American Journal of Botany

American Journal of Botany 97(7): 1220–1228. 2010.

Flower heating following anthesis and the evolution of gall midge pollination in Schisandraceae[1]

Shi-Xiao Luo[2,3], Shu-Miaw Chaw[4], Dianxiang Zhang[2,5], and Susanne S. Renner[3]

[2]Key Laboratory of Plant Resource Conservation and Sustainable Utilization, South China Botanical Garden, The Chinese Academy of Sciences, Guangzhou 510650, China; [3]Department of Biology, University of Munich, D-80638 Munich, Germany; and [4]Biodiversity Research Center, Academia Sinica, Taipei 115, Taiwan

- *Premise of the study*: Flower heating is known from a few species in 11 of the c. 450 families of flowering plants. Flowers in these families produce heat metabolically and are adapted to beetles or flies as pollinators. Here, we focus on the Schisandraceae, an American/Asian plant family known to exhibit flower heating in some species, but not others, raising the question of the adaptive function of heat production.
- *Methods*: We used field observations, experiments, and ancestral trait reconstruction on a molecular phylogeny for Schisandraceae that includes the investigated species.
- *Key results*: At least two Chinese species of *Illicium* are exclusively pollinated by gall midges that use the flowers as brood sites (not for pollen feeding). Continuous monitoring of flower temperatures revealed that the highest temperatures were attained after the flowers' sexual functions were over, and experiments showed that post-anthetic warming benefited larval development, not fruit development. Midge larvae in flowers with trimmed tepals (and hence a lower temperature) died, but fruit set ratios remained unchanged. Based on the DNA phylogeny, gall midge pollination evolved from general fly/beetle pollination several times in Schisandraceae, with some species adapted to flower-breeding midges, others to pollen-feeding midges.
- *Conclusions*: Flower heating may be an ancestral trait in Schisandraceae that became co-opted in species pollinated by flower-breeding midges requiring long-persistent warm chambers for larval development.

Key words: ancestral state reconstruction; brood chamber; gall midges; pollination; post-anthetic flower heating; Schisandraceae; thermogenesis.

Flower heating (variously referred to as endothermy, thermogeny, thermogenesis, or thermogenicity) is known from 11 of the c. 450 families of flowering plants (Yuan et al., 2008; Seymour et al., 2009; APG III, 2009). Flowers in these families produce heat metabolically and are adapted to beetles or flies as pollinators (Thien et al., 2009; Endress, 2010). Experiments support that heat can be a direct energy reward for ectothermic pollinators (e.g., Seymour et al., 2003), increase the volatilization of chemicals directed at pollinators (Seymour and Schultze-Motel, 1998; Seymour et al., 2009), help mimic mammalian feces or carrion in saprophilic flowers (e.g., Yafuso, 1993; Seymour et al., 2003; Angioy et al., 2004), and enhance the respiratory release of CO_2, which, in combination with other volatile chemicals, may stimulate fly oviposition (Patiño et al., 2000, 2002). In the North American *Illicium floridanum* (now in Schisandraceae; APG III, 2009), which is primarily pollinated by nectar-foraging flies, flower heating may aid pollen tube growth or seed development (Thien et al., 2009). When temperatures in this species were recorded over 24 h, that is, during the flower's female phase and portions of its male phase, highest overall temperatures occurred in the pedicels of male-phase flowers. Thien et al. (2009, p. 174) therefore suggested that "Thermogenesis, however, in *I. floridanum* does not cease with fertilization, but continues during fruit (seed) development. During development of the fruit, the pedicel produces temperatures 8° C above ambient temperature as do the young fruits (Fig. 4; L. Thien, personal observation)."

Here we report the first data on thermogenesis and pollination in any Asian *Illicium*, focusing specifically on the precise pattern of heat production. We also report on experiments addressing the adaptive significance of flower heating and place our observations in an evolutionary context, using a molecular phylogeny for Schisandraceae. This family comprises *Illicium*, *Kadsura*, and *Schisandra*, with 90 species altogether (APG III, 2009). *Illicium*, with 42 species, occurs in Southeast Asia and the southeastern United States, Mexico, and the Greater Antilles; *Schisandra*, with 25 species, occurs in tropical Asia, but also has one species, *S. glabra*, in the southeastern United States and Mexico; *Kadsura*, with 22 species, is endemic in tropical Asia (Saunders, 1998, 2000). Schisandraceae thus have their center of diversity in Southeast Asia. The reproductive biology of five of the 90 species has previously been studied. The New World *Illicium floridanum, I. parviflorum*, and *Schisandra glabra* are pollinated predominantly by flies, with beetles as copollinators (Thien et al., 1983; White and Thien, 1985; Dieringer et al., 1999; Liu et al., 2006), while the Asian *Schisandra henryi* and *Kadsura longipedunculata* are exclusively pollinated

[1] Manuscript received 23 February 2010; revision accepted 23 April 2010.

The authors thank G. Yao and N.-Cai Pei for help in the field, R. Gagné (Systematic Entomology Laboratory, U.S. Department of Agriculture) for identifying the gall midges, and P. Endress and reviewers J. Ollerton and L. Thien for helpful comments on the manuscript. Financial support came from the National Science Foundation of China (grant no. 30700044), the Natural Science Foundation of Guangdong Province (grant no. 7301182), the Knowledge Innovation Program of the Chinese Academy of Sciences (grant no. SCBG-200734), and the Biodiversity Research Center, Academia Sinica to S.M.C.

[5] Author for correspondence (e-mail: dx-zhang@scbg.ac.cn)

doi:10.3732/ajb.1000077

by pollen-eating *Megommata* gall midges (Cecidomyiidae; Yuan et al., 2007, 2008). Indeed, pollen consumption in gall midges was first discovered in these two species.

The center of Schisandraceae species diversity is China, which harbors 54 species representing all three genera (Xia and Saunders, 2009; Xia et al., 2009). None of the Chinese *Illicium* species had been studied in terms of its pollination biology, and we therefore selected two species from this genus, *I. dunnianum* and *I. tsangii*. Questions we wanted to answer were: (1) Given that some Schisandraceae exhibit flower heating (Dieringer et al., 1999; Liu et al., 2006; Yuan et al., 2008; Thien et al., 2009), do Asian *Illicium* species also possess this trait? (2) Does any flower heating continue after a flower's sexual function is over, and if so, what is the adaptive significance of postanthetic flower heating? (3) Are Asian *Illicium* species pollinated by gall midges, or do they show "generalized" fly and/or beetle pollination similar to New World *Illicium*? And (4) are flower heating and midge pollination functionally correlated?

MATERIALS AND METHODS

Study species and sites—*Illicium dunnianum* Tutch. is a small shrub (Fig. 1A), 0.5–2 m high, that occurs in Guangdong, Guangxi, Hunan, Guizhou, and Fujian (Xia and Saunders, 2009). Its habitats are riverbanks in wooded ravines at elevations between 300–750 m a.s.l. Each flower has 19–31 oblong stamens with fleshy filaments and eight subulate styles. Observations were made from mid March to late April in 2008 and 2009 on 38 and 8 individuals at two sites near Shiheqiguan (in Nankunshan National Forest Park, Guangdong Province), about 1 km apart (c. 113°53′E, 23°38′N, ~350 m a.s.l.). A third site was located about 2 km north (408 m a.s.l.) and contained another five individuals. A voucher specimen, Luo 447, has been deposited in the herbarium IBSC. *Illicium tsangii* A. C. Smith is a shrub or small tree up to 10 m (Fig. 1Q). Its habitats are mixed forests or thickets between 500–800 m a.s.l. Flowers are similar to those of *I. dunnianum* but have only 7–10 stamens. Observations were made from early April to late May in 2008 and 2009 on 56 individuals along a 3-km stretch of road through Nunkunshan park (voucher: Luo 448, IBSC).

Floral development, function, and temperatures—Flower development in *I. dunnianum* was monitored in 20 flowers on 10 plants selected at random. Flowers were observed with a 5× hand lens for the following traits: relative position and color of tepals, stamens, and styles; presence or absence of a secretion; and the timing of style movements, anther dehiscence, and floral organ wilting and abscission. The onset of stigmatic receptivity and its duration were assessed with 3-4,5- dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT; Sigma, St. Louis, Missouri, USA) as recommended by Rodriguez-Riano and Dafni (2000). The onset of the male phase was readily apparent from the release of pollen. The thickness of the filaments (measured at the filament mid point) and the presence of secretions were recorded in open-pollinated flowers, in experimentally cross-pollinated flowers, and in flowers that had been bagged prior to anthesis. Sample sizes are given in the Results.

In 2008, ambient temperatures and floral temperatures in open-pollinated flowers were recorded for a total of 50 h with a TR-71U thermo recorder (T&D Corp., Matsumoto, Japan), accurate to ±0.1°C. Readings were taken every 5 s, from 1900–0500 hours or from 1930–0630 hours. In male-phase flowers and nursing-phase flowers, one of the two temperature sensors was inserted between the inner filaments and the carpel; the second sensor was placed in the air, about 1 cm above the flower. In female-phase flowers, one sensor was inserted amid the carpels, the other in the air as described.

One-way ANOVA *F*-tests and *t*-tests were carried out with the statistical package SPSS. The *G*-test was carried out in Microsoft (Redmond, Washington, USA) Excel using the Poptools 3.0 statistical package add-in (http://www.cse.csiro.au/poptools). Measurements are reported with means and standard errors throughout.

Plant mating system—Controlled pollinations were carried out at peak flowering, using the following four treatments on freshly opened (unvisited) flowers: (1) randomly selected flowers were marked as controls; (2) flowers were pollinated with pollen from a male-phase flower of the same individual and then enclosed in polyethylene bags, (3) flowers were bagged to test for agamospermy (strong protogyny precluded autonomous self-pollination), (4) flowers were cross-pollinated with pollen from another individual and then bagged. Emasculation of flowers was not possible because emasculated flowers invariably withered and abscised. We counted pollen grains and ovules in 10 flowers from 10 individuals to calculate pollen to ovule (P/O) ratios (Cruden, 1977).

Visitors and pollinators—Diurnal and nocturnal observation of flower visitors were made over 150 h, covering the entire flowering period, from tepal spreading to the end of the staminate phase. Day observations on *I. dunnianum* were made on 18–21 March and 1, 13, 17, and 22 April 2008 and on 29 March and 15 and 27 April 2009. Day observations on *I. tsangii* were made on 2, 3, 18, and 24 April and 6, 15, and 24 May 2008 and 10–11 April 2009. Night observations on *I. dunnianum* were made on 18–25 March, 1–3, 13–19 and 20–23 April 2008 and on 27–31 March and 12–16 and 20–24 April 2009. Night observations on *I. tsangii* were made on 2–5 and 18–24 April and 4–7, 24–16, and 22–24 May 2008 and on 10–13 April 2009. Kinds and numbers of visitors, duration of visits, and insect behavior were recorded. To investigate the flowers' functional phases, we (1) monitored visitor behavior inside the flowers (with the tepals trimmed to expose stigmas and stamens), (2) bagged flowers at the end of the female phase ("interim phase-bagged"), (3) bagged flowers at the end of the male phase ("male phase-bagged"), (4) trimmed the tepal tips in male-phase flowers, and (5) trimmed the tepal tips at the beginning of the nursing phase. Gall midge larvae in the treated flowers as well as in controls were counted immediately and/or 2–3 d after the manipulation. Pollen grains on gall midges and on stigmas were studied with a stereoscope at high magnification, and randomly collected flowers were checked for midge eggs, midge larvae, and pollen grains on stigmas. Insects collected for identification were preserved in alcohol, and voucher specimens are now deposited in the collection of R. Gagné, Systematic Entomology Laboratory, Agricultural Research Service–U. S. Department of Agriculture.

Molecular phylogenetics and ancestral trait reconstruction—To infer the distribution of gall midge pollination in Schisandraceae, we sequenced the complete internal transcribed spacer of ribosomal DNA (ITS1-5.8S-ITS2) and part of the chloroplast *trnL* region, using the methods described in Morris et al. (2007). Sequences of the study species were added to those of Hao et al. (2000, 2001), Liu et al. (2006), and Morris et al. (2007). Accepted species names are those of Xia et al. (2009), and newly generated sequences were submitted to GenBank (accessions GU354242, GU354243, GU354244, and GU354245). The sister group of Schisandraceae is *Trimenia*, for which no ITS and *trnL* sequences are available. We therefore coded the *Trimenia* DNA sequences as "nnnn" except for the first 88 nucleotides of *trnL*, which we copied from *Illicium angustisepalum*; this had the desired effect of pulling *Trimenia* to Schisandraceae

→

Fig. 1. Flowers and pollinators of (A–P) *Illicium dunnianum* and (Q–S) *I. tsangii* in South China. (A) Branches of *I. dunnianum* with flowers. (B) The different states of styles and anthers at four flower phases: 1, bud; 2, female phase; 3, male phase; and 4, nursing phase. (C) Stamens in female-phase flowers (left), male-phase flowers (middle), and nursing-phase flowers (right). (D–F) *Clinodiplosis* gall midge visiting a virgin flower. (G) The same flower with a *Clinodiplosis* egg amid the carpels (white arrow), pollen grains on the stigmas (black arrow) and still closed anthers. (H) A *Clinodiplosis* visiting a female-phase flower on which the tepal tips have been trimmed. (I) *Clinodiplosis* and a predacious gall midge visiting a male-phase flower (with tepals trimmed). (J) An ovipositing *Clinodiplosis*. (K) Eggs of *Clinodiplosis* on stamens and carpels (arrows). Dorsal views of (L) female-phase and (M) male-phase flowers, with eggs of *Clinodiplosis* in the carpel chamber (arrow). (N) A nursing-phase flower with larva of *Clinodiplosis* in the carpel chamber (arrows). (O, P) Larvae of *Clinodiplosis* feeding on the filament secretion. (Q) Branches of *I. tsangii* with flowers. (R) Visited flower with pollen grains on the stigmas (black arrow) and the indehiscent anthers. (S) The same flower with eggs of *Clinodiplosis* on the carpels (arrows). Scale bars: C–K, S = 3 mm long; L–P: 2 mm; Q = 0.1 mm; S = 1 mm.


A
B
1
2
3
4
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S

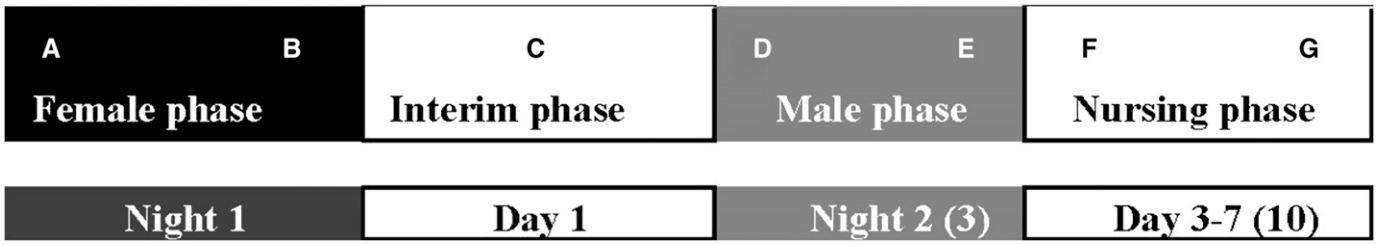

Fig. 2. Flower functional phases in *Illicium dunnianum* and *I. tsangii*. (A) Initiation of stigmatic receptivity. (A)~(B) Tepal spreading creates a small orifice at the top of the floral egg-laying chamber. (B) Cessation of stigma receptivity and 90° movement of styles into an upright position to form a carpel chamber in which the gall midge eggs hatching and larvae develop. (C) Further expansion of flower tepals. (D) Onset of anther dehiscence. (E) Cessation of male function. (E)~(F) Closing of inner tepals and forming a larval nursing chamber; production of a secretion by filament surfaces and inner tepal bases. (G) Abscission of stamens and tepals.

without affecting relationships in the ingroup. The final data set comprised 43 species and 2197 aligned nucleotides. Maximum likelihood (ML) analysis under the GTR + G model of substitution were performed using the program RAxML (Stamatakis et al., 2008). Bootstrap support values were estimated in RAxML with 100 replicate heuristic searches under the same model as used in the tree searches.

We used maximum likelihood as implemented in the program Mesquite 2.7.2 (Maddison and Maddison, 2009) to infer ancestral states, using the Markov *k*-state one-parameter model (Lewis, 2001), which assumes a single rate for all character state transitions. The input phylogeny was the ML tree for the Schisandraceae with branch lengths (the outgroups *Trimenia* and *Austrobaileya* were assigned identical branch lengths), and the traits "pollination mode" and "flower heating" were coded with the following states: (0) exclusive pollination by pollen-feeding midges (*Megommata)* or flower-breeding midges (*Clinodiplosis*); (1) copollination by diverse insects, mostly flies, but also beetles and bees; and (2) unknown; (0) temperature increase absent, (1) temperature increase present, and (2) unknown. *Trimenia* was coded as copollinated by flies and bees (Bernhardt et al., 2003) and *Austrobaileya* as copollinated by flies and beetles (Endress, 2001; Thien et al., 2009). Flower temperatures in neither genus have been investigated. Pagel's (1994) character correlation test was not used because it requires characters with two states, while our characters each have three states (the third being "unknown"). Alternatively, we could have pruned the phylogeny to the six species in which both traits, "mode of pollination" and "heating/no heating," are known (several species have only been studied for one of these traits). A statistical analysis of the correlation between two traits in six species, however, seemed inappropriate because a rule of thumb is that one should have least 10 data points (i.e., recorded trait states) per estimate.

RESULTS

Flower morphology, phenology, and secretions—From mid March to late April, flowers of *I. dunnianum* were produced in large numbers. Buds and flowers were always oriented toward the ground. Flowers had eight carpels and 12–29 stamens with broad, fleshy filaments (Fig. 1B, C, G). The eight styles were pointed and curved backward (Fig. 1B, flower number 2). While open-pollinated flowers lasted 7–10 d, bagged flowers wilted after 3–4 d. No floral odor was detected.

Figure 2 shows a diagram of the flower functional phases. First-night flowers were female (Fig. 2A, B), and based on the MTT test ($N = 20$ flowers) their stigmas were fully receptive (Fig. 1B-2, G, L). In female-phase flowers, the tepals left only a small orifice (1.3 ± 0.1 cm [mean ± SE] in diameter, $N = 5$). During the second night, flowers entered their male phases, beginning with anther dehiscence and lasting until all pollen grains had been released, which lasted 2–3 nights. During this phase, the stigma crests folded in, and the styles moved 90° to an upright position, forming a chamber around the midge eggs (Fig. 1M). The flowers then entered the nursing phase, which on average lasted 7 d. At this stage, the inner tepals closed, forming a chamber, and the adaxial filament surfaces and inner tepal bases produced a secretion. The filaments also increased in diameter (Fig. 3; $t = 2.8$, df = 10, $P = 0.018$). This only occurred in open-pollinated flowers, not in bagged flowers, the filaments of which ceased thickening and wilted (Fig. 3).

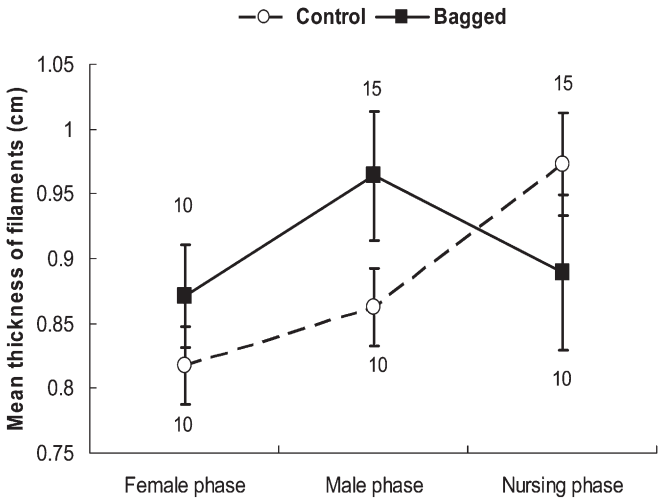

Fig. 3. Mean thickness of filaments in naturally pollinated (control) and bagged flowers of *Illicium dunnianum*. Numbers refer to sample sizes.

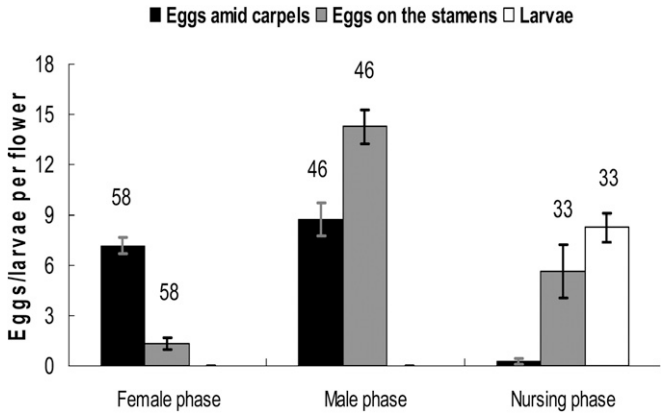

Fig. 4. Mean numbers of gall midge eggs and/or larvae in different floral phases of *Illicium dunnianum*. Numbers refer to sample sizes.

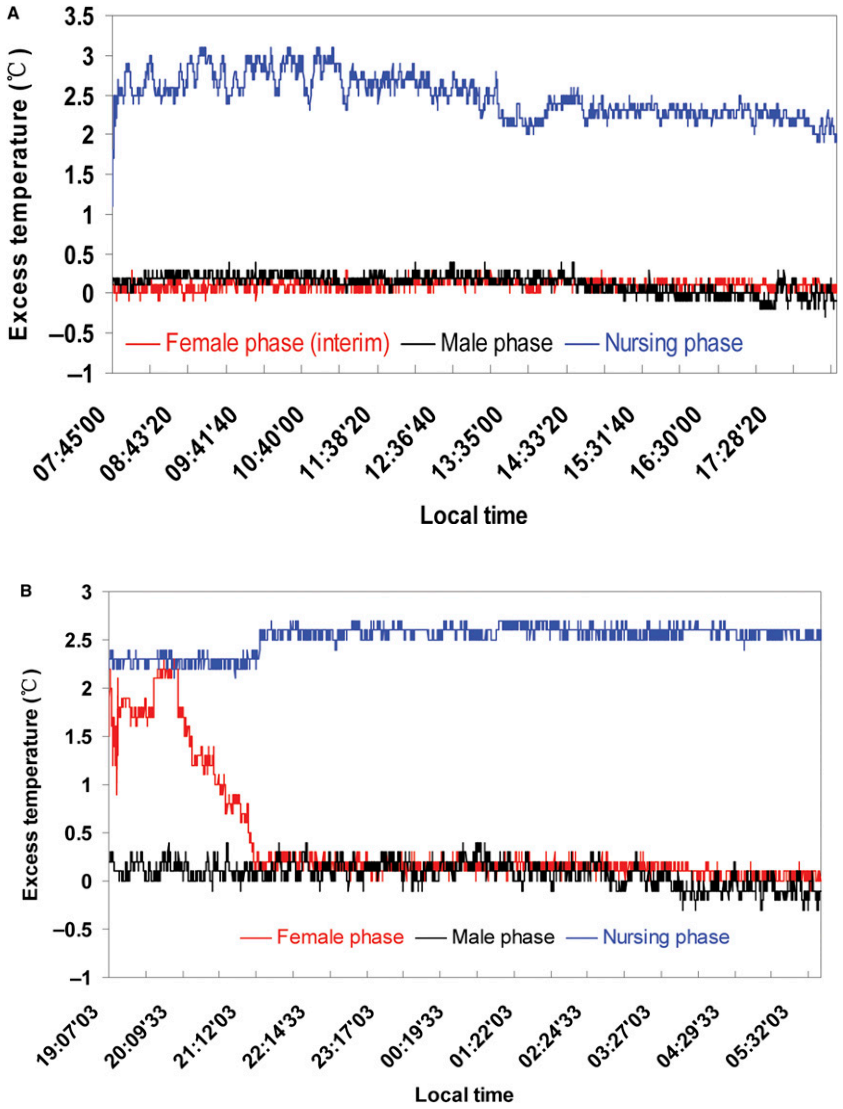

Fig. 5. Above-ambient temperatures in *Illicium dunnianum* flowers during the day and at night. Mean above-ambient temperatures in female-phase flowers, male-phase flowers, and nursing-phase flowers during the day were 0.4 ± 0.07 (mean ± SE, $N = 7735$), 0.073 ± 0.001, and 2.52 ± 0.002 and at night 0.12 ± 0.001 (interim), 0.13 ± 0.001, and 2.49 ± 0.003. Mean above-ambient temperature during the first 2 h of the female phase was 1.6 ± 0.1 (5A: 19:07–21:12 hours, $N = 1441$).

The flowering season of *I. tsangii* lasted from early April to late May. Flower morphology and phenology resemble that of *I. dunnianum* (Fig. 1Q, R), although *I. tsangii* flowers are smaller and have only 7–10 stamens (Fig. 1R). The first-night female phase and second-night male phase are again separated by an interim phase during the day (Fig. 2C) and followed by 3–4 d of a nursing phase.

Visitors and pollinators—As soon as the tepals of fresh flowers of *I. dunnianum* had spread sufficiently for a small orifice to appear (around 1900–2100 hours), the first gall midges approached (Fig. 1D). They would land on a tepal and after a few seconds would climb into the flowers (Fig. 1E). Single midges (apparently females) would enter the same flower on average 9 ± 0.9 times (range from 3 to 14, $N = 10$) before leaving (Fig. 1F). At any one time, a flower would contain but one midge. If other midges landed on a flower that was being visited, they would circle its orifice and then fly away (this was observed in 10 flowers that contained a first-visiting gall midge). The time that a midge spent on visiting a virgin flower varied from 10 min to several hours. Because the diameter of the floral orifice is small, it was difficult to observe the midge behavior. However, following visits, we could readily see midge eggs (Fig. 1G and L) and pollen grains on stigmas, which in female-stage flowers could only come from visiting midges. Single midge visit resulted in the deposition of 26 ± 3 pollen grains ($N = 10$; Fig. 1G), and of 30 captured midges (10 caught on virgin flowers and 20 on female-phase flowers, all of them females) each carried numerous pollen grains on their bodies, all of which belonged to the studied species (the pollen could easily be recognized as belonging to the study species).

The pollinating midges almost certainly belong to a new species of *Clinodiplosis* (R. J. Gagné, Systematic Entomology

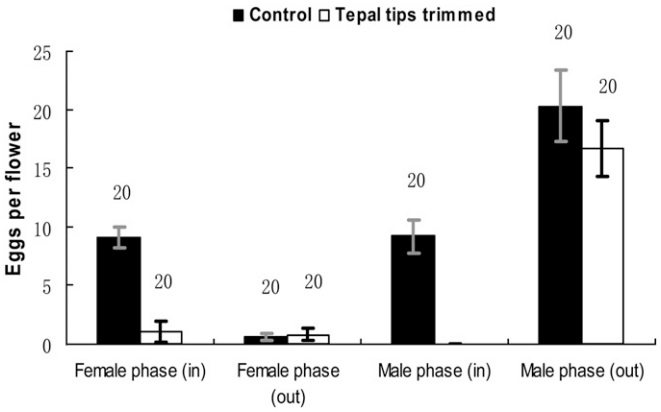

Fig. 6. Mean number of gall midge eggs in female- and male-phase flowers of *Illicium dunnianum* under two treatments. Female phase (in) and Male phase (in) refer to eggs amid the carpels of female-phase and male-phase flowers; female phase (out) and male phase (out) refer to eggs on the stamens. Numbers refer to sample sizes.

Laboratory, U. S. Department of Agriculture; personal communication). Predacious gall midges that also occurred in the flowers were likewise sent to R. Gagné for identification. Visits by other insects were not seen in ~70 h over 10 d of observations.

The next evening, when the anthers dehisced (around 1830–1930 hours), the tepals opened slightly wider, and midges again visited to oviposit. Many of them also seemed to spend the day inside *Illicium* flowers. The time spent by the midges in a male-phase flower varied from a few seconds to several minutes, and four or five midges sometimes visited the same male flower. The midges never tried to enter nursing-phase flowers, although a few midges could still have been hiding in these flowers. Larvae in nursing-phase flowers could be seen feeding on the filament and tepal secretion (Fig. 1O and P). The mean number of eggs in female-phase flowers was 8 ± 0.8 (N = 58), in male-phase flower 23 ± 1.4 (N = 46) and that of larvae in nursing-phase flowers 8.3 ± 0.8 (N = 33, Fig. 4).

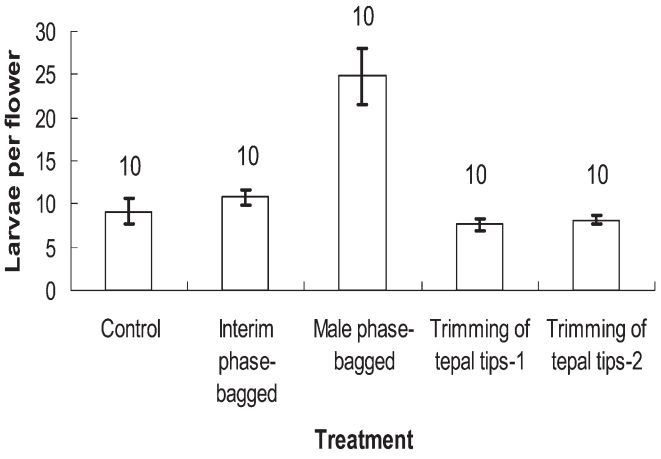

Fig. 7. Mean number of gall midge larvae in flowers of *Illicium dunnianum*. Control: open-pollinated flowers; interim-phase-bagged: flowers bagged from the interim phase until the end of the nursing phase; male phase-bagged: flowers bagged from the end of the male phase until the end of the nursing phase; trimming of tepal tips-1: trimming of tepal tips in late male-phase flowers, trimming of tepal tips-2: trimming of tepal tips in nursing-phase flowers. Larvae in tepal-trimmed flowers soon died. Numbers refer to sample sizes.

During the 80+ h spent observing *I. tsangii*, *Clinodiplosis* was the sole visitor and pollinator (N = 5, Fig. 1R). The midges laid eggs between or on the carpels (Fig. 1S) and visited male and female flowers. Pollination occurred mostly at night and in the same manner as described above for *I. dunnianum.* The mean number of pollen grains deposited by the first visitor was 28 ± 6 (N = 5). The egg numbers in female-phase and male-phase flowers were 5.9 ± 0.5 (N = 10) and 10 ± 2.4 (N = 10), respectively. The average number of larvae in nursing phase flowers was 5.3 ± 0.5 (N = 10).

Floral heating and its adaptive significance—Temperatures inside the flower chamber are shown in Fig. 5. During the first 2 h of the female phase (Fig. 5B), flowers had a temperature of 19–23.7°C, which was significantly higher than the ambient temperature of 18.8–22.3°C (average above-ambient temperature, 1.6 ± 0.1, N = 1441 temperature records, t = 121.1, df = 1523, $P < 0.0001$). Chamber temperature then dropped to ambient temperature (Fig. 5B), and during the day and the male stage, flowers produced little heat. With the onset of the nursing phase, chamber temperature increased, becoming even higher than during the female phase (Fig. 5A and B).

Trimming of tepal tips in female-phase flowers negatively affected midge visiting and egg laying (t = 19, df = 9, $P < 0.0001$; Fig. 1H; Fig. 6), while trimming of tepals in male-phase flowers had no significant effect (t = 0.9, df = 9, P = 0.38; Fig. 6). Bagging of interim-phase flowers (flowers between their female and male phases) had little effect on eggs hatching and larval development (t = 0.99, df = 9, P = 0.345), while bagging of flowers from the end of the male phase until the end of the nursing phase increased the number of surviving larvae (t = 4.7, df = 9, P = 0.001; Fig. 7). Trimming of tepal tips in male-phase flowers and nursing-phase flowers barely affected oviposition (i.e., numbers of eggs and juvenile larvae) but caused older larvae to die (Fig. 7), presumably because of the resulting drop in temperature. Trimming of tepal tips during the flowers' three phases (female, male, nursing) did not differentially affect seed development (Table 1; G = 0.97, df = 1, P = 0.32).

Plant mating system—Bagged flowers set no fruit (Table 1), indicating that a pollen vector is necessary for fruit set. Experimental self-pollination also yielded no fruit (Table 1), showing that *I. dunnianum* is self-incompatible. Fruit set in open-pollinated flowers ranged from 47 to 100%, and fruit set in open-pollinated vs. experimentally cross-pollinated flowers were 82% vs. 87% (Table 1). In *I. tsangii*, natural fruit set was 67 ± 8% (39 flowers from 9 individuals), while none of the bagged flowers set fruits (29 flowers from 5 individuals). Numbers of pollen grains per flower in *I. dunnianum* and *I. tsangii* were 46760 ± 2992 and 33520 ± 9270 (mean ± SE, N = 10), respectively, yielding P/O ratios of 5845 ± 374 and 4190 ± 1158. Both species have 8 ovules/flower.

Phylogenetic distribution of midge pollination and flower heating in Schisandraceae—Figure 8 shows the Schisandraceae phylogeny with the inferred evolutionary shifts in pollinators and flower heating inferred under maximum likelihood; Table 2 summarizes all pollination-relevant traits, such as pollinator rewards, flower heating, and taxonomic groups of pollinators

based on our new data and earlier studies. Pollination by gall midges evolved several times (Fig. 8A), possibly from general fly pollination (sometimes with beetles as copollinators; Table 2), but this inference has weak support because so few species have yet been investigated. Flower heating may be an old trait in the family (Fig. 8B), but again this inference is weakly supported. Comparison of the ancestral state reconstructions for the two traits (Fig. 8A and B) shows that gall midge pollination and flower heating are not strictly correlated (also Table 2 and Discussion).

DISCUSSION

As far as we are aware, this study provides the first evidence for postsexual phase flower heating as a pollinator reward. Our experiments (Table 1, row 4; Figs. 6 and 7) show that postsexual phase heating in *Illicium dunnianum* does not benefit seed development, but is essential for the midge larvae, which can only develop in heated flowers (and fed by a floral secretion). Ancestral state reconstruction suggests that flower heating evolved early during the evolution of Schisandraceae and thus may be preadaptation that became co-opted in flowers pollinated by flower-breeding midges. We now develop a working hypothesis about the evolution of pollination adaptations in Schisandraceae.

An outstanding trait in the Asian *Illicium* we investigated is the long life span of the flowers (up to 10 d of which only 2[–3] involve sexual function), heat production after the flowers' sexual phase, and secretion of exudates for the midge larvae. These features constitute the most intricate adaptation to gall midge pollination so far known in the angiosperms. Gall midges also pollinate, or copollinate, *Amborella* (Amborellaceae; Thien et al., 2003), *Artocarpus* (Moraceae; Sakai et al., 2000), *Clavija* (Theophrastaceae; Gagné et al., 1997), *Piper* (Piperaceae; Ollerton, 1996), *Siparuna* (Siparunaceae; Feil and Renner, 1991; Feil, 1992; Renner et al., 1997), and *Theobroma* (Sterculiaceae; Young, 1985). None of these cases, however, are known to involve flower heating and food secretion after the flower's sexual function is over.

In Schisandraceae, five species are now known to exhibit flower heating (Dieringer et al., 1999; Liu et al., 2006; Yuan et al., 2008; Thien et al., 2009; our Table 2). However, this is the first study to continuously record temperatures over a flower's life span, enabling us to pick up the rise in temperature after pollination has taken place. In species that are copollinated by flies and beetles or by pollen-feeding *Megommata* midges, floral heating likely helps odor emission and, thereby, pollinator attraction (Yuan et al., 2008). Thien et al. (2009) hypothesized that in *Illicium floridanum* flower heating may also aid pollen tube growth and that the heated pedicels of this species might help seed development. Our experimental reduction of flower temperatures in *I. dunnianum*, however, did not affect fruit set (while drastically reducing larval survival), demonstrating that at least in this species postanthetic flower warming mainly benefits the pollinating midges. The midges are reliable pollinators; fruit set in open-pollinated flowers is high (Table 1), and the P/O ratios of both investigated *Illicium* species are typical of obligately outcrossed plants (Cruden, 1977). Obligate outcrossing may be enforced by self-incompatibility, and detailed studies on this are much needed (cf. Koehl et al., 2004).

The demonstration that at least in *I. dunnianum*, flower heating is a reward for flower-breeding midges raises the question of the selective factor(s) behind the evolution of this trait. Of the closest relatives of Schisandraceae, *Trimenia* is fly pollinated with copollination by bees (Bernhardt et al., 2003), and *Austrobaileya* is copollinated by flies and beetles (Endress, 2001; Thien et al., 2009). Neither has been investigated for possible flower heating. A plausible scenario thus is that early Schisandraceae were pollinated by flies and/or beetles and that midges were simply copollinators as is still the case in New World *Illicium* (Table 2). Flower heating would have benefited odor emission to attract scent-oriented pollinators. Some midges,

TABLE 1. Fruit set ratio in natural and manipulated treatments of *Illicium dunnianum.*

Treatment	No. flowers (*N*)	No. fruit	Fruit set ratio (mean ± SE, %)	Fruit set ratio (range, %)
Natural pollination	127 (10)	100	82 ± 5.7	47–100
Bagged	102 (10)	0	0	0
Assisted self-pollination	43 (5)	0	0	0
Assisted cross-pollination	55 (5)	48	87 ± 4	60–100
Trimmed tepal tips (lowered flower temperature)	36 (5)	28	79 ± 4	66–100

Note: N = number of plant individuals.

TABLE 2. Pollination mode and flower heating in the Schisandraceae

Species	Pollinators	Pollinator reward	Thermogenesis	Reference
Illicium arborescens	Unidentified Cecidomyiidae	Brood site	Not investigated	SMC, personal observation
I. dunnianum	*Clinodiplosis* (Cecidomyiidae)	Brood site (warm, with secretion for larvae)	Investigated, present	This study
I. floridanum	Various insects, particularly Diptera (including *Clinodiplosis, Giardomyida, Lestodiplosis*, Cecidomyiidae)	"Nectar"	Investigated, present	Thien et al., 1983, 2009; Dieringer et al., 1999
I. parviflorum	Various insects, particularly Diptera	"Nectar"	Not investigated	White and Thien, 1985
I. tsangii	*Clinodiplosis* (Cecidomyiidae)	Brood site (warm, with secretion for larvae)	Investigated, present	This study
Kadsura longipedunculata	*Megommata* (Cecidomyiidae)	Pollen (in male flowers; deceit in female flowers)	Investigated, present	Yuan et al., 2008
Schisandra glabra	Diptera: Chironomidae, Ceratopogonidae, but also beetles	Brood site (warm)	Investigated, present	Liu et al., 2006
Schisandra henryi	*Megommata* (Cecidomyiidae)	Pollen (in male flowers; deceit in female flowers)	Investigated, absent	Yuan et al., 2007

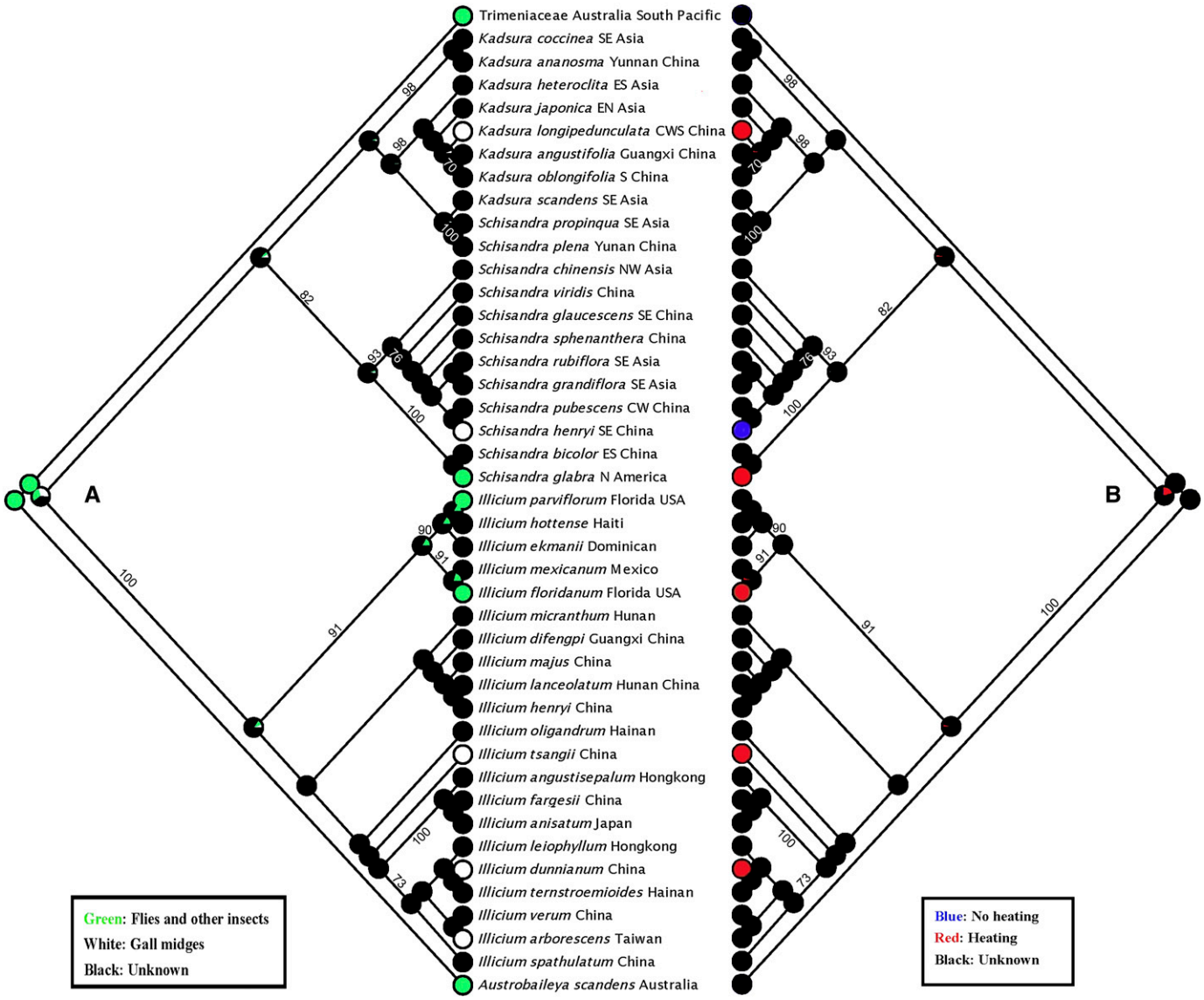

Fig. 8. Maximum likelihood (ML) phylogeny for Schisandraceae based on combined nuclear and chloroplast data (2197 aligned nucleotides), with the maximum likelihood ancestral-state reconstruction of (A) pollination modes and (B) flower heating shown in color. Numbers at nodes refer to ML bootstrap support from 100 replicates. For the three genera to become mutually monophyletic, two species of *Schisandra* will need to be transferred to *Kadsura*.

such as *Clinodiplosis*, then increasingly used the warm flowers for breeding, which set the stage for reciprocal coevolution between midges selecting for long-heated brood chambers and flowers responding by relying exclusively on *Clinodiplosis* (and excluding other visitors via ± closed tepals, hanging flowers, and the absence of rewards other than a brood site). Adult *Clinodiplosis* take liquid food (R. Gagné, U. S. Department of Agriculture, personal communication), and species of this genus also breed in and pollinate flowers of North American *I. floridanum* (Table 2) and South American Siparunaceae (Feil and Renner, 1991; Feil, 1992). Under this scenario, flower heating is a trait that evolved "for" scent emission and that then became co-opted as a pollinator reward in flowers relying on flower-breeding insects, the larvae of which require moist, warm chambers for the duration of their development.

That Schisandraceae adapted to gall midges several times (Fig. 8A), and in different ways, fits with the findings of other phylogenetic analyses of the evolution of insect–plant interactions (Futuyma and Agrawal, 2009). To test the scenario for the evolution of flower heating in Schisandraceae proposed here (namely, that it is an ancestral trait that became coopted in species adapting to flower-breeding midges) more species of *Illicium* will need to be investigated. It is clear, however, that in *I. dunnianum* flower temperatures are highest after the flowers' sexual function is over and that this constitutes an adaptation to the species' exclusive pollinators, *Clinodiplosis* midges.

LITERATURE CITED

ANGIOY, A. M., M. C. STENSMYR, I. URRU, M. PULIAFITO, I. COLLU, AND B. S. HANSSON. 2004. Function of the heater: The dead horse arum revisited. *Proceedings of the Royal Soceity of London, B, Biological Sciences* 271 (Supplement): S13–S15.

APG [ANGIOSPERM PHYLOGENY GROUP] III. 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. *Botanical Journal of the Linnean Society* 161: 105–121.

BERNHARDT, P., T. SAGE, P. WESTON, H. AZUMA, M. LAM, L. B. THIEN, AND J. BRUHL. 2003. The pollination of *Trimenia moorei* (Trimeniaceae): Floral volatiles, insect/wind pollen vectors and stigmatic self-incompatibility in a basal angiosperm. *Annals of Botany* 92: 445–458.

CRUDEN, R. W. 1977. Pollen–ovule ratios: A conservative indicator of breeding systems in flowering plants. *Evolution* 31: 32–46.

DIERINGER, G. L., R. CABRERA, M. LARTA, L. LOYA, AND P. REYES-CASTILLO. 1999. Beetle pollination and floral thermogenicity in *Magnolia tamaulipana* (Magnoliaceae). *International Journal of Plant Sciences* 160: 64–71.

ENDRESS, P. K. 2001. The flowers in extant basal angiosperms and inferences on ancestral flowers. *International Journal of Plant Sciences* 162: 1111–1140.

ENDRESS, P. K. 2010. The evolution of floral biology in basal angiosperms. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 365: 411–421.

FEIL, J. P. 1992. Reproductive ecology of dioecious *Siparuna* (Monimiaceae) in Ecuador—A case of gall midge pollination. *Botanical Journal of the Linnean Society* 110: 171–203.

FEIL, J. P., AND S. S. RENNER. 1991. The pollination of *Siparuna* (Monimiaceae) by gall-midges (Cecidomyiidae): Another likely ancient association. *American Journal of Botany* 78: 186 [Abstract].

FUTUYMA, D. A., AND A. A. AGRAWAL. 2009. Macroevolution and the biological diversity of plants and herbivores. *Proceedings of the National Academy of Sciences, USA* 106: 18054–18061.

GAGNÉ, R. J., C. OTT, AND S. S. RENNER. 1997. A new species of gall midge (Diptera: Cecidomyiidae) from Ecuador associated with flowers of *Clavija* (Theophrastaceae). *Proceedings of the Entomological Society of Washington* 99: 110–114.

HAO, G., M. L. CHYE, AND R. M. K. SAUNDERS. 2001. A phylogenetic analysis of the Schisandraceae based on morphology and nuclear ribosomal ITS sequences. *Botanical Journal of the Linnean Society* 135: 401–411.

HAO, G., R. M. K. SAUNDERS, AND M. L. CHYE. 2000. A phylogenetic analysis of the Illiciaceae based on sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA. *Plant Systematics and Evolution* 223: 81–90.

KOEHL, V., L. B. THIEN, E. G. HEIJ, AND T. L. SAGE. 2004. The causes of self-sterility in natural populations of the relictual angiosperm, *Illicium floridanum* (Illiciaceae). *Annals of Botany* 94: 43–50.

LEWIS, P. O. 2001. A likelihood approach to estimating phylogeny from discrete morphological character data. *Systematic Biology* 50: 913–925.

LIU, Z., G. HAO, Y. B. LUO, L. B. THIEN, S. W. ROSSO, A. M. LU, AND Z. D. CHEN. 2006. Phylogeny and androecial evolution in Schisandraceae, inferred from sequences of nuclear ribosomal DNA ITS and chloroplast DNA *trnL-F* regions. *International Journal of Plant Sciences* 167: 539–550.

MADDISON, W. P., AND D. R. MADDISON. 2009. MESQUITE: A modular system for evolutionary analysis, version 2.6. Computer program and documentation distributed by the author, website: http://mesquiteproject.org/mesquite/mesquite.html [accessed August 2009].

MORRIS, A. B., C. D. BELL, J. W. CLAYTON, W. JUDD, D. E. SOLTIS, AND P. S. SOLTIS. 2007. Phylogeny and divergence time estimation in *Illicium* with implications for New World biogeography. *Systematic Botany* 32: 236–249.

OLLERTON, J. 1996. Interactions between gall midges (Diptera: Cecidomyiidae) and inflorescences of *Piper novae-hollandiae* (Piperaceae) in Australia. *Entomologist* 115: 181–184.

PAGEL, M. 1994. Detecting correlated evolution on phylogenies: A general method for the comparative analysis of discrete characters. Philosophical Transactions of the Royal Society, B. *Biological Sciences* 255: 37–45.

PATIÑO, S., T. AALTO, A. A. EDWARDS, AND J. GRACE. 2002. Is *Rafflesia* an endothermic flower? *New Phytologist* 154: 429–437.

PATIÑO, S., J. GRACE, AND H. BÄNZINGER. 2000. Endothermy by flowers of *Rhizanthes lowii* (Rafflesiaceae). *Oecologia* 124: 149–155.

RENNER, S. S., A. E. SCHWARZBACH, AND L. LOHMANN. 1997. Phylogenetic position and floral function of *Siparuna* (Siparunaceae: Laurales). *International Journal of Plant Sciences* 158 (6 Supplement): S89–S98.

RODRIGUEZ-RIAÑO, T., AND A. DAFNI. 2000. A new procedure to assess pollen viability. *Sexual Plant Reproduction* 12: 241–244.

SAKAI, S., M. KATO, AND H. NAGAMASU. 2000. *Artocarpus* (Moraceae)—Gall midge pollination mutualism mediated by male-flower parasitic fungus. *American Journal of Botany* 87: 440–445.

SAUNDERS, R. M. K. 1998. Monograph of *Kadsura* (Schisandraceae). *Systematic Botany Monographs* 54: 1–106.

SAUNDERS, R. M. K. 2000. Monograph of *Schisandra* (Schisandraceae). *Systematic Botany Monographs* 58: 1–146.

SEYMOUR, R. S., E. MAASS, AND J. F. BOLIN. 2009. Floral thermogenesis of three species of *Hydnora* (Hydnoraceae) in Africa. *Annals of Botany* 104: 823–832.

SEYMOUR, R. S., AND P. SCHULTZE-MOTEL. 1998. Physiological temperature regulation by flowers of the sacred lotus. *Philosophical Transactions of the Royal Society, B, Biological Sciences* 353: 935–943.

SEYMOUR, R. S., C. R. WHITE, AND M. GIBERNAU. 2003. Heat reward for insect pollinators. *Nature* 426: 243–244.

STAMATAKIS, A., P. HOOVER, AND J. ROUGEMONT. 2008. A rapid bootstrap algorithm for the RAxML web-servers. *Systematic Biology* 57: 758–771.

THIEN, L. B., P. BERNHARDT, M. S. DEVALL, Z. D. CHEN, Y. B. LUO, J. H. FAN, L. C. YUAN, AND J. H. WILLIAMS. 2009. Pollination biology of basal angiosperms (ANITA grade). *American Journal of Botany* 96: 166–182.

THIEN, L. B., T. L. SAGE, T. JAFFRE, P. BERNHARDT, V. PONTIERI, P. H. WESTON, D. MALLOCH, ET AL. 2003. The population structure and floral biology of *Amborella trichopoda* (Amborellaceae). *Annals of the Missouri Botanical Garden* 90: 466–490.

THIEN, L. B., D. A. WHITE, AND L. Y. YATSU. 1983. The reproductive biology of a relict: *Illicium floridanum* Ellis. *American Journal of Botany* 70: 719–727.

WHITE, D. A., AND L. B. THIEN. 1985. The pollination of *Illicium parviflorum* (Illiciaceae). *Journal of the Elisha Mitchell Scientific Society* 101: 15–18.

XIA, N. H., Y. H. LIU, AND R. M. K. SAUNDERS. 2009. Schisandraceae. *In* Z. Y. Wu, P. H. Raven, and D. Y. Hong [eds.], Floral of China, vol. 7, 39–47. Science Press, Beijing, China and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

XIA, N. H., AND R. M. K. SAUNDERS. 2009. Illiciaceae. *In* Z. Y. Wu, P. H. Raven, and D. Y. Hong [eds.], Flora of China, vol. 7, 32–38. Science Press, Beijing, China and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

YAFUSO, M. 1993. Thermogenesis of *Alocasia odora* (Araceae) and the role of *Colocasiomyia* flies (Diptera: Drosophilidae) as cross-pollinators. *Environmental Entomology* 22: 601–606.

YOUNG, A. M. 1985. Studies of cecidomyiid midges (Diptera: Cecidomyiidae) as cocoa pollinators (*Theobroma cacao* L.) in Central America. *Proceedings of the Entomological Society of Washington* 87: 49–79.

YUAN, L. C., Y. B. LUO, L. B. THIEN, J. H. FAN, H. L. XU, AND Z. D. CHEN. 2007. Pollination of *Schisandra henryi* (Schisandraceae) by female, pollen-eating *Megommata* species (Cecidomyiidae, Diptera) in south central China. *Annals of Botany* 99: 451–460.

YUAN, L. C., Y. B. LUO, L. B. THIEN, J. H. FAN, H. L. XU, J. YUKAWA, AND Z. D. CHEN. 2008. Pollination of *Kadsura longipedunculata* (Schisandraceae), a monoecious basal angiosperm, by female, pollen-eating *Megommata* sp. (Cecidomyiidae: Diptera) in China. *Biological Journal of the Linnean Society* 93: 523–536.