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Morphological and molecular characterization of underutilized medicinal wild ginger (*Zingiber barbatum* Wall.) from Myanmar

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Abstract

Zingiber barbatum Wall. (family Zingiberaceae), is an underutilized medicinal plant and commonly known as 'Meik tha-lin' in Myanmar where it is used in the indigenous system of medicine. In the present study, 19 accessions of *Z. barbatum* from five provinces in Myanmar have been utilized to characterize and assess genetic diversity. Twenty-nine morphological characters were noted, including growth habit, leaf, pseudo-stem and rhizome characters. Fifteen primer sets of P450-based analogue (PBA) markers were used to reveal molecular characteristics. Of the 29 morphological characters, 22 showed a high degree of variation within wild ginger accessions, whereas 20 of these characters contributed significantly to morphological variation. Eleven amplified primer sets gave a total of 175 bands and exhibited 92.15% polymorphism across *Z. barbatum* accessions. Based on morphological characters and PBA markers, 19 accessions can be divided into two morphotype groups with comparatively higher genetic diversity. This information can be applied in future crop improvement, proper conservation and better use of this underutilized medicinal species.

Keywords: genetic diversity; morphological and genetic characterization; wild ginger; *Zingiber barbatum*; Zingiberaceae

Introduction

Myanmar possesses a unique traditional culture of using herbal medicines (Awale *et al*., 2006) and has rich phytodiversity. It is located in the Indo-Burmese biodiversity hotspot (Myers *et al*., 2000) in a monsoon area of Southeast Asia (10°–28°N and 92°–101°E), with the south having a tropical climate and the north having a temperate climate. Several studies have been conducted using Myanmar germplasm from crops such as banana (Wan *et al*., 2005), tomato (San-San-Yi *et al*., 2008), mango (Hirano *et al*., 2010), *Curcuma amada* (Jatoi *et al*., 2010) and rice (Yamanaka *et al*., 2011). Myanmar is also one of the main diversity centres of the *Zingiber* genus (Ravindran *et al*., 2005), with high genetic diversity observed among *Zingiber* species collected from the genebank, farms and rural markets (Jatoi *et al*., 2008).

The genus *Zingiber* consists of 100–150 species (Thelaide, 1999; Wolff *et al*., 1999; Ravindran *et al*., 2005), which are used as spice, essential oil or herbal medicine.

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Zingiber officinale is used as food flavouring, flowers of *Zingiber mioga* as a vegetable in Japan and *Zingiber zerumbet* and *Zingiber montanum* are widely used as traditional medicines (Ravindran *et al.*, 2005). *Zingiber* is mostly propagated vegetatively through rhizomes. The flower and seed set are rare due to climatic and photoperiodic factors, as well as the high sterility among *Zingiber* flowers (Ravindran *et al.*, 2005).

One of the underutilized medicinal *Zingiber* species in Myanmar is *Zingiber barbatum*, locally called 'Meik tha-lin' (MAS, 2000). This species is commonly used as a traditional medicine for treating gout, by topical application or oral ingestion. The main function of this traditional medicine is anti-inflammatory and analgesic (Awale *et al.*, 2006). Farmers and other local people in Myanmar identify *Z. barbatum* based on leaf and rhizome characters. However, cultural changes in many areas in Myanmar have reduced the demand for medicinal plants (Awale *et al.*, 2006), including *Z. barbatum*. Lack of information and interest in studying this underutilized crop threatens its existence. Characterization and diversity analysis of *Z. barbatum* will provide invaluable information for better utilization and conservation.

Morphological characters have been used to characterize and evaluate genetic diversity in Zingiberaceae species, such as *Curcuma* spp. (Velayudhan *et al.*, 1999; Sasikumar, 2005; Hussain *et al.*, 2008; Keeratinijakal *et al.*, 2010), *Alpinia* spp. (Hussin *et al.*, 2000) and *Zingiber* spp. (Ravindran *et al.*, 1994; Kladmook *et al.*, 2010), as well as other crop species such as kale (Cartea *et al.*, 2002), *Vitis vinifera* (Ortiz *et al.*, 2004), greater yam (Hasan *et al.*, 2008), watermelon (Szamosi *et al.*, 2009), melon (Oumouloud *et al.*, 2009), wheat (Dos Santos *et al.*, 2009) and cowpea (Ghalmi *et al.*, 2010). However, morphological characters, especially quantitative traits, are not stable due to the high dependence on genetic × environment effects (Hussain *et al.*, 2008; Oumouloud *et al.*, 2009). Molecular markers are beneficial complements since these are independent of environmental effects (Solmaz *et al.*, 2010). The combination of morphological characters with molecular markers will provide more comprehensive information about the underutilized *Z. barbatum*.

Limited molecular markers are available for underutilized species, especially Zingiberaceae. The functionality of using randomized amplified polymorphic DNA (RAPD) has been reported for *Z. montanum* (Bua-in and Paisooksantivatana, 2010) and *Z. officinale* (Kizhakkayil and Sasikumar, 2010; Sajeev *et al.*, 2011), inter simple sequence repeats (SSR) for *Z. officinale* (Kizhakkayil and Sasikumar, 2010) and rice SSR markers as an RAPD marker for Zingiberaceae (Jatoi *et al.*, 2006). However, these markers only characterize the neutral region (Yamanaka *et al.*, 2003) and do not cover the entire genome (Karp, 2002). Our laboratory has developed P450-based analogue (PBA) markers, a functional marker system based on cytochrome P450 (Yamanaka *et al.*, 2003), which plays an important role in oxidative metabolism of endogenous and exogenous lipophilic compounds (Inui *et al.*, 2000) as well as the biosynthesis of secondary metabolites in higher plants (Teutsch *et al.*, 1993; Ohkawa *et al.*, 1998). These markers provided highly informative results in diversity studies of 51 species from 28 different families (Yamanaka *et al.*, 2003), Myanmar banana landraces (Wan *et al.*, 2005), genetic variation within and among the fragmented populations of *Withania coagulans* (Gilani *et al.*, 2009) and *C. amada* accessions (Jatoi *et al.*, 2010). Based on successful cross-amplification to the different species and families, in combination with morphological characters, we applied PBA markers for molecular characterization of *Z. barbatum* from Myanmar.

The objectives of the present study were to evaluate the morphological and molecular characteristics as well as to determine the genetic diversity of *Z. barbatum* in Myanmar.

Materials and methods

Plant material

A total of 19 accessions of *Z. barbatum* were collected from five provinces of Myanmar (Kachin State, Shan State, Mandalay Division, Bago Division and Yangon Division) from 2004 to 2008 (Supplementary Table S1, available online only at http://journals.cambridge.org) and maintained as living collections in the field and greenhouse of Gene Research Center, University of Tsukuba, Tsukuba, Japan. The locations of the *Z. barbatum* collection were scattered from the northern highlands to the southern lowlands of Myanmar (Supplementary Fig. S1, available online only at http://journals.cambridge.org). Four accessions of *Zingiber*, including three accessions of ginger (*Z. officinale*; two from Myanmar and one from Thailand) and one accession of hardy ginger (*Z. mioga*; originally from Tsukuba Japan) were also used as out-group controls.

Morphological studies

This study was conducted during the planting season of year 2009 (March–December). Rhizomes of 23 accessions (30 g each) were planted and grown in plastic pots of 30-cm diameter and 50-cm height containing 2–3 cm of large granule soil (Akadama churyu, Kato Sangyo Co., Kanuma-shi, Japan) at the base for better drainage and

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a pre-mixed soil (Hana to yasai no bayo do, Kato Sangyo Co., Kanuma-shi, Japan) as growth medium. Pots were arranged in the field according to a randomized complete block design with two replications.

Morphological characters were observed following the descriptor list for *Z. officinale* developed by Ahmad (2008), with some modifications. A total of 29 morphological characters including growth habit, plant height, pseudo-stem, leaf sheath, leaf, ligule and rhizome characters (Supplementary Table S2, available online only at http://journals.cambridge.org) were studied. Flower characteristics were not included due to the rarity of flowering during three planting seasons in Japan (from 2006 to 2008).

Eight quantitative characters, i.e. plant height, pseudo-stem width, leaf length, leaf width, leaf length-to-width ratio, number of leaves per tiller, rhizome thickness and rhizome yield per pot, were observed and measured using a ruler, vernier calipers and a balance. Plant height, pseudo-stem width, leaf length, leaf width and number of leaves per tiller were recorded from three to five tillers. Plant height was measured from the soil surface to the point of maximum height. Pseudo-stem width was recorded at the first leaf point at the base of the tiller. Leaf length and leaf width were measured for the four largest leaves for each tiller. Leaf width was measured at three points, i.e. at the centre and each quarter point. The leaf length-to-width ratio was calculated from the average leaf length and leaf width of each tiller. Number of leaves per tiller was counted for the tallest tiller from the first leaf at the base of tiller to the last leaf at the terminal of the tiller. Rhizome thickness was measured at the thickest point of five rhizomes. Rhizome yield per pot was obtained from the weight at harvest time after cleaning the rhizome.

Scoring and all measurements were conducted at the maximum vegetative stage and at harvesting times. The quantitative data were recorded from at least three tillers and averaged for analysis. The data were then recorded directly from the measurement using either a 1–9 scale or binary recording (1 = present, 0 = absent).

DNA extraction and polymerase chain reaction (PCR) amplification

Slight modification of the hexadecyltrimethylammonium bromide (CTAB) method, described by Doyle and Doyle (1990), was used to extract total genomic DNA from dry leaf samples of each accession. Dried leaves (0.05 g) were ground in liquid nitrogen and 700 µl CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris–HCl, pH 8.0) were added with 0.2% 2-mercaptoethanol. The homogenate sample was incubated at 60°C for 30 min with occasional gentle swirling. Extraction was made twice using an equal volume of chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 400 µl cold 2-propanol, washed in wash buffer (76% EtOH and 10 mM ammonium acetate) for 20 min, dried and dissolved in 1 × Tris–EDTA (TE) buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4). The DNA quantity was measured with a spectrophotometer and quality was checked on 1% agarose gels. To purify the DNA from RNA, RNase treatment was applied to all the DNA samples. A final concentration of 10 µg/ml RNase was added to the dissolved DNA and incubated for 30 min at 37°C. The sample was then diluted with two volumes of distilled water, and ammonium acetate added to a final concentration of 2.5 M. The DNA was precipitated using 2.5 volumes of cold 100% EtOH, washed in 70% EtOH, dried and re-suspended in TE buffer. Stock DNA was diluted to 25 ng/µl working solution for PCR analysis.

Eight PBA primers were chosen in this study (Supplementary Table S3, available online only at http://journals.cambridge.org) and, according to Yamanaka *et al.* (2003), 15 primer pairs were used for amplification (Supplementary Table S4, available online only at http://journals.cambridge.org). A PCR cocktail was prepared in a total volume of 20 µl, containing 1 µl 25 ng genomic DNA, 10 × *Ex Taq* buffer, 2.5 mM of each deoxyribonucleotide triphosphate mix, 10 µM of each primer and 0.5 units *Ex Taq* DNA polymerase (Takara, Japan). The PCR cocktail was run in a Gene Amp PCR system 9700 with the following PCR conditions: 94°C pre-denaturation step for 5 min, followed by 32 cycles of 94°C for 1 min, 50°C annealing for 2 min, 72°C extension for 3 min and final extension at 72°C for 10 min, followed by cooling to 4°C. The PCR product was electrophoresed using 1.5% of agarose gels in 0.5 × tris–borate–EDTA buffer and stained with ethidium bromide.

Statistical analysis

Morphological analysis was done based on one planting season data of the respective year. Average, standard deviation and range were calculated for all the quantitative data, i.e. plant height, pseudo-stem width, leaf length, leaf width, leaf length-to-width ratio, number of leaves per tiller, rhizome thickness and rhizome yield per pot. Analysis of variance (ANOVA) was conducted to determine the significance of variation among the accessions using SYSTAT 11 software. The quantitative data were then categorized based on the descriptor list and combined with the qualitative data. Only those data that discriminated between accessions were used for further analysis.

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Amplified fragments were scored in a binary fashion, with 1 and 0 representing presence and absence of DNA fragments, respectively. These scored data were first used to calculate the number of bands, percentage polymorphisms and polymorphic information content (PIC) (Botstein *et al.*, 1980). All data were then standardized and subjected to multivariate analysis using principal components analysis (PCA) for morphological data, principal coordinates analysis for molecular data and cluster analysis using NTSys-pc software (Rohlf, 2000).

Morphological data were used to generate the eigenvalue, percentage variation accumulated for each PCA and load coefficient value of each character in their respective principal components (PCs). The first three PCs were selected, and the characters with load coefficient values ≥ 0.6 were considered as major contributors for that PC and considered informative characters for distinguishing the accessions (Jeffers, 1967). The first two PCs with the highest variations were scattered in the two-dimensional plot to find the accession dispersion.

Table 1. Variability in 22 discriminated qualitative morphological characters based on a modified descriptor list for *Zingiber officinale*

		All accessions		Within *Zingiber barbatum*	
Characters	Description	No. of accession	%	No. of accession	%
Plant growth habit	Erect	20	86.96	17	89.47
	Semi-lodging	2	8.70	2	10.53
	Lodging	1	4.35	0	0.00
Leaf sheath type	Split but not overlapping	16	69.57	12	63.16
	Closed	7	30.43	7	36.84
Leaf sheath attachment pattern	Compact	11	47.83	11	57.89
	Loose	12	52.17	8	42.11
Leaf sheath pubescence	Glabrous	16	69.57	12	63.16
	Low pubescene	5	21.74	5	26.32
	Intermediate pubescene	1	4.35	1	5.26
	Highly pubescene	1	4.35	1	5.26
Leaf sheath colour	Light green	3	13.04	3	15.79
	Green	15	65.22	14	73.68
	Pale green	1	4.35	1	5.26
	Dark green	4	17.39	1	5.26
Leaf sheath margin	Narrow papery margin	12	52.17	8	42.11
	Wide papery margin	11	47.83	11	57.89
Leaf shape	Lanceolate	10	43.48	10	52.63
	Linear	5	21.74	2	10.53
	Elliptic	5	21.74	4	21.05
	Oblong	3	13.04	3	15.79
Leaf apex	Caudate	12	52.17	9	47.37
	Acute	11	47.83	10	52.63
Leaf pubescence	Glabrous	13	56.52	12	63.16
	Lower side pubescene	9	39.13	6	31.58
	Both side pubescene	1	4.35	1	5.26
Leaf colour	Green	6	26.09	3	15.79
	Yellowish green	2	8.70	2	10.53
	Dark green	15	65.22	14	73.68
Ligule shape	Emarginated	14	60.87	11	57.89
	Bi-lobed	9	39.13	8	42.11
Ligule size	Short	9	39.13	8	42.11
	Medium	3	13.04	0	0.00
	Long	11	47.83	11	57.89
Rhizome skin colour	Pale yellow	3	13.04	3	15.79
	Light yellow	10	43.48	10	52.63
	Yellow	6	26.09	6	31.58
Rhizome flesh colour	Pale yellow	7	30.43	3	15.79
	Light yellow	7	30.43	7	36.84
	Yellow	2	8.70	2	10.53
	Dark yellow	7	30.43	7	36.84

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Results

Nineteen accessions of *Z. barbatum* collected from five locations in Myanmar and the out-groups species were characterized based on morphological characters and functional markers. Larger variations in morphological characters were observed between the *Zingiber* species and within *Z. barbatum* accessions in 22 characters (Tables 1 and 2, Supplementary Tables S2 and S5, available online only at http://journals.cambridge.org).

Plant characteristics

The majority of *Z. barbatum* accessions (89.47%) and *Z. officinale* had an erect growth habit, while *Z. mioga* had a lodging growth habit. A semi-erect characteristic was observed in two *Z. barbatum* accessions (Z 189 and Z 192) from Shan State. Tall plants were a common characteristic for *Z. barbatum*, in which 94.74% were more than 90 cm in height. Compared to these accessions, *Z. officinale* and *Z. mioga* were shorter. *Z. barbatum* (78.95%) and *Z. mioga* had a thicker pseudo-stem (>9 mm) compared to *Z. officinale*. There were only three *Z. barbatum* accessions (Z 116, Z 189 and Z 190) that had similar characteristics of pseudo-stem width to *Z. officinale* (Table 1, Supplementary Tables S2 and S5, available online only at http://journals.cambridge.org).

There were two types each of leaf sheath (63.16% split but not overlapping and 36.84% closed), leaf sheath attachment pattern (57.89% compact and 42.11% loose) and leaf margin (57.89% wide papery margin and 42.11% narrow papery margin) within *Z. barbatum* accessions, while within *Z. officinale* and *Z. mioga* only split but not overlapping, loose type and narrow papery leaf sheath margins were found. More than 60% of all accessions had identical leaf sheath pubescence and leaf sheath colour (glabrous, green colour). Pubescence was found only within *Z. barbatum* accessions, and Z 154 from Shan State and Z 145 from Kachin State showed prominent pubescence on the leaf sheath surface (Table 1, Supplementary Table S5, available online only at http://journals.cambridge.org).

Simple leaf division, entire leaf margin and cunneate leaf base were observed in all of the investigated accessions. Larger variation in leaf shape was observed within *Z. barbatum* accessions, with shapes of lanceolate, linear, elliptic and oblong. Lanceolate leaf shape was the most common and found in accessions from Shan State and Yangon Division. Oblong leaves were found in accessions from Kachin State (Z 145) and two accessions from Mandalay Division (Z 105 and Z 116). All *Z. officinale* accessions had linear leaf shape, whereas within *Z. barbatum* accessions, only two (Z 154 from Shan State and Z 158 from Mandalay Division) showed this character. Two types of leaf apex, caudate and acute, were found in equal proportions within *Z. barbatum* accessions. *Zingiber officinale* species had a caudate leaf apex, while *Z. mioga* had an acute leaf apex (Table 1, Supplementary Table S5, available online only at http://journals.cambridge.org).

Similar to the leaf sheath pubescence, the majority of leaf surfaces were also glabrous. Only a single accession of *Z. barbatum* from Kachin State (Z 145) had pubescent leaves, and 39.13% of all accessions, including *Z. officinale*, showed pubescence characteristics on the abaxial leaf surface. Dark green was the common leaf colour within *Z. barbatum* accessions, followed by green and yellowish green, which was found only in Z 116 from Mandalay Division and Z 154 from Shan State. Two accessions of *Z. officinale* from Myanmar had green leaf colour, while *Z. officinale* from Thailand had the darker green leaves (Table 1, Supplementary Table S5, available online only at http://journals.cambridge.org).

Leaf size was assessed from leaf length, leaf width and the leaf length-to-width ratio. In most plants, leaf length in *Z. barbatum*, *Z. officinale* and *Z. mioga* accessions ranged from 26 to 35 cm, but shorter leaves were found in two *Z. barbatum* accessions (Z 162 from Bago Divison and Z 111 from Mandalay Division) and one *Z. officinale*

Table 2. Means, standard deviations, ranges and *P*-values of eight quantitative characters within all accessions and *Zingiber barbatum* accessions

	All accessions			Within *Z. barbatum*		
Characters	Mean ± SD	Range	*P*	Mean ± SD	Range	*P*
Plant height (cm)	101.4 ± 15.8	66.8–131.3	0.025	106.2 ± 12.4	84.7–131.3	0.262
Pseudo-stem width (mm)	9.8 ± 1.0	8.2–12.0	0.036	9.9 ± 1.0	8.4–12.0	0.098
Leaf length (cm)	28.1 ± 2.4	23.7–34.2	0.183	28.7 ± 2.2	25.1–34.2	0.424
Leaf width (cm)	3.8 ± 0.8	2.5–5.4	0.000	3.9 ± 0.6	3.1–5.0	0.000
Leaf length-to-width ratio	7.7 ± 1.4	5.0–9.9	0.000	7.6 ± 1.1	5.4–9.3	0.000
Number of leaves per tiller	24.7 ± 4.6	12.3–33.3	0.001	25.4 ± 4.1	17.7–33.3	0.009
Rhizome thickness (mm)	3.2 ± 0.6	1.1–4.1	0.000	3.3 ± 0.4	2.6–4.1	0.000
Rhizome yield per pot (g)	467.3 ± 313.7	100.0–1350.0	0.000	394.3 ± 198.8	167.5–975.0	0.000

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from Thailand. Almost all of *Z. barbatum* accessions had a leaf width ranging from 3 to 5 cm, while only a single accession from Kachin State (Z 145) had broader leaves, similar to *Z. mioga* (Z 201). The ratio of leaf length to width was related to leaf shape. Z 162 had the lowest ratio and an elliptic leaf, similar to *Z. mioga*. The highest ratios were found in Z 155, Z 189 and Z 153, as well as *Z. officinale* accessions, indicating lanceolate to linear leaves. Most *Z. barbatum* and *Z. officinale* produced up to 30 leaves per tiller. There were two *Z. barbatum* accessions that formed fewer leaves per tiller: Z 157 from Mandalay Division and Z 189 from Shan State. *Zingiber mioga* had the lowest number of leaves per tiller, compared to the other two species (Table 1, Supplementary Tables S2 and S5, available online only at http://journals.cambridge.org).

There was no variation in the presence of the ligule and the ligule margin across the investigated accessions. Equal proportions of two ligule sizes and shapes (i.e. short and emarginated; long and bi-lobed) were found within *Z. barbatum* accessions. All the *Z. officinale* accessions had short and emarginated ligules, while *Z. mioga* showed the long and bi-lobed ligule type (Table 1).

Rhizome characteristics

Most of the accessions produced thick rhizomes; only *Z. mioga* produced a very thin rhizome. However, most of the accessions produced less rhizome, and therefore rhizome thickness was not followed by higher rhizomes yield per pot. Higher rhizome yield was observed in two accessions of *Z. barbatum* from Mandalay Division (Z 105 and Z 116), while *Z. officinale* produced >300 g of fresh rhizome per pot (Supplementary Tables S2 and S5, available online only at http://journals.cambridge.org). Moderately larger variation was found in rhizome skin and flesh colour. Rhizome flesh colour ranged from pale to dark yellow and rhizome skin from pale yellow to yellow. *Zingiber officinale* and *Z. mioga* were darker (light brown) than *Z. barbatum* accessions (Table 1).

Variability in quantitative characters

Mean, standard deviation and range were calculated for eight quantitative characters (Table 2). ANOVA was also used to determine the significance of the variation between accessions. Seven quantitative characters – plant height, pseudo-stem width, leaf width, leaf length-to-width ratio, number of leaves per tiller, rhizome thickness and rhizome yield per pot – showed significant variation across all accessions. The variations in plant height, pseudo-stem width and leaf length were not significant within the *Z. barbatum* accessions. Although there was no significant variation observed in plant height, pseudo-stem width and leaf length characters, there was a wide range of minimum and maximum values, indicating higher inter- and intra-specific variability among *Zingiber* species and within *Z. barbatum* accessions.

Molecular characterization

Fifteen PBA primer sets were used to determine the molecular characteristics and genetic diversity of *Z. barbatum* accessions and out-groups (Table 3). Out of 15 primer sets of PBA markers, 11 primer pairs were

Table 3. Frequency of polymorphic bands within all accessions and *Zingiber barbatum* accessions

	All accession			Within *Z. barbatum*		
Primer pairs	No. of bands	Polymorphic bands (%)	PIC value	No. of bands	Polymorphic bands (%)	PIC value
CYP1A1F/CYP2B6R	28	100.00	0.94	23	95.65	0.93
CYP1A1F/CYP2C19R	11	100.00	0.86	9	88.89	0.85
CYP1A1F/heme2B6	19	94.74	0.90	18	88.89	0.90
CYP2B6F/CYP1A1R	21	100.00	0.92	17	88.24	0.89
CYP2B6F/CYP2B6R	20	100.00	0.92	20	100.00	0.92
CYP2B6F/CYP2C19R	23	95.65	0.93	21	76.19	0.93
CYP2C19F/CYP1A1R	8	100.00	0.80	8	87.50	0.79
CYP2C19F/CYP2B6R	20	100.00	0.92	18	100.00	0.91
CYP2C19F/CYP2C19R	20	95.00	0.92	17	88.24	0.90
CYP2C19F/heme2B6	23	100.00	0.93	19	100.00	0.92
CYP2C19F/heme2C19	6	100.00	0.79	5	100.00	0.78
Total	199			175		
Mean	18.1	98.67	0.89	15.9	92.15	0.88

PIC, polymorphic information content.

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successfully amplified and produced 199 and 175 bands with averages of 18.1 and 15.9 bands in all accessions and within *Z. barbatum* accessions, respectively. The primer set CYP1A1F/CYP2B6R gave the highest number of bands in all accessions as well as within *Z. barbatum* accessions and comparatively, primer sets CYP2C19F/heme2C19, CYP2C19F/CYP1A1R and CYP1A1F/CYP2C19R produced fewer bands per primer set. However, all the primers were highly informative, with a high percentage polymorphism and PIC.

Multivariate analysis

Multivariate analysis was used on 19 accessions of *Z. barbatum*, as well as three accessions of *Z. officinale* and one accession of *Z. mioga* as out-groups. PCA and cluster analysis, based on 22 discriminated morphological characters, revealed high genetic diversity in *Z. barbatum*. Generally, the 19 accessions of *Z. barbatum* from Myanmar could be grouped into two morphotypes and were clearly separated from *Z. officinale* and *Z. mioga* (Figs 1 and 2).

The first three PCs, generated from morphological characters, accumulated 72.20% of total variation (Table 4). The first PC gave an eigenvalue of 7.86 and explained 35.73% of total variation. The characters related to leaf sheath (type, attachment pattern, pubescence and margin), leaf (shape, apex, pubescence, width and length-to-width ratio), ligules (shape and size) and rhizome flesh colour were major contributors to this PC (load coefficient correlation >0.6). The second PC was correlated with plant height, leaf sheath colour, leaf length and rhizome skin colour. PC-2 had an eigenvalue of 4.56 and explained 20.73% of total variation. In the third PC (PC-3), plant growth habit, number of leaves per tiller, rhizome thickness and rhizome yield characters made a significant contribution to total variation in PC-3.

The scatter plot of the first two PCs, generated from morphological characters, showed grouping of *Z. barbatum* accessions (Fig. 1(a)). The *Z. barbatum* accessions were clearly separated from *Z. officinale* and *Z. mioga* and further subdivided into two groups. The first group, with eight members, was characterized by loose and split but not overlapping leaf sheathes with a narrow margin, pubescent leaf sheath and leaf surface, elliptic or oblong leaf shape with acute leaf apex, and short and bi-lobed ligule. The second group, with 11 members, was characterized by compact and closed leaf sheath with a wide margin, glabrous leaf sheath and leaf surface, lanceolate leaf with caudate leaf apex, and long and emarginated ligules.

Similar to the multivariate analysis based on morphological characters, principal coordinates analysis based on molecular data produced using 11 sets of PBA primers also exhibited a clear separation between *Z. barbatum* and out-group species from the *Zingiber* genus (Fig. 1(b)). The first two PCs accumulated 43.24% of the total variation, and showed that diversity occurs within *Z. barbatum* accessions. Although it was less clear, *Z. barbatum* accessions could be divided into two groups. This was further confirmed in the cluster analysis.

Cluster analysis based on 22 morphological characters and 11 sets of PBA primers supported the PCA result. Both results showed similar species separation and clustering of *Z. barbatum* accessions (Fig. 2).

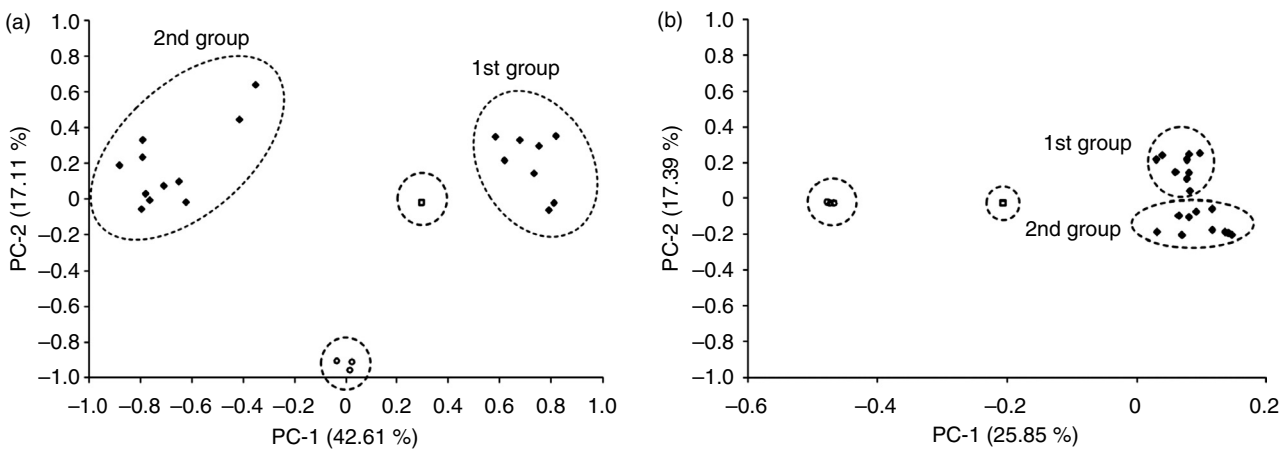

Fig. 1. Two-dimensional plot of 19 *Zingiber barbatum* accessions (■), three *Zingiber officinale* accessions (○) and a *Zingiber mioga* accession (□). (a) PCs generated from 22 morphological characters with contribution rates of PC-1 and PC-2 of 42.61 and 17.11%, respectively. (b) PCs generated from molecular data with contribution rates of PC-1 and PC-2 of 25.85 and 17.39%, respectively.

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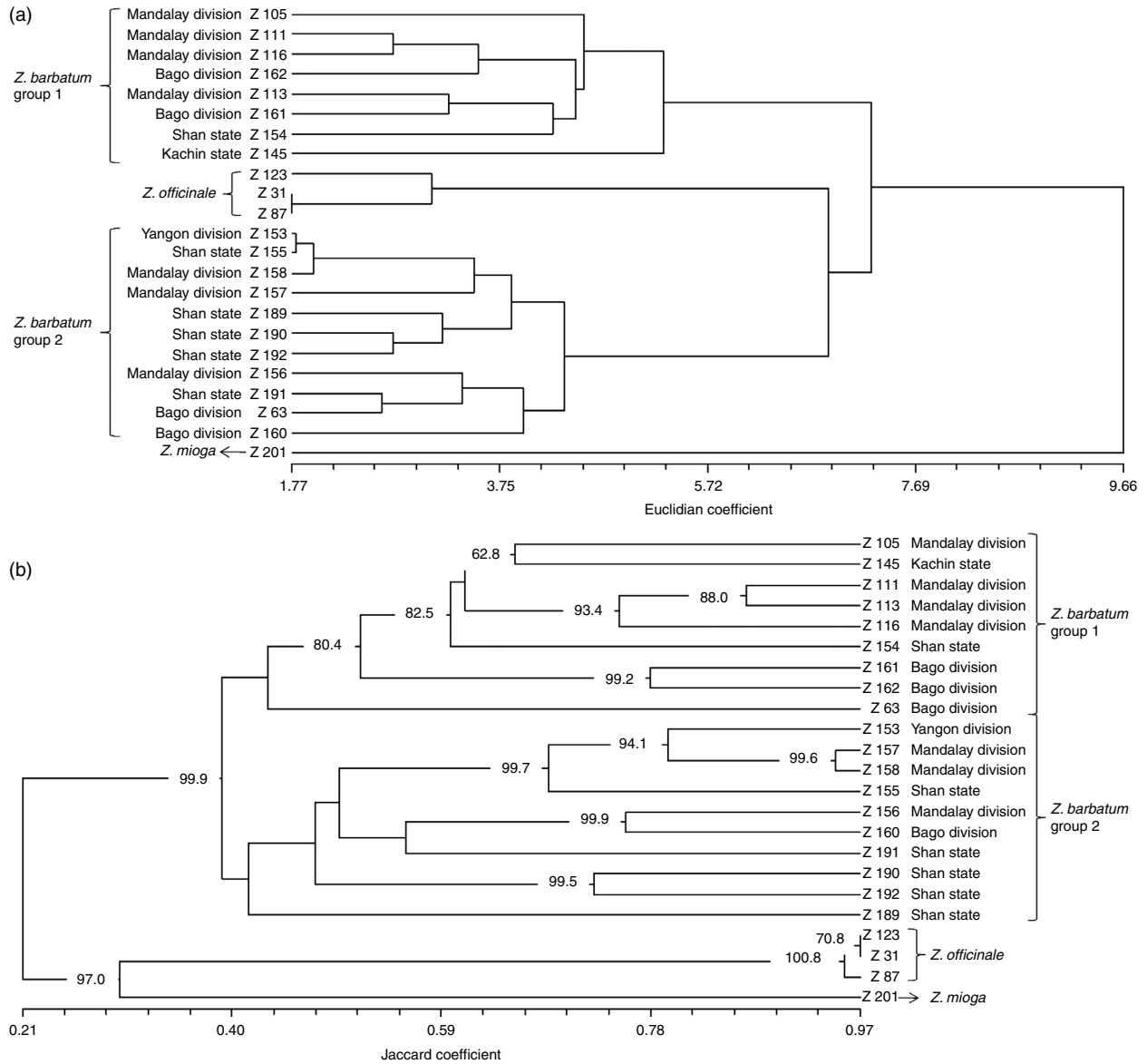

Fig. 2. Unweighted pair group method with arithmetic mean clustering of *Zingiber barbatum, Zingiber offininale* and *Zingiber mioga*. (a) Dendrogram generated from 22 morphological characters using the Euclidean dissimilarity index. (b) Dendrogram generated from 11 P450-based analogue primer sets using the coefficient of Jaccard similarity (confidence interval was tested by bootstrapping with 1000 iterations, and only values $>60\%$ are shown).

The dendrogram gave a clear separation and clustered *Z. barbatum* accessions into two groups, with a Euclidean dissimilarity coefficient ranging from 1.77 to 9.66 (Fig. 2(a)) and Jaccard similarity coefficient from 0.21 to 0.97 (Fig. 2(b)). The dendrogram generated from the 11 PBA primers was also supported by a high bootstrap value (Fig. 2(b)).

Cluster analysis based on 22 morphological characters showed that *Z. barbatum* accessions from Mandalay and Bago divisions were spread in equal proportions within two clusters, while the majority of accessions from Shan State were placed in the second cluster (Fig. 2(a)). Accessions from Kachin State and Yangon Division were placed in group 1 and group 2, respectively. The first cluster (group 1) consisted of four accessions from Mandalay Division (Z 105, Z 111, Z 116 and Z 113), two accessions from Bago Division (Z 162 and Z 161), one accession from Shan State (Z 154) and one accession from Kachin State (Z 145). The second cluster (group 2) contained three accessions from Mandalay Division (Z 158, Z 157 and Z 156), two accessions from Bago Division (Z 63 and Z 160), five accessions from Shan State (Z 155, Z 189, Z 190, Z 192 and Z 191) and one accession from Yangon Division (Z 153). The cluster analysis based

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Table 4. Load coefficient of each discriminated morphological character for the first three principal components (PCs)

Characters	PC-1	PC-2	PC-3
Plant growth habit	−0.0697	−0.2315	*0.8423*
Plant height	0.0862	*0.7016*	−0.5642
Pseudo-stem width	0.3151	0.5266	0.2264
Leaf sheath type	*−0.6671*	0.4055	0.0792
Leaf sheath attachment pattern	*0.8181*	−0.5208	−0.1389
Leaf sheath pubescence	*0.6662*	0.2754	−0.1180
Leaf sheath colour	−0.3384	*−0.6014*	−0.2838
Leaf sheath margin	*−0.8181*	0.5208	0.1389
Leaf shape	*0.8916*	−0.1896	−0.0130
Leaf apex	*0.6622*	0.1433	0.2924
Leaf pubescence	*0.6928*	−0.3543	−0.4219
Leaf colour	−0.0847	0.4957	−0.1581
Leaf length	0.0723	*0.7283*	0.0373
Leaf width	*0.6155*	0.4487	0.5487
Leaf length-to-width ratio	*−0.7217*	−0.2097	−0.4694
Number of leaves per tiller	0.3780	0.2260	*−0.7265*
Ligule shape	*0.9602*	0.0404	0.1373
Ligule size	*−0.9449*	0.2589	0.0025
Rhizome thickness	−0.1835	0.5214	*−0.6458*
Rhizome skin colour	0.3869	*0.7075*	−0.1381
Rhizome flesh colour	*0.7988*	0.4275	−0.2198
Rhizome yield per pot	0.0441	−0.5611	*−0.6342*
Eigen value	7.86	4.56	3.46
Variation (%)	35.73	20.73	15.74
Total variation (%)			72.20

Italics indicates the major contributor (>0.6) that significantly contributed to the variation.

on 11 PBA primers produced similar grouping, except for one accession from Bago Division (Z 63), which originally came from the Myanmar Gene Bank and shifted to the first group (Fig. 2(b)).

Discussion

The broad genetic basis of the germplasm and wide variability in characters provide a basis for sustainable utilization and crop improvement, including clonally propagated plant material (Sasikumar *et al.*, 1999). Characterization and studies of genetic diversity of underutilized crops are critical and will provide invaluable information for planning meaningful breeding strategies (Cooper *et al.*, 2001). Here, we characterized the morphological and molecular characteristics, as well as assessed the genetic diversity, of a clonally propagated traditional medicinal plant from Myanmar, *Z. barbatum*. A wide range of variability was observed within 22 characters across 29 investigated morphological characters, including qualitative and quantitative characters, indicating that a high degree of morphological variation is present among the *Z. barbatum* accessions. This represents a rich *Z. barbatum* germplasm resource in Myanmar.

Generally, *Z. barbatum* growing in Myanmar can be characterized as high and erect plants, which is also a common feature of the *Zingiber* genus (Wolff *et al.*, 1999; Ravindran *et al.*, 2005). The accessions have split, closed and compact or loose leaf sheaths, mostly with a glabrous and green colour. The papery sheath margin is prominent in this underutilized crop. Leaves are lanceolate to oblong with a caudate or acute apex, mostly glabrous or pubescent on the lower surface, green to dark green in colour and medium to long or wide. This species has an emarginate or bi-lobed ligule. Most accessions have a thick rhizome of various skin and flesh colour. The leaf and rhizome characteristics are also used by local people in Myanmar to differentiate *Z. barbatum* from other closely related species.

This study also provided important morphological characters for the study of genetic diversity in *Z. barbatum*. PCA revealed that the most informative characters for diversity are leaf sheath type, leaf sheath arrangement, leaf sheath pubescence, leaf sheath margin, leaf shape, leaf arrangement, leaf pubescence, leaf width, leaf length-to-width ratio, ligule shape and size and rhizome flesh colour. These characters constituted a significant contribution to variation and discrimination within *Z. barbatum* accessions, as well as to differentiating them from other species in the *Zingiber* genus. The pseudo-stem, leaf sheath, leaf and ligule characters have also been used for phylogenetic study of *Zingiber* species in Thailand (Thelaide, 1999) and China (Delin and Larsen, 2000).

Higher polymorphism among morphological characters was also well supported from molecular characterization using PBA markers as functional markers. Higher numbers of bands (15.9 bands per primer set) and percentage of polymorphic bands (92.15% polymorphic bands) across *Z. barbatum* accessions indicate high variability and ample possibilities for finding novel gene combinations. In parallel with this study, high numbers of bands per primer set (13.3) and high percentages of polymorphism (94.58%) were found in a mango ginger collection from Myanmar (Jatoi *et al.*, 2010). A high percentage of polymorphic bands was also observed in a *W. coagulans* population from Pakistan, with an average of 71.95% (Gilani *et al.*, 2009), and a banana collection from Myanmar, with 42 bands and 64.3% polymorphic bands (Wan *et al.*, 2005).

Multivariate analysis using morphological characters and PBA markers produced a clear separation of *Z. barbatum* from the other species examined, as well as similar morphotype grouping. This indicates

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the significance of these two marker groups. Morphological characters and PBA markers may explain genetic diversity at the same level. PBA markers are genome-wide diversity markers that were developed based on the cytochrome P450 gene family and are involved in various important physiological pathways in higher plants (Yamanaka *et al.*, 2005) and probably affect many morphological characters.

Two *Z. barbatum* morphotype groups were found in this study, which could be differentiated by leaf sheath, leaf and ligule characters. It seems that there are two different species that are given the same name by local communities and among which local people could not differentiate. Further confirmation of these two morphotypes could be obtained using molecular analyses, such as sequence analysis of highly conserved genes and inter-genic spacers of chloroplast DNA, to provide a clear picture of this medicinal plant.

High genetic diversity was revealed within the *Z. barbatum* accessions collected from five regions in Myanmar. Low similarity of the Jaccard index and high dissimilarity of the Euclidean index between and within the morphotype groups confirmed the high degree of variability among this clonally propagated plant. In parallel with this study, high genetic diversity was also found within different collection sources of *Z. officinale* (Jatoi *et al.*, 2008), *Z. montanum* from Thailand (Bua-in and Paisooksantivatana, 2010) and different collection sources of *C. amada* from Myanmar (Jatoi *et al.*, 2010). Although *Z. barbatum* is vegetatively propagated, wide morphological and molecular variability and high genetic diversity were observed in this study. Possibly, the long cultivation and utilization history as a traditional medicine in Myanmar by local people and healers, as well as the wide range of local tribes and eco-geographical conditions, has maintained diversity through diversified human selection (Hangelbroek *et al.*, 2002).

Zingiber barbatum accessions used in this study were collected from diverse eco-geographic conditions. Nevertheless, there was no specific relationship between the accession and collection place. *Zingiber barbatum* can be found as a wild plant as well as in the backyards of diverse ethnic groups because of its significant medicinal value. This crop was probably transported and planted from one area to another by users. The same result was reported by Bua-in and Paisooksantivatana (2010) in cassumunar ginger (*Z. montanum*) collected from various locations in Thailand.

In conclusion, this is the first report on the morphological and molecular characterization and genetic diversity of *Z. barbatum*, a medicinal plant from Myanmar. The results showed wide variation and high genetic diversity in this underutilized medicinal crop, representing ample genetic resource availability for crop improvement through breeding programmes. In addition, the important morphological characters identified will be very useful for further genetic diversity studies, not only in *Z. barbatum* accessions but also for other species of *Zingiber*.

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