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MORPHOMETRIC AND GENETIC VARIATION IN *PUERARIA MIRIFICA* CULTIVARS ACROSS THAILAND

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Abstract

Pueraria mirifica is a leguminous herbal plant whose tuberous roots are used in estrogen replacement. Leaves were collected for 39 locations across Thailand with seed pods and flowers also collected when available from a subset of 14 and 11 of these locations respectively. Morphometric analysis revealed a low level of variation between cultivars. Linear regression analysis suggested that leaves trended to decrease in size from the West to the East whilst pods trended to increase in size from the South to the North and also the West to the East. Genetic analysis was conducted by direct sequencing of one nuclear (rDNA ITS region) and one chloroplast (*trn*LF) region, and also by random genome analysis by RAPD-PCR using five primers. All chloroplast sequences obtained revealed a low level of variation between isolates although the rDNA ITS sequences displayed a divergence of up to 25.2 %. All of 93 bands generated by the five RAPD primers were polymorphic. The average genetic distance varied from 0 to 42. NJ based phylogenies derived from ITS and RAPD data revealed poor resolution. In summary, both analyses indicate low variation amongst cultivars.

Introduction

Pueraria mirifica Airy Shaw et. Suvatabandhu or White Kwao Krua is a tropical perennial herbal plant of the family Fabaceae (Suvatti, 1978). The root or tuber extract of *P. mirifica* contains a relatively large amount of several phytoestrogen constituents, that is chemicals whose structure and effect are similar to the female sex hormone, estrogen. It is closely related to *P. lobata* Willd (Kudzu), the native species in southern Japan and southeastern China (Smitasiri & Wungjai, 1986).

P. mirifica is distributed in deciduous or dry forest areas and in mountainous forests with sandy soils at roughly 80-800 meters above the sea level. Interestingly, morphometric diversity exists within and between these different Thai cultivars. For example, cultivars from Kanchanaburi possess darker blue flowers than those from Chiang Mai (Cherdshewasart *et al*., 2007). However, whether this morphometric polymorphism underlies a true genetic biodiversity between cultivars or merely reflect environmental plasticity effects remains unknown, it is important, in the selection of optimal cultivars and best cultivation regimes to yield optimal levels of phytoestrogens as medical dietary supplements.

Morphometric variation has been widely used in determining potential plant diversity. For example, at the visible level in the sea grass *Halodule wrightii* (Creed, 1997) and at the microlevel using SEM on epiphytic orchid seeds (Swarmy *et al.,* 2004).

Genetic variation has been widely used in determining plant diversity including the selection of plant varieties for cultivation. Typically the genetic loci for the desired trait(s) are unknown and thus such approaches require random sampling across the whole genome. Given the cost and time of developing and characterizing a set of comprehensive polymorphic microsatellite (SSR) markers, it is common to employ the more random,

dominant, AFLP or RAPD based approaches and with somewhat related ISSR approach. For example, RAPD have been used in the identification of South African soybean (*Glycine max* (L.) Merrill) for commercial seed production and crop certification (Cherdshewasart *et al*., 2007), with just 14 primers being able to distinguish all 37 cultivars.

In order to determine the genetic diversity in plants, conserved regions of chloroplast DNA ribulose-bisphosphate carboxylase (large subunit) (*rbc*L), chloroplast DNA transfer RNA-Leucine and phenylalanine (*trn*L-F), nuclear ribosomal DNA internal transcribed spacer (ITS) and mitochondrial DNA can be used (Ellison *et al*., 2006). However, despite the clear use of morphometric and genetic analysis in many cultivated plants, to the best of our knowledge, no morphometric or genetic analysis within the genus *Pueraria*, let alone the species *P. mirifica*, has been evaluated despite the potential economic importance of *P. mirifica* as a source of natural phytoestrogens.

There are many cultivars of *P. mirifica* distributed throughout Thailand, and that they apparently differ in their morphology, studies were carried out to characterize the degree of morphological and genetic variation between and, especially within cultivars. For morphological analysis, leaf, seed pod and flower morphometrics were analyzed by cluster and linear regression analyses. For genetic analysis, sequence analysis of the nuclear ribosomal DNA internal transcribed spacer (ITS) and cPCR-RAPD analysis with five primers were used to characterize the genetic variation and to perform phylogenetic analyses by the neighbor-joining method.

Materials and methods

Sampling collection: *Pueraria mirifica* (39 cultivars for leaves, 14 cultivars for pods and 11 cultivars for flowers) were collected from 5 parts of Thailand (i.e., the North, the Northeast, the Center, the West and the South) (Fig. 1 and Table 1). *P. lobata* (Kudzu) was collected from Japan. Cultivar names were ascribed from the locality they were collected from and a sequential number of more than one cultivar (location) within that locality.

Morphometric analysis: Mature leaves (50 leaves/cultivar), plus old brown seed pods (10 pods/cultivar) and blooming flowers (10 flowers/cultivar) where available were collected. Not all the cultivars were in flower or had old seed pods at the time of collection (Table 1). Leaf morphology was measured at nine parameters; the petiole length (PL) and diameter (PD), rachis length (RL), petiolet length (PLL), terminal leaflet length (TLL) and breadth (TLB), stipule length (SPL), angle of first leaf border (AˆB)^{o} and the number of pairs of primary veins (NPV). Seed pods were assayed as pod length (PodL) and width (PodW) and the number of seeds per pod (SNP). Flowers were assayed as pedicel length (PdcL), petal width (PetW) and length (PetL), stamen length (StmL), pistil length (PisL), ovary diameter (OvrD) and calyx length (ClxL). In all cases, numeric data were recorded and used for further statistical analysis.

Morphometric data analysis: The morphometric parameters analyzed were largely similar to other researches (Creed, 1997). However, in order to reduce the morphometric characters or parameters, the data from all the parameters were first analyzed by Principal Component Analysis (PCA) on the raw data of each morphometric character from all the sampled cultivars (Factor; SPSS program for Windows). Factor loading scores were obtained from the output and used to select characters, based upon an Eigen value that was higher than 1.0, to provide a parsimonious reduction in the number of parameters. The selected parameters were then used for between-groups linkage cluster analysis to calculate the relationship between groups and to classify clusters.

Table 1. The plant organs collected and analyzed for each cultivar of *P. mirifica* in Thailand (Nos. 1-39) and *P. lobata* (Japan; No. 40), and those samples for which the chloroplast *trn*L-F (*trn*L-F) and rDNA ITS region (ITS) were sequenced or RAPD-PCR was performed (RAPD). The √ symbol represents a collected and analyzed sample.

No.	Cultivar	Leaf	Pod	Flower	*trn*L-F	ITS	RAPD
1	CM1	√	√		√	√	√
2	CM2	√					
3	CM3	√	√	√	√	√	√
4	CM4	√	√	√	√	√	√
5	CR	√			√		√
6	LPang	√	√		√	√	√
7	MHS	√				√	√
8	LPoon	√			√	√	√
9	Nan	√				√	
10	PY	√	√			√	√
11	P1	√			√	√	√
12	P2	√			√	√	√
13	P3	√	√		√	√	
14	UTRD	√					√
15	KPP	√	√		√	√	√
16	LBR	√	√		√	√	
17	NKSW	√					
18	PBoon	√			√	√	√
19	PSNL	√				√	
20	SR1	√	√		√	√	√
21	SR2	√		√	√	√	√
22	SKHT1	√			√	√	√
23	SKHT2	√			√	√	√
24	UTTN	√			√	√	√
25	KC1	√	√	√	√	√	√
26	KC2	√	√	√	√	√	√
27	KC3	√			√	√	√
28	PCHBR	√	√	√	√	√	√
29	PJKRK	√	√	√	√	√	√
30	RB1	√		√	√	√	√
31	RB2	√		√	√	√	√
32	RB3	√			√	√	√
33	RB4	√					√
34	Tak	√	√		√	√	√
35	CHYP	√		√	√	√	√
36	NKRSM	√			√	√	√
37	SKNK	√			√		√
38	CHPn	√		√	√	√	√
39	SRTN	√			√	√	√
40	*P. lobata*	√	√		√	√	√
Total		**40**	**15**	**11**	**32**	**34**	**34**

No.	Province	Code name
1-4	Chiang Mai	CM1-4
5	Chiang Rai	CR
6	Lampang	LPang
7	Mae Hong Son	MHS
8	Lamphun	Lpoon
9	Nan	Nan
10	Phayao	PY
11-13	Phrae	P1-3
14	Uttaradit	UTRD
15	Kamphaeng Phet	KPP
16	Lopburi	LBR
17	Nakhon Sawan	NKSW
18	Phetchabun	PBoon
19	Phitsanulok	PSNL
20-21	Saraburi	SR1-2
22-23	Sukhothai	SKHT 1-2
24	Uthai Thani	UTTN
25-27	Kanchanaburi	KC1-3
28	Phetchaburi	PCHBR
29	Prachuap Khiri Khan	PJKRK
30-33	Ratchaburi	RB1-4
34	Tak	Tak
35	Chaiyaphum	CHYP
36	Nakhon Ratchasima	NKRSM
37	Sakon Nakhon	SKNK
38	Chumphon	CHPn
39	Surat Thani	SRTN

Fig. 1. Map of Thailand showing the Provinces, with those from where *P. mirifica* samples were collected from being shown by numbers and sample code.

DNA extraction: DNA extraction was performed by using a DNeasy plant mini kit (Qiagen) and Nucleospin® DNA plant kit (Machinery-Nagel) as per the manufacturer's protocol. Eluted DNA was stored at -20^{o}C.

Primer conditions: Primers for PCR amplification of the nuclear rDNA ITS region and the intergenic spacer of transfer RNA-Leucine-Phenylalanine region (*trn*L-F region) in the chloroplast DNA were as reported by (White *et al*., (1990). Each PCR mixture contained 1x PCR master mix (Fermentas Life Science), 2 µM of each forward and reverse primer, 100 ng genomic DNA and d-H_2O to reach a total volume of 20 µl. PCR amplification conditions were as follows: 94°C for 2 min 30 sec and 40-45 cycles of 94°C for 1 min., 55-60°C for 1 min and 72°C for 3 min., with a final extension at 72°C for 10 min. PCR products were resolved by 0.8-1.0% (w/v) agarose TBE gel electrophoresis.

RAPD PCR was performed as above except each reaction contained 20 ng of genomic DNA and one of the primers from OPA-07, OPA-12, OPD-02, OPD-16 and OPE-01 (Taberlet *et al*., 1991), in a total volume of 10 µl and was cycled for 94°C for 2 min., 30 sec., followed by 45 cycles of 94°C for 1 min., 36°C for 1.5 min., and 72°C for 3 min., and resolved through 2 % (w/v) agarose TBE gels.

Sequencing: PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sent to the Bioservice Unit (BSU), National Science and Technology Development Agency (NSTDA), Thailand, for direct DNA sequencing.

RAPD amplicon analysis: For the RAPD analysis for each of the five primers used the amplicons attained from each sample were analyzed by scoring the presence (1) or absence (0) of each clear band, defined as reproducible and easily and reliably discriminated from other bands. These were recorded and subsequently used for phylogenetic analysis.

Phylogenetic analysis: All sequences obtained were aligned using the multiple sequence alignment program Clustal W (http://www.ebi.ac.uk/clustalw), and further analyzed by neighbor-joining (NJ) using the Phylogenetic Analysis Using Parsimony (PAUP*4.0b10) program (Swofford, 2000). The genetic distance calculations were based on the K2P distance (for sequencing) and Nei-Li distances (for RAPD) and were performed before generating the phylogeny. Bootstrapping with 1000 replicates was conducted in order to indicate the robustness of the phylogenetic trees, with values as % displayed above each node if > 50%. *P. lobata* (Kudzu) was used as the out-group in order to determine the intra-specific relationships within *P. mirifica* cultivars.

Results and Discussion

Pueraria mirifica were more widely distributed in the Northern, Central and Western Thailand than in other parts of Thailand, because the habitat is most suitable for this species (deciduous or dry forested mountainous areas) mainly located in these three regions. In contrast, *P. mirifica* was rarely found in the East and the South which are mainly composed of evergreen forests. In this study, 39 cultivars were collected from within 27 provinces of Thailand (Fig. 1 and Table 1). Leaves were found throughout the year (Cherdshewasart *et al*., 2007), but *P. mirifica* are usually distinguished by their distinctive flowers which are not always evident. If the flowers were not available, tuberous roots were obtained and cut to investigate the species. The distribution of *P. mirifica* found in this survey (Fig. 1) concurs with that in a previous report (Cherdshewasart *et al*., 2007).

Morphometric factor analysis of leaf, pod and flower morphometry: Factor analysis using the data of selectable morphometric parameters and scatter plots of two factor scores generated by Principal Component Analysis (PCA) revealed that there was no grouping structure within *P. mirifica* in Thailand. However, considering flower morphometry, the KC1 cultivar from Thongphaphum district in Kanchanaburi province was clearly separated from the rest as an outlier but this was not mirrored in the other characters analyzed from this cultivar.

Three dendrograms were constructed using the Between-groups linkage method of Cluster analysis on Squared Euclidian distance. The factor score values from Factor analysis were used for leaf and flower morphometrics whilst the standardized data were used for pod morphometry. All three dendrograms revealed that all the *P. mirifica* cultivars could be clustered differently. For leaf morphometrics (Fig. 2), the 39 cultivars of *P. mirifica* were classified into 5 groups comprised of one large (24 cultivars) and four small (5, 4, 5 and 1 cultivar) groups. The sole cultivar of the last group, Uthai Thani, may reflect the small leaf size due to the local climate which was a rather high humidity location in contrast to the other sites where *P. mirifica* was found in its more typical low humidity habitats.

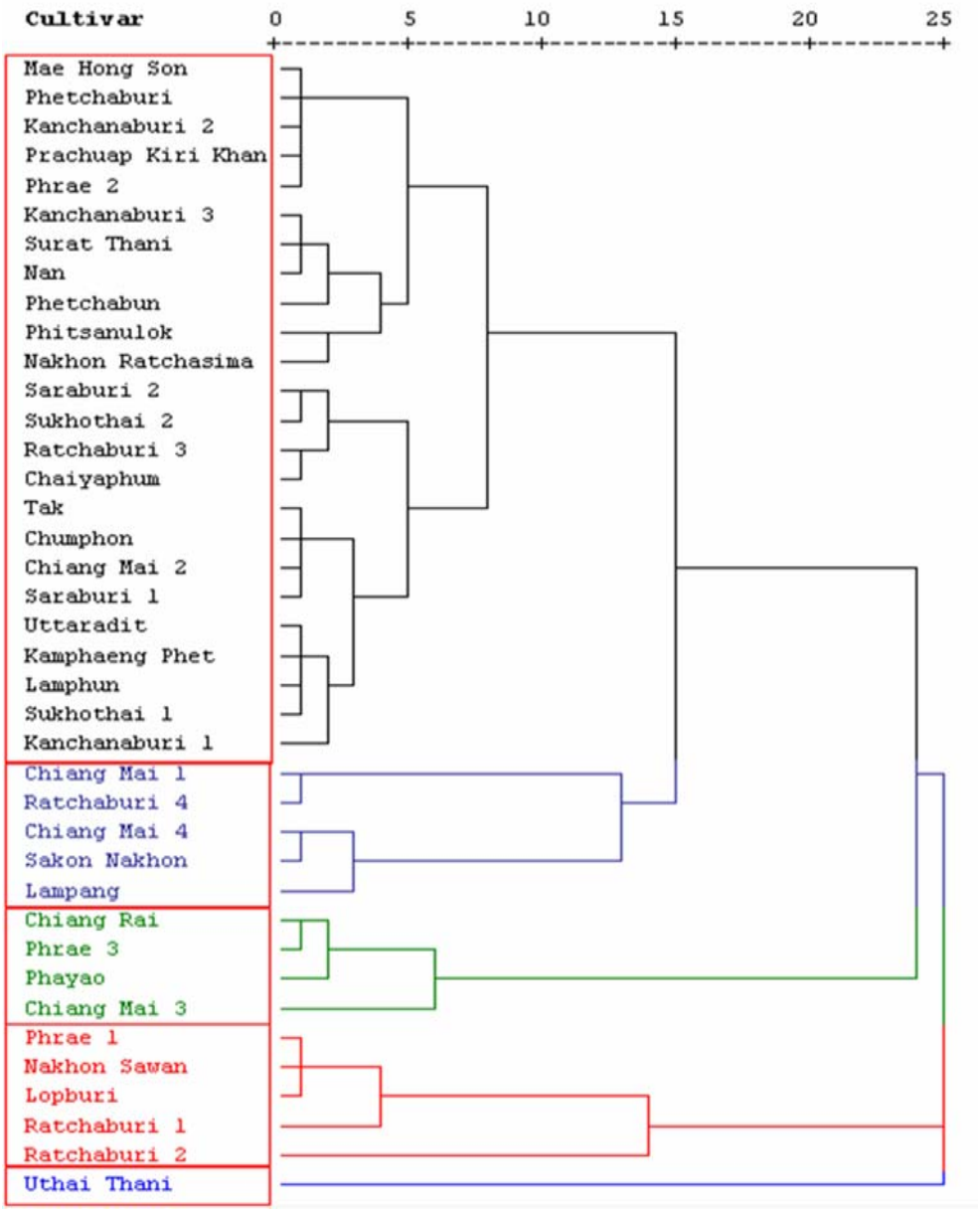

Fig. 2. Leaf morphometric dendrogram created by the Between-groups linkage method of Cluster analysis. *P. mirifica* is classified by the province of collection and sequential number (See Fig. 1).

Characterization of *P. mirifica* in Thailand: An apparent clinal pattern of morphological characterization of *P. mirifica* cultivars in Thailand was revealed when the factor scores for leaf or flower morphometrics and the standardized data of pod morphometry were plotted against latitude and longitude.

Considering first leaf morphometrics, there were statistically significant correlations between both factors 1 (PD, TLB, PL, RL, TLL, PLL and AˆB parameters) and 2 (NPV and SPL parameters) with both latitude and longitude (Fig. 3). Thus a clinal pattern of leaf characterization for *P. mirifica* in Thailand is suggested with the inference that *P. mirifica* leaf characters (PD, TLB, PL, RL, TLL, PLL and AˆB parameters) tend to increase in size from the South to the North of Thailand (R = 0.229). This may reflect the colder weather and reduced light availability in the forested mountainous terrain in the North which is more suitable for the growth of *P. mirifica*. On the other hand, the other two *P. mirifica* leaf characters (NPV and SPL parameters) decreased in size from the South to the North (R = -0.097), although these do not statistically correspond to the above seven parameters. Moreover, the trend of all nine parameters was to decrease in size from the West to the East of Thailand (R = -0.175 and -0.169, respectively), which is also in keeping with the change in topology (the decrease in altitude and mountainous terrain) and associated light availability and temperature. Again, the dry weather and deciduous forest of the West may be more suitable for the growth of *P. mirifica* resulting in larger stems and leaves.

Seed pod morphometrics was analyzed from a smaller sample size (at 14 *P. mirifica* cultivars compared to 39 for leaves) and lacked samples from the more easterly points (samples 35-37), but still revealed a statistically significant correlation in the Z score of PodL ($p \leq 0.05$), with significant correlations to latitude and longitude (R = 0.209 and -0.096, respectively). *P. mirifica* pods (PodL) tend to increase in length from the South to the North, and decrease in length from the West to the East of Thailand, again as with leaves, in agreement with the change in topology (altitude and mountainous terrain). It is conceivable that the dry, cold climate and deciduous, mountainous forests of the North and the West might lead to a higher metabolic storage in the seeds of pods and to a larger size of seeds and length of pods. However, unlike the PodL Z score, there were no significant correlations for both latitude and longitude ($p \geq 0.05$) for PodW and SNP. Thus, the pod shape and size of *P. mirifica* in Thailand are either not significantly different or the three pod parameters used in this study do not reveal the morphometric variation.

Finally, flower morphometric analysis was restricted to only 11 cultivars and lacked all bar one (sample 35) of the likely informative easterly to north easterly locations as well as having only Chiang Mai to represent the north. Thus, although the analysis revealed no statistically significant correlations between latitude for factors 1 (PetL, StmL, PisL, ClxL, PetW and OvrD parameters) and 2 (PdcL), nor for longitude for factor 2 ($p \geq 0.05$), it is less clear if this is due to the reduced resolution following the limited sampling or from an actual trend. Only factor 1 was significantly correlated to longitude (R = -0.468). Scatter plots of factor scores (1 and 2) against latitude and longitude suggested that the size of factor 1 (PetL, StmL, PisL, ClxL, PetW and OvrD) of the flower tended to decrease from the West to the East of Thailand, and coincided with the flower morphometric dendrogram. The eleven cultivars could be classified into three clusters, although, remarkably, the Kanchanaburi 1 cultivar (Thongphaphum district) could be separated from the others having distinctively larger sized flowers. Other than that, *P. mirifica* cultivars have a low apparent morphometric variation.

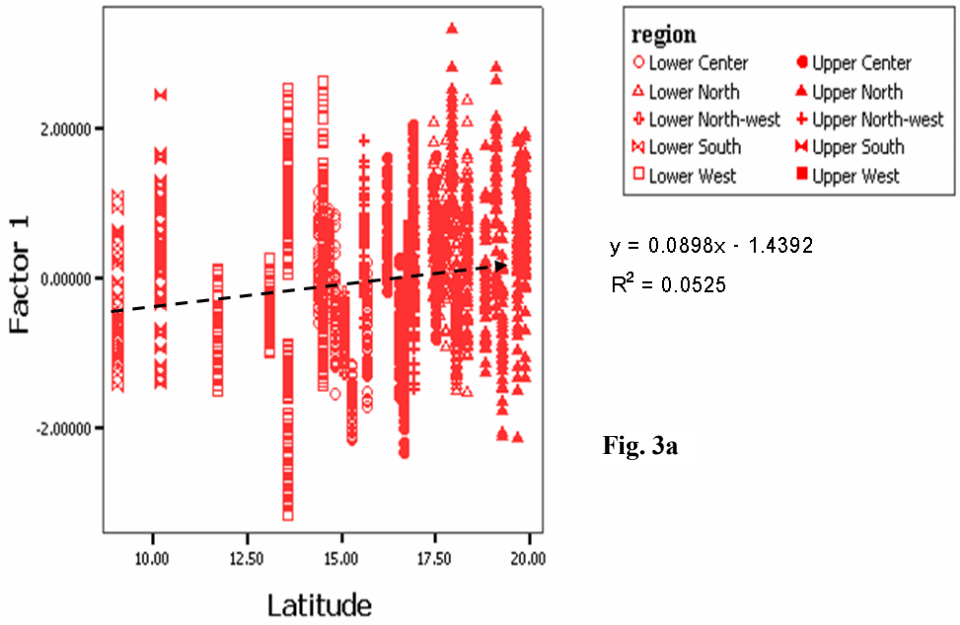
region
Lower Center
Upper Center
Lower North
Upper North
Lower North-west
Upper North-west
Lower South
Upper South
Lower West
Upper West
y = 0.0898x - 1.4392
R2 = 0.0525
Factor 1
2.00000
0.00000
-2.00000
10.00
12.50
15.00
17.50
20.00
Latitude

Fig. 3a

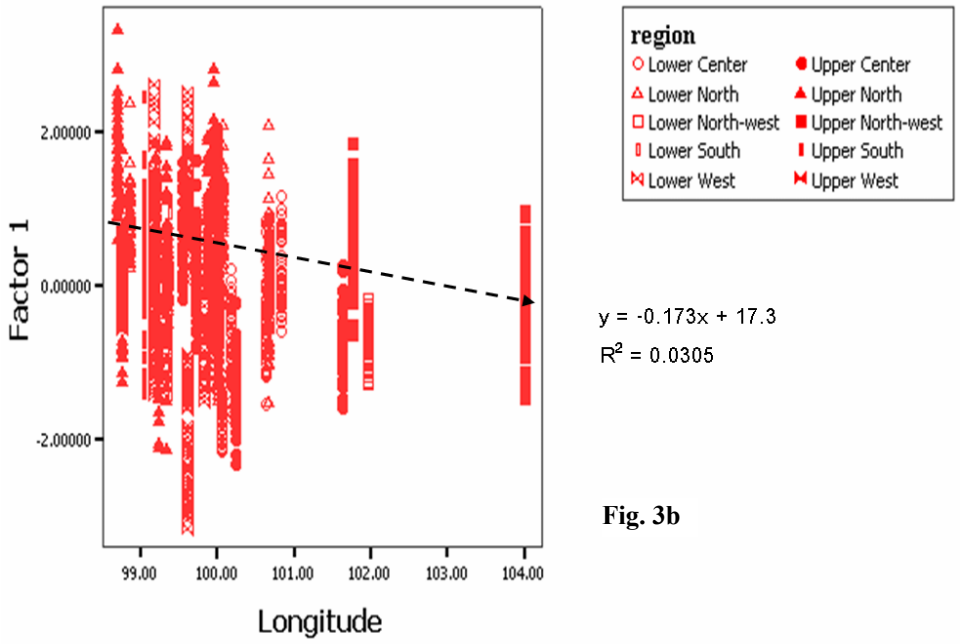
region
Lower Center
Upper Center
Lower North
Upper North
Lower North-west
Upper North-west
Lower South
Upper South
Lower West
Upper West
y = -0.173x + 17.3
R2 = 0.0305
Factor 1
2.00000
0.00000
-2.00000
99.00
100.00
101.00
102.00
103.00
104.00
Longitude

Fig. 3b

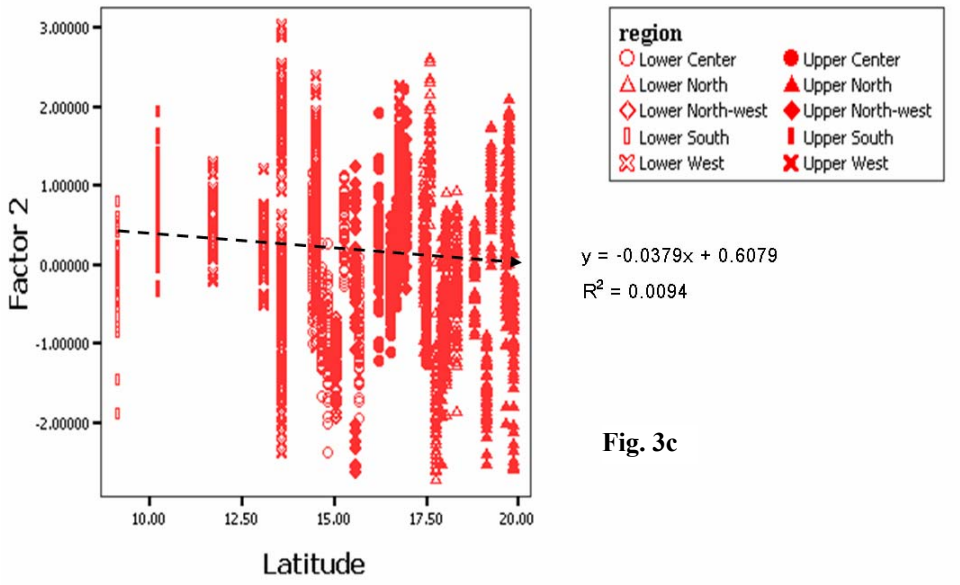

Fig. 3c

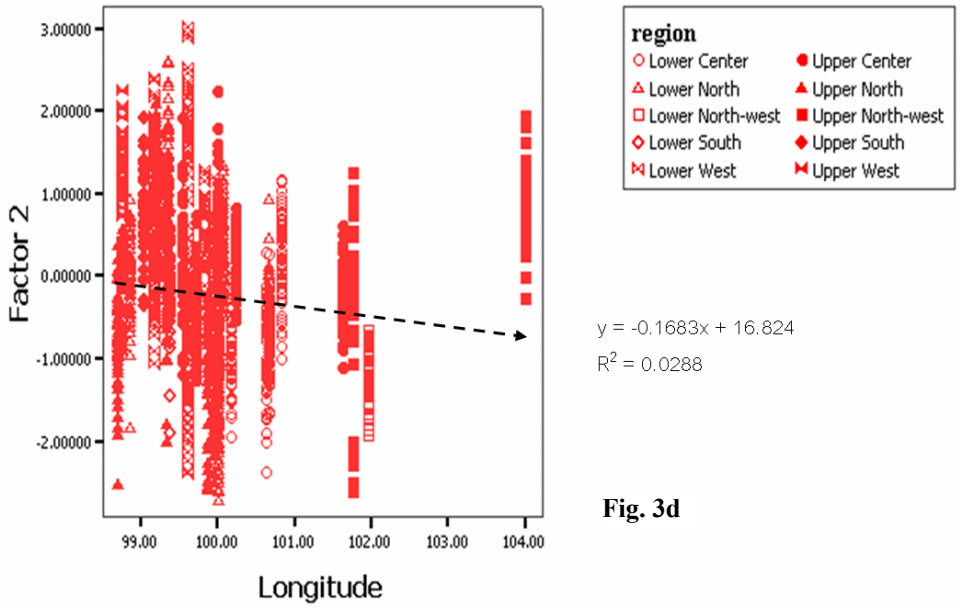

Fig. 3d

Fig. 3. Geographic trends in leaf morphometry of *P. mirifica* in Thailand. Latitude (A & C) and Longitude (B & D) against the factor score 1 (A & B) or 2 (C & D) derived from PCA. Value labels refer to regions.

Genetic variation: All desired amplicons from the PCR amplification of the ITS and *trn*L-F gene fragments of *P. mirifica* from 33 collected sites in Thailand, and *P. lobata* from Japan as an out-group, were purified and directly sequenced. For each cultivar the sequences were completely trimmed aligned and the consensus was kept yielding 687 and 731 bp for the ITS and *trn*L-F regions, respectively. Pairwise and multiple sequences alignment comparisons for the *trn*L-F regions were highly similar (93-100% across all 39 *P. mirifica* samples and 93-98% between the *P. mirifca* samples and *P. lobata*) with a low sequence divergence (0-5.4% across all 39 *P. mirifica* samples and 1.0-4.7% between the *P. mirifica* samples and *P. lobata*), as expected given the highly conserved nature (slow mutation rate) of this chloroplast gene. However, given the inability to discriminate between *P. lobata* and *P. mirifica* samples the *trn*L-F sequences cannot be used to support the identification of all *P. mirifica* samples as likely to be valid, since it clearly does not discriminate between related species. With respect to the ITS sequences of each cultivar of *P. mirifica*, a fair degree of polymorphism was observed with similarity percentage (72-100%) and sequence divergence (0-25.2%) values within the range typically seen within a species but suggestive of a fair degree of cultivar dependent genetic variation. However, very oddly and almost inexplicably, no discrimination between *P. lobata* and *P. mirifica* was noted, questioning how far diverged these two species really are ITS sequence variation is typically representative of only one locus (due to concerted evolution across tandem repeats and loci), and often does not show equal mutation rates across isolates (patterns that are not in accord with the molecular clock). Therefore to sample a larger representation of the genome we used PCR-RAPD analysis with five random primers (Cherdshewasart *et al*., 2007). The five selected primers all successfully amplified *P. mirifica* DNA and revealed polymorphic banding patterns between some *P. mirifica* cultivars as well as the out-group (*P. lobata*), allowing discrimination of individual cultivars (Fig. 4).

In total 93 easily distinguished RAPD bands were reliably amplified and resolved by the 5 primers under these conditions. Of these, none were monomorphic across all 33 *P. mirifica* cultivars. A representative example of amplicons derived from some *P. mirifica* samples with one primer is shown in Fig. 4, whilst a summary for each primer is displayed in Table 2. Interestingly, as can clearly be seen in the summary table, all primers amplified only polymorphic bands meaning not a single amplicon was conserved across all the 39 cultivars.

Phylogenetic analysis: The sequences of PCR products (ITS) and RAPD patterns of *P. mirifica* from the 33 different Thai *P. mirifica* cultivars assayed (Table 1) and from the one isolate of *P. lobata* from Japan (as the out-group) were used for phylogenetic analysis.

Considering the ITS sequence derived NJ tree, although there are many minor groups of with minor bootstrap support, there is no clear resolution except for one group comprised of two apparent sister clades (Fig. 5). The former group is comprised of cultivars of Lamphun and Phrae 3 (Wang Chin district), firmly paired and grouped together with Phrae 2 (Song district), Ratchaburi 2 and Kamphaeng phet whilst the sister clade to this, comprised of Kanchanaburi 2 (Sai Yok district) and Chumphon cultivars also has good support (91%). However, of unclear reason and some cconcern, the out-group (*P. lobata*) failed to out-group but instead was resolved within the *P. mirifica* samples and especially cultivar Phrae 1.

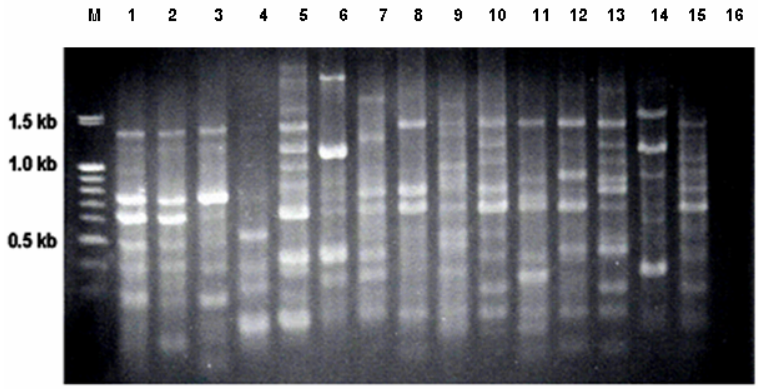

Fig. 4. RAPD patterns amplified by OPA-12 primer and resolved on 2.0 % (w/v) agarose gel. Lane M; 100 bp ladder as DNA marker. Lanes 1-15; the resolved OPA-12 RAPD amplicons from 15 different cultivars of *P. mirifica* in Thailand. Lane 16; the RAPD product of *P. lobata* as an out-group.

Table 2. Total number of distinctive (reliably scored) and reproducible RAPD bands scored for the five RAPD primers using *P. mirifica* genomic DNA as template. Polymorphic bands are scored from across the 33 cultivars of *P. mirifica* in Thailand.

RAPD primer	Number of total band	Number of polymorphic band	Amplicon size range (bp)
OPA-07	21	21 (100 %)	250-1500
OPA-12	20	20 (100 %)	250-1500
OPD-02	17	17 (100 %)	250-1300
OPD-16	20	20 (100 %)	250-1500
OPE-01	15	15 (100 %)	250-1300
Total	**93**	**93 (100 %)**	**250-1500**

As expected for a highly conserved gene within a species, phylogenies based upon the *trn*LF gene fragments revealed no significant discrimination, including between the *P. mirifca* samples and the out-group *P. lobata* (not shown).

For the RAPD derived data the genetic distances (pairwise distances between taxa based on Nei-Li distance in PAUP program), which were further used in NJ phylogenetic construction, varied from 0 to 0.4381 (Mean = 0.1861), suggesting that the 5 primers used in this study could provide moderate information on the genetic variation of *P. mirifica* cultivars. This is not inconsistent with data from the related species, *P. montana*, where RAPD analysis revealed a high degree of genetic variation [17]. The NJ derived phylogram from the RAPD data (Fig. 6) was separated into many inconsistent branches with some weakly supported branches. As with the ITS based phylogeny, again no consistent geographical pattern was evident and cultivars from the same Province often varied from each other as much as those from other Provinces. Again of concern was the lack of separate resolution of the out-group (*P. lobata*) from the *P. mirifica* cultivars, although this time it grouped close to the *P. mirifica* Sukhothai 1 and 2 cultivars and not Phrae 1. Although the RAPD results presented only moderate genetic differentiation and revealed no clear phylogenetic pattern, they were potentially efficient enough for genetic classification among cultivars.

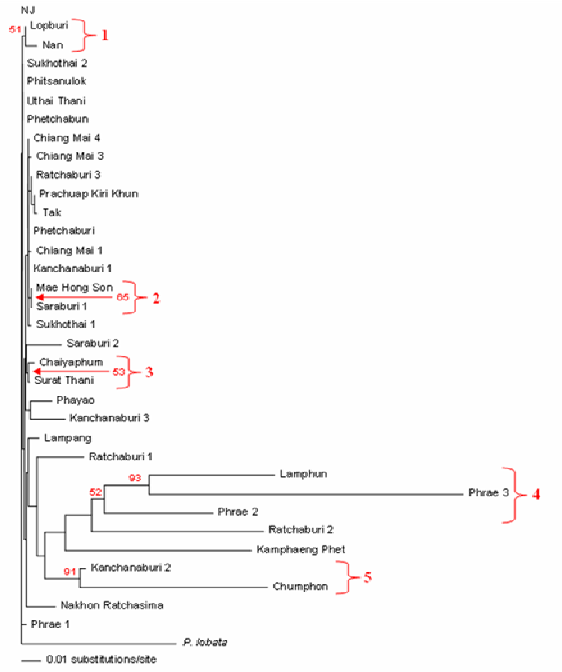

Fig. 5. Rooted NJ tree of the ITS region (33 cultivars of *P. mirifica* plus the out-group *P. lobata*) based on K2P genetic distance. Numbers above the branches indicate bootstrap values of more than 50 % derived from 1000 replicates.

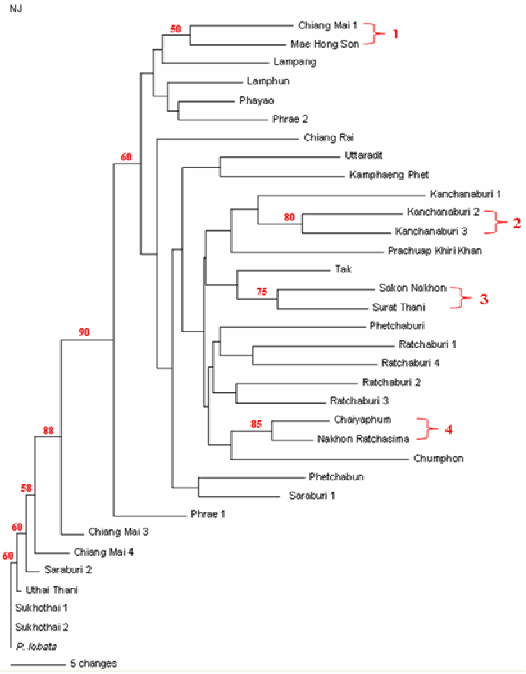

Fig. 6. Rooted NJ tree based on Nei-Li genetic distances among 33 cultivars of *P. mirifica* and 1 cultivar of *P. lobata* as an out-group derived from RAPD patterns. Bootstrap values are shown above the nodes when > 50 %.

Conclusions

Although *P. mirifica* cultivars have a low overall morphometric variation (Fig. 2), potential clinal patterns were still observed by linear regression analysis (Fig. 3) with latitude and longitude changes having an apparent influence on the leaf, seed pod and flower morphologies of this species. In addition, the low level of genetic variation amongst cultivars of *P. mirifica* was supported by sequence analysis of the rDNA ITS region and by RAPD analysis. Although some cultivars clearly differed from the others, such as Kanchanaburi 2, no clear geographical or isolation by distance was observed.

Morphometric and genetic analyses revealed different patterns, as also did genetic analysis derived from the ITS sequence data and the RAPD derived data. Since RAPDs can amplify genomic DNA randomly and assay any genetic variation more apparently, the five primer sets utilized could detect high levels of variation in *P. mirifica*.

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