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Phylogenetic fragrance patterns in *Nicotiana* sections *Alatae* and *Suaveolentes*

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Abstract

We analyzed floral volatiles from eight tobacco species (Nicotiana; Solanaceae) including newly discovered Brazilian taxa (*Nicotiana mutabilis* and "Rastroensis") in section Alatae. Eighty-four compounds were found, including mono- and sesquiterpenoids, nitrogenous compounds, benzenoid and aliphatic alcohols, aldehydes and esters. Floral scent from recent accessions of *Nicotiana alata*, *Nicotiana bonariensis* and *Nicotiana langsdorffii* differed from previously published data, suggesting intraspecific variation in scent composition at the level of biosynthetic class. Newly discovered taxa in Alatae, like their relatives, emit large amounts of 1,8-cineole and smaller amounts of monoterpenes on a nocturnal rhythm, constituting a chemical synapomorphy for this lineage. Fragrance data from three species of *Nicotiana* sect. *Suaveolentes*, the sister group of Alatae, (two Australian species: *N. cavicola*, *N. ingulba*; one African species: *N. africana*), were compared to previously reported data from their close relative, *N. suaveolens*. Like *N. suaveolens*, *N. cavicola* and *N. ingulba* emit fragrances dominated by benzenoids and phenylpropanoids, whereas the flowers of *N. africana* lacked a distinct floral scent and instead emitted only small amounts of an aliphatic methyl ester from foliage. Interestingly, this ester also is emitted from foliage of *N. longiflora* and *N. plumbaginifolia* (both in section *Alatae* s.l.), which share a common ancestor with *N. africana*. This result, combined with the synapomorphic pattern of 1,8 cineole emission in *Alatae* s.s., suggests that phylogenetic signal explains a major component of fragrance composition among tobacco species in sections *Alatae* and *Suaveolentes*. At the intraspecific level, interpopulational scent variation is widespread in sect. *Alatae*, and may reflect edaphic specialization, introgression, local pollinator shifts, genetic drift or artificial selection in cultivation. Further studies with genetically and geographically well-defined populations are needed to distinguish between these possibilities.

Keywords: *Nicotiana* sp.; Solanaceae; GC–MS; Hawkmoths; Headspace; Hummingbirds; Pollination

1. Introduction

During the heyday of biochemical systematics, Adams (1975; Adams et al., 1983), Mabry (Mabry et al., 1962; Mabry, 1977), Rodman (1979) and others explored the extent to which specific classes of plant secondary metabolites provide taxonomically useful information. One major lesson from these studies was that widespread compounds (e.g. monoterpenes, anthocyanin pigments) are relatively uninformative at higher taxonomic levels (e.g. family), whereas less ubiquitous compounds (e.g. glucosinolates and betalain pigments) provide stronger taxonomic signal (Alston and Turner, 1963; Harbourne, 1977; Seigler, 1979). Such compounds tend to be more informative when they are chemically derived or are expressed in an unusual context, as are the heartwood oil components (cedrenes and cedrol) present in the needles of Eastern Hemisphere *Juniperus* (Adams, 1999). Recent studies investigating the

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phylogenetic distribution of floral volatiles reveal similar patterns, but differ in that shared-derived traits are more likely to be identified at or below the genus level (Gerlach and Schill, 1989; Nogueira et al., 2001). Accordingly, Levin et al. (2001) found that floral emission of unique phenylpropanoids and lactones characterized a cluster of *Acleisanthes* and *Selinicarpos* (Nyctaginaceae) species that were later shown with DNA markers to be sister taxa. In contrast, a ubiquitous defense-signaling compound, *cis*-jasmone, was emitted by flowers of all lineages of Nyctaginaceae studied and likely represents an ancestral condition in the family (Levin et al., 2003). Finally, Knudsen and Ståhl (1994) identified unusual oxoisophorone-related odors from closely-related *Jacquinia* (Theophrastaceae) species whose deep orange flowers were pigmented with the putative precursor of these compounds, β-carotene. The consensus among such studies is that floral volatiles generally are so homoplaseous as to be ill-suited to phylogenetic reconstruction (Azuma et al., 1997; Barkman et al., 1997; Williams and Whitten, 1999). Instead, such data should be mapped onto phylogenetic trees generated by independent data sets (Givnish and Sytsma, 1997; Levin et al., 2003). The growing body of research adopting this approach provides evidence for convergent evolution of odors associated with specific pollinator classes (Knudsen and Mori, 1996; Kite and Hetterschieid, 1997; Jürgens et al., 2003), but also suggests that the fragrances of related plants with similar pollination biology tend to be species-specific (Dobson et al., 1997; Jürgens et al., 2000), and that some plants whose pollination biology does not require floral scent still produce it (Levin et al., 2001; Knudsen et al., 2004). Clearly, pollinator-mediated selection is only one of several forces that shape the evolution of fragrance as a component of floral phenotype.

In a previous paper (Raguso et al., 2003), we characterized the chemical composition and emission rates of floral scent from one lineage of tobacco (*Nicotiana* sect. *Alatae*; Solanaceae) and several outgroup species with varying degrees of relatedness. Most of our study plants were grown from seed accessions originally collected by Goodspeed (1954) and propagated either at the University of California, Berkeley Botanical Garden or at the Tobacco Research Station of the United States Department of Agriculture, in Oxford, NC (USA). Subsequent research has revealed substantial variation in floral morphology among natural populations of different species in *Alatae*, including nectar tube length and anther placement in *Nicotiana longiflora*, flower size in *N. bonariensis* and green-to-red corolla coloration in *N. langsdorffii* (T.P. Holtsford, unpublished data). In addition, two new species with unusual floral morphology have been discovered during recent field work in southeastern Brazil. One species, *N. mutabilis*, produces white flowers similar to *N. bonariensis* in size, which turn rose-lavender in color by the second day (Stehmann et al., 2002), a pattern similar to (but opposite) that shown in the related genus *Brunfelsia* (Plowman, 1974; Weiss, 1995). Limited observations at the type locality revealed hummingbird pollination of the white flowers, with no moths observed during evening watches (Stehmann et al., 2002). Flowers of the second putatively novel species, provisionally referred to as "Rastroensis" (Kaczorowski et al., 2005) are morphologically similar to those of *N. bonariensis*, but are coral-pink to pale red in color, like those of *N. forgetiana*, which are pollinated by hummingbirds and also are occasionally visited by small hawkmoths (*Callionima nomius*; Ippolito et al., 2004). Additional field observations on the reproductive ecology of these new taxa are sorely needed.

Our previous study investigated patterns of scent chemistry associated with putative reproductive strategies and floral morphology. Hawkmoth-pollinated species were characterized by nitrogenous compounds, linalool and/or aromatic esters, but did not all produce the strongest odors, in terms of emission rates (toluene equivalents of total ion current) per flower or unit floral mass. Further, patterns of scent production, in some cases, were more indicative of evolutionary history than of pollinator spectrum, such that flowers of all species in sect. *Alatae* sensu strictu emitted large amounts of 1,8-cineole along with lower levels of related monoterpenoids nocturnally, from the limb (distal portion) of the fused corolla (Raguso et al., 2003). This "cassette" of correlated volatiles is present in the same relative ratios as the major and minor products of the 1,8-cineole synthase enzyme in *Salvia officinale* (Lamiaceae) (Wise et al., 1998). Thus, a parsimonious hypothesis is that the nocturnal, corolla-limb specific expression of a 1,8-cineole synthase-like enzyme is a synapomorphy (shared-derived trait) in *Nicotiana* sect. *Alatae* s.s. The persistence of this phenotype in *N. langsdorffii* and *N. forgetiana*, two species whose flowers are pollinated at least in part by hummingbirds, who generally do not use odor to find flowers (rev. by Knudsen et al., 2004), suggests that pollinator-mediated selection alone is insufficient to explain patterns in fragrance evolution among these tobaccos. Finally, the closest outgroup species, *N. suaveolens*, from the Australasian section *Suaveolentes*, produced a markedly different pattern of scent chemistry, dominated by cinnamic acid-derived phenylpropanoid compounds and aromatic esters.

Here we extend these analyses to include five additional species from *Nicotiana* sections *Alatae* and *Suaveolentes* and three additional populations of previously studied species from section *Alatae* (Table 1). Within this framework, we address three specific questions:

(1) Is the nocturnal monoterpenoid emission pattern dominated by 1,8-cineole common to all species and accessions of *Nicotiana* section *Alatae* s.s.?
(2) Do the additional accessions of three species from section *Alatae* produce measurably different fragrances from those previously studied?
(3) Are the fragrances of three additional species from section *Suaveolentes* dominated by aromatic compounds, as is the fragrance of *N. suaveolens*?

Table 1
Floral biology and taxonomic affiliation of *Nicotiana* species included in this study

Species	Flower color, depth (*N*)	Pollinators	Evidence
Section *Alatae* s.s.			
N. alata	White, 82.4 ± 1.8 (10)	Hawkmoth	Ippolito et al. (2004)
N. bonariensis	White, 20.2 ± 0.2 (10)	Moth	Kaczorowski et al. (2005)
N. langsdorffii	Red and green, 24.9 ± 0.5 (10)	Hummingbird, bee	Kaczorowski et al. (2005)
N. mutabilis	White, pink, 21.7 ± 0.4 (10)	Hummingbird	Stehmann et al. (2002)
"Rastroensis"	Coral pink, 20.9 ± 0.2 (10)	Hummingbird, bee	Kaczorowski et al. (2005)
Section *Suaveolentes*			
N. africana	Green, 44.2 + 0.36 (10)	Sunbird?, self?	Morphology
N. cavicola	White, 31.5 ± 1.0 (10)	Hawkmoth, self?	Morphology
N. ingulba	White, 31.3 ± 1.0 (4)	Hawkmoth, self?	Morphology

Measurements of flower depth are means ± S.E. in mm.

Studies of fragrance chemistry from related species within a lineage provide primary information about the phylogenetic distribution of floral scent chemistry and promote the testing of alternative hypotheses about how such patterns may have evolved.

2. Results and discussion

2.1. Sources of variation in scent complexity and composition

We identified a total of 84 volatile compounds from floral and vegetative (leaf or stem) organs of seven *Nicotiana* species (Table 2). As in our previous study, more than half (46) of the identified volatiles were terpenoids, including 21 sesquiterpene hydrocarbons and alcohols. The remaining 38 compounds included 25 common benzenoid alcohols, aldehydes and esters and seven nitrogenous volatiles, all of which are derived from amino acid metabolism. Of the nine fatty acid-derivatives detected, seven were restricted to *N. bonariensis* and have mass spectra that suggest unsaturated long-chain acetates, whereas the other two are methyl esters of aliphatic acids (Table 2). Benzaldehyde and benzyl alcohol were present in nearly all species, as were 1,8-cineole and several monoterpene hydrocarbons.

Scent complexity varied considerably between taxa (mean ± SE = 23 ± 4 compounds), ranging from one compound in *N. africana* to 35 compounds in *N. alata* and *N. bonariensis*. This mean is lower than that measured in our previous study (31 ± 5 compounds), probably due to the great disparity between the two accessions of *N. alata* (TW7, 69 compounds; Rio Pelotas, 35 compounds) (see Section 2.4). Variation in fragrance complexity also was observed for four species in section *Alatae* in terms of the mean number of compounds emitted during night (24 ± 5) vs. day (16 ± 2). In one extreme case, flowers of *N. bonariensis* produced twice as many volatiles at night (35) than during the day (18), including all aldoximes and nitriles, most phenylpropanoids and aromatic esters (Table 2). The two new taxa in section *Alatae* s.s., *N. mutabilis* and "Rastroensis", produced relatively weak fragrances consisting of terpenoids and aromatic alcohols and aldehydes (Table 2). SPME analyses controlling for flower mass and equilibration time revealed that pink (two day old) flowers of *N. mutabilis* were twice as strongly scented as white (one day old) flowers during the evening, and nine times more strongly scented during the morning, largely due to increased amounts of linalool in pink flowers (data not shown). Putative new species "Rastroensis" was found to emit methyl nicotinate, a compound that was otherwise emitted only by flowers of *N. cavicola* and *N. suaveolens*, both from section *Suaveolentes* (Raguso et al., 2003).

Five or fewer compounds were shared between floral and vegetative tissues in any one species, and few volatiles unique to vegetation were detected under our analytical conditions, which were identical to those reported by Raguso et al. (2003). Nitrogenous compounds, sesquiterpenes and all fatty acid derivatives except the methyl ester found in *N. africana* were emitted exclusively by floral tissues, as were 4-oxoisophorone, its hydrogenation product (2,6,6-trimethyl-1,4-cyclohexanedione) and epoxide derivative (1,3,3-trimethyl-7-oxabicyclo[4.1.0] heptan-2,5-dione). The oxophorone-related compounds were originally described from *Buddleija* species (Loganiaceae) by Tabacchi et al. (1986) and Schulz et al. (1988) and now have been found in a wide variety of angiosperm families, including orchids (Kaiser, 1993). Acyclic C_{11} (*E*-4,8-dimethylnona-1,3,7-triene) and C_{16} homoterpene hydrocarbons (*Z*,*E* and *E*,*E*-4,8,12-trimethyl-1,3,7,11-tridecatetraene and a putative epoxide) comprised more than 15% of emissions by *N. alata* flowers (Table 2). These compounds are nearly universal components of herbivore-induced leaf emissions (rev. by Dicke, 1994) and also are constitutive fragrance components of diverse families of night-blooming, moth-pollinated plants (Gäbler et al., 1991; Kaiser, 1991; Levin et al., 2001; Svensson et al., 2005).

2.2. Sources of variation in scent emission rates

Emission rates for total floral scent, expressed as toluene (internal standard) equivalents of total ion current, are summarized in Table 3, including corrected values for those species described in the previous paper (Raguso et al., 2003; see Section 2.3 for correction). Previously, we

Table 2
Volatile compounds emitted by *Nicotiana* species

Compound	RT	# Spp.	*Suaveolentes*				*Alatae*								
			N. africana		*N. cavicola*	*N. ingulba*	*N. alata*	*N. bonariensis*		*N. langsdorffii*		*N. mutabilis*		"*Rastroensis*"	
			PM $n=5$	AM $n=4$	PM $n=1$	PM $n=1$	PM $n=4$	PM $n=3$	AM $n=3$	PM $n=2$	AM $n=2$	PM $n=4$	AM $n=4$	PM $n=4$	AM $n=4$
Total no. compounds (84)			1	1	17	9	35	35	18	29	21	20	13	13	13
No. shared by flowers and veg. tissues			1	1	2	0	3	1	1	0	0	0	0	3	3
No. only floral compounds			0	0	14	9	32	31	12	28	21	20	13	8	9
No. only veg. compounds			0	0	1	0	0	3	5	1	0	0	0	2	1
Monoterpenes (7)															
α-Pinene*	2.76	5					1.34 ± 0.10	ND	*15.07 ± 5.32*	0.57 ± 0.02	4.11 ± 0.20	3.21 ± 0.23	6.14 ± 1.02	3.76 ± 0.27	2.44 ± 2.44
β-Pinene*	4.08	5					0.99 ± 0.35	ND	*0.72 ± 0.21*	0.40 ± 0.02	1.69 ± 0.14	1.74 ± 0.17	1.83 ± 0.25	2.24 ± 0.21	0.60 ± 0.37
Sabinene*	4.31	5					**3.06 ± 0.68**	*0.01 ± 0.01*	*3.22 ± 0.30*	1.18 ± 0.07	4.91 ± 0.03	5.85 ± 0.41	6.69 ± 0.41	8.29 ± 0.58	6.68 ± 3.92
β-Myrcene*	5.07	5					**3.10 ± 0.76**	*0.01 ± 0.01*	*1.59 ± 0.59*	3.19± 0.29	7.86 ± 0.04	4.92± 0.37	3.19 ± 0.11	6.05 ± 0.17	3.35 ± 0.51
Limonene*	5.70	5					**2.53 ± 0.51**	*0.06 ± 0.02*	*2.91 ± 1.04*	1.29 ± 0.11	5.45 ± 0.22	5.34 ± 0.40	8.65 ± 0.92	**7.40 ± 0.95**	**36.20 ± 9.29**
Z-β-ocimene*	6.36	2					0.01 ± 0.01			0.03 ± 0.01	ND				
E-β-ocimene*	6.67	5					1.11 ± 0.32	ND	0.33 ± 0.33	*1.65 ± 0.16*	2.55 ± 0.05	0.17 ± 0.06	ND	0.09 ± 0.06	
Oxygenated monoterpenoids (9)															
1,8-Cineole*	5.69	6				4.98	29.20 ± 7.06	**0.29 ± 0.09**	**14.62 ± 4.92**	12.09 ± 0.89	56.49 ± 0.46	54.75 ± 2.11	55.76 ± 3.87	**69.10 ± 0.54**	**31.63 ± 5.15**
Linalool*	10.95	2					0.09 ± 0.05					18.79 ± 1.79	12.91 ± 1.13		
136 (11), 93 (25), 81 (43), 59 (100), 43 (64), 41 (43)	12.54	3					0.13 ± 0.08					0.22 ± 0.14	ND	0.15 ± 0.09	ND
α-Terpineol*	12.82	4					0.99 ± 0.65			0.09 ± 0.02	0.24 ± 0.08	1.02 ± 0.31	0.26 ± 0.26	2.15 ± 1.28	0.22 ± 0.22
E-2,6-dimethyl 3,7 octadiene-2,6-diol**	15.42	1												ND	6.37 ± 5.68
139 (7), 121 (7), 81 (45), 69 (26), 55 (11), 43 (100)	15.70	2								0.18 ± 0.02	0.54 ± 0.05	0.29 ± 0.14	ND		
82 (16), 81 (25), 80 (14), 59 (100), 55 (14), 43 (22), 41 (20)	16.26	2								0.09 ± 0.09	0.73 ± 0.06	0.52 ± 0.30	0.59 ± 0.59		
81 (34), 69 (78), 55 (100), 43 (58), 41 (88)	16.95	2								0.06 ± 0.06	0.28± 0.02	0.14 ± 0.08	ND		
Z-2,6-dimethyl-2,7 octadiene-1,6-diol**	17.30	1												ND	3.40 ± 3.20
Irregular terpenoids (9)															
E-4,8-dimethylnona-1,3,7-triene**	7.46	1					6.33 ± 1.38								
Chrysanthemone isomer	10.92	1								0.06 ± 0.02	ND				
Chrysanthemone	11.96	2								0.16 ± 0.08	0.36 ± 0.06	0.54 ± 0.34	ND		
1,3,3-Trimethyl-7-oxabicyclo[4.1.0] heptan-2,5-dione**	12.52	1						63.56 ± 5.85	10.83 ± 3.43						
2,6,6-Trimethy-2-cyclohexene-1,4-dione (4-oxoisophorone)**	12.83	1						1.89 ± 0.76	1.60 ± 0.55						
2,6,6-Trimethyl 1,4-cyclohexanedione**	13.80	1						0.31 ± 0.09	1.72 ± 0.87						

Z,E-4,8,12-trimethyl-1,3,7,11-tridecatetraene**	13.85	1			0.01 ± 0.01								
E,E-4,8,12-trimethyl-1,3,7,11-tridecatetraene**	14.14	2	2.21		8.96 ± 2.50								
150 (28), 121 (20), 95 (22), 93 (26), 91 (44), 84 (32), 82 (30), 81 (80), 79 (48), 77(27), 69 (100), 53 (38), 46 (22), 41 (64)	15.55	1			0.32 ± 0.14								
Sesquiterpenes (*14*)													
α-Cedrene*	11.19	1						0.21 ± 0.15	0.81 ± 0.20				
E-α-bergamotene**	11.54	4	0.14					0.87 ± 0.11	6.39 ± 0.62	0.93 ± 0.48	2.66 ± 1.47		
β-Caryophyllene*	11.74	4	**1.24**	10.90	0.09 ± 0.09					0.49 ± 0.28	ND		
147 (27), 119 (7), 105 (85), 91 (62), 79 (17), 77 (20), 56 (77), 55 (69), 43 (68), 41 (100)	11.78	1										0.06 ± 0.06	ND
Z-β-farnesene	12.13	1						0.15 ± 0.03	0.72 ± 0.09				
161 (100), 105 (60), 91 (53), 121 (47), 119 (63), 105 (27), 93 (71), 91 (42), 81 (38), 79 (67), 67 (40), 55 (39), 53 (43), 41 (100)	12.25 12.44	1 2						0.04 ± 0.01 0.30 ± 0.06	 1.59 ± 0.09	 0.37 ± 0.18	 0.54 ± 0.54		
E-β-farnesene**	12.46	1			3.33 ± 1.94								
α-Humulene*	12.62	1	0.07										
119 (100), 93 (62), 77 (19), 69 (30), 42 (36), 41 (62)	12.64	2						0.12 ± 0.02	0.88 ± 0.01	0.23 ± 0.11	0.34 ± 0.34		
Germacrene D*	13.09	1			0.02 ± 0.02								
Z,E-α-farnesene**	13.29	1						0.14 ± 0.02	0.84 ± 0.09				
E,E-α-farnesene*	13.47	1			0.05 ± 0.05								
204 (M+, 58), 189 (25), 161 (100), 147 (13), 133 (31), 119 (52), 105 (76), 93 (29), 91 (44), 81 (79), 79 (26), 77 (28), 55 (34), 41 (73)	13.79	1						0.12 ± 0.01	0.50 ± 0.001				
Oxygenated sesquiterpenoids (*6*)													
93 (33), 91 (10), 79 (5), 59 (22), 55 (56), 43 (100), 41 (61)	16.10	1						0.24 ± 0.19	ND				
E-nerolidol*	16.52	1			7.24 ± 1.05								
farnesol isomer?	18.69	1			0.15 ± 0.07								
E,E-farnesal	18.72	1			0.07 ± 0.05								
E,E-farnesol*	19.46	1			0.53 ± 0.32								
Farnesol isomer*	21.04	1			0.03 ± 0.03								
Aromatic alcohols and aldehydes (*14*)													
Benzaldehyde*	10.69	7	**0.73**	2.43	0.44 ± 0.18			0.13 ± 0.001	0.69 ± 0.34				
75.77 ± 1.08					3.06 ± 0.46	0.32 ± 0.06	ND	**0.62 ± 0.24**	**2.44 ± 1.19**				
Phenylacetaldehyde*	12.20	2			0.06 ± 0.06	0.11 ± 0.09	ND						
Salicylaldehyde*	12.70	1	*0.35*										
Veratrole*	13.15	1	0.40										
Benzyl alcohol*	14.83	6	0.92	12.70	0.02 ± 0.02	0.37 ± 0.11	0.02 ± 0.02	0.34 ± 0.28	ND			ND	1.39 ± 0.82
2-Phenylethanol*	15.22	3				0.66 ± 0.43	ND	0.13 ± 0.07	ND			*<0.01*	5.30 ± 2.91
Methyl isoeugenol*	16.25	1			0.02 ± 0.02								
Cinnamic aldehyde*	16.60	2		3.90		0.04 ± 0.04	ND						
Hydrocinnamic alcohol	16.83	1	0.01										
m-Cresol*	16.90	2	0.80			0.01 ± 0.01	ND						

(*continued on next page*)

Table 2 (*continued*)

Compound	RT	# Spp.	*Suaveolentes*				*Alatae*									
			N. africana		*N. cavicola*	*N. ingulba*	*N. alata*	*N. bonariensis*		*N. langsdorffii*		*N. mutabilis*		"*Rastroensis*"	
			PM $n=5$	AM $n=4$	PM $n=1$	PM $n=1$	PM $n=4$	PM $n=3$	AM $n=3$	PM $n=2$	AM $n=2$	PM $n=4$	AM $n=4$	PM $n=4$	AM $n=4$
Eugenol*	17.78	1						1.55 ± 0.33	ND						
E-cinnamic alcohol*	18.83	2				6.34		0.13 ± 0.09	ND						
Chavicol	19.31	1						0.02 ± 0.02	ND						
E-isoeugenol*	19.43	1				<0.01									
Aromatic esters (*9*)															
Methyl benzoate*	11.95	2			15.74			0.32 ± 0.12	2.99 ± 1.51						
Phenylmethyl (benzyl) acetate*	13.21	2			65.20			18.97 ± 3.12	17.66 ± 2.50						
Methyl salicylate*	13.82	4			7.79					0.26 ± 0.16	ND	0.19 ± 0.11	0.45 ± 0.45	*<0.01*	*<0.01*
2-Phenylethyl acetate*	14.21	1						1.00 ± 0.79	ND						
Amyl benzoate*	15.32	3					0.04 ± 0.03	0.17 ± 0.06	ND	0.13 ± 0.06	ND				
Cinnamyl acetate*	17.64	2				1.29		0.43 ± 0.23	ND						
Benzyl benzoate*	21.84	4			2.47		0.02 ± 0.02	5.52 ± 1.87	4.36 ± 1.00	0.35 ± 0.35	0.30 ± 0.06				
2-Phenylethyl benzoate	22.59	1						0.05 ± 0.03	ND						
Benzyl salicylate*	23.13	1			0.87										
Fatty acid derivatives (*9*)															
Isoamyl acetate*	4.38	1						0.01 ± 0.01	ND						
Methyl heptanoate	6.29	1	**100**	**100**											
97 (7), 83 (17), 69 (21), 61 (23), 57 (27), 56 (23), 55 (36), 43 (100), 41 (36)	17.10	1						0.11 ± 0.11	1.86 ± 0.28						
96 (16), 95 (18), 82 (32), 81 (29), 69 (17), 68 (19), 67 (38), 55 (72), 54 (22), 43 (100), 41 (63)	17.49	1						0.06 ± 0.06	0.35 ± 0.35						
143 (7), 129 (1), 101 (3), 97 (3), 87 (58), 74 (100), 69 (12), 59 (13), 57 (20), 55 (39), 43 (68), 41 (54)	18.31	1			1.03										
109 (8), 98 (2), 87 (13), 85 (14), 71 (32), 56 (29), 43 (100), 41 (52)	18.98	1						0.36 ± 0.07	ND						
125 (2), 111 (6), 97 (13), 83 (18), 70 (11), 69 (19), 61 (25), 57 (26), 56 (20), 55 (34), 43 (100), 41 (40)	19.03	1						1.34 ± 0.48	19.35 ± 4.25						
82 (24), 68 (19), 67 (31), 55 (55), 43 (100), 41 (36)	19.23	1						0.01 ± 0.01	ND						
110 (2), 96 (21), 95 (16), 82 (36), 67 (35), 55 (80), 43 (100), 41 (69)	19.41	1						0.08 ± 0.08	ND						
Nitrogenous compounds (*7*)															
Nitro-3-methylbutane**	7.94	1					0.84 ± 0.23								
2-Methylbutyloxime*	10.20	3				49.49	1.63 ± 0.17	0.83 ± 0.26	ND						
3-Methylbutyloxime*	10.26	2					15.71 ± 3.02	0.66 ± 0.43	ND						
2-Methylbutyloxime*	10.43	3				7.97	0.54 ± 0.10	0.19 ± 0.06	ND						
3-Methylbutyloxime*	10.75	2					11.24 ± 2.29	0.46 ± 0.33	ND						
Methyl nicotinate*	13.79	2			0.10										
0.10 ± 0.06	ND														
Phenylacetonitrile*	15.59	1						0.05 ± 0.02	ND						

Numbers represent mean percentage (out of 100%) ±SE of total scent emitted. Compounds marked with * were identified by co-chromatography with known standards, those with ** using essential oils or natural products for which published GC–MS data are available. For remaining compounds, putative names are provided when MS were >90% identity with NIST and Wiley library spectra. MS of unidentified compounds are given in descending order of *m*/*z*, with % abundance relative to the base peak (100) in parentheses. Italics indicate compounds emitted only by vegetative tissues, bold face indicates compounds emitted by both flowers and non-floral tissues. ND, compound not detected using our protocols.

identified striking nocturnal rhythms in floral scent emissions across several sections of *Nicotiana*, even among species with diurnal pollinators (Raguso et al., 2003). Consistent with this pattern, we observed 7–10-fold increased odor emission at night from flowers of *N. langsdorffii*, *N. mutabilis* and "Rastroensis", three putatively hummingbird pollinated taxa. The rank orders of odor intensity were not significantly correlated with the predicted order based on pollination strategy (hawkmoths > noctuid moths and bees > hummingbirds = autogamy) at night (Spearman's Rho (ρ) = 0.415, $p = 0.21$ per flower, $\rho = 0.037$, $p = 0.91$ per g dry mass) or during day ($\rho = 0.295$, $p = 0.44$ per flower, $\rho = 0.143$, $p = 0.71$ per g dry mass) (SPSS, 1999). Interestingly, an alternative predictive order based on flower size was no better at explaining observed emission rates during night ($\rho = 0.491$, $p = 0.13$ per flower, $\rho = 0.164$, $p = 0.63$ per g dry mass) or during day ($\rho = 0.517$, $p = 0.15$ per flower, $\rho = 0.283$, $p = 0.46$). Thus, variation in the strength of fragrance emission in different species of *Alatae* does not simply reflect putative pollinator affinities or flower size, and instead may reflect selective pressures exerted by herbivores in certain habitats (see Baldwin et al., 1997).

2.3. Is the "cineole cassette" present in all species of sect. Alatae?

Flowers of the two new species and the two new accessions of section *Alatae* produced relatively large amounts of 1,8-cineole with smaller amounts of α- and β-pinene, sabinene, β-myrcene, limonene and α-terpineol, a pattern not observed in any species from section *Suaveolentes* (Table 2, also see Raguso et al., 2003). Interestingly, this odor blend accounted for a majority of all scent emitted by the putatively hummingbird pollinated *N. mutabilis* and "Rastroensis" during day (83, 66%) and night (77, 98%), respectively, with a similar pattern in red-flowered *N. langsdorffii*, except for its unexpected large nocturnal emissions of benzaldehyde (76% of total scent; Table 2). Fatty acid derivatives, nitrogenous aldoximes and nitriles were entirely absent from these species' fragrances. Flowers of *N. mutabilis*,"Rastroensis" and *N. langsdorffii* emitted monoterpenoids in 3–10-fold greater amounts per hr at night than during the day, consistent with patterns observed throughout section *Alatae*, including *N. bonariensis* accession TW28 (Raguso et al., 2003). In contrast, flowers from the Santa Tereza population of *N. bonariensis* emitted only half the amount of monoterpenoids per flower at night than during the day, and α- and β-pinene were not detected at night (Table 2). Headspace analysis using solid phase microextraction (SPME) revealed that 1,8-cineole was the only monoterpenoid emitted by detached *N. bonariensis* flowers at night (data not shown). In Fig. 1 we compare the mean relative ratios of monoterpenoids detected in the floral headspace of each species with the ratios of major- and minor-products of the *Salvia officinalis* 1,8-cineole synthase enzyme (Wise et al., 1998). Outside of *N. bonariensis*, the floral scent patterns are consistent with the hypothesis that an enzyme similar to 1,8-cineole synthase is responsible for the biosynthesis of one major and several minor monoterpenoid products (Fig. 1). As before, the moderate excess of sabinene, β-myrcene and limonene in the tobacco headspace samples is correlated with emissions of these compounds from non-floral tissues (Table 2), and may reflect diverse enzymatic or regulatory mechanisms. It is not known whether patterns such as this are common in floral scent blends, because few studies include enzymological data (e.g. Negre et al., 2003). Interestingly, the volatiles emitted by flowers and leaves of *Mentha × piperita* show a strikingly similar pattern to that presented here (Rohloff, 1999; his Fig. 2), suggesting the action of at least two orthologous enzymes (limonene synthase and 1,8-cineole synthase) known to produce major and minor products in *Mentha* and other Lamiaceae (Colby et al., 1993; Wise et al., 1998). In their *Salvia* study, Wise et al. (1998) explicitly determined the chirality of 1,8-cineole synthase products as racemic blends of α- and β-pinene and limonene and only (+) sabinene, whereas we did not have this capability. Chiral analyses of floral and vegetative headspace from species of *Nicotiana* sect. *Alatae*, along with enzymological studies, will be necessary to dissect the contributions of different parts of the inflorescence to monoterpenoid emissions in these species.

2.4. Do different populations of species in section Alatae s.s. have different odors?

We studied additional populations of three previously studied species, each of which differed in floral size or color from the USDA accessions studied by Raguso et al. (2003). The Morro da Igreja population of *N. langsdorffii* differs from accession TW74 in its red (rather than green-yellow) flowers, which, in a "pollinator syndrome" context might suggest greater reliance on hummingbird pollination. Surprisingly, scent emissions in the red-flowered population were twofold greater at night than the green-flowered population, but only one fourth to one half as great as green-flowered plants during the day (Table 3). Furthermore, the red-flowered population produced twice as many scent compounds as the green-flowered population, including several novel sesquiterpenes, benzaldehyde (75.8% of emissions) and other aromatics during the night (Table 2). Further study is required to determine whether these gross differences, comparable to those between distinct species, reflect local pollinator adaptation, genetic drift or pleiotropic interactions with plant defense. Alternatively, these differences may reflect introgression of red pigment and scent traits through hybridization with another *Nicotiana* species such as *N. forgetiana*, the only other taxon in section *Alatae* s.s. with red flowers (Ippolito et al., 2004). Benzaldehyde was not detected in our previous analysis of *N. forgetiana* fragrance (Raguso et al., 2003), but is structurally related to anthocyanin pigments and could represent a pleiotropic or recombinant character trait (e.g. Zuker et al., 2002).

Table 3
Emission rates (mean ± SE) of tobacco floral scent in species of *Alatae* and *Suaveolentes* studied here, as well as and other sections of *Nicotiana*, including corrected values for different taxa and previously published accessions (* Raguso et al. (2003), see Methods)

Section, taxon	Accession	Time, *N*	μg scent/fl/h	μg scent/g/h	μg scent/dry g/h
Incertae cedis					
*N. sylvestris**	USDA	AM, 3	0.036 ± 0.013	0.088 ± 0.031	0.601 ± 0.187
		PM, 10	0.038 ± 0.007	0.092 ± 0.016	0.617 ± 0.105
Rusticae		PM, 10	0.038 ± 0.007	0.092 ± 0.016	0.617 ± 0.105
*N. rustica**	J. Walker	AM, 5	0.059 ± 0.022	0.437 ± 0.153	2.213 ± 0.810
		PM, 6	0.316 ± 0.126	2.320 ± 0.900	11.840 ± 4.716
Suaveolentes					
*N. suaveolens**	B. Piechulla	AM, 4	0.052 ± 0.014	0.372 ± 0.107	2.461 ± 0.704
		PM, 9	0.289 ± 0.053	2.047 ± 0.492	12.814 ± 2.656
N. africana	none	AM, 4	0.002 ± 0.0001	0.006 ± 0.0004	0.033 ± 0.002
		PM, 5	0.001 ± 0.0001	0.004 ± 0.0005	0.022 ± 0.003
N. cavicola	TS 289	PM, 1	0.831	9.832	59.344
N. ingulba	TS 63	PM, 1	0.009	0.172	1.227
Alatae s.l.					
*N. longiflora**	TW78	AM, 3	0.037 ± 0.004	0.095 ± 0.013	0.345 ± 0.047
		PM, 5	0.085 ± 0.021	0.216 ± 0.051	0.784 ± 0.186
*N. plumbaginifolia**	TW106	PM, 8	0.043 ± 0.016	0.673 ± 0.235	3.425 ± 1.549
Alatae s.s.					
N. alata	TW7	AM, 3	0.748 ± 0.089	1.033 ± 0.122	6.706 ± 1.059
		PM, 8	7.405 ± 1.620	10.214 ± 2.235	60.300 ± 13.104
N. alata	Rio Pelotas	PM, 4	0.360 ± 0.099	0.775 ± 0.220	5.503 ± 1.551
N. bonariensis	TW28	AM, 3	0.031 ± 0.004	0.528 ± 0.067	1.332 ± 0.167
		PM, 4	0.121 ± 0.017	2.094 ± 0.285	5.282 ± 0.719
N. bonariensis	Santa	AM, 3	0.003 ± 0.0004	0.039 ± 0.006	0.215 ± 0.026
	Tereza	PM, 3	0.165 ± 0.037	1.941 ± 0.531	10.871 ± 2.878
N. forgetiana	TW50	AM, 10	0.097 ± 0.016	0.542 ± 0.090	2.205 ± 0.358
		PM, 10	0.480 ± 0.056	2.680 ± 0.310	11.476 ± 1.209
*N. langsdorffii**	TW74	AM, 6	0.049 ± 0.017	0.574 ± 0.195	2.432 ± 0.857
(green corolla)		PM, 6	0.129 ± 0.047	1.484 ± 0.535	6.963 ± 2.693
N. langsdorffii	Morro de	AM, 2	0.014 ± 0.005	0.261 ± 0.009	1.162 ± 0.040
(red corolla)	Igreja	PM, 2	0.168 ± 0.007	3.105 ± 0.129	13.809 ± 0.575
N. mutabilis	Quebra	AM, 4	0.004 ± 0.0007	0.032 ± 0.006	0.148 ± 0.028
	Cabo	PM, 4	0.026 ± 0.003	0.228 ± 0.028	1.049 ± 0.139
"*Rastroensis*"	Bom Jardim	AM, 4	0.002 ± 0.0005	0.027 ± 0.009	0.147 ± 0.047
	da Serra	PM, 4	0.011 ± 0.005	0.190 ± 0.095	1.040 ± 0.520

Sections are listed in order of increasing phylogenetic affinity with *Alatae* s.s.

The Santa Tereza population of *N. bonariensis* produces significantly larger flowers than accession TW28 (fresh mass, $T = 8.2$, $DF = 38$, $P < 0.001$; nectar tube, $T = 14.2$, $DF = 38$, $P < 0.001$), with a faint pink hue absent in the uniformly white flowers of TW28. The Santa Tereza plants are twice as strongly scented as TW28 plants at night, with discernible notes of benzyl acetate and benzyl benzoate, but many times more weakly scented than TW28 during the day (Table 3). These accessions also differ dramatically in scent composition, such that only 5 of the 16 aromatic compounds, seven fatty acid derivatives and five nitrogenous compounds emitted by Santa Tereza flowers also are produced by TW28 flowers. Again, there are several possible explanations for these differences, including the loss of many distinctive compounds in accession TW28 due to inbreeding or artificial selection (see Zuker et al., 2002). Alternatively, if introgression is responsible for the addition of such compounds to a TW28-like phenotype, *N. alata* is the only member of section *Alatae* that produces the array of aromatic and nitrogenous compounds unique to the Santa Tereza population of *N. bonariensis*. Scent analyses from artificial hybrids and backcross plants (see Ippolito et al., 2004) between *N. bonariensis* and *N. alata* would provide a direct test of this hypothesis.

Finally, the floral scent of *N. alata* from Rio Pelotas was tenfold less intense than that of accession TW7 at night, on a per dry mass basis (Table 3), and conspicuously lacked the powerful linalool (27% of emissions in TW7) and eugenol notes that distinguish the scent of TW7 flowers to our human noses. Indeed, the floral fragrance of *N. alata* from Rio Pelotas is missing fully half (34) of the 69 compounds detected in TW7 plants, including several monoterpenoids and aromatic compounds (Raguso et al., 2003). However, the scent compounds present in Rio Pelotas *N. alata* flowers (nitrogenous aldoximes, 1,8-cineole, benzyl benzoate, sesquiterpene alcohols) are highly characteristic of

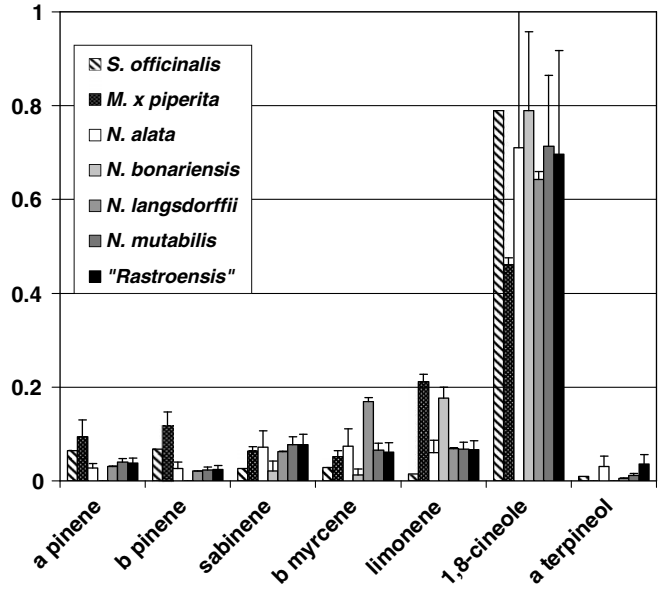

Fig. 1. Relative amounts (*Y*-axis = mean ± SEM proportions) of total monoterpenoids emitted by flowers of *Nicotiana* sect. *Alatae* sensu strictu (solid shaded bars, see legend), in comparison to the major and minor compounds produced by *Salvia officinalis* 1,8-cineole synthase enzyme expressed in *E. coli* (hatched bars; Wise et al., 1998) and the mean ± SD peak areas of SPME headspace from flowers and leaves of *Mentha* × *piperita* (stippled bars; Rohloff, 1999). Data for *Nicotiana* reflect nocturnal emissions from flowers, but in *N. bonariensis* monoterpenes are emitted by both flowers and foliage (Table 2).

hawkmoth pollinated flowers (Knudsen and Tollsten, 1993; Kaiser, 1993) and effectively attracted hawkmoths in field experiments (Ippolito et al., 2004).

2.5. Do species from section Suaveolentes emit aromatic floral odors?

The largely Australasian section *Suaveolentes* consists of 28 species with white, tubular, night blooming flowers, all of which are self-compatible (Goodspeed, 1954). Our previous study included the Australian *N. suaveolens* as an outgroup for comparison with section *Alatae*. The fragrance chemistry of *N. suaveolens* was distinguished from that of other taxa by the dominance of 12 aromatic esters, especially methyl benzoate (57.5%) and benzyl benzoate (18.8% of total emissions), and the presence of cinnamic alcohol and aldehyde (Raguso et al., 2003). We included two additional Australian species from section *Suaveolentes* (*N. cavicola* and *N. ingulba*) in this study, and asked whether they also produced aromatic-dominated floral scents. Indeed, 11 of 16 floral compounds in *N. cavicola* were benzenoids, including benzyl acetate (65.2%) and methyl benzoate (15.7% of total emissions: Table 2). Similarly, six out of nine floral compounds in *N. ingulba* were benzenoids, including cinnamic alcohol, aldehyde and acetate (c. 12% of total emissions). The dominant scent compound in *N. ingulba* (2-methylbutyraldoxime; c. 50% of total emissions) also was present in *N. suaveolens* (Raguso et al., 2003).

We had an opportunity to study *N. africana*, the sole representative of the genus native to sub-Saharan Africa (Chase et al., 2003), in cultivation at the Munich Botanical Garden. This recently described species (Merxmüller and Buttler, 1975) has been identified, through phylogenetic studies of chloroplast and nuclear gene sequences, as the sister taxon to all Australasian members of section *Suaveolentes* (Buckler et al., 1997; Chase et al., 2003). *Nicotiana africana* is native to Namibia and northwestern South Africa, and produces large sickle-shaped, greenish-yellow flowers whose morphology suggests sunbird pollination (Johnson, 1996; Hargreaves et al., 2004). This suggestion is supported by the observation that *Nicotiana glauca*, with similarly diurnal, tubular flowers, is effectively pollinated by sunbirds in exotic populations introduced to Israel (Tadmor-Melamed et al., 2004). To our noses, the flowers were nearly scentless, with a faint unpleasant odor common to the foliage of this and other *Nicotiana* species, especially *N. longiflora* and *N. plumbaginifolia*. Not surprisingly, we detected a single compound, whose spectrum suggests methyl-5-methyl hexanoate, with a retention time on carbowax that was intermediate between authentic standards of methyl hexanoate and methyl heptanoate. This compound was emitted in comparable amounts during day and night (Tables 2 and 3) from flowers, stems and leaves. In our extended study, *N. longiflora* and *N. plumbaginifolia* were the only other species that produced this foliage odor (Raguso et al., 2003). These two South American species, which are most closely related to each other (section *Alatae* s.l.), recently were shown through genomic in situ hybridization (GISH) to share one of the two ancestral genomes that contributed to the hybrid origin of the amphidiploid section *Suaveolentes*, including *N. africana* (Chase et al., 2003), as predicted by Goodspeed in 1954. We find it remarkable that the only scent compound detected from *N. africana*, at levels barely exceeding the threshold of detection for our GC–MS, constitutes a phylogenetically informative character.

3. Experimental

3.1. Study taxa

Fragrance was collected from multiple individuals (see Table 2) of each species. Seed accessions obtained by the Holtsford laboratory (University of Missouri, USA) from Brasil were: *N. alata* Rio Pelotas, *N. bonariensis* Santa Tereza, Morra da Igreja, Urubici, *N. langsdorffii* Santa Catarina, *N. mutabilis* Quebra Cabo, "Rastroensis" Bom Jardim da Serra, Rio Rastro. Australian accessions were: *N. cavicola* Badgerrada Range, Western Australia (TS 289), *N. ingulba* Carrieton, South Australia (TS 63). *Nicotiana africana* flowers were sampled from three plants growing in the Münich Botanical Garden, where *N. africana* has been cultivated since its original description by the director of the Garden (Merxmüller and

Buttler, 1975). Although documentation is not available, these plants likely represent offspring of the original type specimens. All remaining species were germinated from seed and grown in the climate-controlled greenhouses of the Ernest and Lotti Sears Plant Growth Facility, University of Missouri, Columbia MO, USA. Seeds were surface-sterilized and germinated on Murashige and Scoog medium with 0.8% agar. The seedlings were transferred to soil at the two leaf stage and fertilized at each watering with Miracle Gro® fertilizer for Azaleas. Seedlings were transplanted to 4 l containers and placed in trays for bottom watering. Greenhouse conditions included 14 h days at 24 °C and 10 h nights at 13 °C. For each sample, the number of flowers included was noted, and fresh masses were recorded for flowers and vegetation enclosed within headspace bags using a Mettler, Inc. analytical balance (to 0.001 g). Subsequently, these plant tissues were dried for 24 h in an oven at 50 °C to obtain dry masses. Vouchers were made for all taxa studied in Missouri and were deposited at UMO and MOBOT.

3.2. Volatile collection

Volatiles were collected using two complementary methods. First, we used dynamic headspace collection methods (see Raguso and Pellmyr (1998) and references therein) to quantify volatile compound emission rates during diurnal and nocturnal periods. Floral volatiles were concentrated within Reynolds, Inc. (nylon resin) oven bags placed over uncut inflorescences (5–35 flowers, depending upon species) and were trapped on adsorbent cartridges using battery-operated Supelco, Inc. Personal Air Sampler pumps. Glass cartridges were packed with 100 mg of SuperQ adsorbent (80–100 mesh, Alltech Associates, Inc.) between plugs of quartz wool and clean air was pulled over the flowers and into the adsorbent trap at a flow rate of ca. 250 ml/min. Fragrance was collected for 8 h within a large climate controlled growth chamber (Conviron, Inc.), with separate day and night collections performed for each accession. Fragrance generally was collected from flowers on the first day/night of anthesis. Flowers of *N. mutabilis* undergo a color change 24–36 h after opening, and separate collections were made from white and pink flowers.

Second, we used solid phase micro extraction (SPME; Zhang and Pawliszyn, 1993) to verify the identity of compounds detected in floral samples and improve the quality of mass spectral signal for low abundance compounds (e.g. sesquiterpenes). Headspace bags were prepared by cutting and re-sealing oven bags to 8×8 cm dimensions, using an American International Electric, Inc. impulse heat sealer. Bags were filled with 10–20 cut flowers and cinched with plastic ties. Simultaneous collections from empty bags and those enclosing vegetative parts were used to distinguish between floral volatiles, vegetative compounds and ambient contaminants.

3.3. Chemical and data analysis

For the dynamic headspace analyses, scent traps were eluted immediately with 3 ml of hexane, and the eluate was stored at −20 °C in Teflon-capped borosilicate glass vials. Before GC–MS analysis, we used a flow of gaseous N_2 to concentrate samples to 75 μl, then added 5 μl of 0.03% toluene (16 ng) as an internal standard. One microlitre aliquots of each sample were injected into a Shimadzu GC-17A equipped with a Shimadzu QP5000 quadrupole electron impact MS as a detector. For SPME, all samples were equilibrated for 30 min, then a SPME fiber coated with polydimethylsiloxane (PDMS, 100 μm film thickness) was exposed within headspace bags for an additional 30 min followed by immediate GC–MS analysis. Wound artifacts such as *Z*-3-hexenyl acetate and other products of the lipoxygenase cascade (Croft et al., 1993) were ignored for SPME analyses of cut flowers.

All GC–MS analyses were done using splitless injections on a polar GC column [diameter 0.25 mm, length 30 m, film thickness 0.25 μm (EC WAX); Alltech Associates, Inc.], with selected samples of each species also analyzed on a non-polar column [diameter 0.35 mm, length 30 m, film thickness 1.0 μm (EC-5)]. The GC–MS operating conditions and temperature programs were exactly as described by Raguso et al. (2003).

Compounds were tentatively identified using computerized mass spectral libraries [Wiley and NIST libraries (>120,000 mass spectra)]. The identity of many compounds was verified using retention times of known standards, or inferred by comparison with published retention indices (Table 2). Peak areas of total ion chromatograms (TIC) were integrated using Shimadzu's GC-Solutions software, and were quantified by comparison with the internal standard according to the following formula:

$$\text{Total floral scent} = \Sigma \text{ peak areas/peak area toluene} * 16 \text{ ng (toluene)} * 80\ \mu\text{L(total volume sample)}/8 \text{ h}$$

Emission rates were expressed in toluene equivalents as ng per flower, per g fresh floral mass and per g dry floral mass. Unfortunately, emission rate data published previously (Raguso et al., 2003) were not back calculated for the entire 80 μl sample, and thus represented the amount injected into the GC–MS only. Corrected data are presented in Table 3 for all *Nicotiana* species studied.

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References

Adams, R.P., 1975. Statistical character weighting and similarity stability. Brittonia 27, 305–316.

Adams, R.P., 1999. Systematics of multi-seeded eastern hemisphere *Juniperus* based on leaf essential oils and RAPD DNA fingerprinting. Biochemical Systematics and Ecology 27, 709–725.

Adams, R.P., von Rudloff, E., Hogge, L., 1983. Chemosystematic studies of the western North American junipers based on their volatile oils. Biochemical Systematics and Ecology 11, 189–193.

Alston, R.E., Turner, B.L., 1963. Biochemical Systematics. Prentice-Hall, Inc., Englewood Cliffs, NJ.

Azuma, H., Toyota, M., Asakawa, Y., Yamaoka, R., Garcia-Franco, J.G., Dieringer, G., Thien, L.B., Kawano, S., 1997. Chemical divergence in floral scents of *Magnolia* and allied genera (Magnoliaceae). Plant Species Biology 12, 69–83.

Baldwin, I.T., Preston, C., Euler, M., Gorham, D., 1997. Patterns and consequences of benzyl acetone floral emissions from *Nicotiana attenuata* plants. Journal of Chemical Ecology 23, 2327–2343.

Barkman, T.J., Beaman, J.H., Gage, D.A., 1997. Floral fragrance variation in *Cypripedium*: implications for evolutionary and ecological studies. Phytochemistry 44, 875–882.

Buckler IV, E.S., Ippolito, A., Holtsford, T.P., 1997. The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. Genetics 145, 821–832.

Chase, M.W., Knapp, S., Cox, A.V., Clarkson, J.J., Butsko, Y., Joseph, J., Savolainen, V., Parokonny, A.S., 2003. Molecular systematics, GISH and the origin of hybrid taxa in *Nicotiana* (Solanaceae). Annals of Botany 92, 107–127.

Colby, S.M., Alonso, W.R., Katahira, E.J., McGarvey, D.J., Croteau, R., 1993. 4*S*-Limonene synthase from the oil glands of spearmint (*Mentha spicata*). The Journal of Biological Chemistry 268, 23016–23024.

Croft, K.P.C., Jüttner, F., Slusarenko, A.J., 1993. Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. Plant Physiology 101, 13–24.

Dicke, M., 1994. Local and systemic production of volatile herbivore-induced terpenoids: their role in plant–carnivore mutualism. Journal of Plant Physiology 143, 465–472.

Dobson, H.E.M., Arroyo, J., Bergström, G., Groth, I., 1997. Interspecific variation in floral fragrances within the genus *Narcissus* (Amaryllidaceae). Biochemical Systematics and Ecology 25, 685–706.

Gäbler, A., Boland, W., Priess, U., Simon, H., 1991. Stereochemical studies on homoterpene biosynthesis in higher plants: mechanistic, phylogenetic and ecological aspects. Helvetica Chimica Acta 74, 1773–1789.

Gerlach, G., Schill, R., 1989. Fragrance analysis, an aid to taxonomic relationships of the genus *Coryanthes* (Orchidaceae). Plant Systematics and Evolution 168, 159–165.

Givnish, T.J., Sytsma, K.J., 1997. Homoplasy in molecular vs. morphological data: the likelihood of correct phylogenetic inference. In: Givnish, T.J., Sytsma, K.J. (Eds.), Molecular Evolution and Adaptive Radiation. Cambridge University Press, Cambridge, UK, pp. 55–102.

Goodspeed, T.H., 1954. The genus *Nicotiana*. Origins, relationships and evolution of its species in the light of their distribution, morphology and cytogenetics. Chronica Botanica Co., Waltham, MA.

Harbourne, J.B., 1977. Introduction to Ecological Biochemistry. Academic Press, Ltd., London.

Hargreaves, A.L., Johnson, S.D., Nol, E., 2004. Do floral syndromes predict specialization in plant pollination systems? An experimental test in an "ornithophilous" African *Protea*. Oecologia 140, 295–301.

Ippolito, A., Fernandes, G.W., Holtsford, T.P., 2004. Pollinator preferences for *Nicotiana alata*, *N. forgetiana* and their F1 hybrids. Evolution 58, 2634–2644.

Johnson, S.D., 1996. Bird pollination in South African species of *Satyrium* (Orchidaceae). Plant Systematics and Evolution 203, 91–98.

Jürgens, A., Webber, A.C., Gottsberger, G., 2000. Floral scent compounds of Amazonian Annonaceae species pollinated by small beetles and thrips. Phytochemistry 55, 551–558.

Jürgens, A., Witt, T., Gottsberger, G., 2003. Flower scent composition in *Dianthus* and *Saponaria* species (Caryophyllaceae) and its relevance for pollination biology and taxonomy. Biochemical Systematics and Ecology 31, 345–357.

Kaczorowski, R.L., Gardener, M.C., Holtsford, T.P., 2005. Nectar traits in *Nicotiana* section *Alatae* (Solanaceae) in relation to floral traits, pollinators and mating system. American Journal of Botany 92, 1270–1283.

Kaiser, R., 1991. Trapping, investigation and reconstitution of flower scents. In: Müller, P.M., Lamparsky, D. (Eds.), Perfumes: Art, Science and Technology. Elsevier Applied Science Ltd., London, pp. 213–250.

Kaiser, R., 1993. The Scent of Orchids: Olfactory and Chemical Investigations. Elsevier, Amsterdam.

Kite, G.C., Hetterschieid, W.L.A., 1997. Inflorescence odours of *Amorphophallus* and *Pseudodracontium* (Araceae). Phytochemistry 46, 71–75.

Knudsen, J.T., Mori, S.A., 1996. Floral scents and pollination in Neotropical Lecythidaceae. Biotropica 28, 42–60.

Knudsen, J.T., Ståhl, B., 1994. Floral odours in the Theophrastaceae. Biochemical Systematics and Ecology 22, 259–268.

Knudsen, J.T., Tollsten, L., 1993. Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. Botanical Journal of the Linnean Society 113, 263–284.

Knudsen, J.T., Tollsten, L., Groth, I., Bergström, G., Raguso, R.A., 2004. Trends in floral scent chemistry in pollination syndromes: floral scent composition in hummingbird-pollinated taxa. Botanical Journal of the Linnean Society 146, 191–199.

Levin, R.A., Raguso, R.A., McDade, L.A., 2001. Fragrance chemistry and pollinator affinities in Nyctaginaceae. Phytochemistry 58, 429–440.

Levin, R.A., McDade, L.A., Raguso, R.A., 2003. The systematic utility of floral and vegetative fragrance in two genera of Nyctaginaceae. Systematic Biology 52, 334–351.

Mabry, T.J., 1977. The order Centrospermae. Annals of the Missouri Botanical Garden 64, 210–220.

Mabry, T.J., Taylor, A., Turner, B.L., 1962. The betacyanins and their distribution. Phytochemistry 2, 61–64.

Merxmüller, H., Buttler, K.P., 1975. *Nicotiana* in der Afrikanischen namib ein pflanzengeographisches und phylogenitisches Rätsel. Mitteilungen der Botanischen Staatssammlung München 12, 91–104.

Negre, F., Kish, C.M., Boatright, J., Underwood, B., Shibuya, K., Wagner, C., Clark, D.G., Dudareva, N., 2003. Regulation of methylbenzoate emission after pollination in snapdragon and petunia flowers. The Plant Cell 15, 2992–3006.

Nogueira, P.C. de L., Bittrich, V., Shepherd, G.J., Lopes, A.V., Marsaioli, A.J., 2001. The ecological and taxonomic importance of flower volatiles of *Clusia* species (Guttiferae). Phytochemistry 56, 443–452.

Plowman, T. 1974. The South American species of *Brunfelsia* (Solanaceae). PhD dissertation, Harvard University, Cambridge, MA.

Rohloff, J., 1999. Monoterpene composition of essential oil from peppermint (*Mentha* × *piperita* L.) with regard to leaf position using solid-phase microextraction and gas chromatography/mass spectrometry analysis. Journal of Agricultural and Food Chemistry 47, 3782–3786.

Raguso, R.A., Pellmyr, O., 1998. Dynamic headspace analysis of floral volatiles: a comparison of methods. Oikos 81, 238–254.

Raguso, R.A., Levin, R.A., Foose, S.E., Holmberg, M.W., McDade, L.A., 2003. Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. Phytochemistry 63, 265–284.

Rodman, J.E., 1979. Divergence, convergence and parallelism in phytochemical characters: the glucosinolate-myrosinase system. In: Young, D.A., Seigler, D.S. (Eds.), Phytochemistry and Angiosperm Phylogeny. Praeger Scientific, Inc., New York, pp. 43–79.

Schulz, S., Francke, W., Edgar, J., Schneider, D., 1988. Volatile components from androconial organs of Danaine and Ithomiine butterflies. Zeitschrift für Naturforsch 45c, 99–104.

Seigler, D.S., 1979. Terpenes and plant phylogeny. In: Young, D.A., Seigler, D.S. (Eds.), Phytochemistry and Angiosperm Phylogeny. Praeger Scientific, Inc., New York, pp. 117–148.

SPSS Inc., 1999. SPSS for Windows, version 10.0.5. SPSS Inc., Chicago.

Stehmann, J.R., Semir, J., Ippolito, A., 2002. *Nicotiana mutabilis* (Solanaceae), a new species from southern Brazil. Kew Bulletin 57, 639–646.

Svensson, G.P., Hickman, M.O., Bartram, S., Boland, W., Pellmyr, O., Raguso, R.A., 2005. Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae). American Journal of Botany 92, 1624–1631.

Tabacchi, R., Garnero, J., Quellet, C. Tsoupras, G., 1986. Study of the essential oil of *Buddleja salvifolia* (River Plant). In: Lawrence, B.M., Mookherjee, B.D., Willis, B.J. (Eds.), Flavors and Fragrances, a World Perspective. Proceedings of the 10th International Congress of Essential Oils, Fragrances and Flavors. Washington, DC.

Tadmor-Melamed, H., Markman, S., Arieli, A., Distl, M., Wink, M., Izhaki, I., 2004. Limited ability of Palestine Sunbirds *Nectarinia osea* to cope with pyridine alkaloids in nectar of Tree Tobacco, *Nicotiana glauca*. Functional Ecology 18, 844–850.

Weiss, M.R., 1995. Floral color change: a widespread functional convergence. American Journal of Botany 82, 167–185.

Williams, N.H., Whitten, W.M., 1999. Molecular phylogeny and floral fragrances of male euglossine bee-pollinated orchids: a study of *Stanhopea* (Orchidaceae). Plant Species Biology 14, 129–136.

Wise, M.L., Savage, T.J., Katahira, E., Croteau, R., 1998. Monoterpene synthases from common sage (*Salvia officinalis*). The Journal of Biological Chemistry 273, 14891–14899.

Zhang, Z.H., Pawliszyn, J., 1993. Headspace solid-phase microextraction. Analytical Chemistry 65, 1843–1852.

Zuker, A.T., Tzfira, T., Ben-Meir, H., Ovadis, M., Shklarman, E., Itzhaki, H., Forkmann, G., Martens, S., Neta-Sharir, J., Weiss, D., Vainstein, A., 2002. Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. Molecular Breeding 9, 33–41.