ORIGINAL ARTICLE

An evaluation of the sexual system of *Garcinia atroviridis* L. (Clusiaceae), based on reproductive features

Sasithorn Pangsuban[1], Noparat Bamroongrugsa[2], Kamnoon Kanchanapoom[3] and Charassri Nualsri[4]

Abstract

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The sexual system of *Garcinia atroviridis* was evaluated regarding the basic structural specialization and reproductive characters under natural conditions. The species is gynodioecious with females (trees producing pistillate flowers), but hermaphrodites (trees producing perfect flowers) co-occurred in the study site. Significant morphological and anatomical variation was found between pure female and hermaphroditic flowers. Hermaphrodites have relatively long-filament flowers and produce abundant fertile pollen grains, whereas the females produce pollenless anthers. They also differ significantly in reproductive characters. Hermaphrodite flowers have more flowers per inflorescence than female flowers, but they gradually drop off before fruit setting. In contrast, female trees had relatively greater ovules per flower, larger fruits and more seeds per fruit than hermaphrodite trees. Moreover, average seed number from female trees was at least 1.7

[1]Ph.D. student in Biology, [2]Ph.D. (Crop Physiology) Assoc. Prof., [3]Ph.D. (Botany) Assoc. Prof., Department of Biology, Faculty of Science, [4]Ph.D. (Agronomy) Assoc. Prof., Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.
Corresponding e-mail: stmppbriy@yahoo.com
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times higher than that of the hermaphrodite trees. Interestingly, the fruit diameter of hermaphrodites was positively correlated to the number of seeds, whereas it was unrelated in females.

Key words : flower variation, sex-specific traits, reproductive characteristics, *Garcinia atroviridis*

บทคัดย่อ

ศศิธร พังสุบรรณ[1] นพรัตน์ บำรุงรักษ์[1] คำนูณ กาญจนภูมิ[1] และ จรัสศรี นวลศรี[2]

การประเมินระบบการสืบพันธุ์แบบอาศัยเพศของส้มแขก *Garcinia atroviridis* L. (วงศ์ Clusiaceae) โดยอาศัยพื้นฐานในการสืบพันธุ์

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การประเมินระบบการสืบพันธุ์แบบอาศัยเพศของส้มแขก (*Garcinia atroviridis*) โดยอาศัยพื้นฐานจากโครงสร้างจำเพาะต่อการสืบพันธุ์และคุณสมบัติในการสืบพันธุ์ตามธรรมชาติ พบว่า ระบบการสืบพันธุ์เป็นแบบ gynodioecious ซึ่งประกอบด้วย ต้นตัวเมียที่ผลิตดอกซึ่งเกสรตัวผู้ไม่สมบูรณ์อยู่ร่วมกับต้นกระเทยที่ผลิตดอกสมบูรณ์เพศในบริเวณที่ศึกษา โดยมีความผันแปรของโครงสร้างทางกายภาพและทางกายวิภาคระหว่างดอกตัวเมียและดอกกระเทยดังนี้ ดอกกระเทยมีก้านเกสรตัวผู้ยาว และอับเรณูสามารถผลิตละอองเรณูที่สมบูรณ์เป็นจำนวนมาก ในขณะที่ดอกตัวเมียผลิตอับเรณูซึ่งไม่มีละอองเรณู อีกทั้งคุณสมบัติบางประการในการสืบพันธุ์ยังมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ โดยดอกกระเทยมีจำนวนดอกต่อช่อมากกว่าดอกตัวเมีย แต่มักจะหลุดร่วงอย่างต่อเนื่องก่อนที่ดอกกระเทยจะเจริญเป็นผล ในขณะที่ดอกตัวเมียมีจำนวนออวุลต่อดอกมากกว่า ผลมีขนาดใหญ่กว่า และมีจำนวนเมล็ดต่อผลมากกว่าดอกกระเทย นอกจากนี้เมล็ดต่อผลโดยเฉลี่ยจากต้นตัวเมีย มีจำนวนมากกว่าเมล็ดต่อผลจากต้นกระเทยอย่างน้อยประมาณ 1.7 เท่า และเป็นที่น่าสนใจว่าเส้นผ่าศูนย์กลางผลจากต้นกระเทยมีความสัมพันธ์กับจำนวนเมล็ด ในขณะที่ไม่พบลักษณะดังกล่าวนี้ในต้นตัวเมีย

[1]ภาควิชาชีววิทยา คณะวิทยาศาสตร์ [2]ภาควิชาพืชศาสตร์ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Garcinia, with over 400 species, is the largest genus in Clusiaceae (Guttiferae) (Cox, 1976). The genus is distributed across the tropical regions of Asia, Africa and Polynesia (Ridley, 1922, Whitmore, 1973), and many species are important for commercial use, as timber, medicine, resin oil, latex (pigment), fodder, and edible fruits (Cox, 1976). *Garcinia atroviridis* Griff. ex T. Anders, a known fruit tree of this genus, is a slow-growing evergreen dioecious tree in which flowers and fruits are presented in the shady understory. It is recognized as an endemic species in Peninsular Malaysia (Whitmore, 1973; Mackeen *et al.*, 2002), where it grows in the wild throughout its lowland forest on the plains and up to 600 m at the mountains. This species is also extensively cultivated in the Thai-Malaysian peninsular up to northern Burma (Whitmore, 1973; Jansen, 1991).

G. atroviridis is widely used by ethno-botanists, and ethno-pharmacists as preservative, for seasoning, and for other medicinal purposes (Mackeen *et al.*, 2002). Dried fruit has been used for improvement of blood circulation, as an expectorant, for treatment of coughs, and as a laxative (Yapwattanaphun *et al.*, 2002). In addition, phytochemical study of the fruit rind has shown it to contain the commercial substance, (-)-hydroxy-citric acid (HCA), which has been shown to inhibit ATP dependent citrate lyase, a key enzyme in diverting carbohydrate to fatty acid, and for the synthesis of cholesterol (Lewis and Neelakantan, 1965). It has also been claimed to be efficacious in health promotion, such as reducing cholesterol in blood, widening blood vessels, and absorbing excess fat (Yapwattanaphun *et al.*, 2002). Accordingly, this species has potential for commercial development in the pharmacological industry. Nowaday, the Forest Research Institute Malaysia (FRIM) and the Thai Ministry of Public Health plan a project to improve the quality and increase the quantity of fruits to achieve a global standard of herbal products for the pharmacological industry (Chokevivat *et al.*, 2005). Recent researches on the pharmaceutical and phytochemical properties are widely reported (Kosin *et al.*, 1998; Mackeen *et al.*, 1997; 2000; Permana *et al.*, 2001; Mackeen *et al.*, 2002; Tisdale *et al.*, 2003; Preuss, 2004). However, there is little information on its horticultural potential, while the demand for its fruit is increasing (Jansen, 1991; Subhadrabandhu, 2001). In particular, knowledge of its sexual and breeding systems, important for designing suitable breeding strategies in order to increase fruit production, is limited.

Flowering plants in tropical rain forest are predominantly hermaphroditic, with most species producing flowers that contain both female (pistils) and male (stamens) sexual organs (Barrett, 2002). However, the incidence of dioecy is quite high (Bawa, 1980; Bawa *et al.*, 1985; Gross, 2005). Detailed records by the earlier researchers suggest that the genus *Garcinia* is also predominantly dioecious, such as *G. mangostana* and *G. scortechnii*, in which the staminate trees are hardly found (Corner, 1952; Jansen, 1991; Thomas, 1997). Several *Garcinia* species tend to display sex ratios that are more female-biased than other dioecious rainy forest trees, which used to be explained referring to the incidence of agamospermy (Richards, 1997; Ramage *et al.*, 2004). However, subdioecy has also been reported in other species, such as gynodioecious in *G. indica* (Rawat and Bhatnagar, 2005), and androdioecious in *G. cambogia* (George *et al.*, 1992). In the case of *G. atroviridis*, flowers contain both androecia and gynoecia structural parts, but the sexual system has been described by taxonomists as dioecious on the basic of differences in the two external features of the plants. The first features of staminate trees, the peduncles, are in short raceme or cyme, but are solitary in pistillate trees. The second features, the anthers of pistillate trees, seem rudimentary. As a result, the type of gender seems to be cryptically dioecious. This means, for instance, that flowers of the individuals retain nonfunctional organs such as gynoecia in functionally staminate flowers and androecia in functionally pistillate (Mayer and Charlesworth, 1991). However, there seems no benefit of the presence of staminate trees, as gardeners reported that stand-alone female trees produced a large number of fruits. Further observations also showed that staminate trees sometimes produce only a few fruits or no fruit-set. If so, this would mean that *G. atroviridis* is not exactly dioecious, but rather possesses a gender system intermediate between hermaphroditism and dioecious. Therefore, the objective of this research was to investigate the sexual system for better understanding of *G. atroviridis* and to answer the following questions: "What is the flower's gender in high fruiting and low or non-fruiting trees under field conditions?", and "Are there different reproductive characteristics among various trees of these gender morphs?"

Material and Methods

Plant material and experimental site

G. atroviridis trees in the forest in Yala and Songkhla provinces were sampled for this study. Individual trees were assigned to two gender classes based on their floral features and the number of fruit production (modified from Gibson and Diggle, 1997). Trees with intact anther, which produced few or no fruit, were designated low-fruiting trees (LF). Trees which had rudimentary anther and produced a satisfactory to high number of fruits (> 100 fruits/tree) were designated high-fruiting trees (HF).

In 2002, 4 HF and 2 LF trees from Songkhla province and 1 LF tree from Yala province were selected, based on accessibility and flowering performance. Several intact flowers and fruits were randomly sampled from various locations around the canopy in the fully flowering trees.

The sex-specific traits by flower morphology

Flowers were examined in situ and in the laboratory, and the general features and anatomical characteristics of flowers were investigated. The morphological differences of HF and LF anthers were obtained from fixed material in FAAII fixative (70% Ethanol 90 ml, Glacial acetic acid 5 ml, Formalin (37% formaldehyde) 5 ml) and stored in 70% alcohol. Fixed specimens were dehydrated through a graded ethanol-xylene series, infiltrated and embedded in paraffin, and sectioned at 10 - 12 micron thickness. These were then pre-stained with safranin and counterstained with fast green or Delafield hematoxylin. The sections were permanently mouted with Permount (Johanson, 1940). Details of the anther anatomy were examined visually under a light and stereo microscope. Dehydrated pollen grains were removed from the anthers and directly mounted on stubs, coated with gold and observed using scanning electron microscopy (SEM). The pollen grains were tested for pollen viability status by using fluorescein diacetate test (Shivanna and Rangaswamy, 1992). They were randomly selected from 10 anthesis flowers per tree. The whole anthers of each tree were crushed in the Petri dishes and then pollen samples were obtained. For fluorescein diacetate test, stock solution of 2 mg fluorescein diacetate (FDA) in 100 ml acetone, and 10% sucrose in distilled water were prepared. FDA was added dropwise to the sucrose solution until milkiness appeared. The fresh solution was applied to the pollen samples, covered with a slip, and allowed to incubate for 10-15 minutes on a moist filter paper in a Petri dish before examination by fluorescence microscopy. The fluorescein-positive cells were considered metabolically alive because they were able to hydrolyze FDA to release fluorescent fluorescein into the cytoplasm through intracellular esterase. These analyses were separated into 3 replications. Pollen viability was calculated by dividing the number of viable pollen grains by the total number of grains counted in the field of view and expressed as percentage.

Some reproductive efforts

The empirical data of the reproductive characters between two gender classes were determined by the flower number per inflorescence, number of ovules per flower, fruit size and seeds per fruit from open-pollination (modified from Connor, 1990; Heenen, 2000). Fruit, throughout the paper, refers to mature fruit which changes from green to orange color at maturity. Seeds with aborted or no embryos could easily be differentiated, since they appeared small and flattened, while seeds with normal embryos appeared cylindrical. Only cylindrical seeds were counted when calculating average seeds per fruit. The percentage of average seeds per fruit was determined by counting the number of cylindrical seeds per fruit divided by the number of total ovules per flower (modified from Fernando and Cass, 1997). However, one LF tree in Songkhla province did not set fruits, hence, only 2 LF trees were examined. Voucher specimen "sThongma01" (for hermaphrodite tree) and "sThongma02" (for pistillate tree) were deposited at the PSU herbarium.

Data analysis

The mean and its standard error were calculated for all measurements. Pollen viability percentage was analyzed using one-way ANOVA. Nonparametric analyses were performed because the data were abnormally distributed and/or variances and were unequal after being transformed. Hence, a nonparametric Mann-Whitney U test was applied to compare the distributions of values of pistillate and hermaphrodites for each reproductive character (Samuels and Witmer, 2003). The relationships between the number of seeds and the fruit size characteristics were tested with the Pearson correlation. Linear regression was also conducted on normally distributed untransformed data (Samuels and Witmer, 2003). All statistical analyses were performed with SPSS v. 10.

Results

There is scant information on the density of *G. atroviridis*. However, available information suggests that wild *G. atroviridis* densities in Thailand are the highest in the southernmost region (Craib, 1931; Subhadrabandhu, 2001). Annual rainfall (in this region) was ranged from 0.5 mm in February to 500 mm in October. The average temperature varied from 25.7°C to 28.8°C and relative humidity varied from 96% to 98% (Pattani Meteorological Station, 2002). *G. atroviridis* trees bear fruits annually, as flowering and fruiting occur once a year. No sex change was observed in any of the trees. This indicates that it had a strong genetic constraint for sex expression under natural condition. The flower buds formed in February and bloomed two months later.

The sex-specific traits by flower morphology

The anthesis flowers from both gender classes were similar in actinomorphism and structurally perfect. Both were conspicuously devoid of odour, and had no clear separation between style and ovary. In addition, their discoidal stigmas were entire and covered by a little amount of glutinous nectar. HF and LF flowers had the same numbers of sepals (4) and persistent petals (4-5). Outer sepals were oblong or orbicular and showed a bright crimson color, while inner ones were larger and thin-edged. Petals were larger and orbicular or obovate (Figure 1A). Flowers from both gender classes contained common gynoecia, and there were no abnormalities that suggested female sterility. The superior ovary comprised a single compound carpel, each containing one axile ovule per locule (Figure 1B). However, the HF and LF flowers could be distinguished clearly by three marked differences of floral traits, such as the inflorescences, the length of stamen, and the dehiscence of anthers.

Firstly, HF flowers occurred single or in clusters of up to 4 flowers at a twig-end or, rarely, lateral. It would be better to say that HF trees had

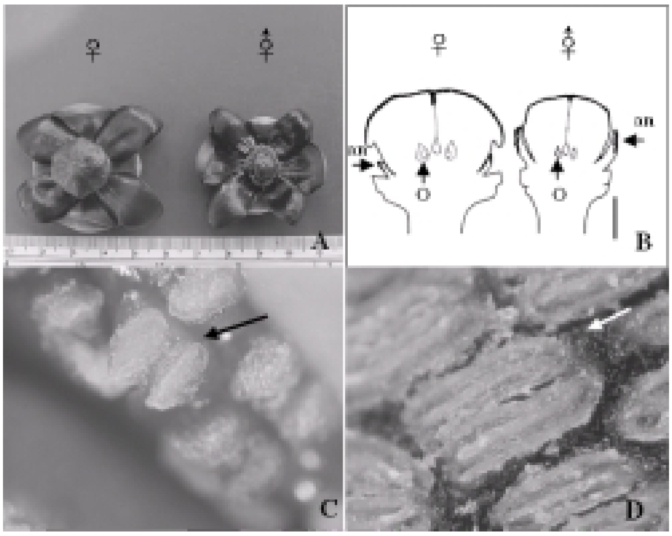

Figure 1. Characteristics of *G. atroviridis* flower: (A) External features of pistillate (♀) and hermaphrodite flower (⚥) with their stigmas covered by glistening exudates. (B) Longitudinal section of receptive flower showing two floral morphs that are ovary filled with healthy-looking ovules (o), but differ in the anther (an) position between pistillate and hermaphrodite morphs. (Bar = 0.5 cm) (C) Anther of the anthesis pistillate flower (arrow) (x 20). (D) The dehiscence anther of the hermaphrodite flower (arrow) (x 20).

usually unbranched inflorescences whereas LF flowers were entirely in short compound cymes (c. 4 cm long), with the central bud opening first, followed by the ones at the two sides. Moreover, all LF inflorescences occurred only at the terminal branches.

Secondly, as can be seen in Figure 1A, the anthers of both gender classes were manifestly numerous and adhered on the fused filaments as a ring around the stigma. However, the level of the distinctive stamens in HF flowers was shorter than LF flowers (Figure 1B). In particular, HF anthers were in general smaller (Figure 1C). Lastly, the LF anther usually shed the dry yellowish pollen through longitudinal slits as soon as the petal discharged (Figure 1D). Due to the sticky exudate, which secreted synchronously from their own stigma, they usually adhered to the sporangial wall

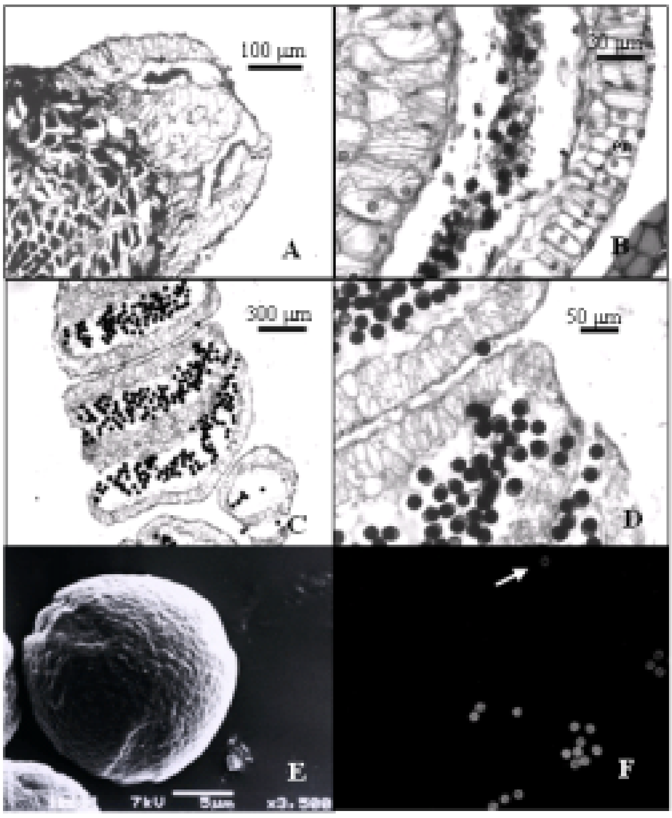

Figure 2. Light micrographs of androecium of *G. atroviridis* female and hermaphrodite flowers: (A) Longitudinal section (L.S.) of female anther stained with safranin showing the mass of collapsed tissue. (B) L.S. of female anther stained with fast green showing endothecium (en) with bars of fibrous thickening, tapetal cells disappeared. The collapsed tissue stained with Delafield hematoxylin shows the residual DNA of degenerated meiocytes. (C) Transverse section (T.S.) of hermaphrodite anther stained with fast green showing bilobed anther and pollen grains. (D) T.S. of hermaphrodite anther stained with Delafield hematoxylin showing prominent nucleus of pollen grains (E) Scanning electron micrograph of pollen grains. (F) Fluorescence micrograph of pollen grains stained with fluorescein diacetate showing many fluorescing grains and non-fluorescing pollen grain (arrow) (x 40).

until pollen vectors effected their dispersal (Figure 1D). In contrast, it was found that dehiscence of the HF anther had not taken place (Figure 1C). From this result, it was inferred that the male sterility could be expressed in the anther, disrupting male function. The internal features of mature anthers from both gender classes were, therefore, affirmed on the basic of histological analysis.

The anatomical characters showed that the mature anthers were bisporangiate. The sub-epidermal cell layer was the endothecium, which developed fibrous thickening, and was stained only with fast-green. These cells reach maximum development after having elongated radially when pollen grains mature. A mass of collapsed tissue, which was stained red with safranin, was usually observed in HF anther (Figure 2A). The result suggests that the structurally hermaphrodite flower displayed the male sterility. This collapsed tissue stained violet with Delafield hematoxylin indicating the residual DNA of the degenerated meiocytes (Figure 2B). It was inferred that the pollen grain probably failed before microspore formation. Hence, the HF flower appeared to be morphologically strictly female and thus undoubtedly lacked self-pollination/fertilization by their pollen. In contrast, most Delafield-hemato-xylin-stained pollen grains were usually found in the locules of LF anthers (Figure 2C, 2D). These tricopolrate pollen grains were small, 20-25 μm in diameter, with a scabrate pattern of exine (Figure 2E). Overall, the HF trees produced the female morph with pistillate flowers, whereas, the LF trees produced the hermaphrodite morph with perfect flowers. Estimation of pollen viability within the LF individuals on the basic of fluorescein diacetate test yielded results shown in Table 1. Based on the criterion that viable pollen grains are fluorescent yellow, which indicating enzyme activity, while a dull brown color indicates non-viable pollen (Figure 2F), LF flowers of *G. atroviridis* were found to have a pollen viability ranging from 90.3% to 92.5%. No significant difference was found within individuals at these study sites.

Some reproductive efforts

The high pollen viability indicates that the gender of *G. atroviridis* significantly affects certain reproductive efforts. The differences between females and hermaphrodites are reported in Table 2. Pistillates had significantly more of ovules per flower, a larger fruit size and more seeds per fruit than hermaphrodites. Hermaphrodites produced greater flowers per inflorescence (range = 4-22) than the pistillates (range = 1-4), however, the majority of hermaphrodite flowers gradually dropped off before initial fruit setting (observed by the unswollen ovary). The result suggests that strong abortion of hermaphrodite flowers seems to occur. Consequently, hermaphrodites usually bear 0, 1, or 2 fruits per inflorescence and thus some of these trees do not bear fruit, whereas pistillates often bear fruits on all inflorescences. Both pistillates and hermaphrodites reproduced many ovules per flower, but not all develop into mature seeds (25% for females and 14% for hermaphrodites). Thus, female fruit contained more viable seeds than hermaphrodite fruit. The average seed number of pistillates was at least 1.7 times higher than hermaphrodites.

In particular, the presence of seedless fruits demonstrated that parthenocarpy occurred in both gender classes, with percentages of 39.6% for hermaphrodites and 18.8% for pistillates (Figure 3). As a result, the majority of hermaphrodite flowers bear twice parthenocarpic fruits than pistillates. There is a positive correlation between

Table 1. Percentage of potential viable pollen grains from hermaphroditic individuals under natural condition of *G. atroviridis.*

Tree No.	Source	Viability (%)
1	Songkhla	92.36
2	Songkhla	90.30
3	Yala	92.54
F-test	-	ns
C.V. (%)	-	4.83

ns = not significantly different at 95% level

Table 2. Comparisons of the intraspecific variation of *G. atroviridis* in four aspects character between pistillates and hermaphrodites under natural conditions.

Character	Female			Hermaphrodite			P-value
	n	mean±SE	range	n	mean±SE	range	
Flowers per inflorescence	65	1.43±0.08	1-4	40	13.10±0.70	4-22	0.000
Ovules per flower	50	13.36±0.17	10-16	30	9.87±0.17	9-12	0.000
Seeds per fruit	30	3.37±0.58	0-11	30	1.37±0.26	0-5	0.009
Fruit diameter (mm)	30	82.07±1.52	59.15-90.55	30	55.46±1.97	40-73.30	0.000

n = sample size. Mean ± SE and results of Mann-Whitney U test are presented.

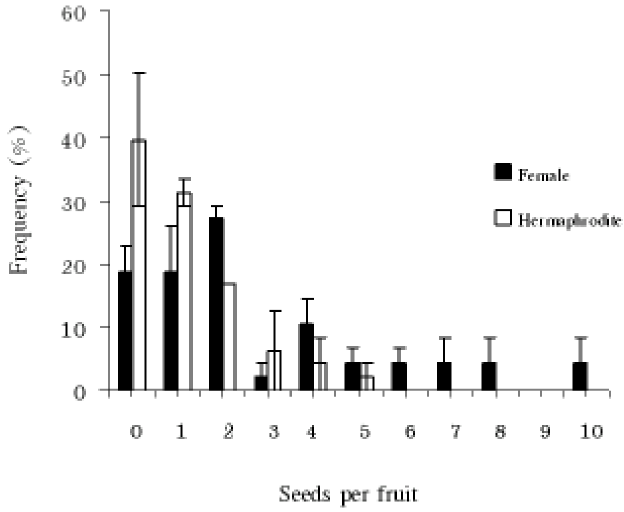

Figure 3. Frequency distribution of the difference in seeds per fruit ratio between female (n = 44) and hermaphroditic (n = 30) fruits under natural condition of *G. atroviridis*. Vertical lines represent standard error (SE).

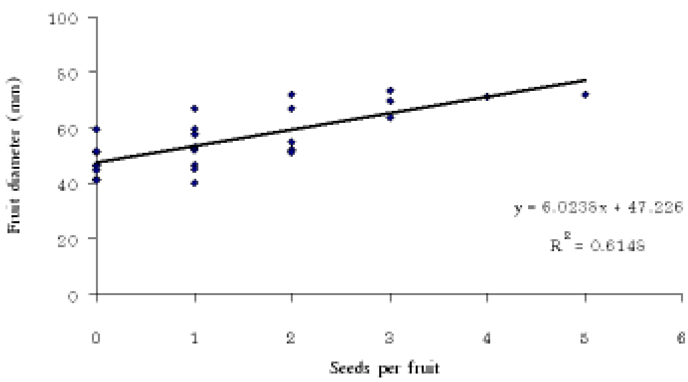

Figure 4. Linear regression of seeds per fruit and fruit diameters (n = 30) of the hermaphrodites. Significantly different at 99% level.

the number of seed and fruit size in hermaphrodites at the 99% confidence interval (Pearson correlation (r) = 0.68; n = 30; P<0.00), while there was no significant correlation in females. (r = -0.07; n = 30; P = 0.711). One can thus say that the fruit size of hermaphrodites increases significantly with the number of seeds (P<0.010), indicating that smaller fruits produce proportionately less seed than the larger ones (Figure 4).

Discussion

This paper reports the first comprehensive study of the sex system of *G. atroviridis*. Individual trees bear either clusters of bisexual or solitary female flowers, in which outcrossing is dominant. Flowers of female trees exhibit sporogenous-type male sterility as stamen form but pollen grain are absent. It seems that the microsporogenous cells degenerate during meiotic division (Kaul, 1988). As a result, dehiscence of the nonfunctional anthers does not take place. These observations are similar to those reported with respect to *G. mangostana* (Lim, 1984) and *G. parvifolia* (Ha *et al.*, 1988). The retention of these nonfunctional organs may be due to not having enough evolution time for their suppression, or due to genetic correlations between androecia and gynoecia, which delayed the suppression of one or the other in functionally unisexual flowers. Another possibility is that these organs are important in the attraction of pollinators to the less rewarding (pollen-lacking) female flowers (Mayer and Charlesworth, 1991).

In the case of hermaphrodites, pollen grains are small and adhere with their own anther wall by the sticky exudate. On this basis, pollen grains would not get wasted by abiotic pollinators such as wind dispersal or gravity (Sedgley and Griffin, 1989). Thus, when flower visitors contact with stigmas and anthers, the stigmatic exudates can support them to attach to the body of the biotic pollinator. This corresponds with the prevailing notion that most *Garcinia* species pollinated by biotic pollinators such as social bees, diverse insects and *Apis* spp. (Richards, 1997; Momose *et al.*, 1998). Therefore, it seems that flowers have conformed to the biotic pollination for fruit setting.

Although pollen grains exhibit high viability, not all of the hermaphrodite flowers set seed because the parthenocarpic fruits appeared. There are two possible factors that might be responsible for this event. The first factor might be due to a partial self-incompatibility effect, which avoids the deleterious effects of inbreeding depression from selfing and promotes heterozygosity, genetic variability and genetic exchange. This would generally be advantageous for the long-term survival and adaptation of the species, because it would cause effective abortion of unfertilized flowers. The second factor might involve the gradual reduction in female fertility through a progression from gynodioecious to full functional dioecy. This is supported by a comparative study which supports the view that dioecy has evolved from an ancestral gynodioecious condition (Dellaporta and Calderom-Urrea, 1993).

In general, females in gynodioecious of several species are reported to produce more seeds than hermaphrodites (Williams *et al.*, 2000; Delph and Mutikainen, 2003; Ramula and Mutikainen, 2003; Chang, 2006). Results from this study also showed that female plants of *G. atroviridis* produce more seeds per fruit than hermaphrodites. A possible reason for this is the fitness of hermaphrodites which is acquired half through seed production and half through gene transmission by pollen. Lacking pollen, females must therefore produce more than twice as many seeds as bisexual plants to achieve higher fitness. Consequently, females also bear larger fruits than hermaphrodites, and seeds are sites where phytohormones are synthesized, so its endosperm in particular contains high levels of plant-growth regulating chemicals, such as auxin, gibberllins and cytokinin (O' Neill and Robert, 2002). As a result, fruit growth, shape and size usually are modified by differences in the number of seeds (Stephenson *et al.*, 1988; Sedgley and Griffin, 1989) and seed genotype (Denny, 1992). Interesting, the fruit diameter of hermaphrodites is also positively correlated with the number of seeds, but it is unrelated in females. As little is known from the literature about this effect, this aspect should be studied further.

Conclusion

In conclusion, the present results suggest that *G. atroviridis* is gynodioecoius in which female and hermaphrodite individuals co-occur. For this reason, cross-pollination should be attempted as primary means for its reproduction.

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