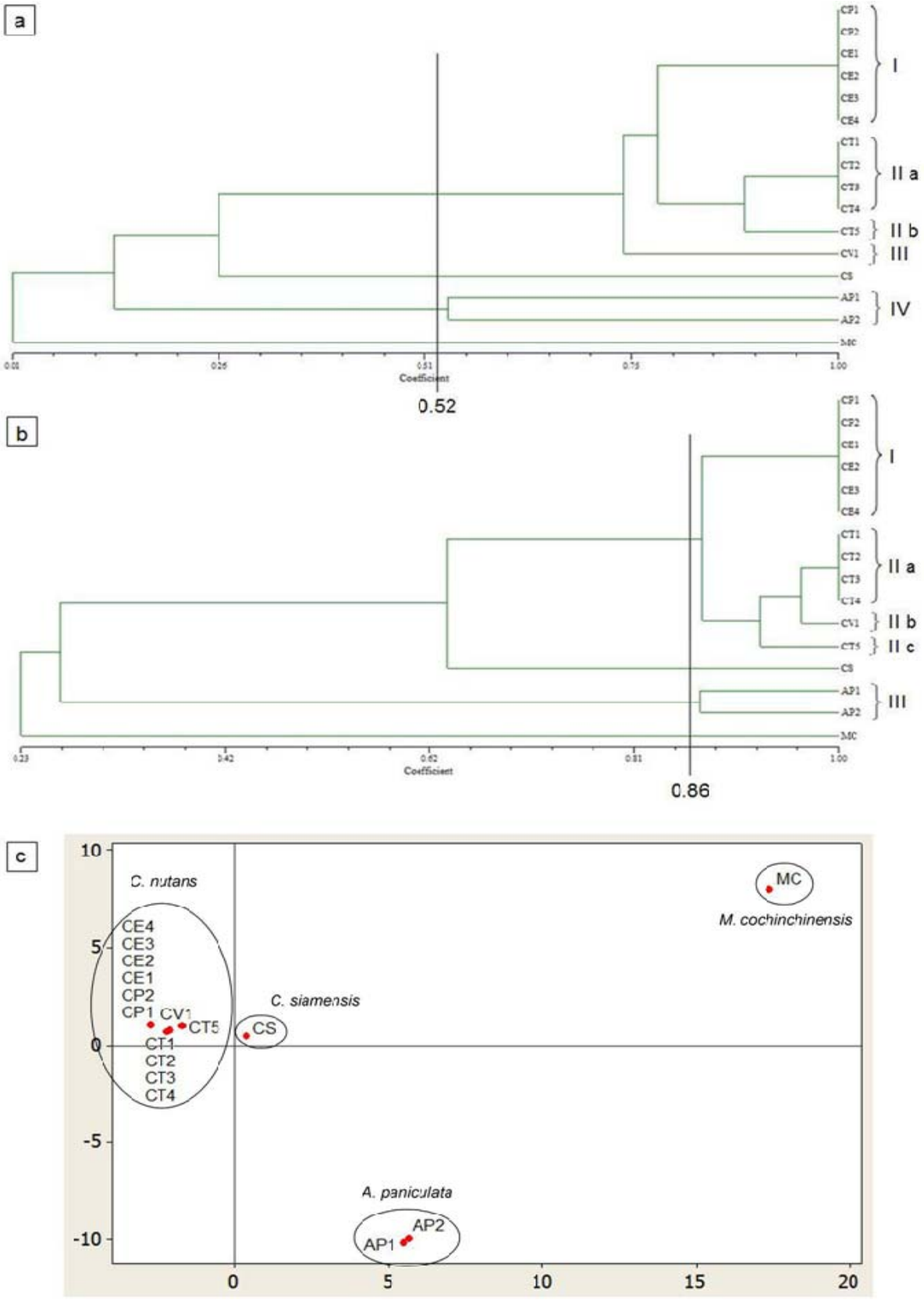

Figure 5. The relationships among 12 *C. nutans*, two *A. paniculata*, one *C. siamensis*, and one *M. cochinchinensis* samples according to UPGMA cluster analysis on RAPD (a) and microsatellite (b) data, and PCA analysis (c) on the combined RAPD and microsatellite data.

markers distinguished differences between and within countries of *C. nutans* and *A. paniculata* including distant, closely related and identical samples.

DISCUSSION

Genetic homogeneity in C. nutans

This is the first study to assess the genetic homogeneity of *C. nutans* samples between and within countries. Low variation was observed between countries and most samples within each country were genetically identical and highly likely to be clones. It is uncertain how these clones could exist from geographically distant sites, borders and terrains such as peninsular Malaysia and Sabah that were separated by the South China Sea. Some possibilities for the genetic homogeneity include: (1) all plants originated from the same parents and have stable persistent genomes that do not evolve rapidly; (2) the genetic diversity of the species may have been low originally and in the long-term domestication process, vegetative reproduction may have contributed to the genetic uniformity or monoculture of the species (Gepts, 2004); (3) inferior or different varieties no longer exist due to agricultural and economic pressures in rural communities; and (4) stem cuttings of the plant was shared between different growers and farms.

C. nutans is vegetatively propagated through stem cuttings (Panyakom, 2006), because the method is easy, economical and produces a high multiplication rate. In addition, flowers are rare, which results in severely reduced sexual reproduction and difficulty in crossing genotypes. The lack of flowers may be due to extended periods of vegetative propagation, selection and harvesting practices, where growers rapidly and constantly harvest the plant during the juvenile stage to meet the consumer demand. This may result in the inability of the plant to reach maturity for sexual reproduction, but is unlikely as some plants selected in this study at mature stages (CT2 sample) and had not flowered in its history (Mr Rattanapong, personal communication, January 27, 2013). Most members of Acanthaceae, especially weedy species produce fruits, or reproduce both by seeds and tubers, for example *Ruellia tuberosa* and some are easily propagated by seeds, which are produced in abundance, for example *Thunbergia fragans* (Meyer and Lavergne, 2004). However, according to Whistler (2000), most ornamental species in Acanthaceae are propagated by stem cuttings or layers in horticulture, and their fruits are rarely formed in cultivation.

Genetic homogeneity is consistent with other studies that have shown no genetic variation in clonal plants in other plant families but not Acanthaceae, for instance *Alternanthera philoxeroides* (Amaranthaceae) (Xu et al., 2003) and *Eichhornia crassipes* (Pontederiaceae) (Li et al., 2006). Other studies have also revealed low levels of genetic diversity in clonal species or in populations of such species with more intense vegetative reproduction such as *Microtis parviflora* (Orchidaceae) (Peakall and Beattie, 1991), *Haloragodendron lucasii* (Haloragaceae) (Sydes and Peakall, 2002), *Allium sativum* (Amaryllidaceae) (Paredes et al., 2008), *Iris sibirica* (Iridaceae) (Kostrakiewicz and Wroblewska, 2008), *Cypripedium calceolus* (Orchidaceae) (Brzosko et al., 2011) and *Chlorophytum borivilianum* (Asparagaceae) (Tripathi et al., 2012).

In contrast, a study on *Poikilacanthus macranthus* (Acanthaceae) revealed high clonal diversity in the species (Bush and Mulcahy, 1999), but this is not the case for *C. nutans*. Other studies have also shown unexpected high levels of genetic diversity in clonal plants such as *Anemone nemorosa* (Ranunculaceae) (Stehlik and Holderegger, 2000), *Vaccinium stamineum* L. (Ericaceae) (Kreher et al., 2000) *Vaccinium myrtillus* L. (Ericaceae) (Albert et al., 2003), *Potamogeton maackianus* (Potamogetonaceae) (Li et al., 2004), and *Goodyera repens* (Orchidaceae) (Brzosko et al., 2013). An hypothesis for the high levels of genetic diversity in clonal species is the presence of sexual reproduction in the founder populations, although it is sporadic and poor (Brzosko et al., 2013). Many populations of clonal species are founded by seeds, which is the most common means of recruitment of individuals to an existing population, but show increasing vegetative reproduction over time, likely due to limited resource availability or changing environmental conditions (Eriksson, 1993). Such populations can be genetically diverse by preserving genetic diversity of the initial population through clonal reproduction to dominate an area (Yeh et al., 1995). In addition, somatic mutations accumulated from constant division of mitotic cells can occur in clonal plants, as observed in *Grevillea rhizomatosa* (Proteaceae) (Gross et al., 2012), which would contribute to genetic diversity, but this has not been well studied in Acanthaceae.

Genetic separation of C. nutans*,* C. siamensis *and* A. paniculata

This is the first study to investigate the genetic differences between *C. nutans, C. siamensis* and *A. paniculata* and can be used as an alternative method to support traditional identification that can be subjective to interpretation such as vegetative characteristics, reproductive morphology and taste (Leyew, 2011). All molecular markers used in this study showed unambiguous differentiation between different genera and species based on DNA fingerprinting. This could be a useful validation tool for ambiguous samples *in situ* and is independent of visual characteristics that may be compromised due to environmental factors. The disadvantage of molecular

differentiation is its limited availability to growers particularly from developing nations compounded by impoverished rural regions where these medicinal plants are most popular. Even the collection of plants used in this study was with great difficulty since farmers only spoke native Asian languages and had minimal literacy skills so the technology may not be embraced widely.

Genetic variation in A. paniculata

By comparison, genotypes of *A. paniculata,* a member of the same family Acanthaceae, which reproduces sexually, were genetically different, although collected from the same farm. Previous studies on the genetic diversity in *A. paniculata* also showed considerable genetic variation within the species using RAPD, SSR and AFLP techniques (Maison et al., 2005; Chua, 2007; Wijarat et al., 2012). White flowers of *A. paniculata* are frequently observed and the plant is commonly propagated by seeds obtained from mature pods (Talei et al., 2011). Cross-pollination and sexual reproduction may contribute to the greater genetic variation within the species compared with vegetative reproduction in *C. nutans*.

Conclusion

This study shows that the genetic diversity of *C. nutans* is low and may affect its long-term survival and evolution if current methods of vegetative propagation from the same parent plant continue. Therefore, understanding and obtaining information on the levels and patterns of genetic diversity are essential for assisting the design of effective species conservation strategies. Studies on genetic diversity are necessary to discover novel factors that can contribute to increased heterogeneity and are valuable for preserving biodiversity. The genetic diversity of *C. nutans* from different countries was assessed and the samples from Malaysia were found to be genetically similar but not identical to those collected from Thailand or Vietnam. Currently, growers are unaware of the genetic similarity of *C. nutans* which may benefit the growing industry since product consistency is more likely. However, genetic differences between different countries as found in this study provides growers with fundamental knowledge on existing cultivars and may assist in future practices and breeding programs to increase biodiversity and favourable traits.

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Conflict of interests

The author(s) have not declared any conflict of interests.

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