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Floral scents of four *Heliotropium* (Boraginaceae) species endemic to Chile: a comparative analysis

[Aromas florales de cuatro especies de *Heliotropium* (Boraginaceae) endémicas de Chile: un análisis comparativo]

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Abstract: The floral volatile chemicals of four *Heliotropium* (Boraginaceae) species endemic to Chile are described and their relationship with an *Heliotropium* phylogeny assessed. Twenty-two compounds were identified; the most prominent family of compounds in *H. floridum*, *H. myosotifolium* and *H. stenophyllum* were benzenoids, and in *H. pycnophyllum* prominence of benzenoids was shared with two monoterpenes, (*E*)-Ocymene and 1,8-cineol. The similarity between chemical mixtures of the species significantly decreased with increasing phylogenetic distance between them. Neighbor-joining analysis produced a tree topology with one clade comprised by *H. myosotifolium* and *H. stenophyllum* and an unresolved relationship between this clade and *H. floridum* and *H. pycnophyllum*; this topology bears a close relationship with molecular phylogeny proposed for the group. These preliminary results presented suggest that the genus *Heliotropium* complies with the condition of phylogenetically-related floral scent interspecific variation, a key condition in studies of plant-pollinator coevolution mediated by them.

Keywords: Floral scents; floral volatiles; native flora of Chile; VOC

Resumen: Se describieron los volátiles florales de cuatro especies de *Heliotropium* (Boraginaceae) y se evaluó su relación con una filogenia del género. Se identificaron 22 compuestos; los bencenoides fueron los más abundantes en *H. floridum*, *H. myosotifolium* and *H. stenophyllum*, acompañados de dos monoterpenos, el (*E*)-Ocimeno y el 1,8-cineol, en *H. pycnophyllum*. La similitud entre los componentes de cada especie aumentó con la distancia filogenética entre ellas. El análisis "neighbor-joining" produjo un árbol con un clado que contenía a *H. myosotifolium* y *H. stenophyllum* y una relación no resuelta entre ese clado y *H. floridum* y *H. pycnophyllum*; esta topología guarda similitud con la hipótesis filogenética molecular propuesta para el grupo. Estos resultados preliminares sugieren que el género *Heliotropium* cumple con la condición de mostrar variación interespecífica en sus volátiles florales, condición básica para estudios de coevolución planta-polinizador mediada por ellos.

Palabras clave: Aromas florales; volátiles florales; flora nativa de Chile; COV

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INTRODUCTION

Plants use various types of signals to guide pollinators to their flowers, visual and olfactory cues being the most important. Early research focused on the importance of visual display (color and shape of flowers) for the attraction of pollinating insects (*e.g.*, Faegri and van der Pijl, 1979; Chittka and Thomson, 2001). Only recently, the advent of sensitive techniques such as gas chromatography – mass spectrometry in its various forms has dramatically enhanced the capacity to detect and identify trace components of mixtures of volatile organic compounds (VOCs) and allowed in-depth studies of floral scents. VOCs in floral scents have been characterized for well over one thousand species of angiosperms (Knudsen *et al.*, 2006; Niemeyer and Teillier, 2007), and scent compounds mediating attraction of potential pollinators have been identified for several insect-plant interactions, many of which are highly specialized (*e.g.*, Borg-Karlson, 1990; Pellmyr *et al.*, 1996; Andersson *et al.*, 2002; Plepys *et al.*, 2002; Stensmyr *et al.*, 2002; Dötterl *et al.*, 2006; Chen *et al.*, 2009; Svensson *et al.*, 2010). Studies using floral scent as a phenotypic trait to test hypotheses regarding coevolutionary aspects of pollinator-plant interactions have been relatively scarce, notwithstanding that floral scents mediate impacts of pollinators and are potentially available for selection (Raguso, 2008). On the other hand, phylogenies and the comparative approach have produced an important impact in studies of mutual adaptation and coevolution of plant/insect interactions (Knudsen and Tollsten, 1993; Dobson, 2006; Schiestl, 2010; Schiestl and Dötterl, 2013).

Of importance to coevolutionary studies of plant pollinator systems based on phylogenies is the occurrence of chemical variation in floral scents related to the phylogeny of the interacting plant group. The genus *Heliotropium* (Boraginaceae) is an attractive system to study this phenomenon because its flowers produce a strong sweet odour and hence their VOCs should be readily detectable and identifiable, and a molecular phylogenetic hypothesis for the genus has been recently proposed (Luebert *et al.*, 2011). In this study, the floral volatile chemicals of four *Heliotropium* species endemic to Chile are described and their relationship with the *Heliotropium* phylogeny assessed.

MATERIALS AND METHODS

Plant material

Fresh flowers of each taxon (between 40 and 70 g fresh wt per sample) were collected from many individual plants occurring within the sites individualized below. Samples were deposited inside a glass jar provided with an inlet and an outlet. A compressed air cylinder containing ultrapure synthetic air was attached to the inlet through a regulator which controlled the air flow and at the outlet, a glass column was attached which contained Porapak Q (*ca.* 100 mg). Volatile entrainment (*ca.* 4 min per gram of sample, with an air flow of 0.5 L/min) was performed immediately after flower collection. The volatiles adsorbed on the Porapak Q were eluted with 1 mL of dichloromethane. These extracts were concentrated under a dry nitrogen flow and injected into the column of a gas chromatograph coupled to a mass detector (GC-MS).

Collection data are as follows: *Heliotropium floridum* near Huasco (28°19,0S - 71°09,2O - 102 msnm), *H. myosotifolium* near Huasco (28°27,5°S; 71°10,9°O; 9 msnm), *H. pycnophyllum* near Chañaral (26°04,3S; 70°31,3O; 339 masl) and *H. stenophyllum* near La Higuera (27°52,2S; 70°59,5O; 96 msnm). Voucher specimens were lodged at the herbarium of Universidad de Concepción (CONC).

Chemical analysis

The GC/MS system consisted of a Hewlett-Packard model HP5891 gas chromatograph with a SPB-5 (25 m x 0.2 mm id) capillary column, and a Hewlett-Packard model HP5972 mass spectrometric detector with integrated data system. Ionization by electron impact (70 eV) was performed at 280° C. The GC oven was programmed to remain at 50° C for 10 min, to increase up to 280° C at a rate of 5° C/min, and then to remain at 280° C for 45 min. The identification of compounds in the chromatographic profiles was achieved by comparison of their mass spectra with a library database, and was confirmed by comparison of retention indices with those of authentic standards or with values from the literature.

Phylogenetic analysis

Percentages of compounds for each individual were converted to discrete characters as follows: 0 = absence, 1 = ≤ 10%, 2 = > 10 - 40%, and 3 = > 40%. These characters were used in neighbor-joining analysis using PAUP* (Swofford, 2002). The statistical support was assessed for each cladogram

by bootstrap consensus trees with over 1000 replications (Felsenstein, 1985). No outgroup was used.

RESULTS AND DISCUSSION

The composition of the VOC mixtures in terms of individual volatile compounds as well as of families of compounds entrained from the headspace of flowers of the species studied are listed in Table 1.

Twenty-two compounds were identified. The most prominent family of compounds in *H. floridum*, *H. myosotifolium* and *H. stenophyllum* were benzenoids, and in *H. pycnophyllum* prominence of benzenoids was shared with two monoterpenes, (*E*)-Ocimene and 1,8-cineol. To the best of our knowledge, only two reports have been published on the chemical composition of the floral scents of *Heliotropium* species.

TABLE 1
Individual volatile compounds and families of compounds entrained from the headspace of flowers of *Heliotropium floridum*, *H. myosotifolium*, *H. pycnophyllum* and *H. stenophyllum*

Compound or Family of compounds	GC area (%)			
	<i>H. floridum</i>	<i>H. myosotifolium</i>	<i>H. pycnophyllum</i>	<i>H. stenophyllum</i>
Methyl angelate	4.6	0	0	1.2
Benzyl alcohol	0	0	0	2.1
Benzaldehyde	14.7	0	31.5	0
Methyl phenylacetate	2.4	0	0	1.1
Methyl cinnamate	1.8	0	0	0
<i>p</i> -Methylanisol	0	69.1	0	88.3
Methyl <i>p</i> -anisate	2	0	0	0
Phenylacetaldehyde	2.1	0	0	0
β -Myrcene	0	0	1	0
Limonene	0.7	0	0	0
Methyl (<i>Z</i>)-hexenoate	0	0	0	0.4
Estragole	0	4.9	0	0
Germacrene D	0.5	0	0	0
(<i>Z</i>)-Ocymene	0	0	0.2	0
Sabinene	0	0	6.7	0
(<i>Z</i>)-3-Hexenyl acetate	0.8	0	0	0
(<i>E</i>)-Ocymene	13.4	26	29.7	2.7
1,8-Cineol	0	0	27.7	0
α -Pinene	0.5	0	0.3	0
(<i>E</i>)-3-Hexen-1-ol	0	0	0	0.6
Methyl benzoate	56.3	0	0	1.4
γ -Terpinene	0	0	0.4	0.7
TOTAL	99.8	100	97.5	98.5
Benzenoids	79.3	74.0	31.5	92.9
Aliphatic	5.4	0	0	1.2
Monoterpenes	14.6	26.0	66.0	3.4
Sesquiterpenes	0.5	0	0	0
TOTAL	99.8	100	97.5	98.5

Hisano *et al.* (1995), using solid phase microextraction techniques, found benzaldehyde, benzyl acetate and p-anisaldehyde as main components of *H. arborescens*, while Kays *et al.* (2005) reported benzaldehyde as the main compound for the same species using headspace collection. This suite of benzenoids are likely responsible for the sweet nature of the flower bouquet.

The relationship between chemical constitution of floral scents and phylogeny of the

plant group was assessed following two approaches. The first approach explored the relationship between phylogenetic distance between species, defined as the number of nodes between species in the phylogeny (Figure 1A), and a similarity index between chemical mixtures, defined as the number of non-zero matches (presence-presence) divided by the number of non matches (presence-absence and *vice-versa*). These two parameters were found to be significantly correlated (Figure 2).

FIGURE 1

A. Phylogenetic relationships within the *Cochranea* clade in the proposed phylogeny of genus *Heliotropium* (Luebert *et al.*, 2011); paths for determining phylogenetic distance by counting nodes (circles) between the species studied are highlighted. **B.** Most parsimonious tree (26 steps) based on the analysis of chemical constituents of the floral scents of *H. floridum*, *H. myosotifolium*, *H. pycnophyllum*, and *H. stenophyllum*.

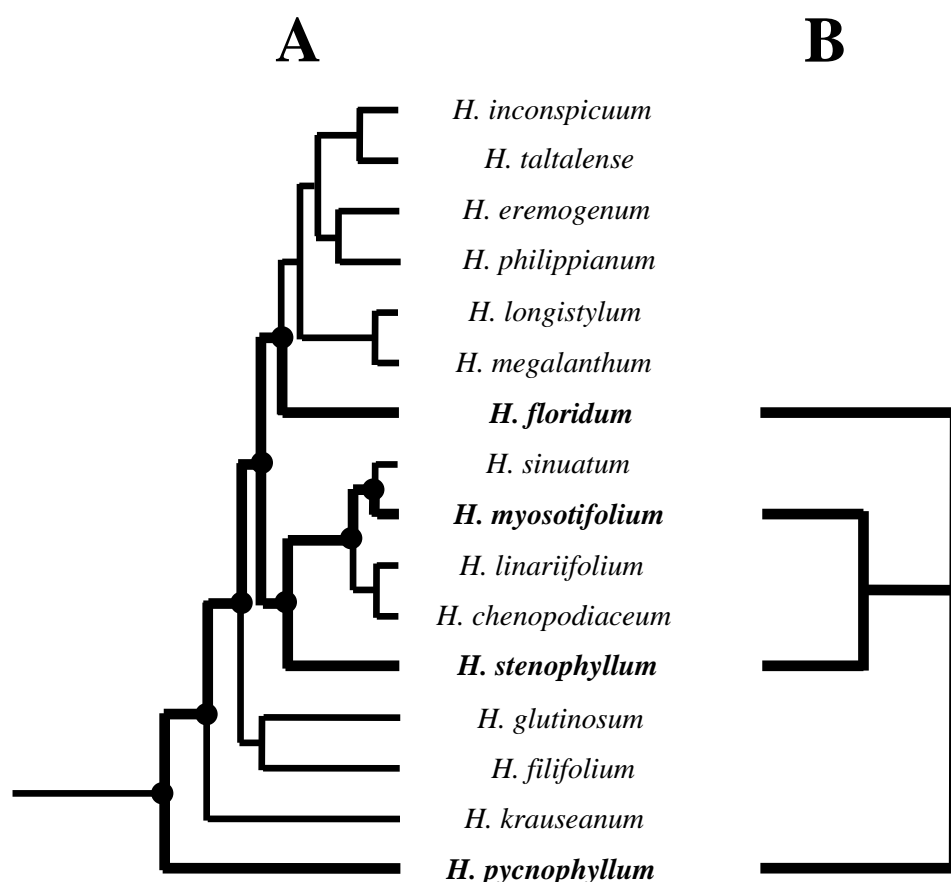
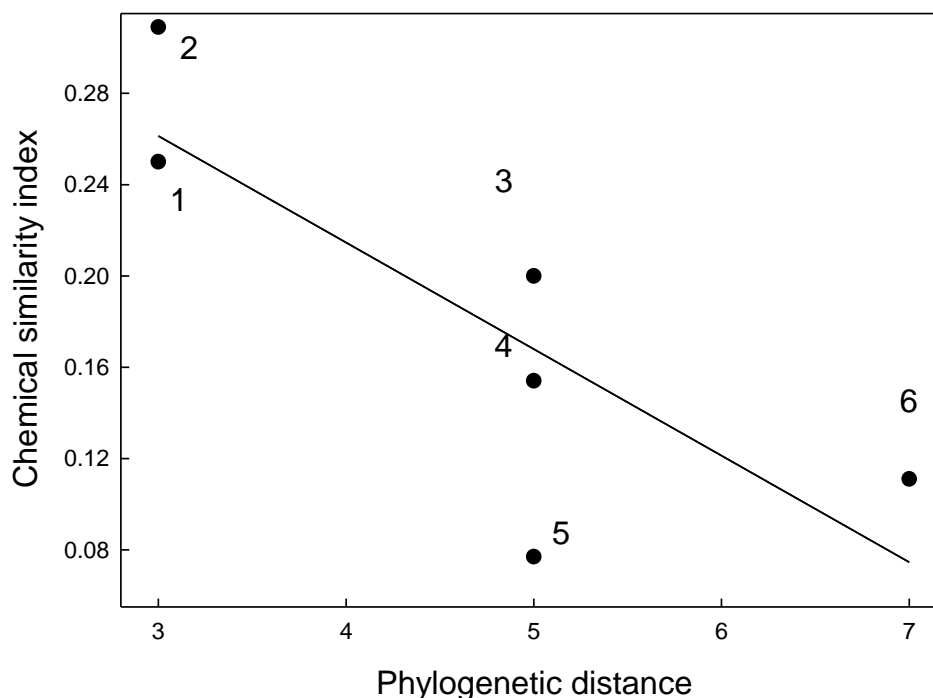


FIGURE 2

Correlation between chemical and phylogenetic distances. 1 = Hm-Hs, 2 = Hf-Hs, 3 = Hf-Hp, 4 = Hp-Hs, 5 = Hf-Hm and 6 = Hm-Hp, with Hf = *H. floridum*, Hm = *H. myosotifolium*, Hp = *H. pycnophyllum* and Hs = *H. stenophyllum*. Pearson correlation: $R = -0.807$, $N = 6$, $P = 0.052$.



The second approach used neighbor-joining analysis, which produced a tree topology with one clade comprised by *H. myosotifolium* and *H. stenophyllum* with a medium bootstrap support (62) and an unresolved relationship between this clade and *H. floridum* and *H. pycnophyllum* (Figure 1B). This topology resembles that of the proposed phylogeny for the group (Luebert *et al.*, 2011).

Although chemical composition of floral scents was correlated with the phylogeny of the group, other factors should be considered if and when this work is expanded to other members of the group, such as: i) floral scents are affected by flower ontology (Steenhuisen *et al.*, 2010), environmental factors (Jakobson and Olsen, 1994), and show variations between individuals (Knudsen, 2002) and populations (Füssel *et al.*, 2007; Anderson *et al.*, 2010). These sources of variation were averaged out in the samples analyzed since they correspond to a

pool of samples collected from individual flowers at a range of stages of development, from numerous plant individuals at different ontogenetic stages, and growing in a range of microenvironments; however, the lack of intraspecific replicates remains a problem, and ii) floral volatiles may change during the course of the day (Dötterl *et al.*, 2012). Flowers of the four species studied in the present work were collected at different times of the day and not necessarily at the peak of scent production; the circadian rhythm of scent production should be considered and scents of different species should be collected at the same time. In spite of these limitations, the preliminary results presented strongly suggest that the genus *Heliotropium* complies with the condition of phylogenetically related flower scent variation, a key condition to test the coevolutionary involvement of floral scents in plant-pollinator interactions. An extension of this work to other species within the

published phylogeny which occur in Chile, should be considered. Of particular interest would be the inclusion of *H. curassavicum* and *H. patagonicum* assigned to the *Platygyne* clade, *H. paranychioides* assigned to the *Plagiomeris* clade, and *H. microstachyum* assigned to the *Hypsogenia* clade of the proposed molecular phylogeny of genus *Heliotropium* (Luebert *et al.*, 2011).

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