Gene xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "*Andrographis paniculata*" to allogamy

Alireza Valdiani [a,*], Daryush Talei [b,c], Arash Javanmard [d,e], Soon Guan Tan [b], Mihdzar Abdul Kadir [f], Mahmood Maziah [a,g,h]

[a] Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia
[b] Department of Cell & Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia
[c] Medicinal Plant Research Center, Shahed University, Tehran, Iran
[d] Department of Animal Science, Faculty of Agriculture, University of Tabriz, Iran
[e] School of Computer Science and Engineering, Faculty of Engineering, The University of New South Wales, Sydney 2052, Australia
[f] Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia
[g] Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia
[h] Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia

ARTICLE INFO

Article history:
Received 5 September 2013
Received in revised form 17 March 2014
Accepted 24 March 2014
Available online xxxx

Keywords:
Crossability (fruit set)
Reproductive biology
RAPD markers
Genetic distance (GD)
Simple Matching (SM)

ABSTRACT

Andrographis paniculata Nees. (AP) is a self-pollinated medicinal herb with a wide range of pharmaceutical properties, facing a low diversity in Malaysia. Cross-pollination of AP accessions leads to considerable rates of heterosis in the agro-morphological characteristics and anticancer phytochemicals of this eminent medicinal herb. However, the poor crossability of the plant at the interpopulation or intraspecific levels is an obstacle from the evolutionary and breeding points of view as an average of 4.56% crossability was recorded for AP in this study. Hence, this research aimed to elicit the impact of parental genetic distances (GDs) on the rate of crossability of AP using seven accessions in 21 possible cross combinations. To this end, a set of 55 randomly amplified polymorphic DNA (RAPD) primers and a total of 13 agro-morphological markers were employed to test the hypothesis. Twenty-two out of the 55 RAPD primers amplified a total of 257 bands of which 107 bands were found to be polymorphic. The principal component analysis (PCA) based on the RAPD markers revealed that the studied AP accessions were distributed to three distinct groups. Furthermore, it was noticed that even a minor increase in GD between two parents can cause a decline in their crossability. Unlike, the morphological-based GDs acted neutrally to crossability. This finding suggests that, despite the low genetic diversity among the Malaysian APs, a population prescreening using RAPD markers would be useful to enhance the rate of fruit set through selecting the genetically adjacent parents.

1. Introduction

Andrographis paniculata Nees. (AP) is a self-pollinated annual herbaceous plant from the family Acanthaceae with a broad range of pharmaceutical properties, which has been stated as a low-diverse, endangered and red-listed species, but at lower risk (Natarajan et al., 2004; Valdiani et al., 2012a). However, the low genetic variation of this species may be associated with its self-pollinated mating system in which flowers in selfing populations receive genetically identical (or nearly so) pollen from a single source (themselves) (Mazer et al., 2010). Perhaps, this type of reproduction mechanism leads to the presence of higher proportions of homozygosity and fixed heterozygosity in its genome composition. Nevertheless, the cryptic feature of Malaysian AP can be also justified due to the historical population bottlenecks which happened after its introduction to the country (Valdiani et al., 2013). Evidently, a better understanding of genetic diversity is essential for conservation of a species (Rao and Hodgkin, 2002). Besides, studying the genetic variation in populations is of great interest in having a clear perspective of the evolutionary history of different organisms (Shringarpure, 2012). Though, the main objective of this study was not only limited to the two aforementioned aspects of

Abbreviations: AP, *Andrographis paniculata* Nees; GD, genetic distance; PCA, principal component analysis; RAPD, randomly amplified polymorphic DNA; SM, Simple Matching; SD, Sorensen–Dice; J, Jaccard; UPGMA, unweighted pair group method with arithmetic mean; NTSYS-Pc, Numerical Taxonomy and Multivariate Analysis System; AFLP, amplified fragment length polymorphism; AAD, arbitrarily amplified dominant; SSR, simple sequence repeats; RCBD, randomized complete block design; ANOVA, analysis of variance; IKI, iodine potassium iodide; PH, plant height; NPB, number of primary branches; PBL, primary branches length; NLN, number of leaves in each node; LL, leaf length; LW, leaf width; SG, stem girth; SIL, shoot internode length; NI, number of internodes; SDW, shoot dry weight; LDW, leaf dry weight; STDW, stem dry weight; L/S, leaf/stem dry weight ratio per plant; P, parent; H, hybrid; SE, Selangor; NS, Negeri Sembilan; PE, Perak; PA, Pahang; KE, Kelantan; TE, Terengganu.

* Corresponding author.
E-mail address: alireza.valdiani@gmail.com (A. Valdiani).

http://dx.doi.org/10.1016/j.gene.2014.03.039
0378-1119/

measuring the genetic distance (GD) as it could not be considered as a novel research topic, but it aimed to study the effect of parental genetic distances on the rate of crossability (cross-compatibility) of AP populations in Malaysia. However, the hypothesis of the present study originated during an independent research that was conducted prior to the current endeavor for realizing the impact of cross-combination (accession), floral morphology (style length) and crossing time on the intraspecific crossability of Malaysian AP accessions (Valdiani et al., 2012c). The results of the aforesaid research showed that AP's response from the crossability point of view was significantly different to each of the applied treatments. Interestingly, it was found out that the rate of crossability can be increased by performing hand-pollination (outcrossing) of the 12 mm styles during early morning or late evening only in some cross combinations. These fluctuating observations took our attention away from the relatively promising results while we conclude that the increasing trend of crossability is limited to some crosses but not to all. In other words, it was noticed that even under the best situation (the proper time of out-crossing and style length), some of the crosses failed to produce a single pod. Thus, it is postulated that the genetic incongruity of the accessions could be potentially involved with this breeding malfunction, while there are credible evidences implying that intraspecific hybridization generates genetic diversification and causes heteroses of the phytochemical and agro-morphological traits in the plant's population in most of the cross combinations (Valdiani et al., 2012b). In consequence, crossability of AP accessions in Malaysia needs to receive a considerable attention to expedite producing prolific varieties of the plant. Hence, troubleshooting of the crossability barrier must be prioritized over other obstacles.

Conceptually, it has been accepted that genetic distance is a useful indicator of connectedness within and between species, and is helpful for reconstructing the historic and phylogenetic relationships among such groups (Shriver et al., 1995). Therefore, we decided to use GD for highlighting the genetic differentiations among the seven Malaysian AP accessions, and to connect them with the crossability issue. Assessment of crossability by employing GD is regarded as a new idea, although the conspecificity of various plant species has formerly been studied by a combination of molecular data such as AFLP (amplified fragment length polymorphism) markers along with additional crossability data (Houghton-Thompson et al., 2005; Koopman et al., 2001). Instances for application of marker systems in the evaluation of plant crossability are not limited to AFLPs, since morphological markers (Shore and Barrett, 1985), allozymes (Irwin, 2001), RAPDs (Bruel et al., 2006; Espinoza et al., 2002; Trame et al., 1995), as well as a combination of morphological and RAPD markers are suggested to study the genetic diversity of plants (Fahmi et al., 2012). To comply with this recommendation, many researchers had employed a series of morphological and RAPD markers together for running different analyses of genetic diversity in AP, prior to the present investigation (Lattoo et al., 2008; Maison et al., 2005; Padmesh et al., 1999; Sabu, 2002; Sharma et al., 2009).

In general, the ease with which arbitrarily amplified dominant (AAD) markers such as RAPDs, AFLPs and ISSRs could be used to create the vast amounts of data led to their rapid application to address a diverse range of biological questions (Bussell et al., 2005). The superiority of RAPD markers compared to that of the SSR (simple sequence repeats), and AFLP markers has been proven for genetic evaluation of AP populations, in recent studies, where the highest and lowest values of the assay efficiency index (A_i) were recorded in the order of 0.145 > 0.0048 > 0.000157, for RAPD, AFLP and SSR markers, respectively (Wijarat et al., 2012). Considering these facts, and taking the unsatisfactory results of the previous efforts into consideration, which disclosed the fully monomorphic pattern of the AP's genome by using 17 sets of RAPD primers (Valdiani et al., 2012b), and 24 sets of microsatellite primers (Valdiani et al., 2013), it was concluded that it is worth to reconsider the RAPD markers in a larger number "as an alternative approach" to study the genetic diversity of AP, as a subsidiary objective. More importantly, this would allow examining the correctitude of the hypothesis whether GD associates crossability, as the main objective of the present study. Hence, triplicating the initial number of the RAPDs used (17 primers) up to 55 along with morphological markers, was considered to increase the credibility of this assessment.

2. Materials and methods

2.1. Plant materials

A total of 400 plants belonging to seven AP accessions including P1 (11179SE), P2 (11216NS), P3 (11261PE), P4 (11313PA), P5 (11322PA), P6 (11344KE) and P7 (11350TE) were collected from six states across Malaysia, and developed for using as the parental sources in the one-way diallel crosses, with the order mentioned previously (Valdiani et al., 2012b, 2012c). The geographical distribution of the selected AP accessions used is presented in Fig. 1.

2.2. Growth condition and transplantations

A two-step germinating procedure was undertaken to increase the seed germination. Accordingly, an adequate number of seeds of the seven parental accessions were surface sterilized using 10% sodium hypochlorite (NaOCl) solution inside separate petri plates for 10 min (Talei et al., 2011). The Whatman No. 2 filter papers were chosen as the seedbeds to support a better germination. To avoid any contamination and humidity loss, all petri plates were sealed carefully (Talei et al., 2012). The sealed petri plates were then placed in a growth chamber with an adjusted temperature between 28 °C and 30 °C (Valdiani et al., 2012c). The first transplantation of the two-leaf seedlings from the petri plates into the Jiffy pots was carried out when the seedlings were just ten-days old. The second transplantation was conducted after one month when the seedlings reached to 6-leaf stage. This time, the seedlings were transferred into 22 × 35 cm polybags (Valdiani et al., 2012c). To enhance the reliability of the outcomes by reducing the environmental effects, the content (soil) of the polybags was homogenized with fine sand, topsoil, and organic material at the ratio of 2:1:1. Macro- and microelement-enriched WELGRO compound fertilizer (NPK 15:30:15) was applied four times during the growth period.

2.3. Experimental design

Since morphological traits in AP are extensively influenced by environmental conditions, the experiment was carried out in a field pot trial. Furthermore, to double-confirm the reliability of the upshots, field experiments based on Randomized Complete Block Design (RCBD) with five replicates were implemented at two planting seasons (ten replicates in total).

2.4. Pollen viability, stigma receptivity test, emasculation and crossing scheme

The viability of pollens was affirmed using IKI solution (iodine + potassium iodide) as described previously (Valdiani et al., 2012c). The hydrogen peroxide test was employed to evaluate the stigma receptivity as explained previously (Valdiani et al., 2012b).

In order to avoid the anther dehiscence, the flowers were emasculated carefully when the bud length was 11 mm, so that the two-celled anthers were removed using sterilized forceps to prevent the accidental self-pollination, as described previously (Valdiani et al., 2012b, 2012c). After emasculation the stigmas were precisely checked using a hand-glass, and the pollen-contaminated stigmas were eliminated, while the clean stigmas were hand-pollinated on several occasions in 12, 13 and 14 mm by fresh pollens. During the emasculation and outcrossing processes, the plants were covered by micro-mesh entirely to evade the unwanted pollination (Valdiani et al., 2012c). The seven

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039

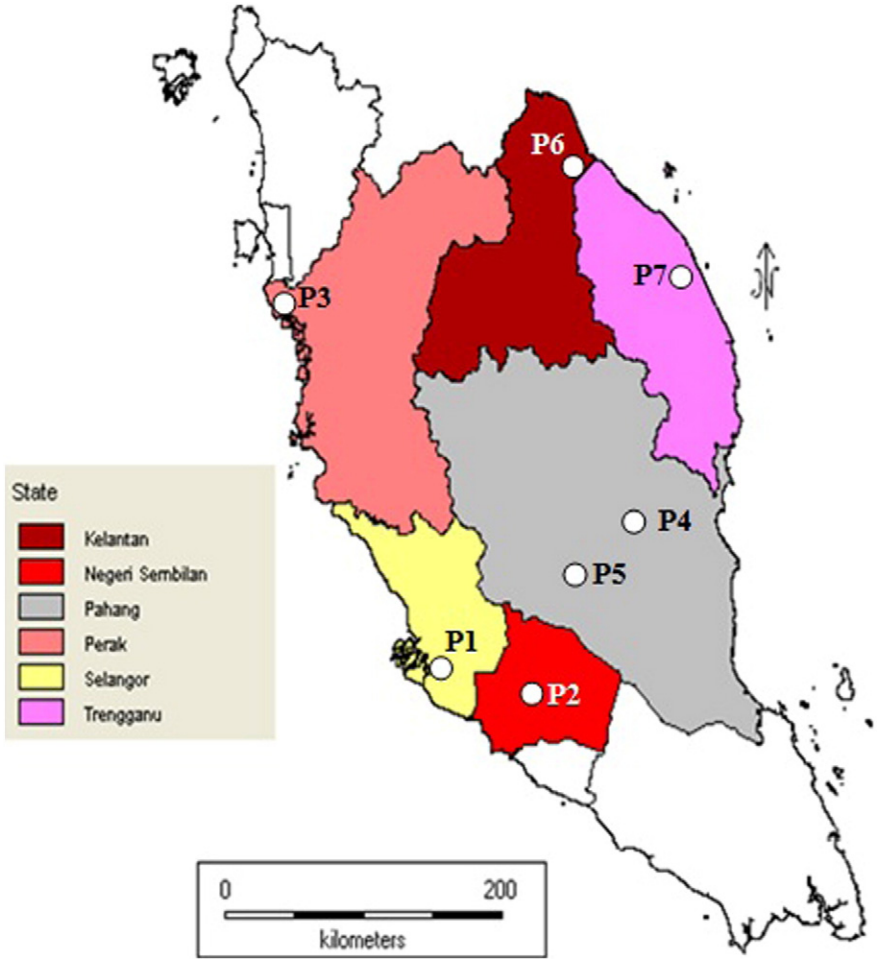

Fig. 1. Geographical distribution of the selected AP accessions across Peninsular Malaysia.

parental individuals of AP were out-crossed in all 21 possible combinations as shown in Table 1. A sum of 8077 crosses was carried out during four months, so that 360 crosses were equally conducted in each cross combination except for two of them including P2 × P6 (602 crosses) and P3 × P6 (635 crosses), as described previously (Valdiani et al., 2012c). The reason of such an inequality in the number of the crosses

Table 1
Cross-pollination scheme among the seven parental AP accessions.

Hybrids	Combination†	Pistillate ♀	Staminate ♂	Number of crosses	Aborted crosses (%)	Fruit set (%)
H1	P1 × P2	11179SE	11216NS	360	92.50	7.50
H2	P1 × P3	11179SE	11261PE	360	94.17	5.83
H3	P1 × P4	11179SE	11313PA	360	95.83	4.17
H4	P1 × P5	11179SE	11322PA	360	95.00	5.00
H5	P1 × P6	11179SE	11344KE	360	95.83	4.17
H6	P1 × P7	11179SE	11350TE	360	95.83	4.17
H7	P2 × P3	11216NS	11261PE	360	98.33	1.67
H8	P2 × P4	11216NS	11313PA	360	98.33	1.67
H9	P2 × P5	11216NS	11322PA	360	97.50	2.50
H10	P2 × P6	11216NS	11344KE	602	99.45	0.55
H11	P2 × P7	11216NS	11350TE	360	97.50	2.50
H12	P3 × P4	11261PE	11313PA	360	98.33	1.67
H13	P3 × P5	11261PE	11322PA	360	96.67	3.33
H14	P3 × P6	11261PE	11344KE	635	99.75	0.25
H15	P3 × P7	11261PE	11350TE	360	99.17	0.83
H16	P4 × P5	11313PA	11322PA	360	90.00	10.00
H17	P4 × P6	11313PA	11344KE	360	89.17	10.83
H18	P4 × P7	11313PA	11350TE	360	90.83	9.17
H19	P5 × P6	11322PA	11344KE	360	95.00	5.00
H20	P5 × P7	11322PA	11350TE	360	86.67	13.33
H21	P6 × P7	11344KE	11350TE	360	98.33	1.67
Total	21	21	21	8077	–	–
Average	–	–	–	384.62	95.44	4.56

H: hybrid, P: parent, SE: Selangor, NS: Negeri Sembilan, PE: Perak, PA: Pahang, KE: Kelantan, TE: Terengganu, ♀: female parent, ♂: male parent.

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039

was the lack of fruit set in the first 360 performed crosses for combinations P2 × P6 and P3 × P6. The hand-pollinated flowers were tagged mentioning the style length, date and time of pollination.

Finally, the fruit set (pod set) was used as an index to determine the amount of crossability of AP accessions on each combination.

2.5. Genomic DNA extraction and assessment

The modified Doyle and Doyle CTAB-based DNA extraction protocol (Doyle and Doyle, 1990) was used to extract the genomic DNA of the seven parental AP plants, as described previously (Valdiani et al., 2012b). Quality and quantity of the genomic DNA of all the seven parental accessions were determined using both gel electrophoresis and spectrophotometric assays. One microliter of each genomic DNA sample was loaded on 2% (w/v) agarose gel and resolved for 45 min at 80 V. The gel was stained using ethidium bromide and visualized under UV light using Gel Documenter (Bio-Rad, USA). Bands of genomic DNA were observed for intensity and any probable contamination. A further quality evaluation of the extracted DNAs was conducted by estimating the OD260/OD280 ratio using a Nanodrop spectrophotometer, model ND1000 (NanoDrop Technologies, Inc., USA).

2.6. RAPD-PCR protocols

The PCR was carried out in a total volume of 25 μL for each microtube, while the final concentration of the PCR master mixes was adjusted at Green GoTaq® Flexi Buffer (1×), PCR nucleotide mix or dNTP (0.2 mM), $MgCl_2$ (1.5 mM), primer (0.4 μM), 0.7 unit of Taq DNA polymerase (GoTaq® PCR Core Systems, Promega, USA) and 50 ng of the genomic DNA. The PCR program was designed with a slight change in Sharma et al. (2009) protocol using a Thermal Cycler machine model TPersonal (Biometra, Germany) as follows: An initial denaturation at 95 °C for 3 min, followed by 40 cycles of 60 s at 94 °C as denaturation, 60 s at 30 °C as annealing, and 2 min at 72 °C as extension. A final extension step at 72 °C for 7 min was defined as the end of the PCR amplification procedure. The PCR products were separated by electrophoresis on 2% (w/v) agarose gel. The electrophoresis was performed for 90 min at 80 V in 1× TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.3). The molecular size of the amplified fragments was estimated using a 100 bp DNA ladder (Fermentas, GmbH, Germany). The gel staining was done using ethidium bromide (0.5 μg/mL) and destained with distilled water. The amplified fragments were visualized under UV light using the same gel documentation instrument (Bio-Rad, USA). The PCR was repeated twice for each primer to confirm the replicability of the markers.

2.7. Molecular markers and data collection

A set of 55 RAPD primers (Operon Technologies, USA) was used for DNA fingerprinting of the seven parental accessions of AP (Supplementary Table 1). Scoring the randomly amplified DNA fragments was performed by using the UVIDoc software version 99.02 for the actual band sizing, while the code "1" was given for the presence of a band and "0" for the absence of the same band.

2.8. Morphological markers and data collection

Data pertaining to 13 agro-morphological characteristics in the seven parental plants including plant height (PH), number of primary branches (NPB), primary branches length (PBL), number of leaves in each node (NLN), leaf length (LL), leaf width (LW), stem girth (SG), shoot internode length (SIL), number of internodes (NI), shoot dry weight (SDW), leaf dry weight (LDW), stem dry weight (STDW) and leaf/stem dry weight ratio per plant (L/S) were collected and analyzed. However, according to the ANOVA results, only nine of these characteristics consisting of PH, NPB, PBL, SDW, LDW, SDW, LDW, STDW and L/S, which were significantly different among the accessions, were used as the morphological markers in this research. The data collection was carried out right before flowering and the plants were harvested immediately after that. The dry weights were measured after harvest.

2.9. Genetic similarity and phylogenetic analysis based on the molecular markers (RAPDs)

The data were analyzed using SIMQUAL (Similarity for Qualitative Data) to generate a similarity coefficient. Similarity matrix from detection of polymorphic fragments was analyzed based on Simple Matching (SM) similarity coefficient (Sokal and Michener, 1958). NTSYS-pc version 2.1 (Exeter Software, USA) was utilized for this part of the analyses (Rohlf, 2000). Complementarily, the genetic similarity calculation resulted in estimating the genetic distance or dissimilarity, as well (GD = 1 − Similarity). However, to confirm the reliability of the SM approach (Dalirsefat et al., 2009; Koopman et al., 2001), the genetic distance was additionally calculated based on Sorensen–Dice (SD) (Dice, 1945; Sorensen, 1948) and Jaccard's similarity coefficients (Jaccard, 1901), but only SM was used for constructing the dendrogram.

The SM, SD and Jaccard's similarities were calculated using the following equations:

$$\text{Simple matching } (SM) = (a+d)/(a+b+c+d)$$

$$\text{Sorensen–Dice } (SD) = 2a/2a+b+c$$

$$\text{Jaccard } (J) = a/a+b+c$$

where;

- a = 1.1 Presence of a specific band in both of the assumed individuals (i and j)
- b = 1.0 Presence of a specific band in individual (i) and absence in (j)
- c = 0.1 Absence of a specific band in individual (i) and presence in (j)
- d = 0.0 Absence of a specific band in both of the assumed individuals (i and j).

Initially, the genetic similarity matrices were calculated by using NTSYS-pc version 2.1 (Exeter Software, USA). As a second step and to generate a dendrogram based on dissimilarities (for a better understanding of the aim of this study) the similarity matrices were converted into symmetric dissimilarity matrices using NT edit 1.07c software and the modified file was saved separately. Finally, the mentioned file was subjected to cluster analysis by employing SAHN (Sequential, Agglomerative, Hierarchical and Nested clustering) and using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath and Sokal, 1973). This method applies sequential clustering, where local relationships are identified. The plot tree option was clicked to display the constructed dendrogram.

2.10. Genetic similarity and phylogenetic analysis based on the morphological markers

As mentioned before, only nine of the morphological characteristics including PH, NPB, PBL, NLN, NI, SDW, LDW, STDW, and L/S were utilized for the phylogenetic analysis. The clustering analysis was performed using NTSYS-pc version 2.1 (Exeter Software, USA). The data were initially standardized and analyzed using SIMINT module. Due to the parametric nature of the data, the Euclidean distance was selected as the coefficient used to express the genetic variation among the studied seven AP accessions. Creating the related dendrogram was accomplished using UPGMA clustering algorithm.

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039

2.11. Linear regression analysis of GDs and crossability

The final step of the analyses was taken in a quest of calculating the simple regression between the morphological-based as well as the molecular-based genetic distances and the crossability percentages of the AP accessions. In general, regression is used to predict the value of one variable based on the value of another variable.

The data for regression consist of pairs in the form (x,y). The GDs were considered as the independent variable (x). Hypothetically, it is assumed that the values calculated for the dependent variable (y) result from the changes in the independent variable (x) (Daniel, 1999). Regression analysis in fact determines the nature and strength of the mentioned relationship (if any).

2.12. Graphical demonstration and slope of the regression line

Typically, data are represented using a plot called a scatter plot or scatter diagram or x–y plot. Consequently, an equation for the regression line that fits the data was generated using the pair points (x,y) by being plotted on the Cartesian coordinate system. The result of such a process was a straight line on the Cartesian coordinate system having the equation $y = mx + b$, where (m) is the slope of the line, and "b" is the y-intercept of the line (Daniel, 1999). Indeed, the slope of the line is always the coefficient of the "x" in the equation, and the equation determines the slope and the y-intercept, anyway. Besides, scatter diagrams are capable of showing a direct relationship between x and y. Hence, when the slope of the line (m) is positive the direct relationship exists ("y" increases as "x" increases, and the function runs so-called "uphill" from left to right), while an inverse relationship exists when the slope of the line (m) is negative ("y" decreases as "x" increases and the function runs alleged "downhill" going left to right). When the slope is zero ($m = 0$) no relationship exists.

2.13. Statistical analyses

The SAS (Statistical Analysis Software) program version 9.1 was used for ANOVA analysis of the morphological traits. Duncan's multiple range test was performed for mean comparison at $\alpha = 0.05$ and 0.01 (SAS Institute Inc., 2003). Principal component analysis (PCA) was performed using NTSYS-pc software version 2.1 (Exeter Software, USA) to visualize the representation of the genetic relationship among the AP accessions in three dimensional graphical features. The linear regression equation and the related graphs were generated using Microsoft Excel version 2010.

3. Results

3.1. Analysis of variance and mean comparison of the morphological traits

As mentioned in Materials and methods section, the field pot trial was undertaken as an efficient strategy to increase the reliability and precision of the experiment. The ANOVA results revealed that the technique was accurate enough as no significant difference was observed in replicates and times for most of the studied traits. Accordingly, the interactions between time and accessions (T × A) for all traits except AGP were found to be non-significant (Table 2). This case proved that the time (planting season) and accession acted perfectly as the two independent factors of this study. In addition, the ANOVA revealed that the accessions were significantly variant ($P \leq 0.01$) in terms of nine morphological characteristics consisting of PH, NPB, PBL, SDW, LDW, SDW, LDW, STDW and L/S, while no significant differences existed among the seven studied accessions in terms of LL, LW, SD and SIL (Table 2). Therefore, these four characters were not used as morphological markers in phylogenetic and GD analyses. Mean comparison results of the morphological traits were in a great agreement with the ANOVA, as the same trends of significance were perfectly repeated by the mean of the traits among the accessions (Table 3).

3.2. Pollen viability and stigma receptivity results

The results of pollen viability and stigma receptivity tests of the current study have been presented previously in an open access publication (Valdiani et al., 2012c). Yet, it is worthwhile to restate that the pollen viability in AP was not significantly different among the accessions ($P > 0.05$), therefore the pollen source is not an issue in this species. However, about the stigma receptivity, strongly positive responses were recorded in 12, 13 and 14 mm flower buds, suggesting that the full receptiveness of the stigma was commenced when buds reached to 12 mm in length (Valdiani et al., 2012c).

3.3. PCR-RAPD analysis

Of 55 decamer primers used to screen the representative DNA samples of the seven parental accessions, 22 primers (40%) were detected as producing polymorphic markers, while 33 of them (60%) were scored as producing monomorphic banding patterns among the seven accessions. The 22 polymorphic primers generated a total of 257 bands of which 107 were polymorphic. The number of bands per accession ranged from 7 to 16, and the amplified bands ranged in size between 176 and 4313 bp (Table 4). The overall mean genetic diversity based on Shannon's index and using 22 polymorphic primers averaged 0.201 (Table 4). Moreover, the lowest and highest gene diversity values were based on the bands produced by primers OPZ-10 (0.0350 ± 0.0889) and OPA-16 (0.3020 ± 0.2212), respectively (Table 4). Accordingly, the lowest and highest Shannon indices as well as the polymorphism percentages were obtained when using the same primers (Table 4). These outcomes are indicative of slight differences among the studied parental accessions, and also revealed the limited genetic source of the Malaysian AP population, which is in a complete agreement with the previous findings (Valdiani et al., 2012b, 2013).

Table 2
Analysis of variance of the agro-morphological traits in the seven parental AP accessions.

Source	df	Mean square												
		PH	NPB	PBL	NLN	LL	LW	SG	SIL	NI	SDW	LDW	STDW	L/S
Replication	4	7.39^{ns}	19.24^{ns}	5.85^{ns}	0.06^{ns}	1.27^{ns}	11.90^{ns}	0.004^{ns}	0.37^{ns}	0.23^{ns}	16.17^{ns}	3.76^{ns}	4.75^{*}	0.01^{ns}
Time (T)	1	58.69^{ns}	35.71^{ns}	0.06^{ns}	0.23^{ns}	0.08^{ns}	11.26^{ns}	0.008^{ns}	0.08^{ns}	0.01^{ns}	6.92^{ns}	0.13^{ns}	8.96^{*}	0.09^{*}
Accession (A)	6	212.93^{**}	98.49^{**}	45.18^{**}	0.91^{**}	1.57^{ns}	10.87^{ns}	0.008^{ns}	0.35^{ns}	6.22^{**}	19.96^{**}	6.56^{**}	10.83^{**}	0.13^{**}
T × A	6	29.22^{ns}	9.35^{ns}	14.41^{ns}	0.23^{ns}	0.44^{ns}	10.08^{ns}	0.004^{ns}	0.19^{ns}	0.74^{ns}	4.06^{ns}	0.88^{ns}	1.42^{ns}	0.01^{ns}
Error	52	19.62	12.85	11.03	0.15	1.08	11.65	0.004	0.16	0.55	4.52	1.72	1.48	0.01

ns: non-significant. PH: plant height (cm), NPB: number of primary branches, PBL: primary branch length (cm), NLN: number of leaf at each node, LL: leaf length (cm), LW: leaf width (cm), SG: stem girth (mm), SIL stem internodes length (cm), NI: number of internodes, SDW: shoot dry weight per plant (g), LDW: leaf dry weight per plant (g), STDW: stem dry weight per plant (g), L/S: leaf/stem dry weight ratio per plant.
** Significant at $P \leq 0.01$.
* Significant at $P \leq 0.05$.

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039

Table 3
Mean comparison test of the morphological traits in the seven parental AP accessions.

Accessions	Mean												
	PH	NPB	PBL	NLN	LL	LW	SG	SIL	NI	SDW	LDW	STDW	L/S
P1	50.6^{a}	39.2^{c}	39.9^{a}	2.8^{b}	9.6^{a}	2.9^{a}	0.46^{a}	2.86^{a}	16.0^{a}	26.3^{bcd}	15.2^{d}	11.2^{a}	1.38^{c}
P2	60.7^{b}	34.5^{b}	45.4^{c}	2.0^{a}	9.1^{a}	3.0^{a}	0.46^{a}	3.07^{ab}	17.8^{bc}	24.1^{a}	12.8^{a}	11.2^{a}	1.15^{ab}
P3	60.9^{b}	29.9^{a}	41.0^{ab}	2.0^{a}	9.5^{a}	3.0^{a}	0.53^{ab}	3.24^{ab}	18.3^{c}	27.7^{cd}	14.4^{bcd}	13.3^{b}	1.09^{a}
P4	62.3^{b}	31.4^{ab}	44.9^{c}	2.0^{a}	10.2^{a}	5.7^{a}	0.52^{ab}	3.22^{ab}	18.3^{c}	28.3^{d}	14.3^{bcd}	13.9^{b}	1.03^{a}
P5	62.7^{b}	32.2^{ab}	42.6^{abc}	2.0^{a}	9.3^{a}	3.0^{a}	0.51^{ab}	3.33^{ab}	17.9^{bc}	25.5^{ab}	13.6^{ab}	11.9^{a}	1.15^{ab}
P6	63.7^{b}	31.2^{ab}	43.3^{bc}	2.0^{a}	9.1^{a}	2.9^{a}	0.47^{ab}	2.87^{a}	17.5^{c}	25.9^{abc}	13.8^{abc}	12.1^{a}	1.15^{ab}
P7	64.2^{b}	31.4^{ab}	45.0^{c}	2.0^{a}	9.9^{a}	3.1^{a}	0.49^{ab}	3.25^{ab}	17.9^{bc}	27.0^{bcd}	15.0^{cd}	12.1^{a}	1.27^{bc}

Different letters indicate significant difference among accessions using Duncan's multiple comparison test at $P \leq 0.01$. PH: plant height (cm), NPB: number of primary branches, PBL: primary branch length (cm), NLN: number of leaf at each node, LL: leaf length (cm), LW: leaf width (cm), SG: stem girth (mm), SIL stem internodes length (cm), NI: number of internodes, SDW: shoot dry weight per plant (g), LDW: leaf dry weight per plant (g), STDW: stem dry weight per plant (g). L/S: leaf/stem dry weight ratio per plant.

Interestingly, the most polymorphic patterns among the seven accessions were profiled in accessions P2 (11216NS) and P6 (11344KE) as presented in Fig. 2.

3.4. Phylogenetic analysis based on the RAPD markers

The UPGMA clustering method when used on RAPD markers (as mentioned earlier in Introduction section) had been largely utilized in the genetic diversity studies of AP. As mentioned previously, the dendrogram was generated based on Simple Matching (SM) coefficient and using symmetric dissimilarity matrices and not similarity matrices. In fact, this strategy was undertaken to generate a dendrogram that arranges the distant parental accessions inside the same or neighboring clusters. Remarkably, two of the three accessions (P2 and P6) that tended hardly to outcross with each other in the combinations P2 × P6 and P3 × P6 were placed in cluster II, while the accession P3 was placed in the neighboring cluster III (Fig. 3). This could presumably cause the low crossability of the aforementioned crosses. According to the clustering analysis results, the Simple Matching coefficient based on symmetric dissimilarity matrices ranged from 0.68 to 0.88 (Fig. 3).

3.5. Phylogenetic analysis based on the morphological markers

Cluster analysis based on the morphological traits and using the UPGMA method ascertained that the geographical distribution of the studied AP accessions and their morphological demonstration are in a great harmony. Interestingly, the morphological traits involved in this study led the seven accessions to be arrayed from one to seven in the dendrogram, respectively (Fig. 4). The phylogeny tree comprised of two main groups with P1 from Selangor state located independently in cluster I, while cluster II consisted of the rest of the selected accessions (six accessions from P2 to P7). The Euclidean distance that ranged from 0.07 to 0.74 was ascribed to the seven AP accessions using the UPGMA method (Fig. 4).

3.6. Principal component analysis (PCA)

PCA analysis based on the RAPD primers revealed that the studied AP accessions were clearly distributed into three distant groups. Considering this, PC1 comprised of accessions P1, P3, P4, P5 and P7 all together, while accessions P2 and P6 represented PC2 and PC3, respectively (Fig. 5). On the other hand, the PCA analysis results and the observed genetic distances between accessions P2 and P6 as well as accessions P3 and P6 were in line with the phylogenetic relationships of these accessions based on RAPD markers (Fig. 3). In other words, accessions P2, P3 and P6 were indicated as the most distant parents, and this could be considered as another indicator for the weak crossability responses of accessions P2 and P3 to accession P6.

Table 4
Polymorphic content of the 22 RAPD markers applied on the seven AP accessions.

	Primer	Size range (bp)	NAL[a]	NPB[b]	Gene diversity	Shannon index	Polymorphism (%)
1	OPA-05	266–3088	13	3	0.0785 ± 0.0949	0.1196 ± 0.1402	23.08
2	OPA-07	389–2000	11	3	0.1262 ± 0.2171	0.1786 ± 0.3066	27.27
3	OPA-10	228–2914	14	3	0.0525 ± 0.1043	0.0879 ± 0.1746	21.43
4	OPA-11	278–3000	9	5	0.1361 ± 0.1291	0.2278 ± 0.2162	55.56
5	OPA-12	217–2629	16	4	0.0918 ± 0.1683	0.1378 ± 0.2501	25.00
6	OPA-16	335–2337	10	7	0.3020 ± 0.2212	0.4338 ± 0.3105	70.00
7	OPA-18	232–2395	14	7	0.1458 ± 0.1607	0.2319 ± 0.2486	50.00
8	OPA-20	227–2452	13	7	0.1319 ± 0.1271	0.2208 ± 0.2128	53.85
9	OPB-01	824–2505	7	1	0.0350 ± 0.0926	0.0586 ± 0.1550	14.29
10	OPB-02	612–3182	10	4	0.1143 ± 0.1549	0.1829 ± 0.2422	40.00
11	OPB-14	239–3923	14	7	0.1749 ± 0.1944	0.2649 ± 0.2859	50.00
12	OPD-16	352–4313	16	8	0.1531 ± 0.1760	0.2392 ± 0.2616	50.00
13	OPH-17	505–2762	7	1	0.0490 ± 0.1095	0.0820 ± 0.1834	20.00
14	OPZ-08	266–2266	14	6	0.1544 ± 0.1872	0.2221 ± 0.2768	42.86
15	OPZ-10	338–3067	14	2	0.0350 ± 0.0889	0.0586 ± 0.1489	14.29
16	OPZ-12	176–1880	8	4	0.1224 ± 0.1309	0.2051 ± 0.2192	50.00
17	OPAW-03	207–3786	8	4	0.1531 ± 0.1822	0.2392 ± 0.2708	50.00
18	OPAW-11	340–3000	13	6	0.1570 ± 0.1899	0.2392 ± 0.2803	46.15
19	OPAO-12	250–2136	14	9	0.2332 ± 0.2021	0.3490 ± 0.2886	64.29
20	OPBB-18	290–3867	13	8	0.1884 ± 0.1776	0.2943 ± 0.2607	61.54
21	OPPO-05	245–3846	10	5	0.1224 ± 0.1291	0.2051 ± 0.2162	50.00
22	OPW-15	346–2000	9	3	0.0816 ± 0.1224	0.1367 ± 0.2051	33.00
Total	22	–	257	107	–	–	–
Overall Mean			11.68	4.86	0.129 ± 0.1527	0.201	41.48

[a] Number of amplified loci (bands).
[b] Number of polymorphic bands.

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039

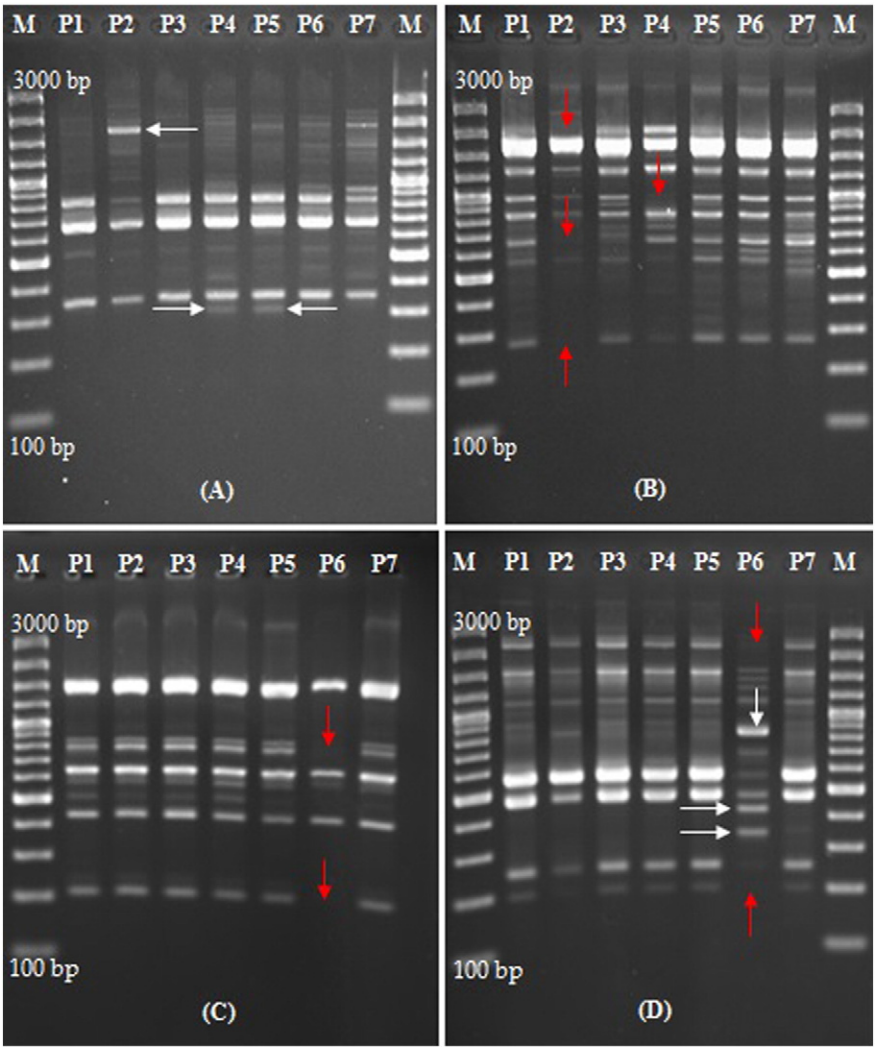

Fig. 2. PCR-RAPD polymorphic DNA fingerprints of seven AP accessions generated by the RAPD primers. (A) OPA-16, (B) OPBB-18, (C) OPAP-20, (D) OPA-18 on 2% agarose gel. Lanes P1–P7 represent the accessions and (M) 100 bp Fermentas size marker. Red arrows highlight the absence of the bands as a symbol of polymorphism, and white arrows demonstrate the presence of polymorphic bands, which happened to be in accessions P2 and P6 mostly.

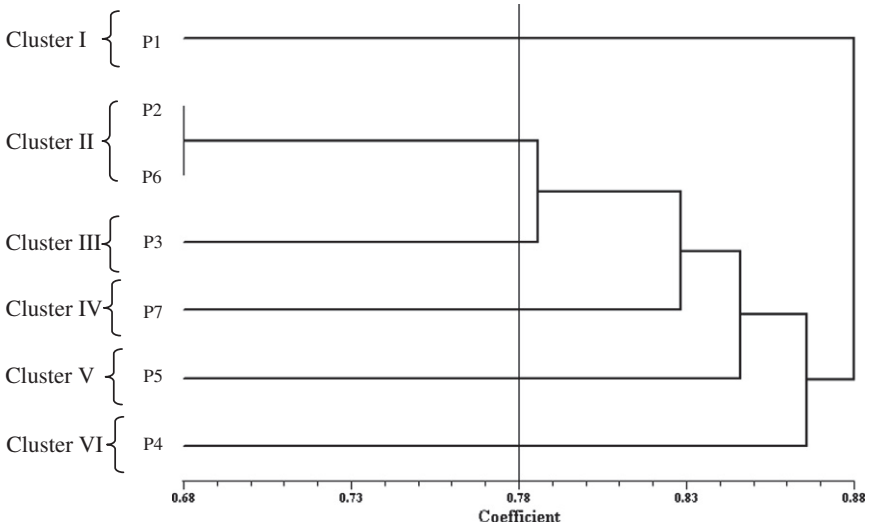

Fig. 3. Dendrogram based on the RAPD markers generated by using UPGMA clustering method and symmetric "dissimilarity matrices" of the Simple Matching (SM) coefficient for seven AP accessions based on RAPD markers.

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039

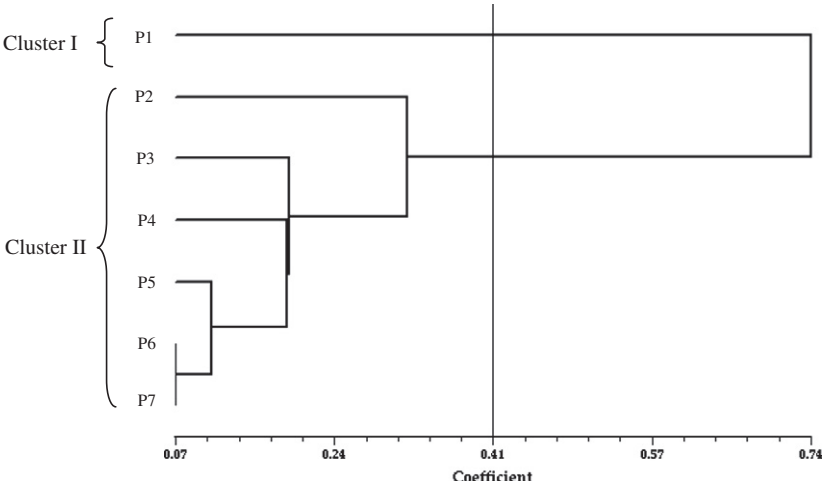

Fig. 4. Dendrogram based on morphological markers. The dendrogram has been generated by using UPGMA clustering method for seven AP accessions based on the morphological markers and using Euclidean distances.

3.7. Relationship between genetic distance (GD) and crossability

To conform to the title of this article, the final aim of the present study was to ascertain if there was any relationship between the genetic distance and the crossability of the AP accessions. To this end, the genetic identities and the distances of the studied accessions were calculated using the molecular and the morphological markers, separately. Tables 5 and 6 demonstrate the genetic distances of the seven accessions using the RAPD and morphological markers, respectively. The correlation between genetic distances (revealed by the RAPD and morphological markers, separately) and crossability percentages of the 21 combinations were merged together to generate a graphical demonstration which could give a better understanding of the relationship between these two (GD and crossability) parameters (Fig. 6A–D). Fig. 6A–C was constructed by employing the RAPD data and based on three different coefficients to confirm the reliability of the results, while Fig. 6D was generated using the morphological data and based on Euclidean distance.

Interestingly, the slope of the regression line was found to be negative when the RAPD-based GDs and the crossability percentages were contrasted with each other. However, very slight differences in the value of the coefficient of determination (R^2) were observed according to each of the coefficients (SM, SD and Jaccard), but it never breached the existence of a negative correlation (or an inverse relationship) between genetic distance and crossability (Fig. 6A–C). Moreover, the low values of the three coefficients of determination ($R^2_{SM} = 0.1908$, $R^2_{SD} = 0.1864$ and $R^2_J = 0.1572$) implied a fragile relationship between the RAPD-based GDs and the crossability of the AP accessions (Fig. 6A–C).

Surprisingly, the most amazing part of the results emerged from the morphological markers. In fact, despite detecting a higher variation and larger GDs among the studied AP accessions when using the morphological markers compared to the RAPD markers (Fig. 4 and Table 6), an almost neutral model was featured, in which no relationship was observed between the morphological-based GD and the crossability of AP. This was shown by the nearly zero value of the coefficient of determination ($R^2 = 0.0018$) (Fig. 6D).

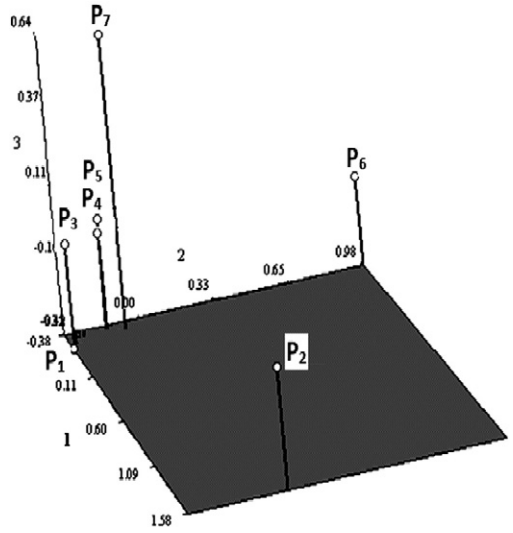

Fig. 5. Three dimensional PCA graph representing the seven AP accessions.

4. Discussion

4.1. Genetic diversity and GD

Understandably, the subject of the genetic diversity in AP is not a preferable issue to be discussed here as a novel topic. However, the low and moderate genetic diversities yielded respectively by the RAPD and morphological markers complied with most of the previously conducted researches using the same (or similar) marker systems in AP (Lattoo et al., 2008; Maison et al., 2005; Padmesh et al., 1999; Prathanturarug et al., 2007; Sakuanrungsirikul et al., 2008; Sharma et al., 2009; Wijarat et al., 2011), and discussing the possible reasons behind the reduced GD and its impact on the crossability of the herb. In line with this, we also realized that except accession P1, the used morphological and RAPD markers were slightly incongruent, meaning that the accessions were grouped into different cluster positions using each of these two markers. A similar situation had been reported by

Table 5
Genetic similarity matrices using RAPD markers based on Simple Matching (SM) coefficient.[a]

Accessions	P1	P2	P3	P4	P5	P6	P7
P1	**1.00**	0.23	0.07	0.07	0.07	0.15	0.05
P2	0.77	**1.00**	0.25	0.27	0.27	0.32	0.26
P3	0.93	0.75	**1.00**	0.09	0.10	0.17	0.10
P4	0.93	0.73	0.91	**1.00**	0.07	0.13	0.09
P5	0.93	0.73	0.90	0.93	**1.00**	0.13	0.10
P6	0.85	0.68	0.83	0.87	0.87	**1.00**	0.14
P7	0.90	0.74	0.90	0.91	0.90	0.86	**1.00**

[a] Genetic dissimilarity (above diagonal) and genetic similarity (below diagonal).

Table 6
Genetic similarity matrices using morphological markers based on Euclidean distance.[a]

Accessions	P1	P2	P3	P4	P5	P6	P7
P1	**1.00**	0.55	0.73	0.71	0.66	0.68	0.71
P2	0.45	**1.00**	0.37	0.31	0.23	0.24	0.27
P3	0.27	0.63	**1.00**	0.11	0.18	0.16	0.16
P4	0.29	0.69	0. 89	**1.00**	0.16	0.11	0.12
P5	0.34	0.77	0.82	0.84	**1.00**	0.07	0.12
P6	0.32	0.76	0.84	0.89	0.93	**1.00**	0.06
P7	0.29	0.73	0.84	0.88	0.88	0.94	**1.00**

[a] Genetic dissimilarity (above diagonal) and genetic similarity (below diagonal).

Maison et al. (2005). Besides, in accordance with the previous researches, it was noticed that less polymorphism could be detected by the RAPD markers compared to the morphological markers.

4.2. Geographical and historical justification of intraspecific phylogenetic relationship

However, according to the morphology-based cluster analysis (Fig. 4), and the morphology-based GDs (Table 6), accessions P1 and P2 appeared to be diverse compared to the other accessions. The morphological similarity of these two accessions was rational and could be justified by the geographical distribution of these two accessions, where the two states Selangor and Negeri Sembilan are closely neighboring each other (Fig. 1). This matter might be connected to the historical introduction of the plant to Peninsular Malaysia through migrant Indians during the British colonial period (Saad et al., 2006; Valdiani et al., 2013). If the port of Malacca is presumed to be one of the main seaports of Peninsular Malaysia and the arrival point of the migrants at that time, then justification of such morphological and also molecular resemblance of the accessions P1 (collected from Selangor state) and P2 (collected from Negeri Sembilan state) will not be difficult. There is another possibility that AP could be imported by the same people, but through Port Klang which is the main gateway by sea into the country located in Selangor state. However, it should be remembered that accession P1 was grouped in a separate cluster based on the RAPD markers, but the UPGMA clustering was constructed based on the symmetric dissimilarity matrices of the SM coefficient (Fig. 3). Therefore, accession P1 should be considered as the closest accession to P2, molecularly. In spite of such similarity, the PCA results based on the RAPD markers determined the genetic distance of accession P1 compared to its closest counterpart accession P2. Unlike the morphological characterization, the molecular classification sets accession P1 in the same group along with accessions P3, P4, P5 and P7 (Fig. 5). As a consequence, the unprecedented fact of the present research appeared when the morphology-based GDs were larger than RAPD-based GDs, despite this fact, the morphology-based GDs acted neutrally contrary to crossability. In other words, if supposedly the crossability was affected by the morphological-based GDs, maternal accession P1 as the most morphologically distant accession should have failed to be crossed with accessions P3, P4, P5, P6 and P7, but this never happened.

Another interpretation of these observations is that the morphological characteristics have not been influenced by the molecular pattern of the plants, and so the morphological differences are not barriers against the crossability of AP. Sabu et al. (2001) also noticed a spectrum of morphological variation among Indian AP accessions grown under uniform environmental condition, while a moderate genetic diversity was revealed by isozymes (Sabu et al., 2001). Simultaneously, he reported a low crossabiliy at the rate of 5.8% for the plant, where 32 out of the 34 conducted crosses failed (Sabu, 2002).

4.3. Evolutionary impact of the mating system on GD

Crossing leads to alteration in genetic variability of plants (Nakayama et al., 2002). As mentioned earlier, AP is an autogamous (self-pollinated) plant. Therefore, facing with a low level of diversity was not surprising, because the phenomenon autogamy is an ordinary mating system in plants and is known to decrease genetic diversity, increase genetic structure and potentially put populations at greater risk

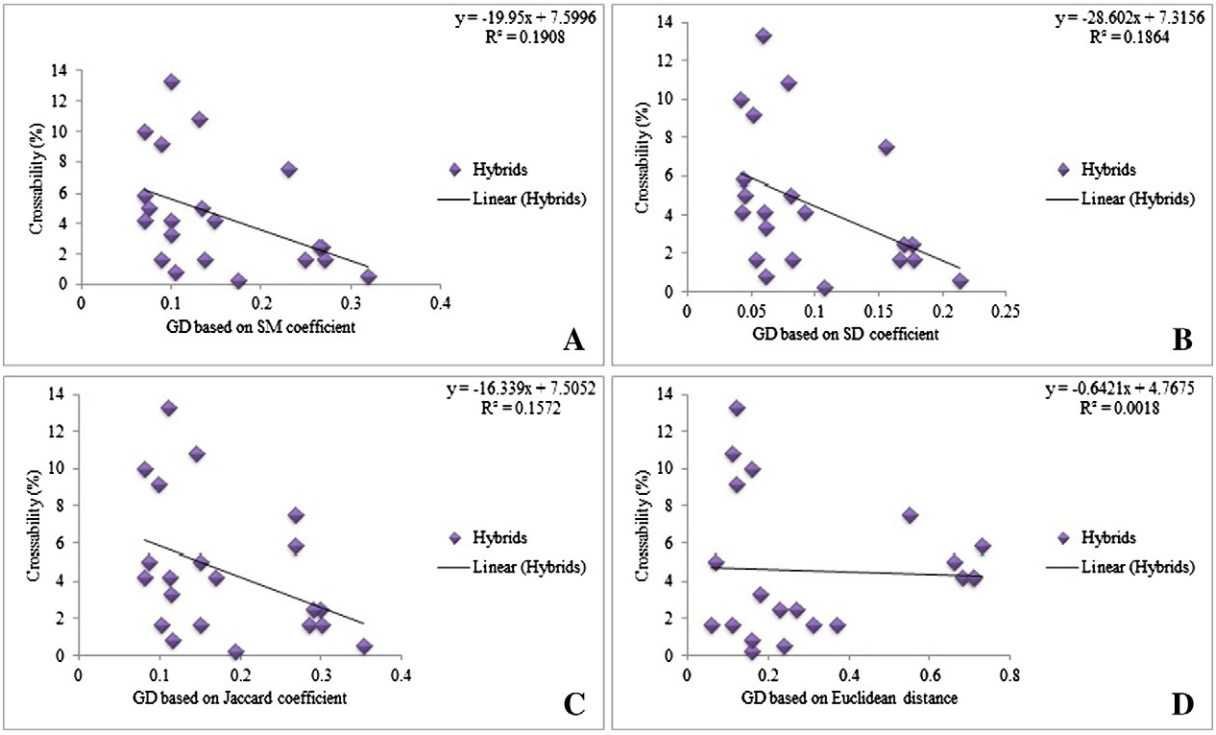

Fig. 6. Graphical demonstration of the linear regression between the crossability of the AP accessions (x-axis) and their genetic distances (GD) (y-axis) revealed by (A) the RAPD markers based on the Simple Matching (SM) coefficient, (B) based on the Sorensen–Dice (SD) coefficient, (C) based on the Jaccard's coefficient, and (D) the morphological markers based on Euclidean distance.

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039

of extinction (Koelling et al., 2011). In addition, we are aware that hybridization at both intra- and interspecific levels is being followed by many researchers as the major tool of plant breeding, since the 18th century (Kölreuter, 1761). Thus, intraspecific hybridization (or so-called outcrossing) using conventional crossing can be carried out to produce new prolific varieties with high andrographolide content. But, this approach has been hampered by a serious obstacle namely low intraspecific crossability of AP. Frequently, the weak response of AP accessions to hand-pollination and difficulties in the manual emasculation of the species have been reported (Sabu, 2002; Valdiani et al., 2012b, 2012c). It seems that these complications led researchers to take troubleshooting steps, in this regard. For instance, nuclear emasculation using gamma ray has been employed as an alternative strategy instead of manual emasculation for producing male sterile progenies (M_1) and studying the genetics and mechanism of induced male sterility of AP (Lattoo et al., 2006). However, the nuclear methods cannot be suggested as cheap, safe and routine techniques in conventional genetic studies and the produced results and analysis of the related data will be questioned strongly. Typically, the engendered data obtained by such process cannot be subjected to any basic analysis like diallel approach and moreover, the heterosis (if any) derived from the nuclear radiation cannot be trusted as the real genetic potential of the plant. As a result, the necessity of conducting safe research to solve or at least facilitate the crossability problem in AP is well justified (Sabu, 2002; Valdiani et al., 2012c).

Discussing the impact of GD takes a special place in such an exploration as the impact of the GD factor on the crossability of AP has never been scrutinized before. Also, it is worthwhile to bring up the reasons that might be causing the low genetic diversity as well as the little GD values in AP first. Outbreeding and inbreeding depressions with respect to hybrid performance in different plants and its association with GD were the concerns of many researchers (Liu et al., 2002; Mable and Adam, 2007).

In justification of low genetic diversity of AP accessions in Malaysia, it is believed that the current population of the plant in the country has been descended from a core and relatively small Indian AP population imported into Malaysia by Indian migrants (Valdiani et al., 2013). On the other hand, it has been proven that the small size of the population especially in self-compatible and autogamous plants may lead to a reduced outcrossing rate (Routley et al., 1999). This could be a reasonable evolutionary episode about AP that its self-compatible and habitual inbreeder style (Lattoo et al., 2006) caused the plant's population to lose a big part of its genetic variation. Consequently, the GD among the Malaysian AP accessions decreased over time.

4.4. Criteria of crossability

Obviously, the evaluation of crossability is not only limited to fruit set, and the case can be deepened further by continuing the analyses in higher levels such as diallel analysis. In diallel studies, more characteristics of the F_1 offspring can be used as an evidence for assessing the quantity and quality of the crossability of parents (Koopman et al., 2001). Different indices such as fruit set, number of F_1 seedlings grown, and percentage of F_1 plants with seeded fruit and without seeded fruit are alternatively used for studying the degree of crossability relationship (Behera and Singh, 2002). Indeed, in many researches crossability is referred to the viability traits and hybrid vigor of the F_1 offspring. Nevertheless, the significant point is that the GD between parental populations and its impacts on crossability are still considered as idiosyncratic issues (Pélabon et al., 2005; Riday et al., 2003).

4.5. GD and crossability

Seemingly, AP is extremely sensitive even to the minor decrease of GD between the (pairs of) parents by showing a negative feedback in terms of crossability (fruit set). As a general rule, hybrid seed production in self-pollinated plants may be difficult to a certain extent (Chrispeels and Sadava, 2003; Patil et al., 2011). However, heterosis may be fixed by using double haploid formation or by using an apomixis technique (Patil et al., 2011). Hence, high self-pollinating rate of AP defends the low crossability of the herb.

As reviewed by Ritland and Ganders (1987), the effect of genetic relatedness between mates (that in the present study is referred to as GD) on the quantity and quality of progenies is usually concluded to be deleterious in plants (Crumpacker, 1967; Darwin, 1867; Grant, 1975; Levin and Kerster, 1974; Wright, 1977). In human (Morton et al., 1956), some insect species (e.g. *Drosophila pseudoobscura* and *Tribolium*) (Dobzhansky et al., 1963; Lavene et al., 1965), and a plant species, namely, *Phlox drummondii* (Levin, 1984) have been indicated that viability (or crossability) depression incurred by mating to close relatives reduces by decreasing relatedness between mates. The same goes about the monkey-flower (*Mimulus guttatus*) (Ritland and Ganders, 1987). In other words, in these species a higher GD is suitable for enhancing crossability, and this is exactly opposed to AP's situation, where higher RAPD-based GDs decreased the crossability rate. However, some references are in agreement with our findings, where they have noted that as parental plants become genetically even less related (existence of high GD), depression effects on crossability may start to increase (Muller, 1883; Price and Waser, 1979). Promising results of hybridization experiments are often used in biosystematic studies to understand phylogenetic relationships, although crossability is fundamentally a plesiomorphic character. Crossability can be used as a rough estimation of GD in systematics and phylogenetics research (Hart, 1997). Principally, this was experienced in the present experiment, when the low crossable parents had the highest GDs compared to those compatible combinations. One more fact which should be technically taken into consideration is that, in the case of using the agro-morphological traits, environmental conditions base most of the initial and basic hypotheses stated on the correlation of GD and crossability. Therefore, electrophoretic markers are alternatively suggested as efficient tools for clarification of the correlation between GD and crossability. The reliability of this recommendation was verified in the present study by utilizing RAPD markers.

4.6. Importance of the similarity coefficients for GD estimation

Although, molecular markers have multiple roles in breeding of inbred annual crops, information about genetic diversity and genetic distance are the most applicable examples. Apart from the importance of GD, it can be utilized in different ways in molecular breeding (Langridge and Chalmers, 2004). GD is a simple quantitative measure of biological relatedness (Soumpasis, 1980), and after proving the impact of GD on crossability, discussion on employing an efficient coefficient to calculate the GD is the most critical issue. In this respect, the three different similarity coefficients including SM, SD and Jaccard's coefficient are recurrently suggested to be employed in dominant marker-based studies (Dalirsefat et al., 2009; Koopman et al., 2001). The SM coefficient considers that absence corresponds to homozygous loci. Hence, it can be used with dominant markers data such as RAPD and AFLP, because the absences could correspond to homozygous recessives (Dalirsefat et al., 2009). Despite the low values of the calculated coefficients of determination in all three approaches, the SM-based regression was slightly stronger than the other two indices SD and Jaccard. This is important because the nature of the relationship is discussed as a part of correlation (Daniel, 1999).

4.7. Efficacy of the used markers in crossability

Regardless of revealing a higher polymorphism by the morphological markers, the RAPD markers were more explanatory in the crossability of AP. At one glance, the negative regression between RAPD-based GDs and crossability complies with the negative response of AP

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039

(in terms of crossability) to genetic incongruity and this could be considered as a symptom of occurrence of outbreeding depressions in the herb, which is in agreement with self-pollinating nature of the herb. Therefore, those genetically far accessions refused to show a positive response to allogamy by yielding a low fruit set in combinations such as P2 × P6 and P3 × P6. Outstandingly, the genetically adjacent accessions in combinations such as P3 × P4 and P3 × P7 produced a higher crossability. So, this was regarded as a negative impact of increasing genetic distance on intraspecific crossability of AP accessions. On the other hand, according to the results of our parallel studies, we believe that AP accessions suffer from a subtle inbreeding depression despite their self-pollinating mating system (Valdiani et al., 2012b, 2014). This conflict in terms of the equivocal response of plant crossability to GD has been reported in the other herbs such as *Agave schottii*, while the verdict on such situation tends to the presence of inbreeding depression and a simultaneous tendency toward outbreeding depression (Trame et al., 1995). Overall, when both inbreeding and outbreeding depressions occur within a population, there is likely to be an intermediate distance at which two mating plants experience an optimal degree of outbreeding (Price and Waser, 1979; Waser, 1993). Fortunately, parallel studies showed that selecting the genetically contiguous parental APs for outcrossing aims, is not in any contradiction with the occurrence of heterosis in important characteristics of the herb (Valdiani et al., 2012b, 2014).

Eventually, it must be highlighted that, the arrangement of the accessions in the RAPD-based clusters was in agreement with our hypothesis (existence of a negative relationship between GD and crossability) that showed the precision of RAPD markers and the reliability of the UPGMA method. Although, in most of the combinations, the parents with lower GDs resulted in higher crossabilities, nevertheless, outcomes of a combination of multi-marker systems or a higher number of RAPD primers would be a better proposal to scrutinize the problem. Despite the low quantity of the coefficient of determination ($R^2_{SM} = 0.1908$) revealed by contrasting the crossability and GD, the function of RAPD markers as an informative tool to this approach should not be neglected. In addition, it is strongly suggested that a higher number of AP population should be subjected to a similar experimentation to prove the reliability of the present outcomes. One of the ambiguities of the present research was that due to the nature of the experiment design (one-way diallel) it could not be clearly realized that the repulsive feedback of the AP accessions was mostly controlled by maternal effects or paternal effects. For this reason, it is suggested that a two-way diallel study consisting of further analyses on the F_1 and reciprocals should be carried out using the AP accessions.

5. Conclusion

Even with the low amount of genetic variation (at the molecular level) among the studied AP accessions, the genetically distant individuals get in trouble during intraspecific hybridization, by refusing to get out-crossed with each other. The best examples of this case were the combinations P2 × P6 and P3 × P6. The effect of the genetic distance revealed by the morphological characteristics on the crossability of the accessions was found to be neutral (Fig. 6D). As mentioned before, the crossibility referring to only fruit set might not be enough to estimate the actual crossability potential of AP. However, our interpretation from crossability (fruit set) of the herb could be considered as the initial sign of low crossability and repulsive feedback of AP to allogamy. This point can be explored further by classical genetic examinations on the F_1 progenies such as diallel analysis. Even though the RAPD marker system might not be a master key to open the lock of inbreeding depression in this autogamous plant, it definitely can give an initial clue on how to choose the most suitable parental plants to fit in a breeding program on AP. In contrast, morphological characteristics were not found useful enough as a marker system to determine the tendency of successful crossability in AP.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.03.039.

Conflict of interest

The authors declare that no conflict of interest exists.

Acknowledgments

The authors would like to thank Universiti Putra Malaysia for supporting the present research.

References

Behera, T.K., Singh, N., 2002. Inter-specific crosses between eggplants (*Solanum melongena* L.) with related *Solanum* species. Scientia Horticulturae 95, 165–172.
Bruel, D.C., Carpentieri-Pípolo, V., Gerage, A.C., Júnior, N.S.F., Prete, C.E.C., Ruas, C.F., et al., 2006. Genetic distance estimated by RAPD markers and its relationship with hybrid performance in maize. Pesquisa Agropecuária Brasileira Brasília 41 (10), 1491–1498.
Bussell, J.D., Waycott, M., Chappill, J.A., 2005. Arbitrarily amplified DNA markers as characters for phylogenetic inference. Perspectives in Plant Ecology, Evolution and Systematics 7, 3–26. http://dx.doi.org/10.1016/j.ppees.2004.07.001.
Chrispeels, M.J., Sadava, D.E., 2003. Plants, Genes, and Crop Biotechnology, 2nd edn. Jones and Bartlett Publishers, Inc., Sudbury, United States.
Crumpacker, D.W., 1967. Genetic loads in maize (*Zea mays* L.) and other cross-fertilizd plants and animals. Evolutionary Biology 1, 306–324.
Dalirsefat, S.B., da Silva Meyer, A., Mirhoseini, S.Z., 2009. Comparison of similarity coefficients used for cluster analysis with amplified fragment length polymorphism markers in the silkworm, *Bombyx mori*. Journal of Insect Science 9, 71. http://dx.doi.org/10.1673/031.009.7101.
Daniel, W.W., 1999. Biostatistics: A Foundation for Analysis in the Health Sciences. John Wiley and Sons, New York.
Darwin, C., 1867. On the Effects of Cross and Self-Fertilisation in the Vegetable Kingdom. Murray, London.
Dice, L.R., 1945. Measures of the amount of ecologic association between species. Ecology 26, 297–302.
Dobzhansky, T.H., Spassky, B., Tidwell, T., 1963. Genetics of natural populations. XXXII. Inbreeding and the mutational and balanced geneticloads in natural populations of *Drosophila pseudoobscura*. Genetics 48, 361–373.
Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12, 13–15.
Espinoza, F., Pessino, S.C., Quarín, C.L., Valle, E.M., 2002. Effect of pollination timing on the rate of apomictic reproduction revealed by RAPD markers in *Paspalum notatum*. Annals of Botany 89, 165–170.
Fahmi, A.I., Assal, O.M., Nawar, A.A., El-Hosary, A.A., Mohammed, M.M., 2012. Genetic diversity of *Brassica napus* L. varieties estimated by morphological and molecular markers. International Journal of Plant Breeding and Genetics 6 (2), 83–93.
Grant, V., 1975. Genetics of Flowering Plants. Columbia University Press, New York.
Hart, H., 1997. Evolutionary and systematic significance of hybridization in Mediterranean sedoideae (Crassulaceae). Lagascalia 19 (1–2), 57–58.
Houghton-Thompson, J., Prince, H.H., Smith, J.S., Hancock, J.F., 2005. Evidence of hybridization between *Lythrum salicaria* (Purple Loosestrife) and *L. alatum* (Winged Loosestrife) in north America. Annals of Botany 96, 877–885.
Irwin, R.E., 2001. Field and allozyme studies investigating optimal mating success in two sympatric spring-ephemeral plants, *Trillium erectum* and *T. grandiflorum*. Heredity 87, 178–189.
Jaccard, P., 1901. Étude comparative de la distribuition florale dans une portion des Alpes et des Jura. Bulletin de la Societe Vaudoise des Sciences Naturelles 37, 547–579.
Koelling, V.A., Hamrick, J.L., Mauricio, R., 2011. Genetic diversity and structure in two species of Leavenworthia with self-incompatible and self-compatible populations. Heredity 106, 310–318.
Kölreuter, J.G., 1761. Vorläufige Nachricht von einigen das Geschlecht der Pflanzen betreffenden Versuchen und Beobachtungen, nebst Fortsetzungen 1, 2 und 3. In Der Gleditschischen Handlung, Leipzig.
Koopman, W.J.M., Zevenbergen, M.J., Van den Berg, R.G., 2001. Species relationships in *Lactuca* s.l. (Lactuceae, Asteraceae) inferred from AFLP fingerprints. American Journal of Botany 88 (10), 1881–1887.
Langridge, P., Chalmers, K., 2004. The principle: identification and application of molecular markers. In: Nagata, T., Lörz, H., Widholm, J.M. (Eds.), Biotechnology in Agriculture and Forestry. Springer-Verlag, Berlin Heidelberg, pp. 3–20.
Lattoo, S.K., Khan, S., Dhar, A.K., Choudhary, D.K., Gupta, K.K., Sharma, P.R., 2006. Genetics and mechanism of induced male sterility in *Andrographis paniculata* (Burm. f.) Nees and its significance. Current Science 91 (4), 515–519.
Lattoo, S.K., Dhar, R.S., Khan, S., Bamotra, S., Bhan, M.K., Dhar, A.K., Gupta, K.K., 2008. Comparative analysis of genetic diversity using molecular and morphometric markers in *Andrographis paniculata* (Burm. f.) Nees. Genetic Resources and Crops Evolution 55, 33–43.
Lavene, H., Lerner, I.M., Sokoloff, A., Ho, F.K., Franklin, I.R., 1965. Genetics load in *Tribolium*. Proceedings of the National Academy of Sciences of the United States of America 53, 1042–1050.
Levin, D.A., 1984. Inbreeding depression and proximity-dependent crossing success in *Phlox drummondii*. Evolution 38, 116–127.
Levin, D., Kerster, H.W., 1974. Gene flow in seed plants. Evolutionary Biology 7, 139–220.

Liu, X.C., Ishiki, K., Wang, W.X., 2002. Identification of AFLP markers favorable to heterosis in hybrid rice. Breeding Science 52, 201–206.
Mable, B.K., Adam, A., 2007. Patterns of genetic diversity in outcrossing and selfing populations of *Arabidopsis lyrata*. Molecular Ecology 16, 3565–3580.
Maison, T., Volkaert, H., Boonprakob, U., Paisooksantivatana, Y., 2005. Genetic diversity of *Andrographis paniculata* Wall. Ex Nees as revealed by morphological characters and molecular markers. Kasetsart Journal (Natural Science) 39, 388–399.
Mazer, S.J., Hove, A.A., Miller, B.S., Barbet-Massin, M., 2010. The joint evolution of mating system and pollen performance: predictions regarding male gametophytic evolution in selfers vs. outcrossers. Perspectives in Plant Ecology, Evolution and Systematics 12, 31–41. http://dx.doi.org/10.1016/j.ppees.2009.06.005.
Morton, N.E., Crow, J.F., Muller, H.J., 1956. An estimate of the mutational damage in man from data on consanguineous marriages. Proceedings of the National Academy of Sciences of the United States of America 42, 855–863.
Muller, H., 1883. The Fertilisation of Flowers. Macmillian, London.
Nakayama, Y., Seno, H., Matsudaa, H., 2002. A population dynamic model for facultative agamosperms. Journal of Theoretical Biology 215, 253–262. http://dx.doi.org/10.1006/jtbi.2001.2373.
Natarajan, D., Britto, S.J., Balaguru, B., Nagamurugan, N., Soosairaj, S., Arockiasamy, D.I., 2004. Identification of conservation priority sites using remote sensing and GIS — a case study from Chitteri hills, Eastern Ghats, Tamil Nadu. Current Science 86 (9), 1316–1323.
Padmesh, P., Sabu, K.K., Seeni, S., Pushpangadan, P., 1999. The use of RAPD in assessing genetic variability in *Andrographis paniculata* Nees, a hepatoprotective drug. Current Science 76, 833–835.
Patil, L.C., Hanchinal, R.R., Lohithaswa, H.C., Nadaf, H.L., Kalappanavar, I.K., Megeri, S.N., 2011. Heterosis studies in interspecific hybridization [*Triticum dicoccum* (Schran) Schulb × *Triticum durum* Desf] of tetraploid wheat. Karnataka Journal of Agricultural Sciences 24 (3), 387–389.
Pélabon, C., Carlson, M.L., Hansen, T.F., Armbruster, W.S., 2005. Effects of crossing distance on offspring fitness and developmental stability in *Dalechampia scandens* (Euphorbiacea). American Journal of Botany 92 (5), 842–851.
Prathanturarug, S., Soonthornchareonnon, N., Chuakul, W., Saralamp, P., 2007. Variation in growth and diterpene lactones among field-cultivated *Andrographis paniculata*. Journal of Natural Medicines 61, 159–163. http://dx.doi.org/10.1007/s11418-006-0115-6.
Price, M.V., Waser, N.M., 1979. Pollen dispersal and optimal outcrossing in *Delphinium nelsoni*. Nature 277, 294–297.
Rao, V.R., Hodgkin, T., 2002. Genetic diversity and conservation and utilization of plant genetic resources. Plant Cell, Tissue and Organ Culture 68, 1–19.
Riday, H., Brummer, E.C., Campbell, T.A., Luth, D., Cazcarro, P.M., 2003. Comparisons of genetic and morphological distance with heterosis between *Medicago sativa* subsp. *sativa* and subsp. *falcata*. Euphytica 131, 37–45.
Ritland, K., Ganders, F.R., 1987. Crossability of *Mimulus guttatus* in relation to components of gene fixation. Evolution 41 (4), 772–786.
Rohlf, F.J., 2000. NTSYS-pc: Numerical Taxonomy System. Ver. 2.1. Applied Biostatic, Exeter Software, Setauket, New York 29–34.
Routley, M.B., Mavraganis, K., Eckert, C.G., 1999. Effect of population size on the mating system in a self-compatible, autogamous plant, *Aquilegia canadensis* (Ranunculaceae). Heredity 82, 518–528.
Saad, M.S., Chia, S.H., Jebril, A.A., Ramisah, M.S., Suharni, H., Norila, Y., Milan, A.R., 2006. Genetic diversity in Hempedu Bumi (*Andrographis paniculata*) germplasm in Malaysia as revealed by RAPD polymorphism. Agriculture Congress. Universiti Putra Malaysia, pp. 227–229.
Sabu, K.K., 2002. Intraspecific Variations in *Andrographis paniculata* Nees. (Ph.D. Thesis) Kerala University, Thiruvananthapuram, India.
Sabu, K.K., Padmesh, P., Seeni, S., 2001. Intraspecific variation in active principle content and isozymes of *Andrographis paniculata* Nees (Kalmegh): a traditional hepatoprotective medicinal herb of India. Journal of Medicinal and Aromatic Plant Sciences 23, 637–647.
Sakuanrungsirikul, S., Jetana, A., Buddanoi, P., Dithachaiyawong, J., 2008. Intraspecific variability assessment of *Andrographis paniculata* collections using molecular markers. Acta Horticulturae 786, 283–286.
SAS Institute Inc., 2003. SAS/STAT® User's Guide. Version 9.1. SAS Institute Inc., Cary, NC.
Sharma, S.N., Sinha, R.K., Sharma, D.K., Jha, Z., 2009. Assessment of intra-specific variability at morphological, molecular and biochemical level of *Andrographis paniculata* (Kalmegh). Current Science 96 (3), 402–408.
Shore, J.S., Barrett, S.C.H., 1985. Morphological differentiation and crossability among populations of the *Turnera ulmifolia* L. complex (Turneraceae). Systematic Botany 10 (3), 308–321.
Shringarpure, S., 2012. Statistical Methods for Studying Genetic Variation in Populations. (PhD thesis) Carnegie Mellon University.
Shriver, M.D., Jin, L., Boerwinkle, E., Deka, R., Ferrell, R.E., Chakraborty, R., 1995. A novel measure of genetic distance for highly polymorphic tandem repeat loci. Molecular Biology and Evolution 12 (5), 914–920.
Sneath, P.H.A., Sokal, R.R., 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification. Freeman, San Francisco.
Sokal, R.R., Michener, C.D., 1958. A statistical method for evaluating systematic relationships. University of Kansas Science Bulletin 38, 1409–1438.
Sorensen, T., 1948. A method of establishing groups of equal amplitude in plant sociology based on similarity of species content and its application to analyses of the vegetation on Danish commons. Videnskabernes Selskabs Biologiske Skrifter 5, 1–34.
Soumpasis, D., 1980. Genetic distance: a simple quantitative measure of biological relatedness. Journal of Theoretical Biology 86, 137–147.
Talei, D., Saad, M.S., Khanif, Y.M., Kadir, M.A., Valdiani, A., 2011. Effect of different surface sterilizes on seed germination and contamination of King of Bitters (*Andrographis paniculata*). American-Eurasian Journal of Agricultural & Environmental Sciences 10 (4), 639–643.
Talei, D., Kadir, M.A., Khanif, Y.M., Valdiani, A., Abdullah, M.P., 2012. Physico-protein based dormancy in medicinal plant of *Andrographis paniculata*. Journal of Medicinal Plant Research: Planta Medica 6 (11), 2170–2177. http://dx.doi.org/10.5897/JMPR011.1580.
Trame, A.-M., Coddington, A.J., Paige, K.N., 1995. Field and genetic studies testing optimal outcrossing in *Agave schottii*, a long-lived clonal plant. Oecologia 104, 93–100.
Valdiani, A., Kadir, M.A., Tan, S.G., Talei, D., Puad, M.A., Nikzad, S., 2012a. Nain-e Havandi (*Andrographis paniculata*) present yesterday, absent today: a plenary review on underutilized herb of Iran's pharmaceutical plants. Molecular Biology Reports 39 (5), 5409–5424. http://dx.doi.org/10.1007/s11033-011-1341-x.
Valdiani, A., Kadir, M.A., Saad, M.S., Talei, D., Tan, S.G., 2012b. Intra-specific hybridization: generator of genetic diversification in *Andrographis paniculata* Nees. A bridge from extinction to survival. Gene 505 (1), 23–36. http://dx.doi.org/10.1016/j.gene.2012.05.056.
Valdiani, A., Kadir, M.A., Saad, M.S., Talei, D., Omidvar, V., Chia, S.H., 2012c. Intraspecific crossability in *Andrographis paniculata* Nees. A barrier against breeding of the species. Scientific World Journal 2012, 1–9. http://dx.doi.org/10.1100/2012/297545.
Valdiani, A., Javanmard, A., Talei, D., Tan, S.G., Nikzad, S., Kadir, M.A., Abdullah, S.N.A., 2013. Microsatellite-based evidences of genetic bottlenecks in the cryptic species "*Andrographis paniculata* Nees": a potential anticancer agent. Molecular Biology Reports 40, 1775–1784. http://dx.doi.org/10.1007/s11033-012-2231-6.
Valdiani, A., Talei, D., Tan, S.G., Kadir, M.A., Maziah, M., Rafii, M.Y., Sagineedu, S.R., 2014. A classical genetic solution to enhance the biosynthesis of anticancer phytochemicals in *Andrographis paniculata* Nees. PLoS ONE 9 (2), e87034. http://dx.doi.org/10.1371/journal.pone.0087034.
Waser, N.M., 1993. Population structure, optimal outbreeding and assortative mating in angiosperms. In: Thornhill, N.W. (Ed.), The Natural History of Inbreeding and Outbreeding: Theoretical and Empirical Perspectives. University of Chicago Press, Chicago, pp. 173–199.
Wijarat, P., Keeratinijakal, V., Toojinda, T., Vanavichit, A., Tragoonrung, S., 2011. Genetic diversity and inbreeder specie of *Andrographis paniculata* (Burm. f.) Nees by randomly amplified polymorphic deoxyribonucleic acid (RAPD) and floral architecture analysis. Journal of Plant Breeding and Crop Science 3 (12), 327–334. http://dx.doi.org/10.5897/JPBCS11.066.
Wijarat, P., Keeratinijakal, V., Toojinda, T., Vanavichit, A., Tragoonrung, S., 2012. Genetic evaluation of *Andrographis paniculata* (Burm. f.) Nees. revealed by SSR, AFLP and RAPD markers. Journal of Medicinal Plant Research: Planta Medica 6 (14), 2777–2788. http://dx.doi.org/10.5897/JMPR11.11-1025.
Wright, S., 1977. Evolution and genetics of population. Experimental Results and Evolutionary Deductions, vol. 3. University Chicago Press, Chicago.

Please cite this article as: Valdiani, A., et al., Morpho-molecular analysis as a prognostic model for repulsive feedback of the medicinal plant "Andrographis paniculata" to allogamy, Gene (2014), http://dx.doi.org/10.1016/j.gene.2014.03.039