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Full Length Research Paper

Ethnobotanical and taxonomic screening of genus *Morus* for wild edible fruits used by the inhabitants of Lesser Himalayas-Pakistan

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The present study was focused on ethnobotanical and taxonomic screening of four wild edible fruit species of family Moraceae including *Morus alba* L., *Morus laevigata* Wall., *Morus nigra* L. and *Morus rubra* L. used by the local inhabitants of Lesser Himalayas-Pakistan. Questionnaires based on semi-structured interviews and observations were used to collect data about ethnobotanical importance of these plants. Morphological, including organoleptographical characters of leaves, flowers, fruits and leaf epidermal anatomy were investigated. Leaf epidermal anatomy was carried out under light microscope. All four species showed variations in fruit size, shape, color, in leaf lamina and base shape, floral characters, leaf epidermis cells shape, size and type of stomata. Such taxonomic variations are significant in correct identification of plant species.

Key words: Ethnobotancal, taxonomic screening, *Morus,* Lesser Himalayas.

INTRODUCTION

The Pakistani Himalaya is located south and east of the Indus River, which originates close to the holy mountain of Kailash in Western Tibet, marking the ranges true western frontier. The Himalaya is a totally separate range to the Karakoram which runs parallel to the north. The Himalaya in Pakistan is green and fertile as compared to the arid Karakoram and Hindukush. The Himalaya is spread across three Pakistan's provinces. The Northern area encompasses the Nanga Parbat massif and her surrounding valleys, Azad Jammu and Kashmir and extreme South-east corner of the North West Frontier Province (Pakhtoonkhawa). The Lesser Himalaya is a prominent range 2,000 to 3,000 m high formed along the main boundary Thrust fault zone, with a steep southern face and gentler northern slopes. These Himalayas lie north of the Sub-Himalayan Range and south of the Great Himalayas. In Pakistan these mountains lie just North of Rawalpindi district, covering the districts of

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Batagram, Mansehra and Abbottabad as well as Pakistan Administered Kashmir. These mountains are also home to Pakistan's important hill stations like Murree, Ghora Gali and Nathia Gali. It snows here during a few months of the year but there are no glaciers found in this region. The climate of the Lesser Himalayas ranges from tropical at the base of the mountains to permanent ice and snow at the highest elevations. The amount of yearly rainfall increases from west to east along the front of the range. This diversity of climate, altitude, rainfall and soil conditions generates a variety of distinct plant and animal communities (Anonymous, 2010).

Traditional fruits represent inexpensive but high quality nutrition sources for the poor segment of the population, especially where malnutrition is widespread. Traditional fruits grow wild and are readily available as they do not require any formal cultivation. Wild edible plants (fruits and vegetables) are necessary for the well being of the populations, not only as source of supplemental food, nutritionally balanced diets, medicine, fodder and fuel, but also for developed methods of resource management, which may be fundamental to the conservation of some of the world's important habitats (Chweya and Eyzaguirre, 1999).

Morphology plays a basic role in the field of Taxonomy. The taxonomic keys and identification are mostly the result of morphological studies. Leaf epidermal tissue characteristics have an important role in taxonomy of plant genera and species (Scatena et al., 2005; Strgulc et al., 2006; Uphof, 1962; Yang and Lin, 2005). The epidermis posses a number of important diagnostic characters that offer valuable clues for identification, like size, shape and orientation of stomata, guard cells and subsidiary cells, structural peculiarities of epidermal cell walls, distinctive or specialized form of trichomes (Dickison, 2000). The aim of present investigation was to study ethnobotanical and taxonomic features of four wild edible fruits species of genus *Morus.* No such reference exists on the comparative organoleptographic and taxonomic description with reference to this genus.

METHODOLOGY

Questionnaires and observations were made to record ethnobotanical data about plant species. Fresh samples of about 5 to 6 specimens of each plant were collected from different areas of Lesser Himalayas. Voucher specimens were deposited in Herbarium of Pakistan, Quaid-i-Azam, University, Islamabad after correct identification. Using the modified methodology of Shaheen et al. (2009), fresh leaves were infused in 30% nitric acid with 2.0 g of potassium chloride in a test tube for 2 to 3 min. These leaf pieces were then washed with distilled water twice. Epidermis was peeled off and kept in 60% potassium hydroxide solution for 1 to 2 h. Finally, thin sections of epidermis were suspended in lactic acid on glass slide for light microscopic (LM) study. Microphotographs were taken by using CCD digital camera (Model: DK 5000) fitted on Leica light microscope (Model: DM 1000).

RESULTS

Morus alba L.

Local name: Chitta toot; English name: White mulberry. Distribution: Pakistan, Hazara, Swat, Kashmir and Murree. World: Native to china, Asia, Japan, Malaya, Burma, India, North Africa. Habitat: It grows commonly on the clay soil. Flowering period: February to March. Fruiting period: May to June. Morphology: A monoecious, deciduous, 8 to 15 m or rarely up to 20 m tall tree with a dense, compact leafy crown. Trunk is 1.5 to 2 m in circumference, with dark grey-brown, rough, vertically fissured bark, tender twigs airy to glabrescent. Leaves are green, sticky, herbaceous, 12.1 to 2.9 cm long and 12.5 to 1.6 cm wide, ovate, alternate, somewhat hairy, scabrous, insertion of leaf; ramal, petiolate, lamina narrow to broad, shallowly cordate base, upper surface glabrous, midrib and veins pubescent, margin; regularly serrate, Apex; obtuse, acute or shortly acuminate, stipule; brownish, lanceolate and hairy. Male catkins is 10 to 20 mm long including slender, hairy peduncle, 5.6 mm broad, with flowers. Male flowers: sepals free, broadly ovate, 2.5 mm long, cucullate, obtuse, glabrous to hairy; staminal filaments equal to sepals, with ovate, exserted anthers. Female catkins is ovoid, 5 to 10 mm long, without equally long or slightly longer peduncle. Female flowers: sepals sub orbicular, as long as or slightly larger than male flowers, glabrous or ciliate on margins; ovary with glabrous free styles (Plate # 1).

Organoleptography of fruits: Dark green (unripe), pale yellow to creamy (ripe), edible, sweet, axillary, simple succulent, fleshy, sorosis, drupelets, oval, up to 2.24 cm long, 2.18 cm wide and 3.29 cm circumference. Exocarp, mesocarp and endocarp are indistinct (Plate # 2).

Leaf epidermal anatomy: Costal and inter costal zonation conspicuous. Epidermal cells on the abaxial and adaxial surfaces are variously shaped. The size of epidermal cells; adaxial 33.41 (27.5 to 37.75) × 18.58 (13.25 to 22.5) µm, abaxial 20.83 (12.5 to 27.5) × 10.08 (7.5 to 12.75) µm. Stomata are absent on adaxial surface while on abaxial surface anomocytic to desmocytic stomata are present. Size of stomatal complex is 17.5 (12.5 to 22.5) × 9.58 (6.25 to 12.5) µm while size of aperture is 9.08 (7.5 to 10) × 2 (0.25 to 3) µm. Peltate glands and hooked hairs were also present on adaxial surface. Non-glandular and unicellular trichomes are present on the adaxial surface. The size of trichome is 165 (20 to 250) µm while the base of trichome is rounded

Figure 1. Leaf epidermal anatomy of *Morus alba* L.

Figure 2. Leaf epidermal anatomy of *Morus nigra* L.

having breadth of 22.66 (12.5 to 25.5) µm, while glandular and unicellular trichomes are present on abaxial surface. The size of trichome is 43.33 (37.5 to 47.5) × 7.58 (7.5 to 7.75) µm (Figure 1).

Ethnobotanical uses: Fruits are cool and laxative used to cure sore throat, dyspepsia and melancholia. Wood is used as fuel, in tool handles and construction. Fresh leaves are used as fodder for goats and sheep.

***Morus nigra* L.**

English name: Black mulberry. Local name: Kala Toot. Distribution, Pakistan: cultivated and grows wild through out the country. World: Westwards to Asia Minor, Central and South Europe, North Africa, Central Asia and U.S.A. Habitat: It grows commonly on the clay soil. Flowering period: February to March. Fruiting period: March to April.

Morphology: A monoecious, dioecious, medium or small sized tree, up to 10 m tall, with a compact spreading wide crown. Trunk is 1 to 2 m in circumference with fissured rough bark, tender twigs which are reddish-brown, with densely hairy Leaves that are green, glucose, scabrous above, herbaceous, 11.7 to 4.5 cm long and 8.1 to 4.2 cm wide, insertion of leaf is ramal, petiolate, lamina broad ovate, deeply cordate base, somewhat hairy. Margin: crenate-dentate, Apex: acuminate to caudate, stipules; pale brown, lanceolate, hairy. Male catkins is 25 to 35 mm long, including dense hair, up to 10 mm long peduncle. Flowers: sepals free, broadly ovate, 2.5 to 3 mm long, 23 mm wide, deeply concave, lanate-hairy outside; stamens with boradly oval, exserted anther. Female catkins are oval, 15 to 28 mm long including 6 to 8 mm long, hairy peduncles. Female flowers: sepals broadly elliptic, 3 to 3.5 mm long, 2.5 to 3 mm broad, hairy outside; ovary with densely white hair and divergent styles (Plate 3).

Organoleptography of fruits: Dark green (unripe), black (ripe), edible, sweet, axillary, simple succulent, fleshy, berry, globular, up to 2.59 cm long, 2.29 cm wide and 3.57 cm circumference. Exocarp, mesocarp and endocarp are indistinct (Plate 4).

Leaf epidermal anatomy: Costal and intercostals zonation conspicuous. Epidermal cells on abaxial and adaxial surfaces are variously shaped. The size of epidermal cells; adaxial 25 (22.5 to 27.5) × 16.75 (12.75 to 20) µm, abaxial 21.75 (17.5 to 25.25) × 11.58 (10 to 12.5) µm. Peltate glands and hooked hair are also present on adaxial surface. Stomata are absent on adaxial surface while on abaxial surface anomocytic stomata are present. Size of stomatal complex is 18.41 (12.75 to 22.5) × 6 (5.25 to 7.5) µm while size of aperture is 10.16 (7.5 to 13) × 1.91 (0.25 to 2.75) µm. Glandular and unicellular trichomes having pointed heads are present on the adaxial surface. The size of trichome is 40.91 (40 to 42.5) µm while the base of trichome is rounded having breadth of 9.16 (7.5 to 12.5) µm, while unicellular glandular as well as non-glandular trichomes and hooked hairs are present on abaxial surface. The size of glandular trichome is 17.58 (12.25 to 27.5) and its base is rounded having breadth of 10.75 (7.5 to 12.5) µm. The size of non-glandular trichome on abaxial surface is 216.66 (87.5 to 250) µm, the base of trichome is also rounded having breadth of 52.5 (50 to 55) µm (Figure 2).

Ethnobotanical uses: The leaves are used for feeding silkworms. Leaves infusion is used to control sugar level and blood pressure. Fruits are edible and considered as refrigerant and laxative. Leaves are also used as fodder. Wood is used as fuel and in construction. Young soft and flexible branches are used in making baskets.

Figure 3. Leaf epidermal anatomy of *Morus laevigata.*

***Morus laevigata* Wall.**

Local name: Shah Toot. English name: Long black mulberry. Distribution, Pakistan: Haripur, Abbottabad, Swat, Hazara, Peshwar, Rawalpindi. World: India, Nepal, West and South China and Indo-China. Habitat: It grows rarely on the clay soil. Flowering period: March to April. Fruiting period: May to June. Morphology: A small to large tree, up to 10 m tall with a dense crown. Trunk is 1.5 to 2 m in circumference, with grey smooth bark, young shoots long soft and hairy. Leaves: Dark green, herbaceous, 12.8 to 4.8 cm long and 8.9 to 3.4 cm wide, pubescent petiole, up to 2.5 to 4 cm long, lamina glaucocus, ovate to rotundate, cordate base, sparsely pubescent to glabrous. Insertion of leaf: ramal. Margins: serrate, shortly acuminate, Apex: obtuse to acute, stipules: brownish, lanceolate and hairy. Male catkins are 5 to 10 cm long including a slender 1.5 cm long, pubescent peduncle, densely hairy. Male flowers: Sepals 4, ciliate on margins; staminal filaments as long as sepals. Female catkins are cylindrical, 5 to 12 cm long including 2 cm long peduncle, pendulous, lax-flowered and almost glabrous. Female flowers: sepals 4, imbricate, thin, with outer 2 concave-rotundate, inner ones are plane, floccose-ciliate on margins; ovary with elongated, bipartite style, with stigmas that are patent (Plate 5).

Organoleptography of fruits: Dark green (unripe), yellowish white (ripe), axillary, edible and somewhat sweet, multiple fruits and sorosis, flattened, up to 4.3 cm long, 1.2 cm wide and 2.2 cm circumference. Exocarp, mesocarp and endocarp are indistinct (Plate 6).

Leaf epidermal anatomy: Costal and intercostals zonation are conspicuous. Epidermal cells on the abaxial surface are undulating to irregular shaped while on the adaxial surfaces the cells are variously shaped. The size of epidermal cells; adaxial 36.66 (30 to 42.5) × 21.66 (17.5 to 25) µm, abaxial 41.25 (23.75 to 62.5) × 13.41 (10 to 17.5) µm. Peltate glands and hooked hairs are also present on adaxial surface. The stomata are absent on the adaxial surface while anomocytic to anomotetracytic stomata are present on the abaxial surface. Size of stomatal complex is 20.75 (17.5 to 24.75) × 14.25 (10 to 17.5) µm while size of aperture is 12.5 (10 to 15) × 2.93 (2.75 to 3.25) µm. Glandular and unicellular trichomes are present on adaxial surface. The size of trichome is 45 (43.5 to 46.25) µm, while base of trichome is rounded having breadth of 3.125 (3 to 3.25) µm. On abaxial surface trichomes and glands are absent (Figure 3).

Ethnobotanical uses: Ripe fruits are edible, laxative and refrigerant. Leaves are excellent fodder for goats and sheep. Wood is used in making tool handles and in decoration pieces.

***Morus rubra* L.**

English name: Black mulberry. Local name: Shah Toot Kala. Distribution, Pakistan: Swat, Hazara, northern areas, Murree, Ghora gali. World: Southern New Mexico and Eastern North America. Habitat: It grows rarely on the clay soil. Flowering period: February to March. Fruiting period: April to June. Morphology: A tree up to 12 m tall. Bark is gray-brown with orange tint, furrows shallow, ridges flat, broad. Branchlets are red-brown to light greenish brown, glabrous or with a few trichomes; lenticels are light colored, elliptic, prominent. Buds are ovoid, slightly compressed, 3 to 7 mm, apex acute; outer scales are dark brown, often pubescent and minutely ciliate; leaf scars are oval to irregularly circular, bundle scars are numerous, in circle. Leaves: green, herbaceous, 5.8 to 8.1 cm long and 3.6 to 5.2 cm wide, glucocus, oblong-ovate, insertion of leaf: ramal, petiole up to 2 to 3.3 cm long. Margin: serrate to dentate, Apex: abruptly acuminate, leaf base: broadly ovate. Catkins: peduncle pubescent; staminate catkins 3 to 5 cm; pistillate catkins 8 to 12 × 5 to 7 mm. Flowers: staminate and pistillate on different plants. Staminate flowers: sepals connate at base, green tinged with red, 2 to 2.5 mm, pubescent outside, ciliate toward tip; stamens 4; filaments 3 to 3.5 mm. Pistillate flowers: calyx tightly surrounding ovary; ovary green, broadly ellipsoid or obovoid, slightly compressed, 1.5 to 2 mm, glabrous; style branches divergent, whitish, sessile, 1.5 mm; stigma papillose (Plate 7).

Organoleptography of fruits: Dark green (unripe), red or black (ripe), axillary, edible, sweet, multiple fruit and sorosis, flattened, up to 6 cm long, 1.6 cm wide and 2.6 cm circumference. Exocarp, mesocarp and endocarp are

Figure 4. Leaf epidermal anatomy of *Morus rubra.*

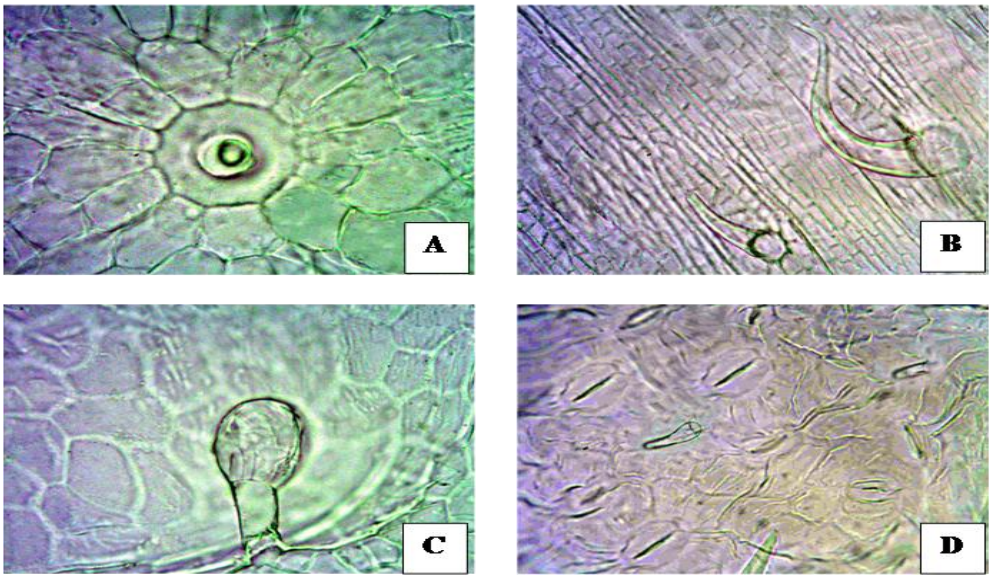

Plate 1. *Morus alba.* A: Peltate glands on adaxial surface (40×), B: Hooked hairs and unicellular trichomes on adaxial surface (20×), C: Glandular trichomes on abaxial surface (40×). D: Stomata and glandular trichomes on abaxial surface (20×).

indistinct (Plate 8).

Leaf epidermal anatomy: Zonal and interzonal cells are conspicuous. Epidermal cells on abaxial and adaxial surfaces are variously shaped .The size of epidermal cells; adaxial 33.33 (30 to 37.5) × 21.75 (20.25 to 22.5) µm, abaxial 30 (27.5 to 32.5) × 14.25 (12.5 to 15.25) µm. Peltate glands and hooked hairs are also present on adaxial surface. The stomata are absent on the adaxial surface while actinocytic and anomocytic stomata are present on the abaxial surface. Size of stomatal complex is 19.88 (19.75 to 20) × 18.16 (17 to 20) µm while size of aperture is 12.33 (12 to 12.5) × 4.91 (4.75 to 5) µm. Unicellular glandular as well as non-glandular trichomes are present on both surfaces. The size of unicellular glandular trichome: Adaxial, 127.91 (112.5 to 157.5) × 26.58 (24.75 to 30) µm, abaxial, 29.91 (27.5 to 32.25) × 13.66 (12.25 to 16.25) µm, while the size of unicellular non-glandular trichomes is: Adaxial, 27.83 (27.25 to 29.75) × 15 (14.75 to 15) µm, Abaxial, 38.25 (27.25 to 29.75) × 14.16 (10 to 20) µm. The base of trichome is rounded and stomata on abaxial surface (20×), D: Non-glandular and Glandular trichomes and stomata on abaxial surface (20×) (Figure 4).

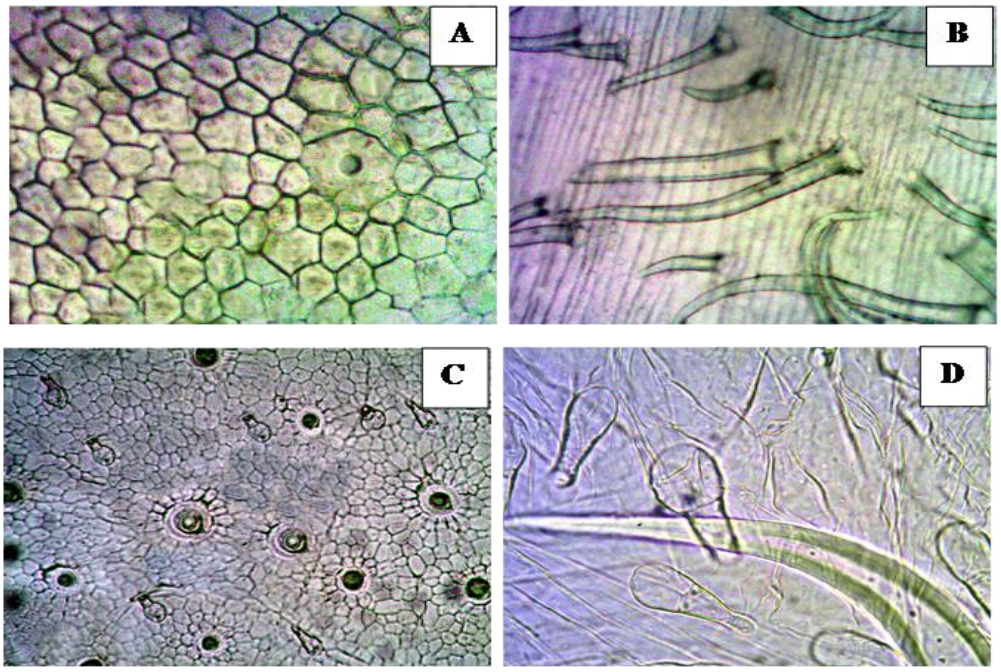

Plate 2. *Morus nigra.* A: Peltate glands on adaxial surface (20×), B: Non-glandular trichomes, hooked hairs on adaxial surface (10×), C: Glandular and unicellular trichomes on abaxial surface (10×), D: Glandular trichomes and hooked hair on abaxial surface (20×).

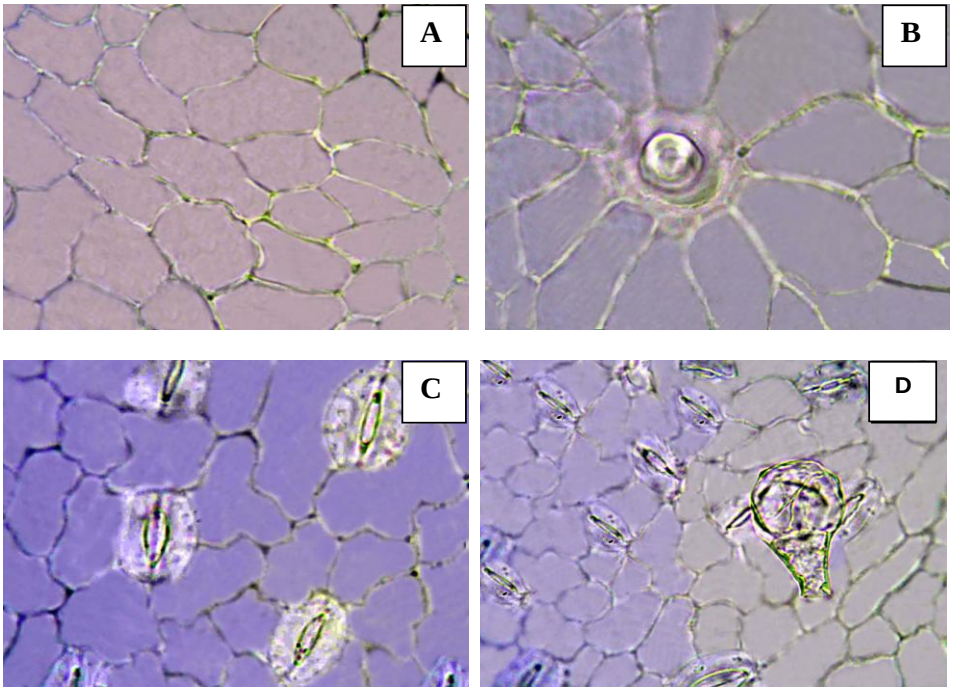

Plate 3. *Morus laevigata*. A: ordinary epidermal cells adaxial surface (40×), B: Peltate glands on adaxial surface, C: Stomata on abaxial surface (40×), Glandular trichomes and stomata on abaxial surface (20×).

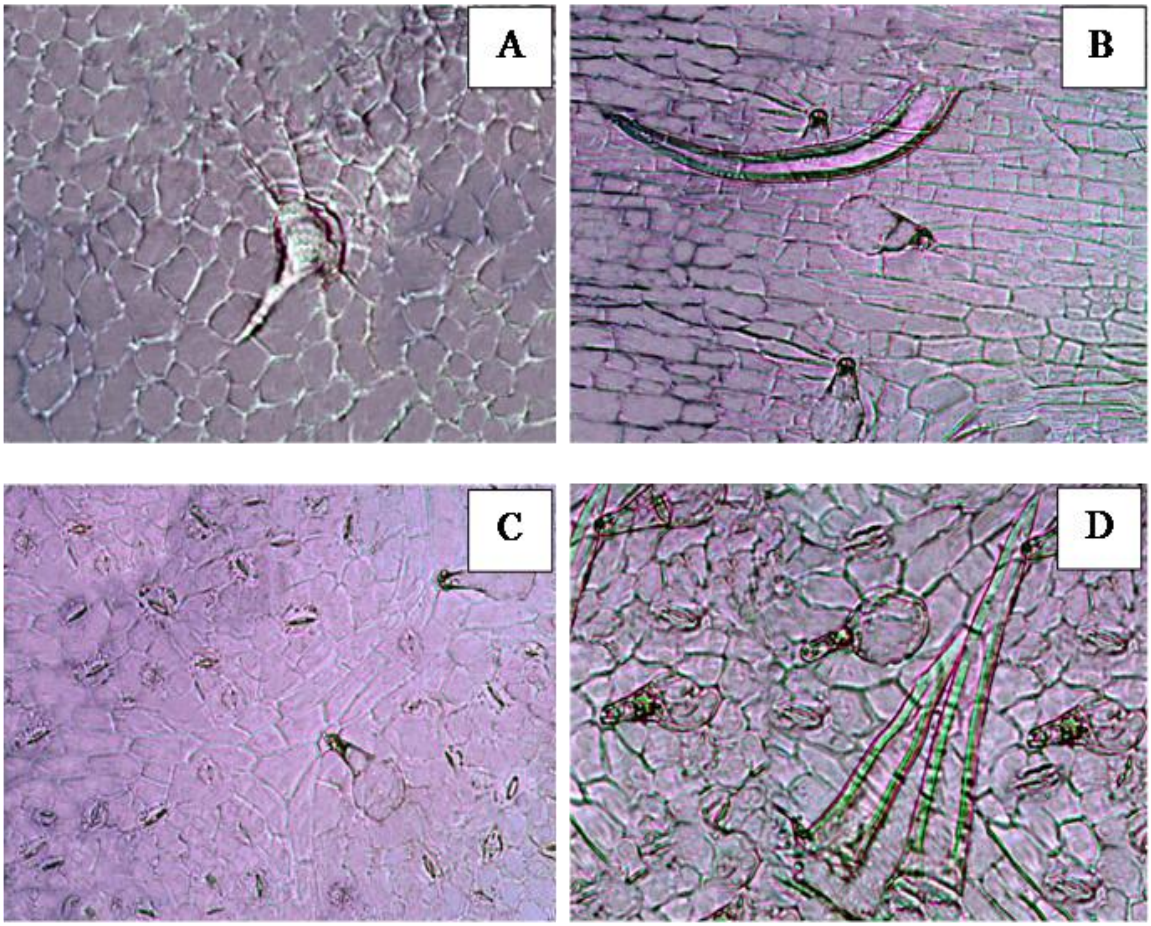

Plate 4. *Morus rubra.* A: Peltate glands and hooked hairs on adaxial surface (20x), B: Hook Ethnobotanical uses: Fruits are laxative and purgative. Root infusion is useful in weakness and urinary problems. Tree sap rubbed directly on the skin as treatment for ringworm. Also used as fodder and in construction.

Plate 5. Organoleptography of fruits of *Morus alba* L.

Plate 6. Organoleptography of fruits of *Morus laevigata*.

Plate 7. Organoleptography of fruits of *Morus nigra* L.

Plate 8. Organoleptography of fruits of *Morus rubra.*

Ethnobotanical uses: Ripe fruits are edible, laxative and refrigerant. Leaves are excellent fodder for goats and sheep.

DISCUSSION

Pakistan is one of the developing countries having great potential of wild flora especially with reference to edible fruit bearing plants. Due to varied edaphic and climatic conditions, about 6000 flowering plant species have been reported from Pakistan. Majority of these plant species have ethnobotanical importance. During present work, preference was given to four edible species of genus *Morus* grown widely through out lesser Himalayas. These plants species not only are sources of edible fruits but they are also used as medicinal plants, fuel wood, fodder, in construction, furniture, tool handles etc. Because of more ethnobotanical importance, these species are under high anthropogenic pressure, which demands their urgent conservation.

The comparative study of plant structure as morphology and anatomy has always been the back bone of the plant systematic, which endeavors to elucidate plant diversity, morphology and evolution. During the present investigation, four species of family Moraceae as *Morus alba, M. nigra, M. leavigata* and *M. rubra* were examined taxonomically which showed diversity in their morphological and anatomical features. Although these four species show some similarities in their characters like catkin inflorescence and sorosis type of fruits,there are remarkable differences in their shape of lamina; as ovate (*M. alba*), broadly ovate (*M. nigra*), ovate to rotundate (*M. laevigata*) and oblong-ovate (*M. rubra*). Lamina base; cordate (*M. alba* and *M. laevigata*), deeply cordate (*M. nigra*) and broadly ovate in *M. rubra.* Similarly, there is great diversity in leaf margin, apex and inflorescence of these species.

Organoleptogrphic characters indicates that fruit color in four species is different as pale yellow (*M. alba*), Black (*M. nigra*), yellow to white (*M. laevigata*) and reddish to black (*M. rubra*). Fruits length also varies as long fruits (4.3 to 6.5 cm) are present in *M. laevigata* and *M. rubra* while small but wide fruits are found in *M. alba* and *M. nigra*.

The leaf epidermal anatomy is one the most significant taxonomic characters studied in number of families based on leaf epidermis anatomy (Bhatia, 1984; Jones, 1986). According to Scotland et al. (2003), rigorous critical anatomical studies of morphological features in the content of molecular phylogenies are fruitful to integrate the strength of morphological data. Leaf epidermis anatomy also shows variation in anatomical features of these species (Table 1). Although there were similarities in epidermal cells in all four species of *Morus* but variation in shapes of epidermis cells can be observed (Figures 1 to 4). Variations can also be observed in the length and width measurement of these epidermis cells (Table 2). Hooked hairs and trichomes have been recorded on both adaxial and abaxial surface of these species except *M. nigra* where unicellular trichomes with pointed ends can be observed (Figure 3). There is also variation in the type and presence of stomata. Mostly, different types of stomata like anomocytic-desmocytic (*M. alba*); anomocytic (*M. nigra*); anomocytic-anomotetracytic (*M. laevigata*) and cyclocytic- anomocytic (*M. rubra*) were observed on abaxial surface (Figures 1 to 4). Presence of different stomata on same leaf indicates the systematic value of this character but its potential value, if properly interpreted and used with other characters, is substantial (Raju and Rao, 1977; Isawumi, 1989).

Table 1. Qualitative analysis of foliar epidermal cells characteristics.

Plant species	Leaf epidermis (Ad/Ab)			Stomata		Trichomes		Glands
	Shape		Margin/wall morphology	P/A (Ab/Ad)	Type	P/A (Ab/Ad)	Type (Glandular/ Non glandular)	
	Ad	Ab						
Morus alba	Variously shaped	Variously shaped	Straight	Ab	Anomocytic to DSesmocytic	P on both	Both	Peltate glands
Morus nigra	Variously shaped	Variously shaped	Straight	Ab	Anomocytic	P on both	Both	Peltate glands
Morus leavigata	Variously shaped	Variously shaped	Straight	Ab	Anomotetracytic and Anomocytic	P on Ad	Glandular	Peltate glands
Morus rubra	Undulating to irregular	Variously shaped	Straight on ab and wavy on ad	Ab	Actinocytic and Anomocytic	Both	Both	Peltate glands

Ad: Adaxial; Ab: Abaxial; P: present; A: Absent.

Table 2. Quantitative analysis of Foliar Epidermal cells characteristics.

Plant species	Surface	Leaf epidermis (µm)	Stomatal complex (µm)	Trichomes (µm)
	Ab/Ad	Length × Width	Length × Width	Length × width
Morus alba	Ab	20.83(12.5 - 27.5)×10.08 (7.5 - 12.75)	17.5 (12.5 - 22.5)×9.58 (6.25 - 12.5)	43.33(37.5 - 47.5)×7.58(7.5 - 7.75)
	Ad	33.41 (27.5 - 37.75)×18.58 (13.25 - 22.5)	Absent	165 (20 - 250)×22.66 (12.5 - 25.5)
Morus nigra	Ab	21.75 (17.5 – 25.25)×11.58 (10 -12.5)	18.41 (12.75 - 22.5)×6 (5.25 - 7.5)	17.58 (12.25 - 27.5)×10.75 (7.5 - 12.5)
	Ad	25 (22.5 - 27.5)×16.75 (12.75 - 20)	Absent	40.91(40 - 42.5)×9.16 (7.5 - 12.5)
Morus leavigata	Ab	41.25 (23.75 - 62.5)×13.41 (10 -17.5)	20.75 (17.5 - 24.75)×14.25 (10 - 17.5)	Absent
	Ad	36.66 (30 - 42.5)×21.66 (17.5 - 25)	Absent	45 (43.5 - 46.25)×3.125 (3 - 3.25)
Morus rubra	Ab	30 (27.5 - 32.5)×14.25 (12.5 - 15.25)	19.88 (19.75 - 20)×18.16 (17- 20)	29.91(27.5 - 32.25)×13.66 (12.25 - 16.25)
	Ad	33.33 (30 - 37.5)×21.75 (20.25 - 22.5)	Absent	127.91 (112.5 - 157.5)×26.58 (24.75 - 30)

Ad: Adaxial; Ab: Abaxial.

Conclusion

The present study was focused on ethnobotanical and taxonomic screening of four species of genus *Morus*. Fruit's organoleptography and leaf epidermal anatomy was studied first time with reference to Pakistan. Variations were observed in morphological and anatomical features of these species. All the taxonomic characters like leaves shape, size, width, types and size of branches, type of stem, floral characters, fruits morphology, leaf epidermis cells and stomata are important tools which tools which play key role in the identification of plant species. Genetic diversity and DNA sequencing may also be helpful in this regard. Ethnobotanical investigation revealed that these plant species not only yield valuable edible and medicinal fruits but also an important source of

fuel, fodder and construction. There is an urgent need for conservation of this nature gift.

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Full Length Research Paper

Hypoglycaemic effect of *Lagerstroemia speciosa* in type 2 diabetic rats

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The objective of this study was to study the hypoglycaemic effect of the aqueous extract of *Lagerstroemia speciosa* in type 2 diabetic rats. Male albino Wistar rats (30) weighing 180 to 190 g were selected and divided into five groups, maintained six rats in each group. Groups I and II were served as normal and diabetic control. Group III was treated with standard drug as glibenclamide 0.25 mg/kg body weight, whereas groups IV and V were administered extract of 100 and 200 mg/kg body weight, respectively. Type 2 diabetes was induced by using streptozotocin (STZ)-nicotinamide (NIA) in rats. Treatment was carried out for 14 days and the fasting serum glucose level, body weight, glycosylated haemoglobin and lipid profile were measured. Data was collected and analysed statistically using one-way analysis of variance (ANOVA) followed by Student's t-test. The statistical data indicated the significant decrease in the fasting serum glucose level in diabetic rats. Glycosylated haemoglobin and lipid profile of diabetic rats also decreased. The aqueous extract of *L. speciosa* has shown hypoglycaemic effect in STZ- nicotinamide induced type 2 diabetic rats.

Key words: Type 2 diabetes, *Lagerstroemia speciosa*, streptozotocin, nicotinamide.

INTRODUCTION

Lagerstroemia speciosa (Lythraceae) is also known as Banaba found in India, Philippines, Southern China, Malaysia and tropical Australia. In recent years, it has been used as a folk medicine for the treatment of diabetes and kidney diseases (Quisumbing, 1978). In Manipur, *L. speciosa* is locally known as Jarol and found abundantly. In KK-Ay diabetic mice, corosolic acid which is present in this plant increased the plasma insulin level resulting in fall in blood glucose level (Kakuda et al., 1996; Miura et al., 2006) as well as stimulate glucose uptake in 3T3-L1 cells (Liu et al., 2001). Furthermore, ellagitannins which were also found in *L. speciosa* extract showed as an activator of the glucose transportation in fat cells (Hayashi et al., 2002). Some of the authors reported that *L. speciosa* found in different countries are equally effective in the treatment of diabetes is not clear (Klein et al., 2007). Furthermore, the antidiabetic effect of *L. speciosa* in different animal diabetic model has been reported, but none of the reports found in streptozotocin-nicotinamide induced type 2 diabetic rats. So, this study was aimed to evaluate the hypoglycaemic effect of *L. speciosa* in

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streptozotocin-nicotinamide induced type 2 diabetic rats.

MATERIALS AND METHODS

Collection of plant

The leaves of *L. speciosa* (Jarol) were collected by Dr. Kula from the hilly areas of Khonghampat, Manipur State, India. It was authenticated by the Department of Botany, Oriental College of Sciences, Manipur, India.

Preparation of extract

Leaves of *L. speciosa* were collected and shade dried avoiding sunlight contact. The leaves were crushed into powder by a mixer. Defatting was carried out by immersing the powdered leaves into petroleum ether for 12 h by regular shaking. Using decoction method, 25 g of defatted leaves were added into 500 ml beaker with 250 ml of water and was heated on a water bath for 30 minutes and filtered. The excess of solvent were removed by simple evaporation technique (Saraswathi et al., 2011).

Animal

Male albino Wistar rats (30) weighing 190 to 200 g were selected for the experiment. The study was conducted after taking the approval of Institutional Animal Ethical Committee of Regional Institute of Medical Sciences, Imphal, India.

Acute toxicity study

According to the OECD guidelines, acute oral toxicity study was performed at a dose of 5000 mg/kg body weight.

Induction of diabetes mellitus

Diabetes mellitus was induced by injecting Nicotinamide 110 mg/kg body weight in normal saline intraperitoneally, after 15 min followed by STZ 65 mg/kg body weight in citrate buffer (pH 4.5). Hyperglycemia was confirmed on day 7 after injection. Animals showing fasting serum glucose level more than 200 mg/dl were selected for the study (Masiello et al., 1998; Punitha et al., 2005).

Experimental design

Animals were divided into five groups of six animals each. Groups I and II were served as normal control and diabetic control. Group III was treated with standard drug as glibenclamide 0.25 mg/kg body weight, whereas groups IV and V were administered with extract of 100 and 200 mg/kg body weight, respectively. Treatment was carried out orally for 14 days. The fasting serum glucose level was estimated on days 7 and 14. Lipid profile, glycosylated haemoglobin and changes in body weight were also measured.

Statistical analysis

All results were expressed as the mean ± standard error of mean (SEM). The statistical significance was evaluated using one- way analysis of variance (ANOVA), followed by Student's t-test using SPSS 20 version software.

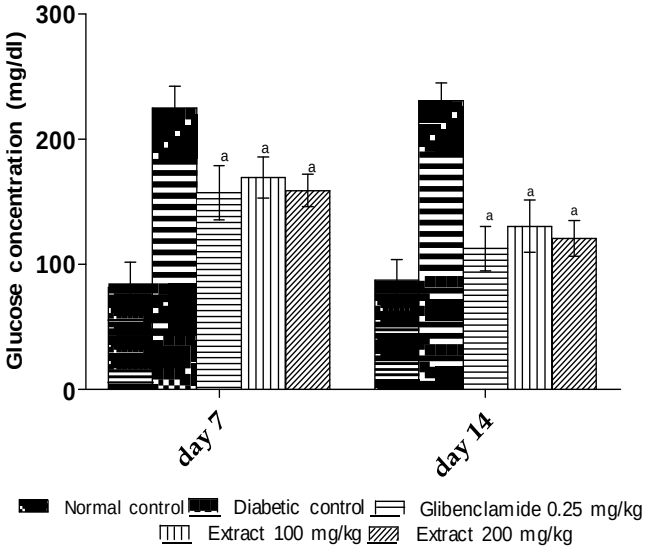

Figure 1. Serum glucose level. Each value is mean ± SEM of six rats in each group. [a]p<0.05 by comparison with diabetic control.

RESULTS

Acute toxicity study

The various observations showed the normal behaviour of the treated animals and no toxic effects. Hence, there was no lethal effect found.

Hypoglycaemic effect of extract

This study showed that at the end of 14 days of treatment, there was a significant decrease ($p<0.05$) of fasting serum glucose level in groups IV (43.51%, 130±21.00) and V (47.71%, 120.66±14.45); the extract had shown a dose dependent, significant decrease of glucose level versus diabetic rats of group II, as indicated in Figure 1. There was a significant fall (51.19%, 112.63±17.74) of the glucose level in standard drug treated group which is comparable with the highest dose of the extract. Thus, the study shows that the extract exhibited significant hypoglycaemic effect in diabetic animals.

Changes in body weight

Body weights were not increased significantly in all the treated groups as compared to diabetic control as shown in Figure 2.

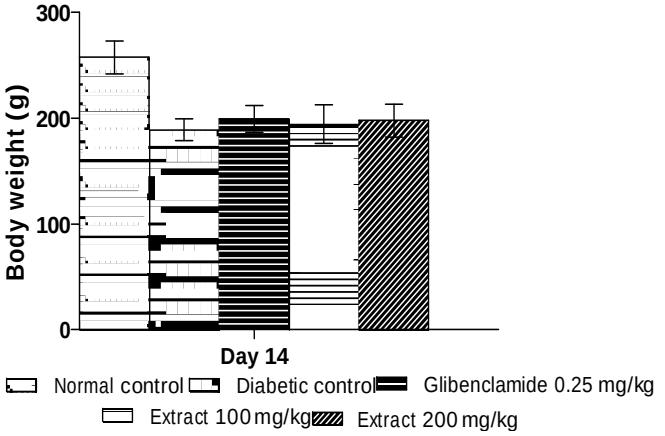

Figure 2. Body weight. Values are expressed as mean±SEM; n=6. The body weight of the treated animals did not improve significantly.

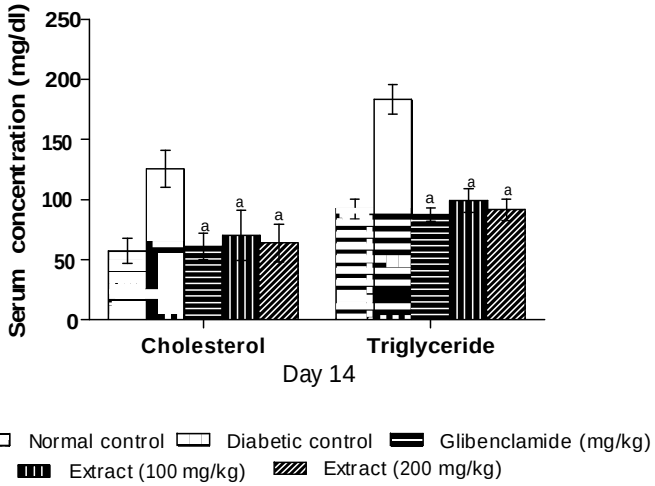

Figure 3. Cholesterol and triglyceride level. Each value is mean±SEM of six rats in each group.
[a]p<0.05 by comparison with diabetic control.

Changes of lipid profile

Both cholesterol and triglyceride level (Figure 3) were found to reduced significantly in extract treated groups IV and V. Percentage of reduction of cholesterol level in groups IV and V were (44.03%, 70.32±21.03) and (49.23%, 63.78±15.88) as compared to diabetic group, whereas triglyceride levels were 45.94% (99.26±10.11) and 50.11% (91.60±8.77), respectively.

Glycosylated haemoglobin level

Glycosylated haemoglobin level in group IV (40.27%, 4.3±0.66) and group V (43.05%, 4.1±0.52) was significantly decreased as compared to diabetic control as shown in Figure 4.

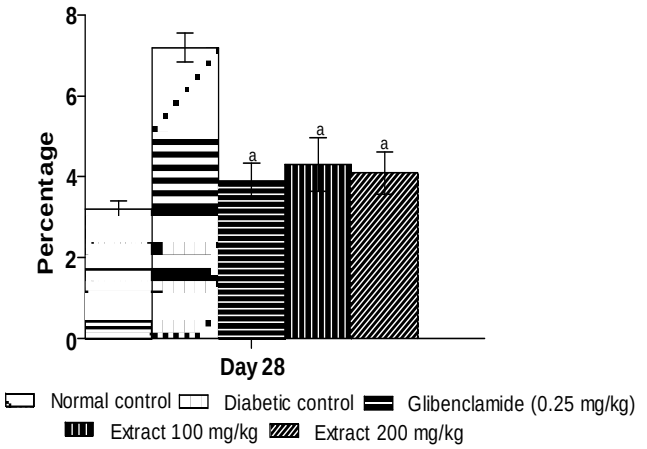

Figure 4. Glycosylated haemoglobin level. Each value is mean±SEM of six rats in each group. [a]p<0.05 by comparison with diabetic control.

DISCUSSION

The protective effect of nicotinamide against the cytotoxicity of STZ in pancreatic beta cells is dose dependent which act as a inhibitor of poly(ADP-ribose) synthetase, a NAD consuming enzyme. A suitable combined dose of nicotinamide and STZ induced a stable moderate hyperglycemia and reduced pancreatic insulin stores (Masiello et al., 1998).

This study showed significant differences in fasting serum glucose level between the diabetic treated group and untreated diabetic rats. However, the higher dose of extract showed more efficiency of hypoglycaemic activity in diabetic rats. It may be due to the influence of insulin release either by regenerating the pancreatic beta cells or by activating pancreatic granules where insulin is stored (Hayashi et al., 2002; Yoshio et al., 1999). It also potentiates translocation of glucose transporter 4 (GLUT4) from intracellular microsomal membrane to plasma membrane (Hattori et al., 2003). Ellagitannins acted as activators of hexose uptake in rat adipocytes like insulin (Judy et al., 2003; Swanston-Flat et al., 1990). The fall of glycosylated haemoglobin in treated diabetic groups may be due to glycaemic effect. Both serum cholesterol and triglyceride level were found to decrease in diabetic induced rats which may probably be due to deterioration of lipolysis as well as cholesterol biosynthesis (De Sereday et al., 2004; Sharma and Narir, 2003).

The aqueous extract of *L. speciosa* has also shown hypoglycaemic effect in STZ- Nicotinamide induced

diabetic rats. However, further study is required to elucidate in controlling diabetes mellitus.

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Full Length Research Paper

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

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

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

Plant

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

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

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

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

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

DNA extraction

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

PCR-RFLP

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

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

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

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

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

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

RAPD amplification

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

Microsatellite amplification

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

Data collection and analysis

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

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

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

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

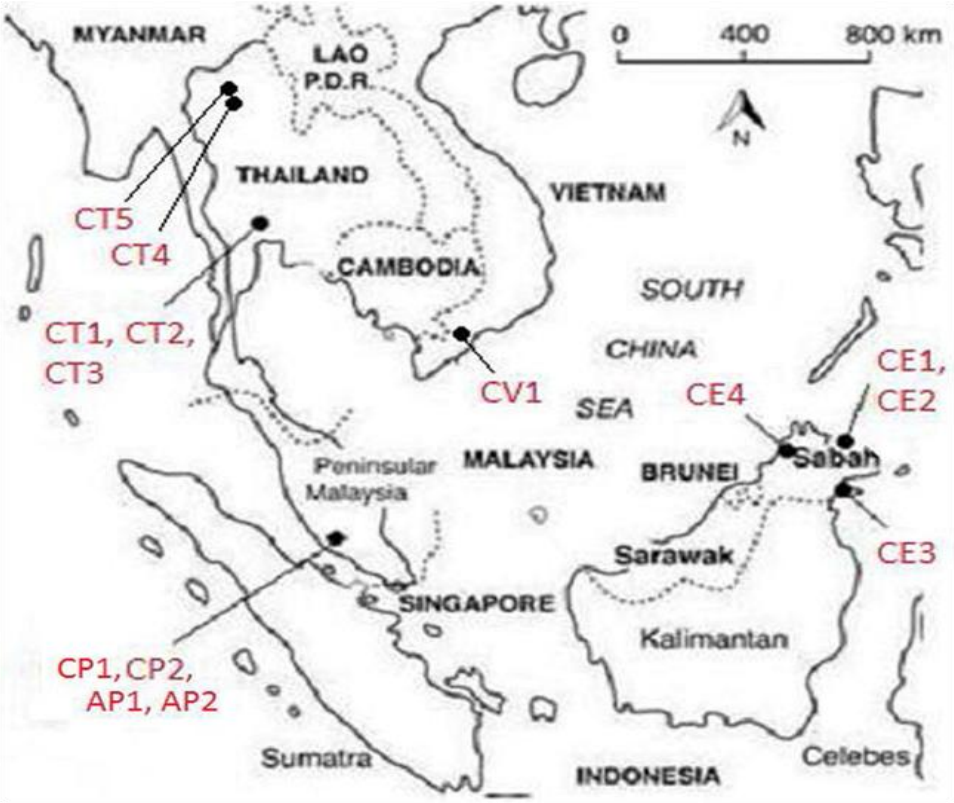

Figure 2. Sampling sites of *C. nutans* and *A. paniculata* in Malaysia, Thailand and Vietnam.

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

RESULTS

PCR-RFLP analysis

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

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

RAPD analysis

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

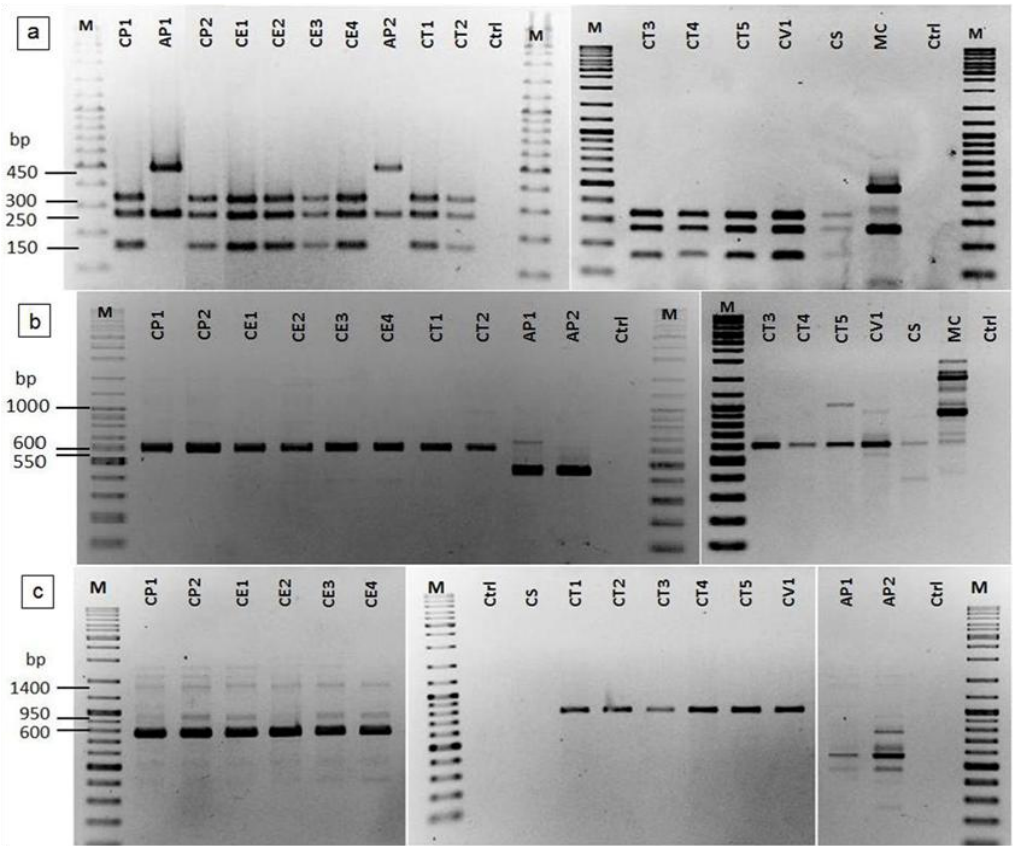

Figure 3. PCR-RFLP patterns of *Taq I* digested rbcL gene (a), RAPD amplification products of OPA-11 (b) and OPA-18 (c). Lane M=100 bp DNA ladder; lanes CP1, CP2, CE1, CE2, CE3, CE4, CT1, CT2, CT3, CT4, CT5, and CV1=*C. nutans*; lanes AP1, AP2=*A. paniculata;* lane CS=*C. siamensis*; lane MC=*M. cochinchinensis*;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

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

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

Microsatellite analysis

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

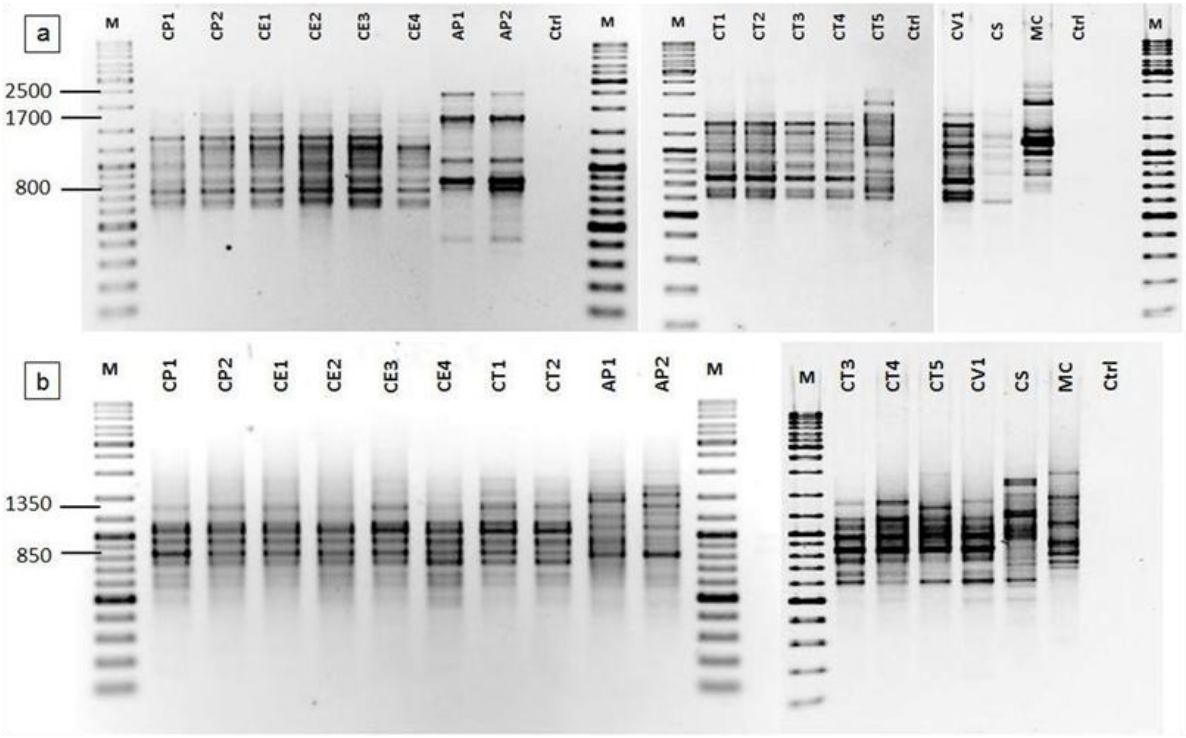

Figure 4. Microsatellite amplification products of GACA4. (a) and GTG5 (b). Lane M=100 bp DNA ladder; lanes CP1, CP2, CE1, CE2, CE3, CE4, CT1, CT2, CT3, CT4, CT5, and CV1=*C. nutans*; lanes AP1, AP2=*A. paniculata;* lane CS=*C. siamensis*; lane MC=*M. cochinchinensis*; lane Ctrl=no template control.

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

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

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

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

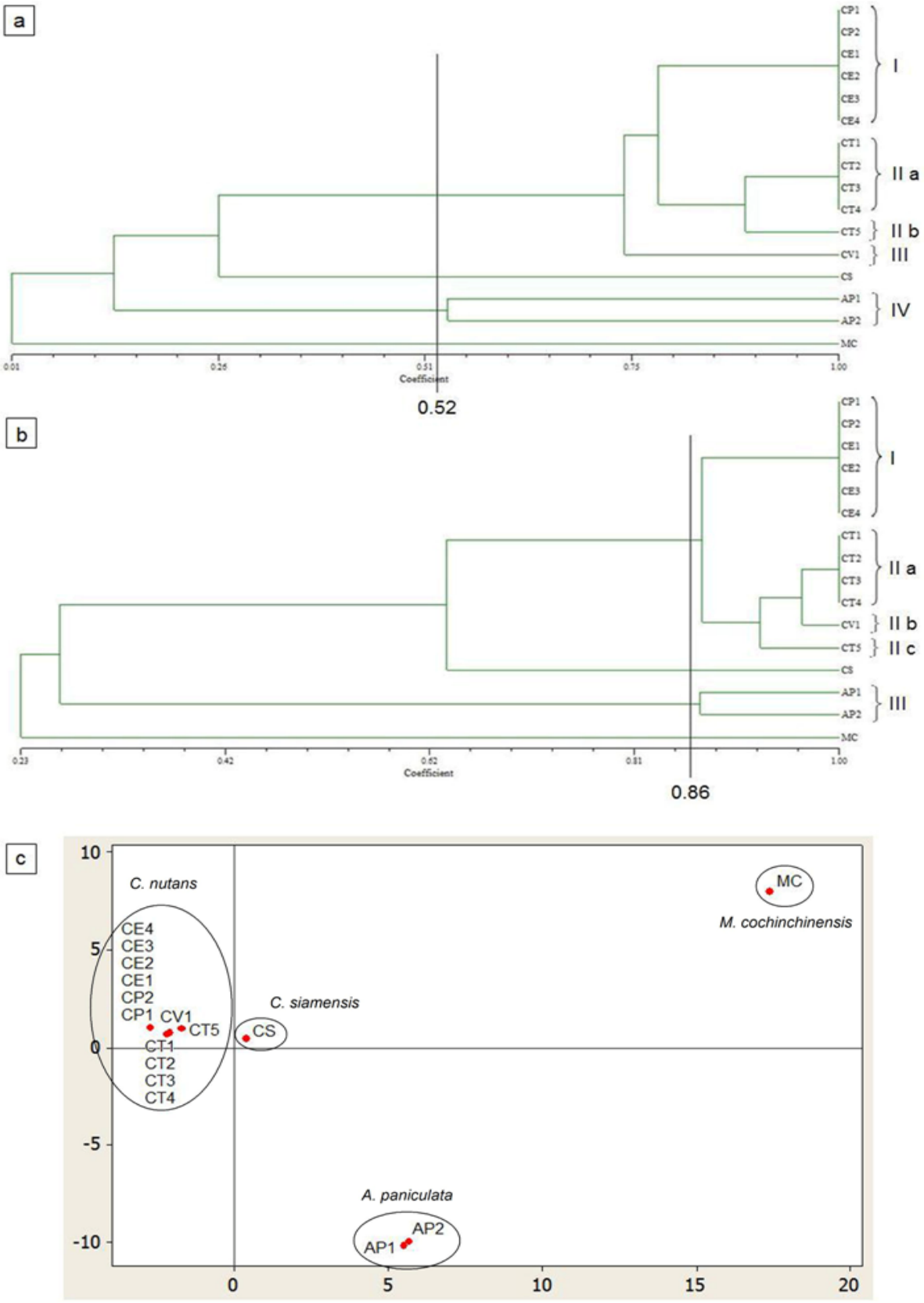

Figure 5. The relationships among 12 *C. nutans*, two *A. paniculata*, one *C. siamensis*, and one *M. cochinchinensis* samples according to UPGMA cluster analysis on RAPD (a) and microsatellite (b) data, and PCA analysis (c) on the combined RAPD and microsatellite data.

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

DISCUSSION

Genetic homogeneity in C. nutans

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

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

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

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

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

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

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

Genetic variation in A. paniculata

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

Conclusion

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

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Conflict of interests

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

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