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The olfactory component of floral display in *Asimina* and *Deeringothamnus* (Annonaceae)

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Summary

- Floral scent is a key component of floral display, and probably one of the first floral attractants linking insect pollinators to the radiation of Angiosperms. In this article, we investigate floral scent in two extra-tropical genera of Annonaceae. We discuss floral scent in the context of differing pollination strategies in these genera, and compare their scent to that of a close tropical relative.
- Floral volatiles were collected for *Annona glabra*, *Asimina* and *Deeringothamnus* whole flowers and dissected floral organs, using a standardized static-headspace solid phase microextraction method. Scents were analyzed using gas chromatography–mass spectrometry, and identified using known standards.
- The floral scents of these species are highly dynamic, varying between floral organs, sexual stages and species. Maroon-flowered species of *Asimina* produce 'yeasty' odors, dominated by fermentation volatiles and occasionally containing sulfurous or nitrogenous compounds. White-flowered species of *Asimina* and *Deeringothamnus* produce pleasant odors characterized by lilac compounds, benzenoids and hydrocarbons. *Annona glabra* produces a strong, fruity-acetonic scent dominated by 3-pentanyl acetate and 1,8-cineole.
- The fermented/decaying scents of maroon-flowered species of *Asimina* suggest mimicry-based pollination strategies similar to aroids and stapeliads, whereas the pleasant scents of white-flowered species of *Asimina* suggest honest, reward-based pollination strategies. The scent of *Annona glabra* is typical of specialized beetle pollination systems common to tropical Annonaceae.

Introduction

The early, rapid radiation of flowering plants has been linked to their mechanisms of pollinator attraction (Ren, 1998; Thien *et al.*, 2000) through combinations of floral scent, color and morphology (van der Pijl, 1960; Stebbins, 1970; Barth, 1991). Historically, such floral traits have been considered important for reproductive isolation and speciation processes (for example, Grant, 1949; Hills *et al.*, 1972), and detailed studies have explored the contributions of floral phenotype to plant reproductive success (Grant & Grant, 1965; Castellanos *et al.*, 2004; Dafni & Potts, 2004). However, until recently, the scent component of floral display has been difficult to analyze objectively. Recently, Knudsen *et al.* (2006) summarized our current knowledge of floral scent diversity, emphasizing that most angiosperm lineages have not yet been investigated for fragrance composition.

Scent is a conspicuous component of floral display for many angiosperms, and probably played a pivotal role in their early evolution (Gottsberger, 1988; Thien *et al.*, 2000; Endress, 2001). Olfactory floral cues are suggested to have evolved from volatile antimicrobial agents and/or herbivore deterrents, pre-dating the evolution of attractive visual cues (Pellmyr & Thien, 1986; Pellmyr *et al.*, 1991; Harrewijn *et al.*, 1994). Reproductive studies of extant taxa from basal angiosperm lineages support the hypothesis that olfactory cues may have played crucial roles as pollinator attractants in early angiosperms, citing floral scent as an attractant in Trimeniaceae (Bernhardt *et al.*, 2003), Nymphaeaceae (Ervik & Knudsen, 2003), Magnoliaceae (Azuma *et al.*, 1999), Aristolochiaceae (Burgess *et al.*, 2004) and Annonaceae (Silberbauer-Gottsberger *et al.*, 2003). Thus, the linking of floral scent (as an attractant) with components of floral morphology that influence visitors' behavior in optimizing pollen

transfer may have been a critical stage in the evolution of functionally integrated blossoms.

In this study, we investigate the olfactory components of floral display in two genera of the basal angiosperm family Annonaceae. Flowers of Annonaceae typically are protogynous, with fleshy petals and strong, distinctive scents (Fries, 1959). Literature on reproductive biology in Annonaceae frequently offers subjective descriptions of scent quality and/or intensity as flowers progress from female to male phases of ontogeny (Armstrong & Marsh, 1997; Momose *et al.*, 1998; Silberbauer-Gottsberger *et al.*, 2003), during which scent often is emitted in distinct diel rhythms, occasionally in concert with thermogenesis (Rogstad, 1994; Gottsberger, 1999; Silberbauer-Gottsberger *et al.*, 2003; Ratnayake *et al.*, 2006). Pollination studies of tropical Annonaceae often link scent to highly specialized pollination syndromes. For example, fruity odors are correlated with small (nitidulid) or large (dynastine) beetle pollination, sour or rotting odors are associated with fly pollination, and spicy odors are associated with pollination by male euglossine bees (Carvalho & Webber, 2000; Jürgens *et al.*, 2000; Silberbauer-Gottsberger *et al.*, 2003; Su *et al.*, 2005; Teichert *et al.*, 2008). The generalization–specialization debate in pollination ecology has generated predictions that stable, tropical habitats favor specialized pollination for the fidelity of pollen transfer, whereas temperate habitats favor generalized strategies for reproductive assurance in the face of spatial and temporal variability in climate and pollinator abundance (Waser *et al.*, 1996, Johnson & Steiner, 2000). In this context, the biogeography of *Asimina* and its sister genus *Deeringothamnus* (Kral, 1960; L. Chatrou, Wageningen University, Wageningen, the Netherlands, unpublished) is particularly interesting, as these represent nontropical genera in an otherwise tropical family. Comparative studies of their floral scent chemistry, in conjunction with that of their tropical relatives, might reveal whether the invasion of temperate habitats by *Asimina* and *Deeringothamnus* (or their common ancestor) was accompanied by shifts to more generalized reproductive strategies (see Ollerton & Cranmer, 2002). Although floral scents are complex blends of pollinator attractants, florivore repellents and adaptively neutral compounds with biosynthetic or phylogenetic significance (Levin *et al.*, 2003; Knudsen *et al.*, 2006; Raguso, 2008), scent chemistry is now understood well enough in a comparative context to generate testable predictions about pollinator spectra (Dobson, 2006; Jürgens *et al.*, 2006).

Asimina and *Deeringothamnus* are distributed across southeastern North America in temperate and/or subtropical habitats (Kral, 1960). Both species of *Deeringothamus* and six species of *Asimina* occur in sandy, well-drained soils of pine flatwoods, scrub and high pine habitats in peninsular Florida, USA, whereas the remaining two species of *Asimina* (*A. triloba* and *A. parviflora*) occur in mesic, organic soils of riparian forests in eastern North America. *Asimina triloba*, *A. parviflora* and two of the Floridian species (*A. pygmaea* and *A. tetramera*) have small maroon flowers (Fig. 1) with a distinctive yeasty odor. The remaining species of *Asimina* and *Deeringothamnus pulchellus* have white flowers with sweet or waxy odors. *Deeringothamnus rugelii* differs from the other species described here, having yellow flowers and a faint rubbery scent. The flowers of sweet-smelling *Asimina* species have large petals, whereas those of *D. pulchellus* and *D. rugelii* are highly reduced in size (Fig. 1).

One other species of Annonaceae, *Annona glabra* (Fig. 1), occurs in southeastern North America. *Annona* is a pantropical genus of 120–175 species, native to tropical America and Africa (Fries, 1959). At its northernmost range, *An. glabra* extends into southern Florida, where it overlaps with the southernmost species of *Asimina*. However, on a finer landscape scale, *An. glabra* occurs in swampy mangrove habitats, not the sandy well-drained soils inhabited by Floridian *Asimina* and *Deeringothamnus*. Flowers of *An. glabra* share the fleshy petals and creamy, off-white coloration typical of many tropical Annonaceae (Fig. 1), with a sharp, acetone-like fragrance whose peak intensity occurs in the evening. This combination of features is typical of the beetle pollination strategy employed by a majority of tropical Annonaceae (Jürgens *et al.*, 2000; Silberbauer-Gottsberger *et al.*, 2003). Existing phylogenetic data show *Annona* to be closely related to *Asimina* (Couvreur *et al.*, 2008).

Previously, we have investigated the floral scent composition of *A. triloba*, the most widely distributed North American species (Goodrich *et al.*, 2006). Its floral odor is dominated by compounds indicative of fermenting sugar (2- and 4-carbon aliphatic alcohols, acetic acid and 3-hydroxy-2-butanone), and is perceived by humans as 'yeasty'. The unusual floral scent of *A. triloba* differs from the fruity alcohol- and ester-dominated scents of tropical Annonaceae studied by Jürgens *et al.* (2000) and Teichert (2008), and from the unusual floral volatiles of *Unonopsis stipitata* and *Duguetia cadaverica*, which attract male euglossine bees and mycetophagous beetles, respectively (Teichert, 2008; Teichert *et al.*, 2008). These 'yeasty' 2- and 4-carbon aliphatic compounds may attract a broader spectrum of saprophilic insects from several orders (see Willson & Schemske, 1980) through generalized mimicry of fermented fruit or sap.

Our study of floral scent in *A. triloba* showed it to be spatially and temporally dynamic, with shifts in composition and quantity between stages of floral ontogeny and floral organs (Goodrich *et al.*, 2006). Are similar compositional and ontogenetic patterns of scent production present in closely related species of *Asimina* and *Deeringothamnus*? If so, what might these patterns suggest about pollination strategies within these species? In this paper, we analyze the fragrance composition for all species of *Asimina* and *Deeringothamnus*, as well as *An. glabra*, using standardized methodology appropriate for scent chemistry in these taxa (see Goodrich *et al.*, 2006). Data were collected from immature-, female- and male-stage flowers in the field and in the laboratory, as well as from dissected floral organs of each floral stage in the laboratory. We identified 272 volatile compounds from 11 species, with complex patterns of floral scent composition emerging between genera, species, floral ontogenetic stages and floral organs within each stage.

Fig. 1 Flowers of *Annona glabra*, *Asimina* and *Deeringothamnus*, and the two habitat types occupied by *Asimina* and *Deeringothamnus*. Flowers of the 'maroon phenotype' in *Asimina* are represented by *A. parviflora* (a1), *A. triloba* (a2), *A. pygmaea* (a3) and *A. tetramera* (a4). Flowers of the 'white phenotype' in *Asimina* include *A. incana* (b1), *A. obovata* (b2), *A. reticulata* (b3) and *A. longifolia* (b4). The genus *Deeringothamnus* consists of *D. rugelii* (c1, c2) and *D. pulchellus* (c3, c4). *Annona glabra* whole flower (d1) and dissected floral organs (d2) are shown, as well as mesic woodland habitats (d3) of *A. triloba* and *A. parviflora* and xeric pine flatwoods and scrub habitats (d4) of other *Asimina* and *Deeringothamnus* species. Bars, 1 cm.

We interpret these data in the light of what is known about odor-mediated pollinator behavior (for example, Dobson, 2006) and the potential for evolutionary shifts in pollination strategies in *Asimina* and *Deeringothamnus* and between tropical and temperate Annonaceae.

Materials and Methods

Field sites

Scent data and voucher specimens for all species were collected from natural populations in Florida, Georgia and South Carolina, USA. Field sites were established near Columbia, SC [*Asimina triloba* (Linnaeus) Dunal], Newberry, SC [*Asimina parviflora* (Michaux) Dunal], Statesboro, GA (*Asimina parviflora*), Starke, FL [*Asimina pygmaea* (W. Bartram) Dunal, *Asimina obovata* (Willdenow) Nash, *Asimina incana* (W. Bartram) Exell], Lake Placid, FL (*Asimina reticulata* Shuttleworth ex Chapman), Jupiter, FL (*Asimina tetramera* Small; harvesting permit # 642), Gainesville, FL (*Asimina longifolia* Kral; state park collection permit # 03280622), New Smyrna Beach, FL [*Deeringothamnus rugelii* (B.L. Robinson) Small], Orlando, FL (*Deeringothamnus pulchellus* Small; harvesting permit # 635) and Boynton Beach, FL (*Annona glabra* Linnaeus). Vouchers for species at each site were collected and placed in the A. C. Moore Herbarium (USCH) at the University of South Carolina.

Field scent collection and analysis

Floral scent was collected in field and laboratory settings using a static-headspace solid phase microextraction (SPME) method, and was analyzed using combined GC–MS, as described by Goodrich *et al.* (2006) in their study of *A. triloba*. Scent was equilibrated in oven bags, adsorbed onto SPME fibers from attached, intact flowers in natural populations and transported to the laboratory in coolers for further analysis. In addition, flowering branches of each taxon, except the two *Deeringothamnus* species, were cut, wrapped in wet newsprint and transported to the laboratory for finer scale scent analysis from dissected flower parts of different ontogenetic stages [for details, see Methods S1 (Supporting Information)].

Scent samples were directly thermally desorbed from SPME fibers into the injection port of a Shimadzu (Shimadzu, Columbia, MD, USA) GC17A gas chromatograph and separated on a polar GC column (EconoCap™ ECwax). Mass spectra were generated using a Shimadzu QP5000 quadrupole, electron impact (70 eV) mass spectrometer, and peak areas were calculated using Shimadzu GCMS Solutions software. Compounds were tentatively identified using mass spectral libraries (see Methods S1 for details) and confirmed using known standards or Kovats indices (Kovats, 1961), whenever possible.

The relative percentage peak area of each compound for each stage of ontogeny within each species was averaged, and the

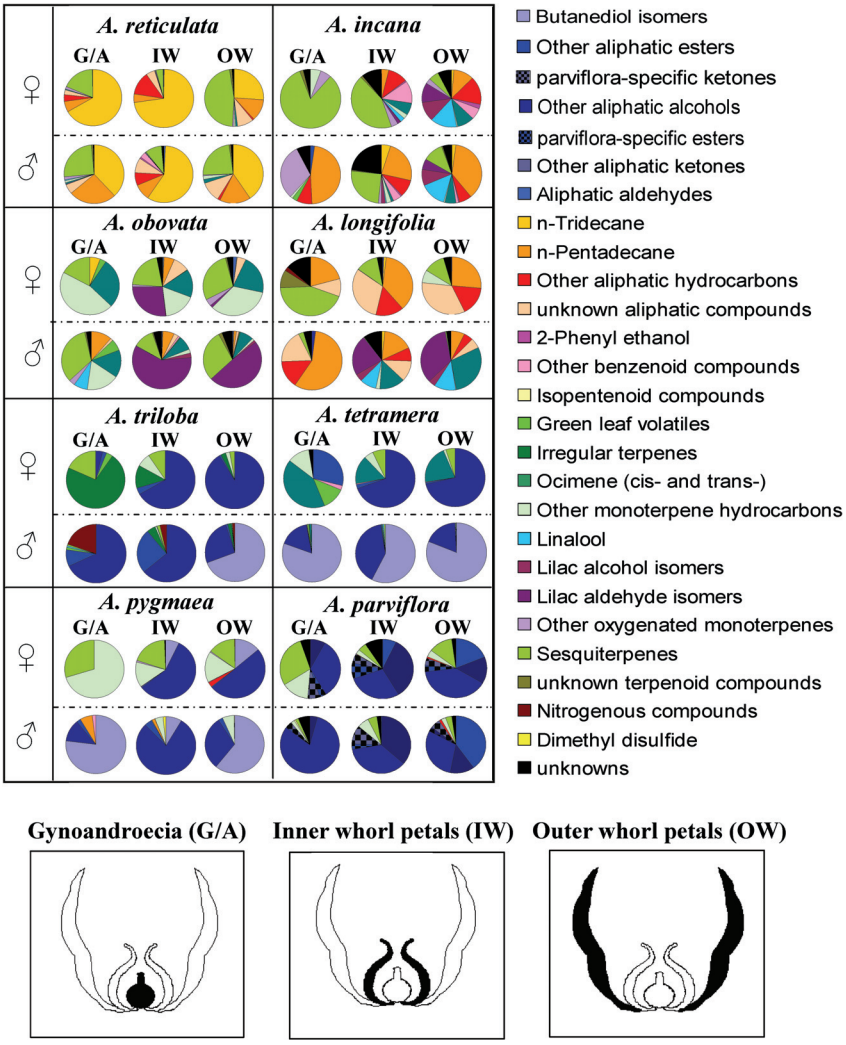

Fig. 2 Relative percentage floral scent composition for each species of *Asimina*, separated by floral organ and ontogenetic stage. G/A, gynoecium/androecium; IW, inner whorl petals; OW, outer whorl petals. Diagrams along the bottom indicate the floral parts dissected for floral scent samples shown in the pie charts.

standard errors of the means (SEMs) were calculated. Compound identifications, average percentage peak areas and SEMs for all species were sorted onto a master table by retention times and identifications (Table S1, see Supporting Information). We excluded compounds which represented less than 0.1% of the total ion current peak area and were only found in a single sample. Compounds identified in the compiled table were then categorized by biosynthetic class, following Knudsen *et al.* (2006). (For additional caveats and limitations of these data, see Methods S1.)

Results

A total of 272 compounds was isolated from study species, and assigned to the following classes: aliphatic compounds, benzenoid compounds, isopentenoid compounds, monoterpenoid compounds, sesquiterpenoid compounds, irregular terpenoid compounds, green leaf volatiles (derived from the lipoxygenase pathway) and nitrogen- or sulfur-containing compounds (Table 1). Each class was further subdivided into alcohols, aldehydes, ketones, esters, ethers and hydrocarbons. The complete list of compounds and classifications is given in Table S1.

Patterns of floral scent within the genus *Asimina*

Asimina exhibits clear differences in scent composition between maroon and white floral phenotypes, between individual species, between floral ontogenetic stages and between floral organs, as highlighted in Fig. 2. Scent data for whole female- and male-stage flowers of *Asimina* species are included in Table S1.

Maroon-flowered species The four maroon-flowered species of *Asimina* share several oxygenated 2- and 4-carbon aliphatic compounds associated with yeast fermentation of sugar, as detailed by Goodrich *et al.* (2006). In these maroon-flowered species, fermentation volatiles represent more than 50% of the total relative peak area of scent for all floral organs except

Table 1 Key compounds and compound classes for female- and male-stage flowers of *Annona glabra*, *Asimina* and *Deeringothamnus*

Key compounds & compound class[1]	*Asimina parviflora*		*Asimina triloba*		*Asimina tetramera*		*Asimina pygmaea*		*Asimina reticulata*		*Asimina longifolia*		*Asimina obovata*		*Asimina incana*		*Deering. pulchellus*		*Deering. rugelii*		*Annona glabra*	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
3-Pentanyl acetate	*	*	*	*	*	*	*	*	*	*	*	0.1	*	*	*	*	*	*	*	*	27.5	32.7
Penta(e)noid alcohols & ketone	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	8.1	9.6
parviflora-specific esters & ketones	9.2	5.3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Butanediol isomers	*	*	*	23.7	*	73.2	4.2	36.5	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Other aliphatic alcohols	52.9	65.1	63.9	56.0	31.5	16.4	21.5	29.4	*	*	0.3	0.3	*	*	*	*	*	*	*	*	*	*
Other aliphatic esters	7.1	8.6	2.1	9.3	5.3	0.1	0.2	1.8	*	*	*	*	0.4	0.2	*	0.4	2.4	0.4	0.0	15.1	*	*
Other aliphatic ketones	*	*	*	*	*	*	0.2	*	*	*	*	*	*	*	*	0.2	*	*	0.9	0.2	*	*
Aliphatic aldehydes	*	*	*	*	*	*	*	*	0.1	0.7	0.2	*	0.5	0.1	0.2	0.3	*	*	*	*	*	*
n-Tridecane	*	*	*	*	*	2.0	0.6	1.7	59.0	49.3	0.4	1.2	1.5	0.1	0.7	1.8	*	*	*	*	*	0.0
n-Pentadecane	*	*	*	*	*	*	0.0	1.7	6.2	12.2	25.3	25.5	7.2	5.4	3.0	26.0	*	*	*	0.5	*	*
Other aliphatic hydrocarbons	*	*	*	*	*	*	1.4	0.3	6.6	2.6	12.2	11.9	3.0	0.4	9.8	9.7	*	*	*	*	*	*
UnID aliphatic compounds	*	*	*	*	0.8	*	0.0	0.0	4.7	8.4	23.6	20.5	6.3	1.3	0.0	0.2	*	*	0.1	*	*	*
2-Phenyl ethanol	*	*	*	*	*	*	*	*	*	0.2	*	*	*	*	5.0	11.8	*	*	*	*	*	*
Other benzenoid compounds	0.1	0.1	*	*	0.8	0.2	0.1	0.4	4.2	10.3	*	*	*	0.4	7.7	8.4	51.9	87.6	7.6	*	*	*
Isopentenoid compounds	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	0.1	*	*	*
Green leaf volatiles	0.1	1.3	0.9	0.4	*	*	*	*	*	*	*	0.1	0.7	1.3	*	0.5	0.9	*	*	*	*	*
Irregular terpenes	2.4	0.4	22.1	1.9	0.1	0.1	5.6	0.1	0.2	0.2	*	0.3	0.0	0.2	0.1	0.2	5.9	5.8	0.1	1.1	*	*
Ocimene (*cis*- and *trans*-)	1.0	2.0	*	*	38.2	1.8	0.0	0.2	0.3	0.7	0.9	7.9	14.3	9.1	4.9	1.9	0.6	*	66.7	5.5	0.9	1.4
Other monoterpene hydrocarbons	0.0	0.0	2.6	0.5	3.8	0.2	20.5	2.7	0.2	0.6	1.3	0.4	19.3	4.3	1.9	1.0	*	*	10.4	37.1	8.3	12.6
Linalool	*	*	0.0	0.6	2.9	1.2	*	*	0.3	0.5	1.0	9.9	*	1.5	17.6	7.7	16.5	*	0.0	5.1	0.0	0.0
Lilac alcohols & aldehydes	*	*	*	*	*	*	*	*	*	*	0.5	12.8	7.7	41.9	6.1	8.3	*	*	*	*	*	*
Other oxygenated monoterpenes	*	*	*	*	*	*	0.3	*	1.4	1.2	*	*	1.0	0.8	3.2	7.2	0.8	*	1.1	0.4	46.7	35.6
Sesquiterpenes	21.8	10.0	8.2	1.3	11.2	3.6	43.0	22.7	15.9	12.0	26.2	3.1	32.1	25.5	32.8	8.9	6.8	3.2	0.1	26.2	0.2	0.1
UnID terpenoid compounds	0.3	0.1	*	*	2.4	0.8	1.7	1.5	0.4	0.4	1.7	0.0	2.3	3.7	0.7	0.2	6.1	*	0.4	1.5	8.2	7.5
Nitrogenous compounds	*	*	*	6.4	0.0	0.1	0.1	0.0	*	0.1	0.4	*	*	*	*	0.4	0.6	1.8	*	0.4	*	*
Dimethyl disulfide	*	*	*	*	*	*	*	0.4	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Unidentified	5.0	7.1	*	*	2.8	0.3	0.7	0.6	0.4	0.7	6.1	6.1	3.7	3.9	6.4	4.9	7.4	1.2	12.6	7.0	0.1	0.3

[1], Values represent average relative percentage floral scent composition. Averages based on relative percentage of total peak areas from scent chromatograms of each sample. Asterisks indicate absence or levels below threshold of detection.

female-stage gynoandroecia. The terpenoid-dominated scents emitted by female gynoandroecia are similar in composition to odors of immature flowers and vegetation. Although these maroon-flowered species share many scent compounds and are perceived as 'yeasty' to the human nose, they vary somewhat in their chemical composition (Fig. 2, Table 1).

The floral scent data for *A. triloba* published in Goodrich *et al*. (2006) are included here for completeness. *Asimina triloba* contains ethyl acetate, ethanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone and a pair of butanediol isomers as its major constituents. *Asimina triloba* also contains several nitriles, aldoximes and other nitrogenous compounds which are either absent or present in limited amounts in the other species. Male-stage flowers of *A. triloba* contain a higher overall percentage of aliphatic alcohols and esters than do female-stage flowers. Ethanol represents a particularly high percentage of the total relative peak area for inner whorl petals of female-stage (57.8%) and male-stage (46.6%) flowers. The nitrogenous compounds (nitriles, aldoximes and nitro-compounds) and butanediol isomers are male-stage specific, and were not found in other species. Within the male-stage flowers, the butanediol isomers are only emitted from the outer whorl petals. A majority of the nitrogenous compounds are emitted by the gynoandroecia of male-stage flowers and may represent pollen-specific odors.

The scent of *A. parviflora* is dominated by the same fermentation compounds as *A. triloba*, but lacks butanediols. The flowers of *A. parviflora* produce aliphatic esters and ketones not found in the other species studied here. *Asimina parviflora* is also unusual in its emission of 15-carbon sesquiterpene compounds, including six isomers of α- and β-farnesene and two isomers of zingiberene. *Asimina reticulata*, a white-flowered species (see below), emits three of the farnesene isomers and both zingiberene isomers (Table S1). All other species in this study produce, at most, two farnesene isomers and no zingiberenes. Gender-specific differences in *A. parviflora* are less marked than in the other maroon-flowered species. The floral scents of female- and male-stage gynoandroecia of *A. parviflora* are dominated by 3-hydroxy-2-butanone (33.2% and 81.0%, respectively), whereas female-stage gynoandroecia emit relatively large percentages of germacrene D (19.6%) and *trans*-β-ocimene (12.6%). Inner whorl petals of female- and male-stage flowers emit primarily 3-methyl-1-butanol and 3-hydroxy-2-butanone. Male-stage inner whorl petals also emit a relatively large percentage of a compound whose spectrum is suggestive of methyl amyl ketone (10.5% of total composition), a compound unique to *A. parviflora*. Outer whorl petals of female- and male-stage flowers are dominated by ethyl acetate (18.9% and 31.7%, respectively), 3-hydroxy-2-butanone (39.9% and 29.7%, respectively) and 3-methyl-1-butanol (13.3% and 11.5%, respectively). Interestingly, the percentage of ethyl acetate exceeds that of ethanol in female and male petals of *A. parviflora*. The opposite trend is observed in other maroon-phenotype flowers of *Asimina*, where ethanol is typically 2–40 times more abundant than ethyl acetate. Most aliphatic esters and ketones unique to *A. parviflora* show no gender-specific emission patterns; instead, they vary spatially, dominating the odor of inner whorl petals of both sexual stages.

Asimina pygmaea and *A. tetramera* share most of the oxygenated aliphatic volatiles found in *A. triloba* (including butanediols), but were the only species in this study found to emit anisole, a benzenoid ether. In general, benzenoid compounds comprise a very small percentage of the floral scent for maroon-flowered species. Both *A. tetramera* and *A. pygmaea* also contain small amounts of aliphatic hydrocarbons, which are major constituents of the floral scent of white-flowered *Asimina* species (see below).

As in *A. triloba*, butanediol isomers are restricted to male-stage flowers of *A. tetramera*. However, unlike *A. triloba*, these isomers occur in, and constitute more than 50% of, the floral scent from all dissected organs of male-stage *A. tetramera* flowers. A small amount of 2-methylbutane nitrile occurs in both female- and male-stage petals of *A. tetramera*, but not in gynoandroecia, indicating that it is unlikely to be a pollen odor. Indole, another nitrogenous compound, is emitted in small amounts from the inner and outer whorl petals of male-stage *A. tetramera* flowers, but is absent from female-stage *A. tetramera* and from all other maroon-flowered *Asimina* species. The gin-scented monoterpenes (*Z*)- and (*E*)-β-ocimene comprise 38.2% of the total relative peak area for female *A. tetramera*, but are absent or present in less than 5% of the peak area for all other maroon-flowered species. The butanediol isomers are present in both sexual stages and all floral organs of *A. pygmaea*, except female gynoandroecia, although their percentage is highest in male gynoandroecia and male outer whorl petals (77.0% and 60.8%, respectively). Male-stage petals of *A. pygmaea* produce a small amount of dimethyl disulfide, a sulfur-containing volatile. Dimethyl disulfide was not detected in whole-flower samples of *A. pygmaea*, possibly as a result of a threshold effect for the compound, as only one to two flowers per sample were used for whole-flower experiments, because of the dispersed spacing of flowers on live plants of *A. pygmaea*. Floral organs of female-stage *A. pygmaea* produce relatively high percentages of α-pinene, β-pinene, α-copaene and β-elemene, terpenoid compounds which appear at smaller percentages in male-stage organs and vegetative tissue (data not shown).

White-flowered species The 'sweet,' 'pleasant' and 'waxy' scents of white-flowered species of *Asimina* differ dramatically from the 'yeasty' scents of maroon-flowered species, and show greater variation between species (Fig. 2). White-flowered species of *Asimina* all contain one or more aliphatic aldehydes, which are absent from other species studied here. Their scents also contain large amounts of aliphatic hydrocarbons (for example, *n*-tridecane, *n*-pentadecane and heptadecene) relative to other species in this study. However, white-flowered species lack the 2- and 4-carbon aliphatic alcohols and ketones found in maroon-flowered species, except for *A. longifolia* which emits a small percentage of 3-methyl-1-butanol. Other compound types

unique to white-flowered species of *Asimina* include lilac alcohols and aldehydes, present in all white-flowered species except *A. reticulata*, and several benzenoid alcohols and aldehydes including benzaldehyde, present in all white-flowered species except *A. longifolia*.

Floral scent composition is strongly species specific in white-flowered species of *Asimina*. The floral scent of *A. reticulata* is unique in its high relative percentage of *n*-tridecane, its diversity of farnesene and zingiberene isomers, its lack of lilac compounds and its organ-specific pattern of benzenoid emissions (Fig. 2). *Asimina reticulata* emits the structurally related compounds benzyl alcohol, benzaldehyde, 2-phenyl ethanol and 2-phenylethyl acetate, predominantly from the inner whorl petals of female- and male-stage flowers. A small percentage of 2-phenyl ethanol (0.3%) was detected from male gynoandroecia, possibly as a result of the absorption and subsequent release of 2-phenyl ethanol from surrounding inner whorl petals by the pollenkit. The high percentage of *n*-tridecane is particularly unusual in the female gynoandroecia of *A. reticulata*, as the female gynoandroecia of all other species of *Asimina* are dominated by terpene hydrocarbons.

The floral scent of *A. incana* is unique in its relatively high percentage of benzenoid compounds and its patterns of emission of linalool and the biosynthetically related (*E*)-furanoid linalool oxide, lilac alcohols and aldehydes. Benzenoid compounds comprise between 1.7% and 13.1% of the total relative peak area of female- and male-stage petals. In both female- and male-stage flowers, the percentage of benzenoid compounds is highest in inner whorl petals. Lilac alcohols and aldehydes are emitted exclusively by petals in female- and male-stage flowers, with ratios of alcohols to aldehydes of approximately 1 : 1. This ratio differs from that of *A. longifolia* and *A. obovata*, where the percentage of lilac aldehydes is substantially higher than that of lilac alcohols (approximately 1 : 7 and 1 : 50, respectively). Linalool comprises a relatively high percentage of the total relative peak area for outer whorl petals in both stages, roughly matching the percentages of lilac alcohols and lilac aldehydes. Finally, (*E*)-furanoid linalool oxide represents 6.0% of the total relative peak area in female gynoandroecium scent and 30.8% of the total relative peak area in male gynoandroecium scent of *A. incana*. Linalool oxide also appears in *A. reticulata* and female-stage *D. pulchellus*, but, in both cases, it represents less than 1% of the total relative peak area. A small percentage of indole is found in the scent of whole male-stage flowers of *A. incana*, although it was not detected in male-stage floral organs. This is probably a result of a threshold effect, with dissected floral organs producing a quantity of indole below our threshold of detection.

The floral scent of *A. obovata* is unique in its relatively high percentages of ocimenes and other monoterpene hydrocarbons, its relatively low percentages of aliphatic hydrocarbons, and its patterns of lilac alcohol and aldehyde emissions. Floral organs of *A. obovata* emit lower percentages of aliphatic hydrocarbons than the other white-flowered *Asimina* species. Monoterpene hydrocarbons comprise more than one-half of the total relative peak area for the scent of gynoandroecia and outer whorl petals in female *A. obovata*. These compounds make up over 30% of the total relative peak area for female inner whorl petals and male gynoandroecia. Monoterpene hydrocarbons represent a much smaller percentage of the scent in male-stage petals, which are dominated by lilac aldehydes. Lilac aldehydes are initially emitted from inner whorl petals during the female stage, but are emitted from inner and outer whorl petals during the male stage. A headspace sample eluted with hexane was determined by Stefan Dötterl to contain four stereoisomers of lilac aldehydes in the following relative proportions: (2S,2′S,5S, 1.7%; 2R,2′S,5′S, 48.2%; 2S,2′R,5′S, 47.0%; 2R,2′R,5′S, 3.1%). Lilac alcohols are also present, but in much smaller relative amounts.

Finally, the floral scent of *A. longifolia* is unique in its relatively large percentages of *n*-pentadecane and heptadecene, and its pattern of (*Z*)-β-ocimene, linalool and lilac compound emissions. In addition to its relatively large percentages of *n*-pentadecane and heptadecene, *A. longifolia* has a large percentage of an unknown compound (possibly heptadecadiene, but see Heinze *et al.*, 1998) which has a Kovats index of 1758 and a putative molecular ion of 236 *m*/*z*. Linalool, (*Z*)-β-ocimene and isomers of lilac alcohol and aldehyde are absent from all floral organs of *A. longifolia* except male-stage petals, where they collectively comprise 50.1% of the total relative peak area for inner whorl scent and 79.1% of the total relative peak area for outer whorl scent. The ratio of lilac alcohols and aldehydes is similar to that seen in *A. obovata*, where lilac aldehydes represent a much larger percentage of the floral scent than do lilac alcohols.

Patterns of floral scent within *An. glabra* and *Deeringothamnus*

The major scent components of female- and male-stage flowers of *An. glabra* are 1,8-cineole (46.7% and 35.6%, respectively), 3-pentanyl acetate (27.5% and 32.7%, respectively) and 3-pentanol (8.1% and 8.8%, respectively) (Fig. 3, Table S1). Both female and male flowers of *An. glabra* are dominated by the same compounds; however, 1,8-cineole represents a considerably larger percentage than 3-pentanyl acetate for the total scent composition for female flowers, whereas 1,8-cineole and 3-pentanyl acetate represent roughly equal proportions of the total scent composition for male flowers. No other compounds exhibit noticeable gender-specific patterns within *An. glabra*. Collectively, the 5-carbon oxygenated aliphatic compounds and 1,8-cineole comprise 82.3% of the average female scent and 77.2% of the average male scent for flowers of *An. glabra*, but are either absent or present only in trace amounts (less than 1%) for the other species studied. The fragrance of *An. glabra* is also distinguished by its lack of benzenoid compounds.

Scent chemistry in *Deeringothamnus* differs from that of the other genera studied here (Fig. 3). The odor of *D. pulchellus*

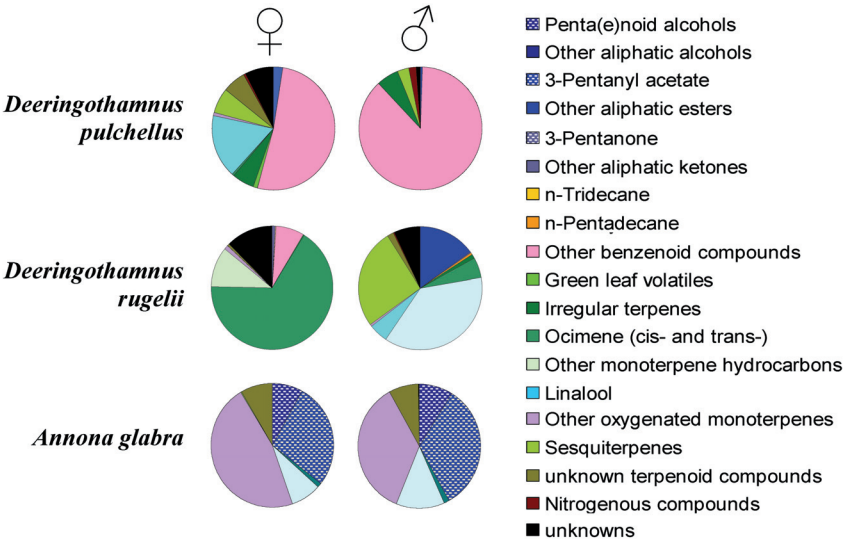

Fig. 3 Relative percentage floral scent composition for female and male ontogenetic stages of *Annona glabra* and *Deeringothamnus*. Key is an abbreviated version of that given in Fig. 2.

is dominated by sweet-smelling benzenoid compounds, which comprise 51.9% of female floral scent and 87.6% of male floral scent. These compounds include veratrole, found only in *D. pulchellus*, and ethyl benzoate, restricted to female-stage flowers of *D. pulchellus* and *D. rugelii*. As flowers of *D. pulchellus* transition to male phase, the percentage of floral scent composed of benzenoids increases substantially (from 51.9% to 87.6%). The fragrance of *D. pulchellus* also contains the pleasant-smelling monoterpene alcohol linalool in female flowers (16.5% of total), but not in male flowers.

The faint, unpleasant scent of *D. rugelii* was highly variable between samples (Table S1). The floral scent of *D. rugelii* is dominated by mono- and sesquiterpene hydrocarbons, including *trans*-β-ocimene, limonene and germacrene D. However, the relative percentage of these compounds varies by ontogenetic stage. The scent of female-stage flowers of *D. rugelii* is dominated by (*E*)-β-ocimene (64.9% of total relative peak area), whereas male-stage scent is dominated by limonene (36.9%), ethyl acetate (15.1%) and germacrene D (13.4%), all of which are absent or present in much smaller percentages in female-stage *D. rugelii* flowers. The scent of *D. rugelii* also contains a relatively large number and percentage of unidentified compounds (40 in total, representing 12.6% of female- and 7.0% of male-stage scent). Both species of *Deeringothamnus* lack bicyclic monoterpenes, such as α-pinene, β-pinene and camphene, which are common in the flowers and vegetation of other species in this study.

Discussion

The floral scent composition of the species included in this study provides a wealth of spatial, temporal and taxon-specific patterns of floral phenotype, suggesting hypotheses about pollination strategies and potential mechanisms of reproductive isolation between species. First, we discuss the scent patterns present in the two broad phenotypes and individual *Asimina* species.

Maroon-flowered species

Asimina parviflora and *A. triloba* differ from the other species of this study by their habitat affiliation (rich organic soils of riparian forests; Fig. 1) and relatively expansive ranges. The floral scents of both species contain compounds that typify fermenting sugars, suggesting that they mimic rotting fruit or sap, which is a likely food substrate and/or brood site for saprophilous insects of temperate forests (Thien *et al.*, 1983). The floral scent of *A. parviflora* also contains several unique aliphatic alcohols and esters. It is possible that these 'ripe fruit' odors are especially attractive to fruit flies and similar insects (Miyake & Yafuso, 2003), whose small body size would render them effective pollinators for the small floral chambers of *A. parviflora*. A pollination study of *A. parviflora* cited drosophilid, milichiid, clusiid and chloropid flies as the most common floral visitors, but larger blowflies (*Lucilia*) and nitidulid beetles were also observed (Norman *et al.*, 1992). Observations of *A. triloba* have shown it to be visited mostly by larger flies (Muscidae and Sarcophagidae), beetles (Coleoptera) and wasps (Hymenoptera) (Willson & Schemske, 1980; M. L. Zjhra, Asian University for Women, Chittagong, Bangladesh, unpublished). Field assays testing insect attraction to alcohols and esters specific to *A. parviflora*, as well as butanediols and nitrogenous compounds specific to *A. triloba*, would directly test the importance of these compounds in insect attraction and their roles in pollinator partitioning between these co-occurring species. Scent emitted from the decaying fruits or sap of co-occurring plant species should also be sampled to determine whether these flowers specifically mimic fermenting substrates within their habitats.

The other maroon-phenotype species of *Asimina* occur in xeric pine-scrub habitats of central and coastal Florida, USA

(Fig. 1). These species share the fermentation volatiles found in *A. triloba* and *A. parviflora*, but also emit compounds absent from the riparian forest species, specifically dimethyl disulfide (*A. pygmaea*) and indole (*A. tetramera*). Dimethyl disulfide and indole contain sulfur and nitrogen, respectively, and are by-products of protein degradation by microbes in carrion or feces (see Jürgens *et al.*, 2006). Their presence in the floral headspace of *A. pygmaea* and *A. tetramera* suggests that these species have expanded beyond the putative mimicry of fermenting fruits suggested for *A. parviflora* and *A. triloba*, to include mimicry of carrion (dimethyl disulfide) and feces (indole), which may be more common and attractive food sources/brood sites to saprophilic insects in xeric pine-scrub habitats. Unlike *A. triloba*, these species truly have 'fetid' scents, as described in Kral's (1960) monograph of *Asimina*. Similar diversity in mimicry-based pollination strategies has been demonstrated in aroids (Kite, 1995; Skubatz *et al.*, 1996; Kite & Hetterschieid, 1997; Stensmyr *et al.*, 2002) and stapeliads (Jürgens *et al.*, 2006). White-flowered *A. incana* emits a small percentage of indole from male-stage flowers; thus, the presence of indole in the rare endemic *A. tetramera* suggests historical gene flow between it and *A. incana* or an extinct relative (see below). *Asimina pygmaea* and *A. tetramera* both contain small percentages of anisole (methoxybenzene). Benzenoid compounds are virtually absent in maroon-flowered species of *Asimina*, and none of the other 'benzenoid-producing' species emit anisole. Finally, both *A. pygmaea* and *A. tetramera* emit small percentages of several aliphatic hydrocarbons, seen at much higher percentages in white-flowered species of *Asimina*. It is possible that these hydrocarbons, together with indole, indicate introgression with co-occurring white-flowered species, as may also be the case for outer whorl petal color in *A. pygmaea* and *A. tetramera*, which is frequently white or white with maroon striations (Fig. 1). Whatever their origin, the volatile hydrocarbons and color variation of these maroon-flowered species may contribute to the attraction of more diverse pollinators in the pine-scrub habitats (as discussed for white-flowered species below), and thus may be maintained by selection. *Euphoria* and *Trichotinus* (Scarabaeidae) are the likely pollinators of *A. pygmaea* (Norman & Clayton, 1986). Species of longhorn beetles (Cerambycidae) have also been observed moving within the inner petals of *A. pygmaea* (K. Goodrich, pers. obs.), but their effectiveness as potential pollen vectors has not been tested. Clearly, more intensive pollinator observations and trapping experiments are needed for *A. pygmaea* to test specific predictions of carrion mimicry.

White-flowered species

The species-specific composition of floral scent in white-flowered species suggests a reward-based pollination strategy ensuring floral constancy (Goulson, 2000), rather than the mimicry-based strategies indicated by yeasty or fetid scents of maroon-flowered species of *Asimina*. Possible floral rewards offered by white-flowered *Asimina* species include copious pollen, floral tissues and exudates secreted by corrugations at the base of inner whorl petals (Norman & Clayton, 1986). The unique scent composition and emission patterns for each of the white-flowered species are outlined above (see Results); here, we discuss the possible implications of pollinator attraction for compounds or classes of compounds present in their floral scents.

First, all white-flowered species contain aliphatic hydrocarbons, which constitute between 6% and 72% of the total relative peak area of their floral scent. Several aliphatic hydrocarbons, including tridecane, pentadecane and heptadecene, are known to be attractive to various beetles (Dobson, 2006) and may mimic insect pheromones (Howard & Blomquist, 2005). Pentadecane and heptadecane are also the main components of the floral scent of *Theobroma cacao*, pollinated by midges (Ceratopogonidae) (Young & Severson, 1994). Alternatively, these waxy hydrocarbons may simply reflect the need to prevent desiccation of larger petals in xeric habitats (Hadley, 1981).

Lilac alcohols and aldehydes make up a substantial (6–14%) percentage of floral scent for *A. incana*, *A. obovata* and male-stage flowers of *A. longifolia*. Lilac compounds are biosynthetically derived from linalool, and are highly attractive to noctuid moths (Plepys *et al.*, 2002; Dötterl *et al.*, 2006). Linalool is common in the scent of many flowers (Raguso & Pichersky, 1999; Knudsen *et al.*, 2006), and linalool and its oxides are dominant components of floral scent for a number of bee-, moth- and butterfly-pollinated flowers (Dobson, 2006). Linalool is present in all white-flowered species, with percentages ranging from 18% to less than 1% of the total relative peak area. No clear patterns are found between linalool and its derivatives; the relative abundance of linalool does not substantially decrease as its derivatives increase, and the highest percentages of linalool (seen in *A. incana*) do not correlate with the highest percentages of linalool derivatives (seen in *A. obovata*). Interestingly, *A. incana* is the only species in this study to emit linalool and both lilac compounds and linalool oxides.

Benzenoid compounds are also characteristic of several white-flowered species of *Asimina*, and are the dominant compounds of white-flowered *D. pulchellus*. Benzyl alcohol, 2-phenyl ethanol, phenylacetaldehyde and benzaldehyde are the primary benzenoid compounds emitted by white-flowered species of *Asimina*, and these compounds are frequently associated with butterfly and moth pollination (Raguso *et al.*, 2003; Huber *et al.*, 2005). These compounds may appeal to a broad spectrum of insects, as they are also emitted from some bee- and beetle-pollinated flowers (Dobson, 2006).

The present knowledge of the compound classes (aliphatic hydrocarbons, monoterpene derivatives and benezenoid compounds) characteristic of white-flowered *Asimina* species suggests that the floral scent of these species may attract a broad assemblage of potential pollinators. However, species-specific odor blends may function primarily through associative learning (Cunningham *et al.*, 2004; Reinhard *et al.*, 2004) to ensure pollinator constancy, or certain odor blends may differentially

attract specific pollinator types within the broader range of potential visitors, allowing at least some degree of pollinator partitioning (Tollsten *et al.*, 1994). In addition, patterns of scent emission between floral organs may act to filter and/or behaviorally manipulate 'appropriate' species-specific pollinators (Dobson *et al.*, 1999).

A study of *A. obovata* reproductive ecology indicates beetle pollination (Norman & Clayton, 1986) and beetles are frequently found within the inner whorl of all white-flowered *Asimina* species (K. Goodrich, pers. obs.). These observations suggest that compounds attractive to beetles should play important roles in the pollination of white-flowered *Asimina* species. In one case, a honey bee was observed entering the inner whorl petals of *A. reticulata* to lick exudates from the corrugations at the base of the petals. The bee traveled between several flowers and had copious pollen on the dorsal surface of its head and thorax (K. Goodrich, pers. obs.). Clearly, more intensive pollinator studies are needed for all white-flowered species of *Asimina* to determine which insects are attracted, and whether they are differentially attracted to the species-specific odor blends. Also, pollinator observations of white-flowered *Asimina* species have not been conducted at night, but the presence of several compounds attractive to moths – especially lilac alcohols and aldehydes – suggests the need for such observations.

Species- and gender-specific patterns of *An. glabra* and *Deeringothamnus*

The abundance of 5-carbon oxygenated aliphatic compounds and 1,8-cineole differentiates *An. glabra* substantially from species of *Asimina* and *Deeringothamnus*. The oxygenated monoterpene 1,8-cineole contributes a strong note of camphor or eucalyptus, whereas 3-pentanyl acetate is suggestive of nail polish products. This latter compound has not been identified in other analyzed floral scents (Knudsen *et al.*, 2006), and its identity was confirmed by consultation with expert chemical ecologists (R. Kaiser, Givaudan, Vernier, Switzerland & W. Francke, Universitat Hamburg, Hamburg, Germany, pers. comm.) and GC–MS verification with a synthesized standard. The scent of *An. glabra* increases in intensity after sunset, becoming exceptionally strong and acetone-like at night. This perceptual description matches those made for many tropical beetle-pollinated Annonaceae (Gottsberger, 1999; Jürgens *et al.*, 2000; Silberbauer-Gottsberger *et al.*, 2003), suggesting that *An. glabra* may also be specialized for beetle pollination. Although beetles have been identified as pollinators for several species of *Annona* (Gazit *et al.*, 1982; Gottsberger, 1989a,b; Nadel & Peña, 1994; Bernhardt, 2000; Tsukada *et al.*, 2005), most pollination studies have been conducted on crops of hybrid *Annona* spp., and pollination studies of native populations of *An. glabra* in Florida are needed.

Species of *Deeringothamnus* (Fig. 1) differ substantially from the floral phenotypes observed in many Annonaceae. Their flowers are small (approximately 1 cm in diameter), with irregular whorls of petals and a markedly reduced number of reproductive organs (Norman, 2003). Such trends typically indicate autogamy (Stebbins, 1970; Lloyd, 1987), but breeding experiments conducted by Norman (2003) indicate a breeding system that is intermediate between autogamy and facultative xenogamy. The floral scent of *D. pulchellus* is not consistent with a shift towards autogamy, as a result of the large amounts of generally attractive benzenoid compounds detectable at a considerable distance. The floral scent of *D. pulchellus* should be attractive to a number of insects, but observations of actual pollinator visits are rare (Norman, 2003). Alternatively, high benzenoid emission may have a repellent effect on destructive herbivorous insects (for example, Borden *et al.*, 2004).

The floral scent of *D. rugelii* is less predictive of reproductive trends. Its GC–MS chromatograms are dominated by pleasant-smelling monoterpenes, but our perception of its floral scent was faint and almost rubbery. This may be a result of the presence of ethyl acetate or as yet unidentified scent components, whose mass spectra are suggestive of sesquiterpene epoxides. We initially considered the possibility that the numerous, low-quantity unknowns were contaminants of the field SPME fibers; however, we re-sampled the floral scent for *D. rugelii* and found identical floral-specific compounds. A high level of variability was noted in the percentages of scent composition between samples of *D. rugelii*. This may be caused by the low volatility and/or low concentration of scent compounds at the detection limits of the SPME/GC–MS methods used for scent collection.

In both species of *Deeringothamnus*, floral visitors are infrequent and fruit set is exceptionally low, between 1.2–1.8% in open-pollinated flowers (Norman, 2003). This poor reproductive success is likely to be a factor in their rarity, and this has led to their both being listed as federally endangered. Although day-time pollinator observations cite very few floral visitors, night-time observations should be carried out for both species. This is especially true for *D. pulchellus*, which has white flowers and a strong jasmine-like fragrance, potentially attractive to moths. Its floral size and architecture (Fig. 1) suggest that noctuid moths would be more likely than sphingid moths to act as pollinators.

Shifts in pollination strategies between temperate and tropical Annonaceae?

The floral scent composition of white-flowered species suggests a generalist pollination strategy, and it would be interesting to test whether certain pollinator affinities exist within a generalist context, as a result of the species-specific floral odors. The maroon-flowered species of *Asimina* are perhaps more functionally specialized, mimicking the scent of decaying organic matter (fruits, and possible carrion or feces), and attracting saprophilous flies and beetles (Willson & Schemske, 1980; Norman *et al.*, 1992; M. L. Zjhra, unpublished). However, both of the floral phenotypes observed in *Asimina* suggest more ecologically generalized pollination strategies than those of most tropical

Annonaceae. Available data reveal floral scent associated with highly specialized pollinator relationships among tropical Annonaceae. Fruity scents dominated by aliphatic or benzenoid alcohols and esters are associated with 'small beetle pollination syndrome' (*sensu* Gottsberger, 1989a) in *Anaxagorea prinoides* (Teichert, 2008), *Anaxagorea brevipes*, *Anaxagorea dolichocarpa*, *Duguetia asterotricha*, *Rollina insignis* and *Xylopia benthamii* (Jürgens *et al.*, 2000). Mushroom-like odors are implicated in the attraction of mycetophagous beetles for both *Duguetia cadaverica* (Teichert, 2008) and *Uvaria elmeri* (Nagamitsu & Inoue, 1997). Spicy floral odors similar to spearmint, lemon grass and vanilla are associated with pollination by perfume-collecting male euglossine bees in *Unonopsis stipitata* (Teichert, 2008) and *Unonopsis guatteroides* (Carvalho & Webber, 2000). Taken together, the patterns above support the hypothesis that temperate species of Annonaceae may adopt more generalist pollination strategies than tropical species, but more data on pollinator spectrum and effectiveness are needed for all species of *Asimina*, as well as for tropical Annonaceae.

Conclusions and prospectus

The data presented here reveal that floral scent in temperate North American *Asimina* and *Deeringothamnus* species shows complex spatial and temporal patterns in nonrandom association with floral architecture and color. These chemical data contain patterns that suggest clear, testable hypotheses about the identity and effectiveness of floral visitors, and indicate at least one major evolutionary transition between honest (white-flowered) and putatively deceptive (maroon-flowered) pollination strategies among *Asimina* species. Detailed pollinator observations are needed for a number of species, together with manipulative experiments testing the interaction between odor and color, or odor differences between floral parts, in pollinator behavior. Eventually, field observations of pollinators for each species will be combined with the floral trait data, as well as a phylogenetic hypothesis. This work will provide a model by which to examine larger questions of angiosperm speciation and evolution.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Detailed methods

Table S1 Average relative percentage scent composition for female and male flowers of *Annona glabra*, *Asimina* and *Deeringothamnus*

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