



FLORAL FRAGRANCE VARIATION IN *CYPRIPEDIUM*: IMPLICATIONS FOR EVOLUTIONARY AND ECOLOGICAL STUDIES

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Abstract—The floral fragrance compositions of eight species (including two putative varieties) of *Cypripedium* were determined by GC-mass spectrometry. Substantial diversity in fragrance compound skeletal type was detected and included benzenoid, terpenoid and fatty alcohol derivatives. Qualitative fragrance variation was investigated within and between two populations of *Cypripedium calceolus* var. *parviflorum*. The clustering algorithm, unweighted pair group method arithmetic average (UPGMA) was used as an objective analytical means to assess population-level fragrance variation relative to species-level variation. Members from two populations of *C. calceolus* var. *parviflorum* formed a single cluster with similarity values ranging from 0.36 to 1.0, indicating a high degree of biogenetic uniformity among some individuals as well as substantial relative divergence between others. The results of this study indicate that conclusions about taxonomic or pollinator relationships, based upon limited infraspecific sampling of floral fragrance, should be considered tentative until the extent of population variation is investigated. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

The taxonomy and floral fragrance composition of *Cypripedium* L., the temperate genus of ladyslipper orchids, has recently received attention. The genus is of interest from a taxonomic standpoint because of unresolved relationships among many of its members. Particularly problematic is the yellow ladyslipper *C. calceolus*. Atwood [1] and Bergstrom *et al.* [2] suggested that two eastern North American taxa, *C. calceolus* var. *parviflorum* and *C. calceolus* var. *pubescens*, be recognized as distinct species on the basis of morphology and floral fragrance compound variation, respectively, while Case [3] recommended varietal status based upon allozyme variation. Ladyslipper floral fragrance composition has been investigated because of the interesting pollination syndrome exhibited by these species. The nectarless flowers reportedly depend upon deception of solitary bees to entice their entrance into the semi-trap labellum [4]. Upon exiting the labellum, insect visitors pass the stigma and anthers, either depositing and/or receiving pollen during the process [5, 6]. Volatile compounds, such as fatty alcohol acetates and farnesene produced by *C.*

calceolus have been implicated in the deception of certain bees which visit this species because the same compounds are used by the insects as pheromones and to signal the presence of nest sites, respectively [3].

While floral fragrance variation has been utilized previously in taxonomic and pollination ecology studies in the Orchidaceae [2, 4, 7, 8], objective methods of analysis of these data are rarely utilized (see refs [9] and [10] for exceptions). In addition, few studies compare the variation within and among populations of the same species or across a wide range of diverse but related taxa. We argue that these comparisons are necessary in order to assess the significance of detected variation. Phenetic analyses utilizing clustering algorithms provide an objective numerical technique for comparing data among taxa or individuals. The results obtained include a phenogram with discrete values representing the relative degree of similarity between any two clusters of taxa [11]. In this paper we used phenetic methods to assess the extent of floral fragrance variation within *C. calceolus*. We examined variation within *C. calceolus* var. *parviflorum* and compared it to that observed between other species and varieties of *Cypripedium*. In addition, we included

data recently published by Bergstrom *et al.* [2] in our analysis.

RESULTS

The fragrance compositions of all taxa investigated varied quantitatively and qualitatively. Table 1 lists all 67 compounds isolated from the nine taxa sampled. Although the compounds are listed in terms of a percentage of the total composition, this quantitative variation was not incorporated in the phenetic analysis due to experimental, environmental, and developmental factors reported to affect it [12–15]. For the purpose of this study 'absence' of a compound is that quantity which is lower than the threshold for detection as determined for our instrument (*ca* 1–5 ng injected for limonene and benzaldehyde using full scanning measurement). No two taxa produced more than five compounds in common. *C. arietinum* produced relatively few compounds (all benzenoid derivatives), which were hardly detectable by our analytical methods. *C. candidum* produced the same compounds found in *C. arietinum* as well as linalool and two additional benzenoid derivatives. The richest floral fragrance was found within *C. acaule*, which produced terpenoid, benzenoid, and fatty alcohol derivatives. This species and *C. macranthum* both produced lilac aldehyde and lilac alcohol isomers. In spite of these similarities they differed in all other compounds produced. *C. acaule* also produced many compounds identified from *C. guttatum*. Although these species look very dissimilar, they both yielded numerous identical fatty acid methyl esters.

C. kentuckiense was the only taxon sampled which produced fatty alcohol acetates. On the basis of floral morphology, this taxon looks similar to *C. calceolus* var. *parviflorum* and *C. calceolus* var. *pubescens*, however, they share few compounds between them. Although the latter two varieties can be difficult to distinguish on the basis of floral characters they appear quite distinct in terms of fragrance chemistry with *C. calceolus* var. *parviflorum* producing a variety of terpenoids, none of which are found in *C. calceolus* var. *pubescens*. Finally, *C. reginae* produced only methyl benzoate and methyl salicylate, both of which were not found in any other taxon. Bergstrom *et al.* [2] reported a similar wide diversity of fragrance compound structures within *Cypripedium*.

Table 2 shows all of the compounds isolated from 15 individuals of *C. calceolus* var. *parviflorum* from two populations. Although relative percentages of compounds differed among individuals, only qualitative compound variation was expressed (either present (1) or absent (0)) due to uncontrolled factors reported to affect quantitative variation. Benzaldehyde and linalool were present in all individuals sampled, but other compounds, such as limonene and amino-benzaldehyde, were isolated from only one clone (R4). Individuals R2 and R3 are nearly identical in sharing 10 of 12 compounds, while R6 and R7 share

only 4 of 12. Although casual inspection of the table allows subjective comparison of individuals, a numerical analysis of all *Cypripedium* species examined as well as those sampled by Bergstrom *et al.* [2] was carried out to assess the significance of the observed variation.

In order to perform the phenetic analysis, compound data were coded as independent characters as recommended by Sneath and Sokal [11]. This coding process required explicit assumptions about the biogenetic origins of the compounds identified. For instance, in several taxa, co-occurring compounds differed only by simple oxidation changes such as that observed between benzaldehyde and benzyl alcohol. These compounds were assumed to be non-independent due to a shared biogenetic pathway and were therefore treated collectively as a single character in the phenetic analysis. The 4-methoxy substitution found on benzaldehyde and benzyl alcohol, however, was treated as an independent character as this is a more significant modification than a dehydrogenation and is found in a restricted subset of taxa. In another case, four stereoisomers of lilac alcohol and lilac aldehyde, respectively, were detected in both *C. acaule* and *C. macranthum*. In this case the isomers collectively were coded as a single character. The other instance in which we treated multiple compounds as a single character involved the fatty alcohol derivatives. In *C. kentuckiense*, fatty acid acetates ranged in length from 10 to 18 carbon atoms. It seems likely that a single enzyme was responsible for the acetylation of these compounds in this taxon and therefore they were treated as a single character in the phenetic analysis. This coding assumption was also used for the fatty acid methyl and ethyl esters found in *C. acaule* and *C. guttatum*, which ranged in length from 8 to 12 carbon atoms. In this way our coding scheme represented putative independent biogenetic steps as characters for analysis. Due to the complex nature of terpenoid production no assumptions were made about the biogenesis and interconversions between these compounds which resulted in each compound coded as a single character. The outline of our coding approach allows other researchers to evaluate alternative assumptions about the biogenesis of these compounds for subsequent analyses.

Phenetic analyses resulted in calculated interspecific similarity values which ranged from 0.50 to 0.00, whereas intraspecific phenetic similarity of *C. calceolus* var. *parviflorum* ranged from 1.0 to 0.30 (a complete list of pairwise similarity values is available upon request). Figure 1 shows the results of the cluster analysis. The tree topology shows that individuals of *C. calceolus* var. *parviflorum* were more similar to each other than they were to any other taxon in the genus. Although the individuals formed a single cluster, no consistent chemical distinction was observed between the two populations sampled. In addition, the individuals of *C. calceolus* var. *parviflorum* and *C. calceolus* var. *pubescens* sampled by Bergstrom *et al.* [2]

Table 1. Taxa sampled within *Cypripedium* with compounds listed as percentages of total composition. Compounds ordered according to relative retention times. *C. calceolus* var. *purviflorum* and *C. calceolus* var. *pubescens* are listed by varietal epithet only

	Skeletal type*	<i>purviflorum</i>	<i>pubescens</i>	<i>candidum</i>	<i>arrietinum</i>	<i>kentuckiense</i>	<i>acule</i>	<i>reginae</i>	<i>guttatum</i>	<i>macranthum</i>
β -pinene	T	<0.5	—	—	—	—	<0.5	—	—	—
limonene	T	—	—	—	—	—	0.5	—	—	—
<i>cis</i> -ocimene	T	—	—	—	—	—	—	—	—	2.1
<i>trans</i> -ocimene	T	—	0.6	—	—	3.8	—	—	—	—
octanoic acid methyl ester	FA	—	—	—	—	—	3.4	—	45	—
<i>trans</i> -linalool oxyd	T	0.6	—	—	—	—	—	—	—	—
4-octenoic acid methyl ester	FA	—	—	—	—	—	0.6	—	—	—
octanoic acid ethyl ester	FA	—	—	—	—	—	1.7	—	—	—
3-octenoic acid ethyl ester	FA	—	—	—	—	—	0.5	—	—	—
nonanoic acid methyl ester	FA	—	—	—	—	—	0.5	—	0.5	—
benzaldehyde	B	<0.5	1.4	28.5	13.1	—	—	—	0.5	—
<i>cis</i> -linalool oxyd	T	2.4	—	17.5	—	0.5	—	—	—	—
linalool	T	37.7	—	—	—	—	—	—	—	—
lilac aldehyde a	T	—	—	—	—	—	4.2	—	—	45.9
lilac aldehyde b	T	—	—	—	—	—	1	—	—	24.1
lilac aldehyde c	T	—	—	—	—	—	<0.5	—	—	9.6
citronellal acid methyl ester	T	—	—	—	—	—	—	—	3.1	—
lilac aldehyde d	T	—	—	—	—	—	1.7	—	—	8.6
decanoic acid methyl ester	FA	—	—	—	—	—	34.5	—	3.7	—
caryophyllene	T	—	—	—	—	—	—	—	3.9	—
4-decenoic acid methyl ester	FA	—	—	—	—	—	3.6	—	3.7	—
benzoic acid methyl ester	B	—	—	—	—	—	—	62	—	—
phenylacetaldehyde	B	—	26.8	25.4	—	—	—	—	—	—
decanoic acid ethyl ester	FA	—	—	—	—	—	18	—	—	—
citronellyl acetate	T	—	—	—	—	—	—	—	1.4	—
3-decenoic acid ethyl ester	FA	—	—	—	—	—	0.9	—	—	—
benzoic acid ethyl ester	B	—	—	—	—	—	0.5	—	—	—
decyl acetate	FA	—	—	—	—	3.5	—	—	—	—
α -humulene	T	—	—	—	—	—	—	—	1.1	—
unknown oxygen containing terpene	T	—	—	—	—	—	0.7	—	1.4	—
unknown oxygen containing terpene	T	—	—	—	—	—	<0.5	—	—	—
geranic acid methyl ester	T	—	—	—	—	—	<0.5	—	2.8	—
lilac alcohol b	T	—	—	—	—	—	<0.5	—	—	<0.5
unknown oxygen containing terpene	T	—	—	—	—	—	<0.5	—	—	—
1,4-dimethoxy benzene	B	15.2	<0.5	—	—	—	—	—	—	—

Table 1.—Continued.

	Skeletal type*	parviflorum	pubescens	candidum	arietinum	kentuckiense	acaule	reginae	guttatum	macranthum
lilac alcohol d	T	—	—	—	—	—	<0.5	—	—	2
citronellol	T	—	—	—	—	—	—	—	5.7	—
methyl salicylate	B	—	—	—	—	—	—	38	—	—
lilac alcohol a	T	—	—	—	—	—	0.5	—	—	2.4
linalool oxide	T	2.6	—	—	—	—	—	—	—	—
nerol	T	—	—	—	—	—	—	—	2.2	—
dodecanoic acid methyl ester	FA	—	—	—	—	—	0.7	—	—	—
lilac alcohol c	T	—	—	—	—	—	—	—	—	0.5
E-cinnamaldehyde	B	—	—	—	—	—	—	—	—	1.3
dodecanoic acid ethyl ester	FA	—	—	—	—	—	<0.5	—	—	—
α -farnesene	T	15.9	—	—	—	13	—	—	—	—
phenyl ethyl acetate	B	—	<0.5	—	—	3.1	—	—	—	—
Z-cinnamaldehyde	B	—	—	—	—	—	—	—	—	0.5
dodecyl acetate	FA	—	—	—	—	27.5	—	—	—	—
benzyl alcohol	B	—	2	6.6	74	—	—	—	17.2	—
3,6-dodecadienoic acid methyl ester	FA	—	—	—	—	—	—	—	3.4	—
phenyl ethyl alcohol	B	—	63.6	2.5	—	—	—	—	—	—
4-phenyl-3-buten-2-one	B	—	—	—	—	—	—	—	—	<0.5
dodecanol	FA	4.7	2.4	—	—	—	—	—	—	—
4-methoxy benzaldehyde	B	6.1	—	13	7	—	—	—	—	—
nerolidol	T	1.8	—	—	—	—	—	—	—	—
tetradecyl acetate	FA	—	—	—	—	4.1	—	—	—	2.5
benzyl benzoic acid	B	—	—	—	—	—	—	—	—	0.5
eugenol	B	—	—	—	—	—	—	—	4.4	—
1-hexadecyl acetate	FA	—	—	—	—	44	—	—	—	—
amino benzaldehyde	B	<0.5	—	—	—	—	17.7	—	—	—
4-methoxy, benzyl, alcohol	B	9.7	—	6.5	5.9	—	—	—	—	—
2-amino benzoic acid ethyl ester	B	—	—	—	—	—	0.5	—	—	—
octadecyl acetate	FA	—	—	—	—	0.5	—	—	—	—
4-methoxy phenyl ethyl alcohol	B	—	3.2	—	—	—	—	—	—	—
2-amino benzene thiol	B	—	—	—	—	—	6.8	—	—	—
indole	B	3.3	—	—	—	—	1.5	—	—	—

* Compounds classified according to general skeletal type; B = benzenoid, FA = fatty acid and T = terpenoid.

Table 2. Individuals sampled from two populations (R = Rose Lake, W = Williamston) of *C. calceolus* var. *parviflorum*. Compounds are scored as present (1) or absent (0) and ordered according to relative retention times

	Skeletal type*	R0	R1	R2	R3	R4	R5	R6	R7	R8	R9	W1	W2	W3	W4	W5
β -pinene	T	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1
limonene	T	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
<i>cis</i> -ocimene	T	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
<i>trans</i> -ocimene	T	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
1-methoxy-4-methylbenzene	B	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
<i>trans</i> -linalool oxyd	T	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0
<i>cis</i> -linalool oxyd	T	1	1	1	1	1	1	0	0	0	0	1	0	1	0	1
benzaldehyde	B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
linalool	T	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1,4-dimethoxy benzene	B	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
α -farnesene	T	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
linalool oxide (<i>cis</i> or <i>trans</i>)	T	1	1	1	0	0	0	1	0	0	0	1	0	1	0	1
<i>Z</i> -cinnamaldehyde	B	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
benzyl alcohol	B	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0
<i>E</i> -cinnamaldehyde	B	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
dodecanol	FA	1	1	1	1	1	1	1	0	0	0	0	0	1	0	0
4-phenyl-3-buten-2-one	B	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
4-methoxy benzaldehyde	B	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
nerolidol	T	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
benzyl benzoate	B	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
amino benzaldehyde	B	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
1,3,5-tri-methoxy benzene	B	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
4-methoxy-benzyl alcohol	B	1	1	1	1	1	1	1	0	0	1	0	1	1	0	1
indole	B	1	1	1	1	1	1	1	0	0	1	0	0	0	0	1

* Compounds classified according to general skeletal type; B = benzenoid, FA = fatty acid and T = terpenoid.

were most similar to each other and did not group with the *C. calceolus* var. *parviflorum* individuals sampled in this study.

DISCUSSION

An important result of this analysis was the clustering coherence of *C. calceolus* var. *parviflorum* individuals in spite of variation in composition evident from inspection of Table 2. This suggests that although population-level qualitative variation in floral fragrance composition appears to be substantial, in this case it is not as great as that observed between species. This conclusion is significant and could not have been made without reference to the other taxa in conjunction with the numerical approach used to quantify the relative variation. Gregg [9] suggested the recognition of chemotypes within *Cynoches* species based upon a numerical analysis of quantitative fragrance variation at the population level which was correlated with floral morphological distinctions. No chemotypes are recognized on the basis of the results presented here as morphological or geographical differences could not be correlated with fragrance variation between these populations.

The fragrance composition found for *C. calceolus* var. *parviflorum* in this study differs substantially from that reported by Bergstrom *et al.* [2]. Monoterpene

hydrocarbons were diverse and in relatively large quantities in the clone sampled in the earlier study whereas the clones examined in the present investigation produced few of these compounds in relatively small quantities. In fact, the highest similarity value obtained between individuals of *C. calceolus* var. *parviflorum* between the two studies is 0.44. This is striking considering that the two morphologically distinct species, *C. candidum* and *C. arietinum*, sampled here share a fragrance similarity of 0.50. In addition, an individual of *C. calceolus* var. *pubescens* was reported to produce a scent dominated by 1,3,5-trimethoxy benzene [2], while the individual sampled in our study yielded predominantly phenyl ethyl alcohol and phenylacetaldehyde. The similarity value obtained between these two individuals is only 0.09. Although differences in data collection exist between the studies and could be a cause of disparity, the results presented here suggest that extensive variation is present not only within *C. calceolus* but within and between populations of the two putative varieties as well. Taken in a broader context, this detected variation could affect conclusions drawn in both taxonomic and pollination ecology studies. Consider for example the potential recognition of the varieties of *C. calceolus* as separate species by several authors [1, 2]. Conclusions based upon the clustering patterns shown in Fig. 1 would be ambiguous concerning the

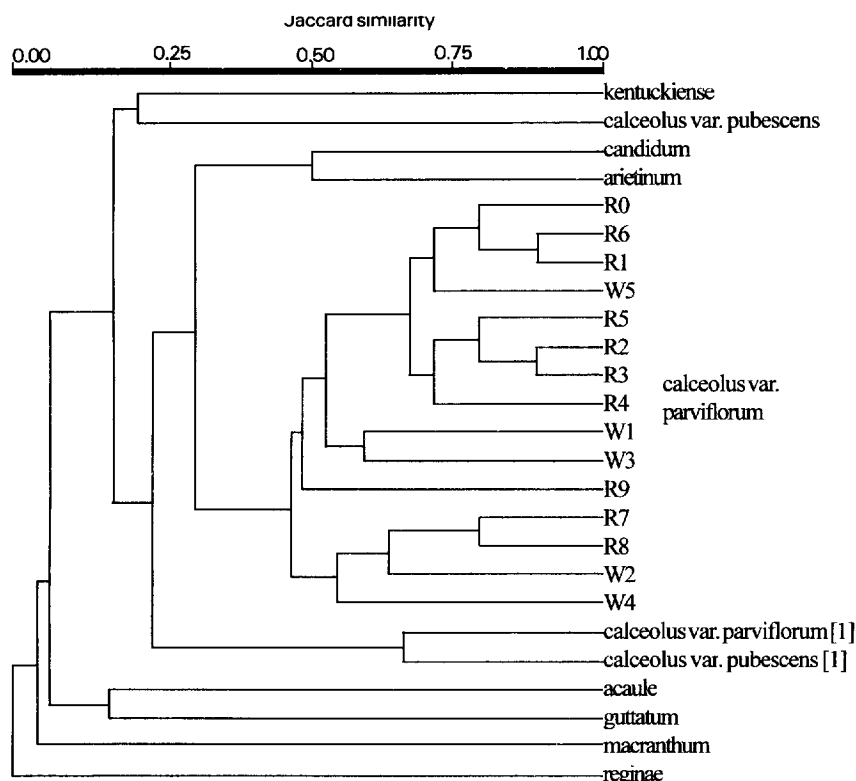


Fig. 1. UPGMA-derived phenogram representing fragrance similarity relationships among all individuals and taxa sampled within *Cypripedium*. Species are indicated by specific epithet and individuals of *C. calceolus* var. *parviflorum* are represented by letters corresponding to population of origin (W = Williamston, R = Rose Lake). Individuals of *C. calceolus* var. *parviflorum* and *C. calceolus* var. *pubescens* sampled by Bergstrom *et al.* [1] are clearly labelled.

specific status of *C. calceolus* var. *parviflorum* and *C. calceolus* var. *pubescens* due to the equivocal positions occupied by the latter variety. In addition, conclusions about pollination biology may be affected by a consideration of infraspecific variation. The implicated role of α -farnesene in deceiving bees of the genus *Andrena* [4] would be applicable to all individuals of *C. calceolus* except the clone of *C. calceolus* var. *pubescens* sampled in this study as it completely lacks terpenoid production. This finding supports the hypothesis of Bergstrom *et al.* [2] that evolutionary differentiation of fragrance composition in response to pollinator selection may occur throughout the range of these taxa due to differing pollinator guilds. Although there is little empirical evidence for selection among fragrance types within a species, there is evidence which suggests pollinators are biased towards visiting different coloured flowers in polymorphic populations of *Raphanus sativus*, potentially causing observed frequency differences between them [16].

The results of the phenetic analysis suggest that it is necessary to determine the extent of qualitative fragrance variation within species in order to make evolutionary or ecological conclusions about them. In addition, the extent of variation within a species should be compared with that occurring between related species because the latter serves as a relevant

basis for comparison. These types of comparisons are most effectively made when combined with a numerical method of analysis. One caveat to our analysis is that we were unable to control many environmental variables which may have been responsible for part of the fragrance variation observed, and therefore the amount of variation attributed to intraspecific variation may be over-estimated.

Although we report a method for the analysis of fragrance compound variation, limitations exist for its use in proposing evolutionary relationships. The accurate coding of the compounds as independent characters is difficult, because many serve as precursors in the biogenesis of others. It may be more useful to represent the entire biogenetic pathway as a character to be used in comparisons. In addition, the clustering algorithm unweighted pair group method arithmetic average (UPGMA) may have limitations for constructing branching patterns that reflect evolutionary relationships [17]. Therefore, we plan to explore alternative coding procedures for these data to be used in cladistic analyses utilizing parsimony algorithms. The method presented here is superior, however, at assessing levels of intraspecific variation, as cladistic algorithms are not well suited to studies at this level [18]. Finally, the variation present within a taxon over the entirety of its geographic range, as

indicated by *C. calceolus*, indicate that extensive sampling is required to use these types of data in taxonomic or pollination studies.

EXPERIMENTAL

All plants were sampled outdoors. *Cypripedium macranthum*, *C. guttatum*, *C. candidum*, *C. calceolus* var. *pubescens*, and *C. kentuckiense* were sampled in the garden of Frederick and Roberta Case (Saginaw Co., MI). The original sources of material in the garden are available upon request. *C. arietinum*, *C. acaule*, and *C. reginae* were sampled in natural populations throughout Michigan. Fifteen *C. calceolus* var. *parviflorum* clones were sampled from two populations, Rose Lake Research Station (R) and Williamston (W) (private land), both in Ingham County, MI. Only one individual was sampled for each taxon except in *C. calceolus* var. *parviflorum*. Voucher specimens are deposited in the Beal-Darlington Herbarium, Michigan State University (MSC).

For field sampling studies, one flower on an intact inflorescence was enclosed in a Plexiglas[®] box for approximately 1 hr prior to sampling. The fragrance-laden air was drawn through a sorbent tube, containing 99 mg of XAD-2 polymers (Supelco, Inc.) using a portable battery-powered sampling pump. Our preliminary studies on this system showed that this sorbent resulted in the best recovery of volatiles, although charcoal, Carbotrap[®] and tenax gave comparable qualitative results. External atmospheric samples were drawn simultaneously as controls. Sampling was initiated at 9.00 and ended at 18.00 each period, with a flow rate of ca 60 ml min⁻¹. Trapped fragrance compounds were removed from the sorbent tubes using 1 ml hexane. Initial development of a sampling methodology for these taxa demonstrated that additional volumes of solvent or different solvents such as pentane or diethyl ether did not result in the additional recovery of compounds from these species. The eluate was coned prior to analyses to a volume of ca 300 µl using compressed N₂ gas. This methodology was developed based upon considerations outlined in Hills and Schutzman [19].

Samples were analysed by GC-MS. Injector temp. was 240° and chromatographic sep'n was performed using a temp. program initiated at 40° for 2 min followed by a ramp to 240° at 7° min⁻¹. The final temp. was held for 2 min. The capillary column (DB-Wax 0.32 mm i.d. × 30 m) was directly interfaced into the ion source of a mass selective detector (MSD). Spectra were acquired from 40 to 300 mass units. The threshold of detection of this instrument was 1–5 ng for limonene and benzaldehyde using full scanning measurement. The MS obtained were compared to published spectra available in a computer database [20] using the search algorithm BenchTop/PBM version 3.0 and original literature reports [21, 22]. Compound identification was based upon the combination

of comparisons of relative retention times and spectral profiles.

The determination of overall chemical similarity between taxa was calculated using Jaccard's coefficient of similarity. UPGMA was chosen as the clustering algorithm due to its widespread use in phenetic studies. The software package NTSYS, version 1.7 [23], was used for the clustering analyses.

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