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Original Article

Identity and localization of floral scent components in an androdioecious species, *Chionanthus retusus* (Oleaceae)

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ABSTRACT

Chionanthus retusus Lindl. & Paxton (Oleaceae) is an androdioecious species, in which male individuals coexist with hermaphroditic individuals. Even though the floral scent dimorphism of dioecious taxa has been reported elsewhere, little is known about the floral scent compounds of androdioecious species. The aim of the present study was to elucidate the identity and localization of floral scent components in *C. retusus* in both male and hermaphrodite. The headspace of whole flowers exhibited quantitative and qualitative floral scent dimorphism. Among several constituents, including phenylethyl alcohol, benzeneacetonitrile, 2-hexen-1-ol were the main fragrance component of both sexual morphotypes. In particular, the abundance of phenylethyl alcohol (2-phenylethanol), which is one of the compounds in pollen and pollenkitt, in both sexual morphotypes might provide indirect evidence for the viability and functionality of pollen grains. However, 3-hexen-1-ol, 1-hexanol, 3-hexenyl 2-metylbutanoate, alloaromadendrene, and farnesol were only detected in hermaphrodites. These patterns of floral scent difference suggest that several compounds solely detected in each sexual type are associated with specific reproductive organs. Comparative micromorphological and ultrastructural studies of floral surfaces (e.g. floral stomata, calyx glands) and pollen grains (especially pollenkitt) revealed that all represent a putative secretory structures and materials.

Introduction

In many angiosperms, floral volatiles play important roles in the chemical communication involved in the interactions of plants and insects (e.g. pollinators, herbivores; Pellmyr and Thien 1986; Knudsen and Tollsten 1993; Dobson 1994; Miyake et al 1998; Dobson and Bergström 2000; Custódio et al 2006; Tsuji and Sota 2010, 2013; Dötterl et al 2014). Floral scents contain a variety of volatile organic compounds (VOCs), which are classified into seven major groups (aliphatics, benzenoids, and phenylpropanoids, C5-branched compounds, terpenoids, nitrogen-containing compounds, sulfur-containing compounds, and miscellaneous cyclic compounds). Furthermore, more than 1700 compounds have been identified through the study of 990 taxa from 90 families and 38 orders (Knudsen et al 2006).

The spatial and temporal variation of floral VOCs has been studied, especially in regard to different floral parts (Dobson et al 1990; Knudsen and Tollsten 1991; Pichersky et al 1994; Bergström et al 1995; Mactavish and Menary 1997; Dötterl and Jürgens 2005; Custódio et al 2006) and different flowering stages (Schade et al 2001; Kumano and Ymaoka 2006; Wang et al 2018). Furthermore, sexual dimorphism in floral scent has also been reported for a variety of flowering plants, especially dioecious taxa (Tollsten and Knudsen 1992; Flamini et al 2002; Dufa et al 2004; Tsuji and Sota 2010; Okamoto et al 2013; Dötterl et al 2014; Milet-Pinheiro et al 2015). In angiosperms, the evolution of separate sexes (dioecy) from combined sexes (hermaphroditism) is often accompanied by sexual dimorphism in floral scent, as well as in phenotype (Ashman 2009).

However, the floral scent dimorphism of intermediate sexual systems that include both hermaphrodites and males (androdioecy) or both hermaphrodites and females (gynodioecy) have a received little attention (Nogueira et al 2001; Ashman et al 2005), and few studies have performed comparative analyses of floral scent compounds or the localization of scent-emitting structures.

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The Chinese fringe tree *Chionanthus retusus* Lindl. & Paxton (Oleeae-Oleaceae) is a deciduous tree that reaches 20 m in height and is mainly distributed in Eastern Asia (Chang et al 1996) and cultivated in various countries as an ornamental tree because of its production of small, white, fringe-like flowers (Huxley et al 1992). This species has oblong to ovate or obovate leaves with anomocytic stomata in abaxial and peltate glands and nonglandular trichomes in both surfaces. Inflorescences can be cymose panicles, and fruits are bluish black or black drupes (Song et al 2011). Recently, reproductive morphological study of the *C. retusus* suggest that this species is an androdioecious species in which male individuals coexist with hermaphroditic individuals, and both morphotypes produce fertile pollen grain (Song et al 2016).

The aim of the present study was (1) to confirm the floral scent dimorphism of *C. retusus* by comparing the main VOCs of hermaphrodite and male individuals and (2) to identify scent-emitting structures using electron microscopic analyses. These data will be valuable for future research regarding floral scent dimorphism and the microstructures involved in intermediate sexual systems.

Material and methods

Materials

The present study was based on the investigation of living material (Figure 1), mainly collected from Wolgok-dong (37°35'54.8"N; 127°02'32.2"E) in Seongbuk-gu, Seoul, Korea, and all vouchers were deposited in KHUS (Herbarium of Kyung Hee University, Seoul, Korea).

Floral scent analyses

For floral scent analyses, three flowers were randomly sampled from each morphotype. Whole flowers (0.3 g) of each morphotype were hermetically sealed in a 10-ml vial that was equipped with a silicone septum and aluminum cap (crimp cap; Supelco, Bellefonte, PA, USA). Polydimethylsiloxane solid-phase microextraction fiber (100 μm) was conditioned in a GC injector at 270°C for 30 min and then exposed to the headspace of the sample vial at 50°C for 30 min. Gas chromatography-mass spectrometry analyses were carried out using a gas chromatograph (6890 Series; Hewlett-Packard, Palo Alto, CA, USA) and a mass selective detector (Agilent-5973 N; Agilent Technologies, Wilmington, DE, USA) that was equipped with 5% phenyl polydimethylsiloxane (DB-5MS 30 m × 0.25 mm i.d., film thickness 0.25 μm). The oven temperature was initially maintained at 40°C for 5 min and then programmed, heated to 200°C for 10 min at rate 5°C/min. The temperatures of the injector, ion source, and quadrupole were set to 270°C, 230°C, and 150°C, respectively, and He (99.999%) was used as the carrier gas at 1.0ml/min under split mode (split ratio 10:1). The mass spectrometer was run in the electron impact mode with electron ionization energy at 70 eV, scanning 40 to 400 amu.

Microscopic analyses

For microstructural observations, all floral parts (calyx, corolla, anther, filament, and ovary) of the individuals used for floral scent analyses were dehydrated using an acetone series (30–60 min each in 50, 70, and 90% acetone, followed by ~60 min in absolute acetone). The completely dehydrated materials were immersed in carbon dioxide for critical point drying (SPI-13200J-AB; SPI Supplies, West Chester, PA, USA). The resulting dried materials were fixed to aluminum stubs using double adhesive carbon tape, and the stubs were then coated with gold (Au) using an ion-sputtering device (E-1045; Hitachi, Tokyo, Japan). Finally, the floral organs were visualized using a field-emission scanning electron microscope (S-4700; Hitachi) at an accelerating voltage of 10 kV with a 10–13 mm working distance.

For ultrastructural observations of the pollen, anthers of each morphotype were fixed in modified Karnovsky's (1965) fixative at room temperature for 2 h, rinsed with 0.05 M sodium cacodylate buffer (pH 7.2), and then postfixed in 1% osmium tetroxide (OsO_4)

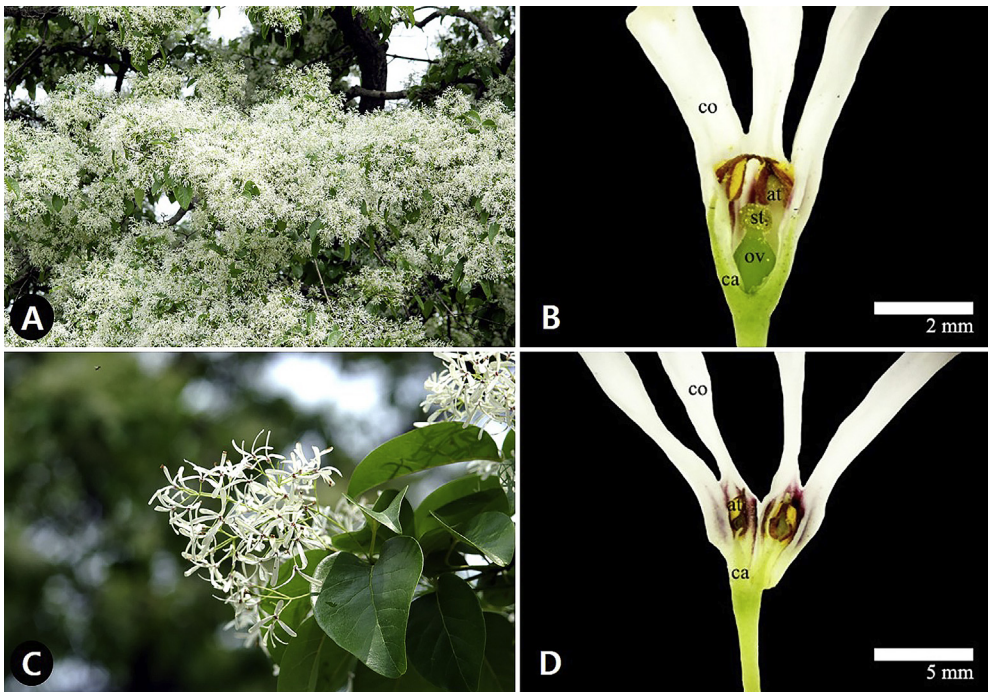

Figure 1. Photographs of *Chionanthus retusus*: A and B, Hermaphrodites; C and D, Males; A and C, Habit; B and D, Flower; at = anther; ca = calyx; co = corolla; ov = ovary; st = stigma.

at 4°C for 2 h. *En bloc* staining was carried out overnight in 0.5% uranyl acetate at 4°C. The stained anthers were then dehydrated in a graded ethanol series, following a step with propylene oxide for transition and infiltrated and polymerized using Spurr's (1969) resins at 70°C for 24 h. Sections were cut using an MT-X Ultramicrotome (RMC; Boeckeler Instruments, Tucson, AZ, USA), stained in 2% uranyl acetate for 7 minutes at room temperature and stained again in Reynold's lead citrate for 7 min at room temperature. The stained sections were placed on copper grids, examined using an energy-filtering transmission electron microscope (TEM, LIBRA-120; Carl Zeiss, Oberkochen, Germany), and photographed.

Results

Floral scent composition

Through the use of headspace solid-phase microextraction–gas chromatography-mass spectrometry analysis, the present study detected and identified 14 main compounds and 10 compounds, respectively (Table 1 and Figure 2). The headspace of whole hermaphrodite flowers was mainly composed of aliphatics, which accounted for 43.90%, whereas that of male flowers was benzenoids/phenylpropanoids, which accounted for 68.62% of total floral scent emission. Five of these compounds (3-hexen-1-ol, 1-hexanol, 3-hexenyl 2-metylbutanoate, alloaromadendrene, farnesol) were only detected in the hermaphrodite flowers, whereas one (phenylacetaldehyde) was only detected in male flowers (Table 1 and Figure 2). In addition, the abundance of aliphatic compounds was greater in the scent of the hermaphrodite flowers (43.9%) than in that of the male flowers (8.64%), whereas the abundance of was greater in male flowers (68.62%) than in hermaphrodite flowers (35.64%). The main floral scent components of the hermaphrodite and male flowers were phenylethyl alcohol (15.59 and 41.42%, respectively), benzeneacetonitrile (20.05 and 23.16%), 2-hexen-1-ol (11.48 and 5.29%), and 3-hexen-1-ol (21.95% and not detected; Table 1 and Figure 3).

Microstructural and ultrastructural features

Except for the ovaries of hermaphrodite flowers, the microstructures and ultrastructures of floral scent-emitting parts of the hermaphrodite and male flowers were very similar (Table 2). Capitate-sessile multicellular glandular trichomes and floral stomata were found on the outer calyx surfaces of both morphotypes (Figures 4A–B and 5A–B) but not on the inner calyx surfaces of either (Figure 4C). The floral stomata were distributed on the outer corolla epidermis of both morphotypes (Figures 4D and 5C). There were no structures on the inner corolla, which was composed of papillose epidermal cells (Figure 5D). Floral stomata were also found on the filaments of both the hermaphrodite and male flowers (Figure 4E). The floral stomata were also found on the surface of the hermaphrodite ovaries (Figure 4F). The pollen grains of both morphotypes were tricolpate with reticulate sexine ornamentation (Figures 4G and 5E), and pollenkitt was accumulated in the infratectum of the pollen grains (Figures 4H and 5F).

Table 1. Constituents and average compositional percentage of the floral scent of whole flowers from hermaphrodite and male in *Chionanthus retusus* by HS-SPME-GC/MS.

Class	Compound	Composition (%)	
		Hermaphrodite	Male
Aliphatics	3-hexen-1-ol	21.95	-
	2-hexen-1-ol	11.48	5.29
	1-hexanol	6.64	-
	1-octanol	3.83	3.35
Benzenoids and phenylpropanoids	phenylacetaldehyde	-	4.04
	phenylethyl alcohol	15.59	41.42
	benzeneacetonitrile	20.05	23.16
C5-branched chain compounds	3-hexenyl 2-metylbutanoate	6.31	-
Terpenes (Sesquiterpenes)	alloaromadendrene	3.34	-
	farnesol	2.50	-
Unknown	unknown alkane 1	4.51	5.44
	unknown alkane 2	0.91	1.23
	unknown alkane 3	1.26	11.97
	unknown aldehyde	1.63	4.1

HS-SPME-GC/MS = headspace solid-phase microextraction–gas chromatography-mass spectrometry.

Discussion

The present study demonstrated that *C. retusus*, an androdioecious species (Figure 1B, D), exhibits both quantitative and qualitative floral scent dimorphism and identified putative emission structures in both hermaphrodite and male flowers.

The evolution of dioecy from hermaphroditism is related to sexual dimorphism in floral scent (Ashman 2009). Furthermore, these sexual dimorphic scents can be associated with specific reproductive organs, and emitted compounds can vary among floral organs (Dobson and Bergström 2000). Despite the high degree of similarity in the floral scent composition of sexual morphs, differences have also been reported (Tollsten and Knudsen 1992; Ervik et al 1999; Knudsen et al 1999; Grison-Pigé et al 2001; Flamini et al 2002; Dufa et al 2004; Custódio et al 2006; Tsuji and Sota 2010; Okamoto et al 2013; Dötterl et al 2014; Milet-Pinheiro et al 2015). However, comparative scent analysis of androdioecious species has only been reported for one androdioecious species, *Clusia nemorosa* G. Mey. (Clusiaceae) and has been briefly discussed their sexual variations of floral scent (Nogueira et al 2001).

The present study demonstrated that *C. retusus* exhibits both qualitative and quantitative floral scent dimorphism (Figures 2 and 3; Table 1). The qualitative differences suggest that the emission of certain compounds are organ-specific (Vogel 1990; Dudareva and Pichersky 2000; Miyake and Yafuso 2003; Ashman et al 2005). In particular, Pichersky et al (1994) suggested that floral scent compounds emitted by female organs are distinctly different from those emitted by stamens. To verify organ-specific scent emission, it would be necessary to analyze isolated flower organs.

In the present study, the most abundant floral scent compounds in *C. retusus* were phenylethyl alcohol and benzeneacetonitrile (Table 1), and a quantitative difference was observed between the phenylethyl alcohol (2-phenylethanol) emission of hermaphrodite and male flowers (Figure 3). This compound has been described previously, as a generalist-attracting compound, from the flowers of *Rosa rugosa* Thunb. (Dobson et al 1990), *Laurus nobilis* L. (Flamini et al 2002), and *Trimenia moorei* (Oliv.) Philipson (Bernhardt et al 2003). Moreover, Dobson and Bergström (2000) suggested that phenylethyl alcohol is one of the compounds in pollen and pollenkitt, which is a pollen-binding agent found in almost all entomophilous plants (Hesse 1993; Pacini and Hesse 2005). The present study determined that the pollenkitt of *C. retusus* accumulates in the infratectum of both hermaphrodite and male pollen grains (Figures 4H and 5F). Moreover, the abundance of phenylethyl alcohol was greater in male flowers than in hermaphrodite flowers (~2.66 times), and it is possible that this difference is related to pollen production because pollen production was ~1.8 times higher in the male flowers than in the hermaphrodite flowers (Song et al 2016). Therefore, phenylethyl alcohol might be associated with pollen and pollenkitt production and could indicate the viability and functionality of pollen grains. Phenylethyl alcohol is reportedly

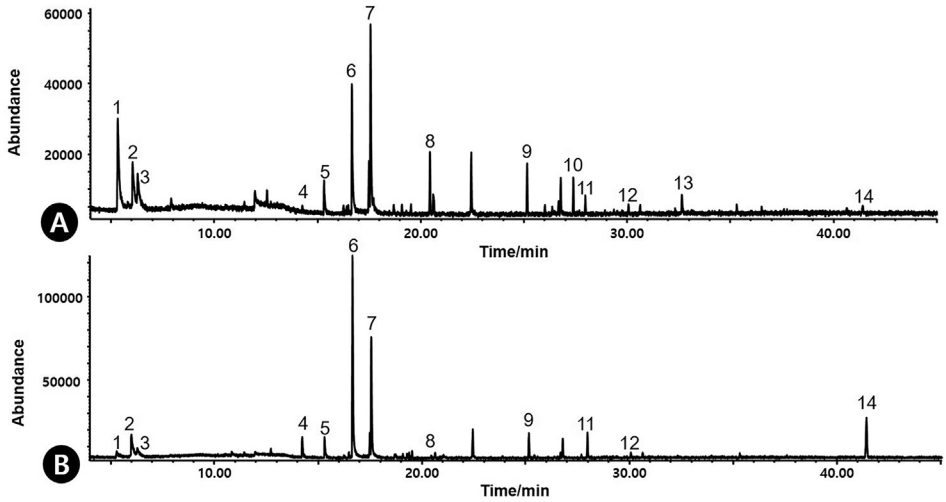

Figure 2. Chromatograms of the volatiles of whole flowers by GC-MS: A, Hermaphrodites; B, Males. Peak Identification; 1, 3-hexen-1ol; 2, 2-hexen-pl; 3, 1-hexenol; 4, phenylacetaldehyde; 5, 1-octanol; 6, phenylalcohol; 7, benzeneacetonitrile; 8, 3-hexenyl 2-methylbutanoate; 9, unknown alkane 1; 10, alloaromadendrene; 11, Unknown aldehyde; 12, unknown alkane 2; 13, farnesol; 14, unknown alkane 3.

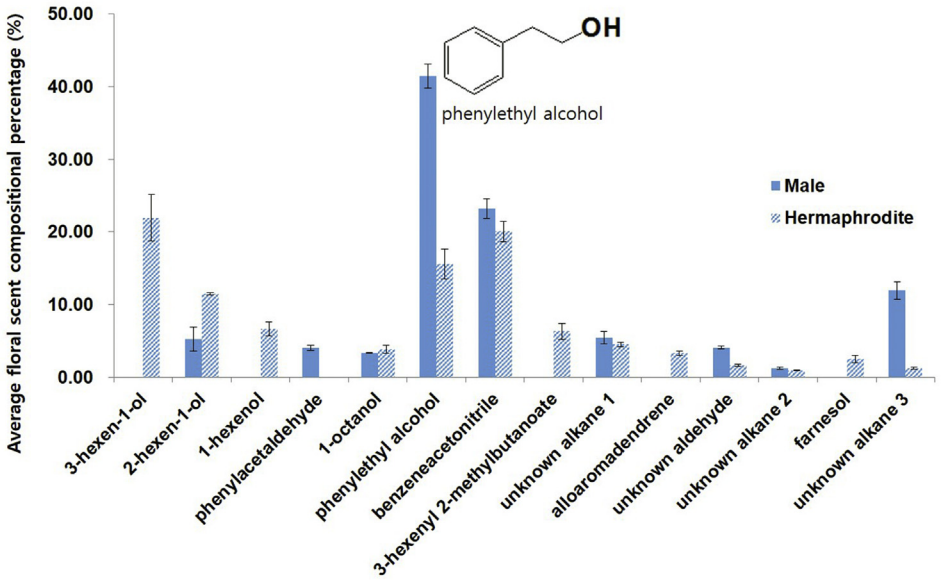

Figure 3. Average percentage of major floral volatiles composition emitted by flower of different sexual morphs in *Chionanthus retusus* (hermaphrodites $n = 3$; males $n = 3$).

one of the most attractive scents to bees and a variety of other flower-visiting insects (Raguso 2004). Although no pollinators were observed during the present study, several previous reports have suggested that *C. retusus* is pollinated by bees (Elisabeth Carey Miller Botanical Gardens 2019; The National Gardening Association 2019). A previous study of the pygmy-fringe tree (*C. pygmaeus* Small), failed to document any diurnal pollinators, which suggested that the species could be visited by nocturnal moth pollinators (Stout 1993). However, Wunderlin et al (1980) reported that honey bees (*Apis mellifera*) visited the *C. pygmaeus* flowers. Therefore, further studies that include field observations of pollinators are needed to clarify the plant-pollinator interactions of *Chionanthus* species.

Table 2. Putative floral scent emission structures in flowers from hermaphrodite and male in *Chionanthus retusus*.

Floral organs	Calyx		Corolla		Filament	Pollen	Ovary
	Outer surface	Inner surface	Outer surface	Inner surface			
Hermaphrodite	g, s	-	s	-	s	pk	s
Male	g, s	-	s	-	s	pk	N/E

g = glandular trichome; s = floral stomata; pk = pollenkitt, - = absent; N/E = not applicable.

Benzeneacetonitrile, which was detected for another abundant floral scent compound, is found in various aromatic flowers such as *Pachira aquatica* Aubl. (Malvaceae) (Zoghbi et al 2003), *Garcinia macrophylla* Mart. (Clusiaceae) (Andrade et al 2007), *Moringa oleifera* (Moringaceae), and *Persea americana* (Lauraceae) (Stashenko and Martínez 2018). Besides, the highest amounts, 3-hexen-1-ol and 2-hexen-1-ol in aliphatics are known as green leaf volatiles (Matsui 2006). These compounds are related to fresh green fragrance associated with fruits and vegetables (Jensen et al 2001;

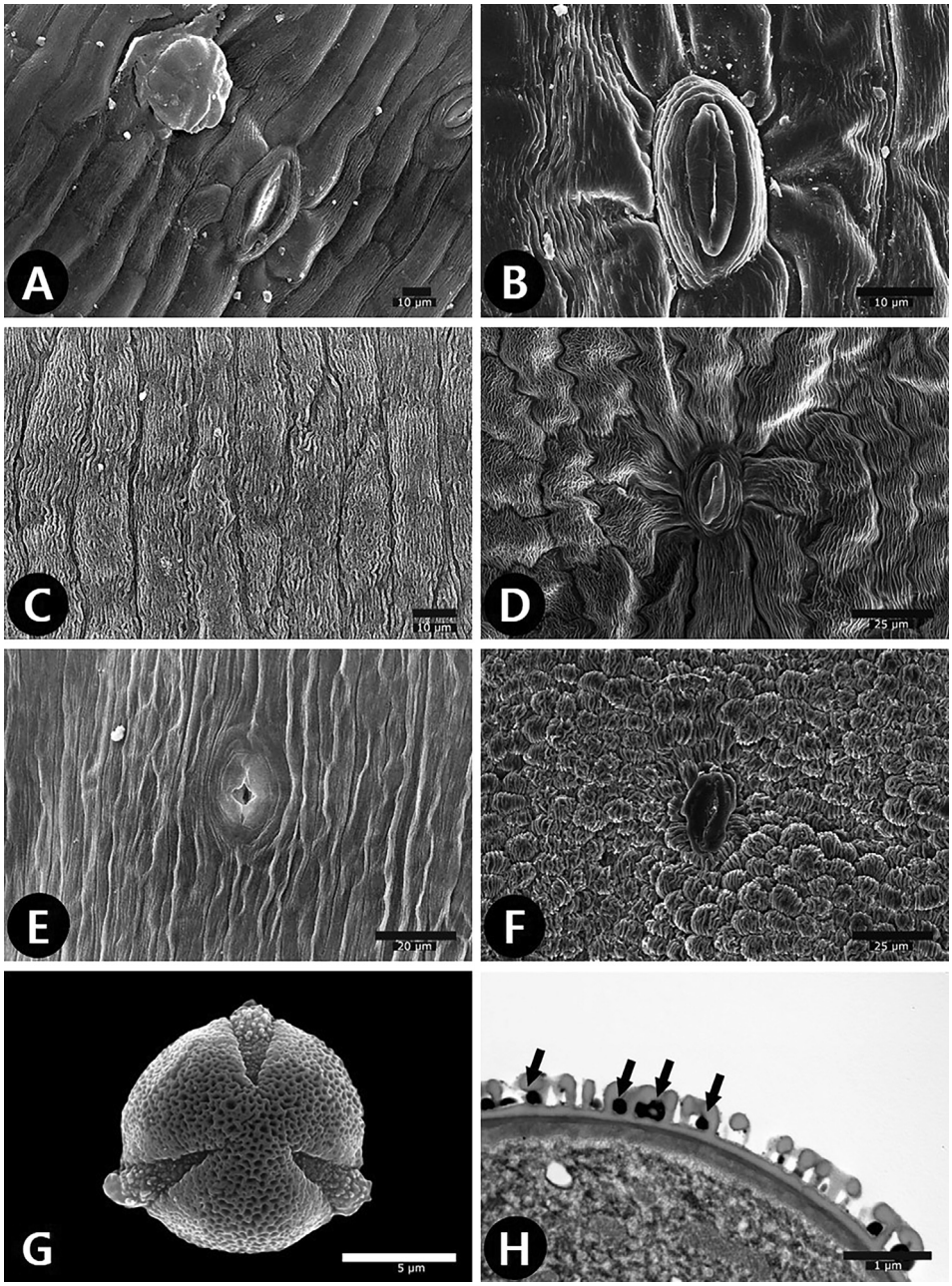

Figure 4. Scanning electron microscope and transmission electron microscope micrographs of floral scent emission structures of hermaphrodites in *Chionanthus retusus*: A and B, outer surface of calyx; A, glandular trichome and floral stomata; B, floral stomata; C, inner surface of calyx; D, outer surface of corolla (floral stomata); E, surface of filament (floral stomata); F, ovary surface (floral stomata); G, pollen grains (polar view); H, wall stratification of pollen grain (black arrows indicate pollenkitt).

Poll and Lewis 1986). Thus, 3-hexen-1-ol, which is solely detected in hermaphroditic flowers, and 2-hexen-1-ol, which is detected in both male and hermaphrodite, may be the scent that is emitted from gynoecium and calyx, respectively.

The present study identified two putative floral scent-emitting structures: floral stomata and capitate-sessile multicellular glandular trichomes (Figures 4 and 5; Table 2). The presence of stomata among the epidermal cells of flowers is not that uncommon (Effmert et al 2005). However, the floral stomata identified here were frequently observed on the outer surfaces of both the corolla and calyx. Vogel (1962) and Skubatz et al (1995) suggested that floral stomata on abaxial surfaces are involved in gas exchange, owing to the intensive metabolism caused by volatile production. Although the present study did not find that floral stomata are directly involved in volatile emission, the structures could still be related to floral scent emission. Several taxa of the Lamiaceae, Solanaceae, and Orchidaceae store various amounts of secretory materials in glandular trichomes on their corollas (Effmert et al 2006). Moreover, glandular trichomes may be a source of floral scents (Effmert et al 2006) and, as primary and secondary attractants, may mediate interactions with floral visitors (Possobom et al 2015). In the present study, capitate-sessile multicellular glandular trichomes were only found on the outer calyx surfaces (Figures 4A and 5A). These glands may mediate interaction with pollinators.

In conclusion, the present study demonstrated that *C. retusus* exhibits both quantitative and qualitative floral scent dimorphism.

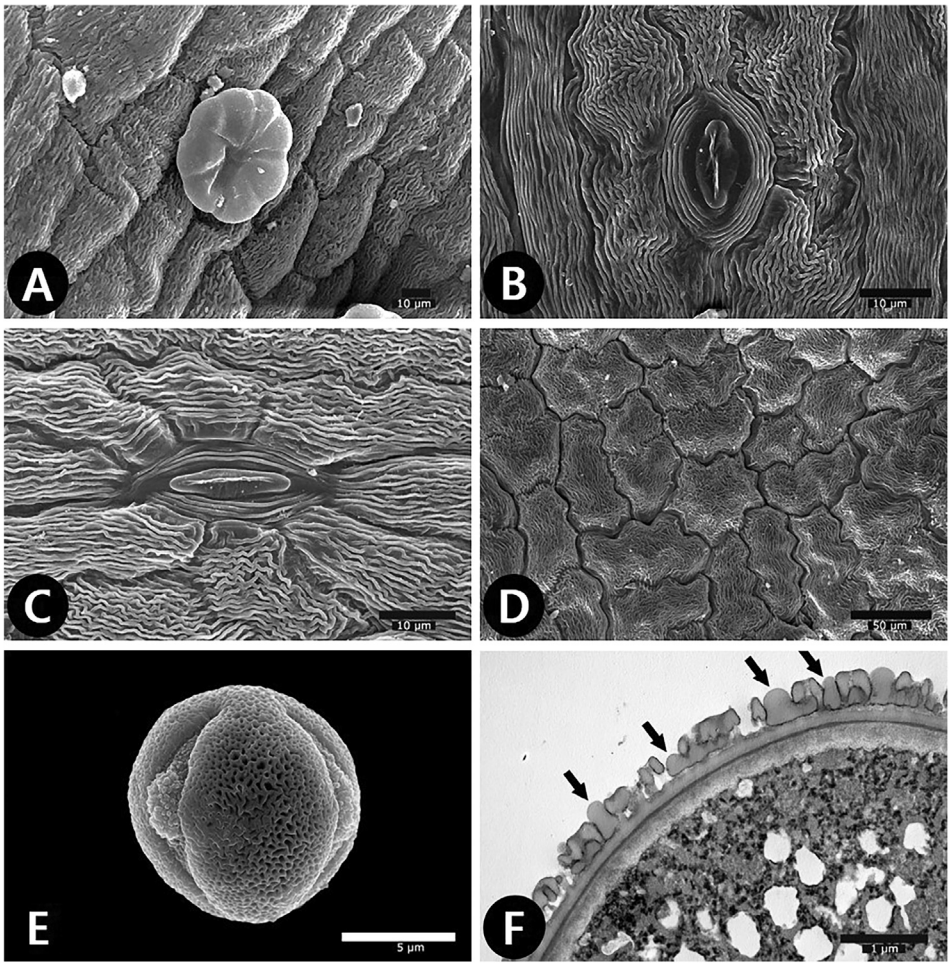

Figure 5. Scanning electron microscope and transmission electron microscope micrographs of floral scent emission structures of males in *Chionanthus retusus*: A and B, outer surface of calyx; A, glandular trichome; B, floral stomata; C, outer surface of corolla (floral stomata); D, inner surface of corolla; E, pollen grains (equatorial view); F, wall stratification of pollen grain (black arrows indicate pollenkitt).

Moreover, the abundance of phenylethyl alcohol (2-phenylethanol) was identified as one of the compounds in pollen and pollenkitt. It is necessary to assess the chemical profiles of both isolated flower organs (e.g. anther, pollen, pistil, corolla, and calyx) and the sexual morphotypes to further elucidate the association between specific scent compounds and organs. Even though the present study does not provide evidence for a direct association between floral scent and either floral stomata or calyx glands, the identification of scent compounds should facilitate future studies of floral scent diversity and scent-emitting structures in sexually dimorphic species.

Conflict of interest

The authors declare that there is no conflict of interest.

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