Orchid Fragrance Complexity as a Mechanism for Euglossine Bee Pollinator Specialization

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ABSTRACT

Neotropical orchids of subtribes Stanhopeinae and Catasetinae are exclusively pollinated by euglossine bees (Euglossini). This study determines whether orchids present specific combinations and proportions of chemical odors in order to attract a limited subset of euglossine bee species, thereby avoiding hybridization that may result from the attraction of too many pollinators. Chemical baits of cineole, benzyl acetate, methyl salicylate, cineole/benzyl acetate (a Stanhopea tricornis fragrance analogue), and cineole/methyl salicylate/methyl benzoate (a Catasetum gnomus fragrance analogue) were used to attract fifteen species of euglossine bees in the San Luis Valley, Monteverde, Costa Rica. The diversity of bee species attracted to the cineole/benzyl acetate solution was significantly lower than either cineole or benzyl acetate (Shannon-Weiner index; for cineole/benzyl acetate: $H' = 0.89$; for cineole: $H' = 1.91$; for benzyl acetate: $H' = 1.82$; for cineole/benzyl acetate and cineole: $F = 4.89, df = 68.00$; for cineole/benzyl acetate and benzyl acetate: $F = 5.66, df = 64.29, p < 0.05$). The diversity of species attracted to the cineole/methyl salicylate/methyl benzoate solution was significantly lower than cineole (Shannon-Weiner index; for cineole/methyl salicylate/methyl benzoate: $H' = 0.82$; for cineole: $H' = 1.91$; $F = 6.16, df = 61.25$, $p < 0.05$). The results provide evidence that orchid fragrance complexes are designed to attract specific euglossine species that are both effective pollinators and specialized enough to reduce risk of interspecific outcrossing.

RESUMEN

Las orquideas neotropicales de las subtribus Stanhopeinae y Catasetinae son exclusivamente polinizadas por abejas euglosinas (Euglossini). Este estudio determinó si las orquideas presentan combinaciones y proporciones de olores químicos específicos con el fin de atraer un grupo limitado de abejas euglosinas, para evitar hibridización que puede resultar de la atracción de muchos polinizadores. Sebos de cineol, bencyl acetato, metil salisilato/metil benzoato (un análogo de la fragancia de Stanhopea tricornis), y cineol/metil salicilato/metil benzoato (un análogo de la fragancia de Catasetum gnomus) fueron utilizados para atraer quince euglossinas en el valle de San Luis, Monteverde, Costa Rica. La diversidad de especies de abejas atraídas al cineole/benzyl acetato fue significativamente menor que el cineole o benzyl acetato (Indice Shannon-Weiner para cineole/benzyl acetato $H' = 0.89$; for cineole $H' = 1.91$; for benzyl acetato $H' = 1.82$; for cineole/benzyl acetato y cineole $F = 4.89, df = 68$; for cineole/benzyl acetato y benzyl acetato $F = 5.66, df = 64.29, p < 0.05$). La diversidad de especies atraídas al cineole/metil salicilato/metil benzoato fue significativamente menor que el cineole (Indice de Shannon-Weiner para cineole metil salicilato/metil benzoato $H' = 0.82$; for cineole $H' = 1.91$; $F = 6.16 df = 61.25, p < 0.05$). Los resultados presentan evidencia que los complejos de fragancias de las orquideas están diseñados para atraer especies específicas de abejas euglosinas que sean polinizadores efectivos y suficientemente especializados para reducir el riesgo de exocruzamiento interespecífico.

INTRODUCTION

Many plants depend on their ability to attract pollinators so that outcrossing may occur, thereby increasing genetic variation in the population (Dressler 1981). In the tropics, plant species are typically rare with scattered distributions, making it necessary to specialize with specific animal pollinators to ensure that pollen will effectively reach conspecifics (Schatz 1990). As vessels of both pollen distribution and reception, flowers are characterized by specific morphological and
chemical traits that result from coevolution with their respective pollinators (Dressler 1993, Raguso 2008). By catering to the preferences of their pollinator organism, plants receive a means for outcrossing via the resulting pollen removal. In turn, pollinators use the flower rewards of nectar, pollen, etc. to maximize their own fitness, making this evolutionary relationship vital for both plant and animal (Raguso 2008).

Some of the most interesting and highly evolved pollination syndromes are those of Family Orchidaceae. Orchids comprise the most species rich of any plant family, with 20,000 species known worldwide and 7,000 in the Neotropics (Roubik & Hanson 2004). Despite high biodiversity, orchids have particularly low biomass, scattered distributions, and inconspicuous microhabitats, necessitating some of the most highly coevolved plant-pollinator relationships known (Raguso 2008). Orchid pollinators seek out conspecifics in search of nectar (the most common reward), oil, pseudopollen, or perfume, and therefore provide a mechanism for the transfer of pollinia over long distances and varied microhabitats (Dressler 1993, Raguso 2008).

The interaction between Neotropical orchids and male euglossine bees (Family Apidae; tribe Euglossini), or orchid bees, is a well-studied example of a specialized orchid-pollinator relationship and is estimated to have arisen repeatedly over the last 20 million years (Engel 1999, Roubik & Hanson 2004). As a result of this long time period, male euglossine bees have coevolved extensively with their orchid partners and currently pollinate up to ten percent of Neotropical orchid species from subtribes Catasetinae, Stanhopeinae, Lycastinae, Zygopetalinae, and Oncidiinae (Ramirez et al. 2002; Roubik & Hanson 2004). This highly evolved pollination syndrome is unique in that (1) each partner confers greater reproductive fitness to the other and (2) fragrance is the currency (and sole reward) involved in these mutualisms. Thus while a male bee is collecting fragrances for use in mate attraction, the orchid is increasing its chances of outcrossing by transferring pollinia to the body of the bee (Dressler 1993; Roubik & Hanson 2004).

Though outcrossing is the essential goal of pollination from the perspective of the orchid, there is risk of hybridization via uptake of pollinia from dissimilar species (Roubik & Hanson 2004). Evolutionarily, hybridization is harmful in that it leads to homogenization of species, thereby decreasing genetic variation and leading to simpler systems that lower population resilience and decrease community function. Therefore, in order to coexist in the same habitat and remain separate species, orchids utilize a variety of reproductive isolating mechanisms, including temporal strategies as well as complex and varying floral morphology (Dressler 1981). Given such mechanisms, only euglossine bee species that are of a certain size or that exhibit particular behavior can successfully transfer the pollen of specific orchids. However, since many species of euglossine bees may visit a variety of orchid species for fragrance collection, the chance of transgenic pollinia uptake and subsequent hybridization may be increased. To avoid hybridization, orchid species may produce different fragrance combinations and/or proportions of combinations to further narrow the specificity of bee species, therefore using fragrance as yet another reproductive isolating mechanism (Hills et al. 1972; Roubik & Hanson 2004, Raguso 2008).

There is strong evidence to suggest that orchids produce and bees collect specific fragrance combinations. At least 60 compounds have been identified in the odors of euglossine orchids, but individual orchid species often contain fewer than 12 of these, with domination by one or two chemical components (Gerlach & Schill 1991; Eltz et al. 2005). Also, it is well documented that euglossine males display interspecific fragrance bouquet preference (Hills et al. 1972; Roubik & Hanson 2004; Ackerman et al. 2005; Eltz et al. 2005). However, past studies have shown contradictory results in testing the preference by male bees for chemical baits of single or multiple fragrances (Dodson et al. 1969; Schiestl & Roubik 2003). Modifier chemicals
(which are found in many orchid fragrance complexes, but do not by themselves act as attractants) such as alpha-pinene are known to reduce attractant potential, but it is not as certain whether a combination of successful attractants also alters the composition of pollinators. This study set out to determine whether orchids, specifically Stanhopea tricornis and Catasetum gnomus of Costa Rica, present specific combinations and proportions of chemical odors to attract a limited subset of Euglossine bee species. These two orchid species serve as representatives of the subtribes Stanhopeinae and Catasetinae, the only subtribes pollinated exclusively by male Euglossine bees (Williams & Whitten 1983). I predicted that more complex combinations of fragrances would attract fewer Euglossine bee species as a mechanism to attract a limited set of effective pollinators, thereby increasing chances of outcrossing and reducing chances of orchid hybridization.

METHODS

General

This study was conducted in the Puntarenas province of Costa Rica in the San Luis Valley, Monteverde, Costa Rica (1000 meters above sea level). Sampling was done in primary forest from 800 to 1200 (period of greatest fragrance collection by male Euglossine bees, Williams & Whitten 1983) for 15 days from October 27 to November 16, 2009. My sampling consisted of two separate parts (1) Fragrance and (2) Visitation of species.

Fragrance Combinations

In order to test for specificity of fragrances and pollinator attraction, I mimicked the fragrance combination of two orchid species, Stanhopea tricornis and Catasetum gnomus. Stanhopea tricornis produces a fragrance that is one part cineole and 39 parts benzyl acetate (Dodson et al. 1969), so I mixed 0.2 mL cineole and 7.8 mL benzyl acetate to create its fragrance analogue. Catasetum gnomus produces a fragrance that is 59% methyl salicylate, 10% cineole, and 9% methyl benzoate (Hills et al. 1972), so I created the analogue with 1.5 mL methyl salicylate, 0.25 mL cineole, and 0.23 mL methyl benzoate. For each orchid species I conducted trials for each separate fragrance and for the specific combination of fragrances.

Visitation of Species

Only one fragrance or combination was tested in a day. For each trial, I saturated cotton balls with three drops of the appropriate fragrance or solution, hung them from shrubs, and identified the species that visited the bait. Baits were replenished with fragrance halfway through the trial period. Some Euglossine bee species pairs were difficult to distinguish in the field and were recorded as either/or, including Euglossa cybelia and maculilabris, Euglossa deceptrix and tridentata, Euglossa dodsoni and erythrobolora, and Eulaema meriana and bombiformis. Each bee observed was marked with a paint pen to avoid recounts. Abiotic conditions, including temperature, humidity, and general weather conditions (sunny, windy, raining, etc.) were also recorded with each observation.

Statistical Analysis
A template was used to calculate Shannon-Weiner diversity index for the euglossine bee community visiting each chemical fragrance. These values were compared using modified t-tests.

**RESULTS**

**Statistical Results**

A total of 191 euglossine bees were observed across 15 species of all four genera (Table 1). The most frequent visitors to the fragrance cineole were *Euglossa imperialis* and *Euglossa cybelia/maculilabris* and the most frequent visitors to benzyl acetate were *E. imperialis*, *Euglossa deceptrix/tridentata*, and *Eulaema meriana*. *Eulaema meriana* was the most frequent visitor of cineole/benzyl acetate and, along with *E. imperialis*, to methyl salicylate and cineole/methyl salicylate/methyl benzoate. *Euglossa championi* and *Euglossa dodsoni/erythrochilora* were both attracted to cineole and benzyl acetate when these chemicals were presented alone, but not in combination. *Euglossa championi* was similarly attracted to methyl salicylate alone, but not to the cineole/methyl salicylate/methyl benzoate solution (Table 1).

For the test of *S. tricornis* orchid fragrances (cineole, benzyl acetate, and the cineole/benzyl acetate analogue solution), the diversity of species that visited cineole alone was not different from the diversity of species that visited benzyl acetate alone (Shannon-Weiner index; for cineole: $H' = 1.91$; for benzyl acetate: $H' = 1.82$; $F = 0.62, df = 33.80, p = 0.54$; Figure 1). Both cineole and benzyl acetate alone attracted a greater diversity of species than the cineole/benzyl acetate solution (Shannon-Weiner index; for cineole: $H' = 1.91$; for benzyl acetate: $H' = 1.82$; for cineole/benzyl acetate: $H' = 0.89$; for cineole and solution: $F = 4.89, df = 68.00$; for benzyl acetate and solution: $F = 5.66, df = 64.29, p < 0.05$).

For the test of *C. gnomus* orchid fragrances (cineole, methyl salicylate, methyl benzoate, and the cineole/methyl salicylate/methyl benzoate analogue solution), the diversity of species that visited cineole alone was greater than the diversity that visited methyl salicylate alone (Shannon-Weiner index; for cineole: $H' = 1.91$; for methyl salicylate: $H' = 0.96$; $F = 5.04, df = 64.86, p < 0.05$; Figure 1). Diversity of visitors to cineole was also greater than the diversity of visitors to the cineole/methyl salicylate/methyl benzoate solution (Shannon-Weiner index; for cineole: $H' = 1.91$; for cineole/methyl salicylate/methyl benzoate: $H' = 0.82$; $F = 6.16, df = 61.25, p < 0.05$). Methyl salicylate and the cineole/methyl salicylate/methyl benzoate solution did not attract different species diversities (Shannon-Weiner index; for methyl salicylate: $H' = 0.96$; for cineole/methyl salicylate/methyl benzoate: $H' = 0.82$; $F = 0.97, df = 72.43, p = 0.33$).

For other comparisons, the diversity of species attracted to benzyl acetate alone was greater than the diversity attracted to methyl salicylate alone as well as to the cineole/methyl salicylate/methyl benzoate solution (Shannon-Weiner index; for benzyl acetate: $H' = 1.82$; for methyl salicylate: $H' = 0.96$; for cineole/methyl salicylate/methyl benzoate: $H' = 0.82$; for benzyl acetate and methyl salicylate: $F = 6.07, df = 74.84$; for benzyl acetate and cineole/methyl salicylate/methyl benzoate: $F = 7.63, df = 86.30, p < 0.05$; Figure 1). There was no difference between the diversity of species attracted to methyl salicylate and the cineole/benzyl acetate solution (Shannon-Weiner index; for methyl salicylate: $H' = 0.96$; for cineole/benzyl acetate: $H' = 0.89$; $F = 2.31, df = 65.05, p = 0.15$). The diversity of euglossine bee visitors to the cineole/benzyl acetate solution was greater than the diversity for the cineole/methyl salicylate/methyl benzoate solution (Shannon-Weiner index; for cineole/benzyl acetate: $H' = 0.96$; for cineole/methyl salicylate/methyl benzoate: $H' = 0.82$; $F = 5.04, df = 64.86, p < 0.05$).
0.89; for cineole/methyl salicylate/methyl benzoate: $H' = 0.82; F = 2.31, df = 60.77, p = 0.02$; Figure 1).

The range of ambient temperatures with the highest species richness was between 21 and 24 °C (Figure 2) and with the highest species abundances was between 20 and 24 °C (Figure 3).

**Additional Observations**

Of the 191 bees observed, ten were carrying pollinia. All bees with pollinia were *E. meriana/bombiformis* except for one *Eufrisea surninamensis*. Seven bees carried the pollinia attached to their cleypeus, which could have been from orchid genera *Aspasia*, *Notylia*, or *Thrichophilia* (Roubik & Hanson 2004). Three bees, including *E. surninamensis*, carried the pollinia on the frontal thorax, indicating that the visited orchids could have been *Sobralia*, *Houlettia*, and/or *Kegeliella* genera.

**DISCUSSION**

Each orchid analogue experiment supported my original hypothesis. For the *Stanhopea* analogue, cineole and benzyl acetate by themselves attracted a greater diversity of bees than when the chemicals were combined in specified proportions found in *S. tricornis*. *Eulaema meriana*, the known pollinator of this orchid, was the most frequent visitor to the analogue solution. Four *Euglossa* species also visited the cineole/benzyl acetate baits, but these are all too small to be effective pollinators for *S. tricornis* (Hills et al. 1972). It is likely that these bees visited because the true fragrance composition in nature is much more complex and includes modifiers such as alpha- and beta-pinene that may normally ward off such visitors. Furthermore, given that cineole and benzyl acetate attract the same diversity of euglossine bee species, it is unlikely that fewer bees were attracted to the combined solution due to a difference in concentration.

For *Catasetum*, the combination of cineole, methyl salicylate, and methyl benzoate found in *C. gnomus* attracted a lower diversity of bees than cineole alone. As with the *Stanhopea* analogue, the most frequent visitor was *E. meriana*. *Euglossa imperialis* was also a relatively frequent visitor. What is surprising is that the diversity of bees attracted to methyl salicylate was not statistically different from the cineole/methyl salicylate/methyl benzoate solution. This could be due to the frequency of methyl salicylate in nature. Fourteen of the 39 compounds produced by *Catasetum* orchids have been identified with gas-liquid chromatography. Of the 22 *Catasetum* species analyzed by Hills *et al.* (1972), cineole is present in 64 percent of the species compared to methyl salicylate, which is only present in 14 percent of the species. Cineole is also the dominant component in 14 percent of the species while methyl salicylate is the dominant fragrance in 9 percent. Furthermore, in a meta-analysis on orchid floral fragrances as euglossine bee attractants done by Williams and Whitten (1983), 60 chemicals were identified and rated in attractiveness and abundance. Cineole was marked as a good (as opposed to moderate) attractant, is known to be present in a relatively large number of orchid species, and makes up 21 to 90 percent of the fragrance composition of most species in which it is present. Therefore cineole is more commonly found in nature than methyl salicylate and acts as a stronger attractant. It is reasonable to conclude that the inclusion of methyl salicylate in a floral presentation already acts to attract a limited subset of euglossine species in comparison to cineole. Further studies should be done to test whether the assumption that frequency in nature and attractive ability are indeed positively correlated.
For the chemical baits presented, simple and complex alike, *E. meriana* and *E. imperialis* made up a large portion of the bee visitors. It is possible that the orchid fragrances are not only selectively attracting species, but that these bees species are simply more generalist. For *E. meriana* individuals with pollinia, the placement on the body suggests that these individuals received the pollen from at least two and at most six different orchid genera, none of which are *Catasetum* or *Stanhopea*. Furthermore, it is not unusual for certain euglossine species to be attracted to a wide variety of odors. In nature, euglossine males have been observed collecting odors from a diversity of locations, including rotting fruit and wood, tree bark, and exposed roots (Ackerman 1989). Furthermore, researchers often successfully attract euglossines using chemicals that have not been found in natural orchid fragrance complexes (e.g. vanillin, skatole, p-crestyl acetate) (Williams and Whitten 1983). One study even reports technical grade DDT used for malaria control as an attractant for male *Eufriesea purpurata* in Amazonian Brazil (Roberts et al. 1982). Along with possibly being more generalist in fragrance preference, *E. meriana* and *E. imperialis* bees are larger in body size and may be able to better withstand abiotic conditions of rain, wind, or temperature. It would be interesting to test this by controlling for scent preference and observing whether fewer of the small *Euglossa* bees are attracted in harsher weather conditions.

Both *E. championi* and *E. dodsoni/erythrochlora* were attracted to cineole and benzyl acetate separately, but not to the combined solution. *Euglossa championi* was also attracted to methyl salicylate, but not to the cineole/methyl salicylate/methyl benzoate solution. This is not necessarily a rare occurrence. In a previous study, Williams and Calaway (1972) found that *E. meriana* is attracted to cineole and methyl salicylate separately, but is attracted to the combination in very low numbers. Further study is needed to determine by what mechanism a combination of fragrances reduces attractiveness. Currently, the most convincing hypothesis is that reduction in attraction is due to a synergistic inhibition of receptor responses at the peripheral and central nervous system (Williams & Calaway 1972; Schiestl & Roubik 2003). This could occur via binding interactions, where each chemical inhibits the other from binding to a receptor (Schiestl & Roubik 2003). The fact that two attractive chemicals become unappealing to certain bee species when combined demonstrates the importance of chemical blends and proportions in orchid fragrances.

This study provides support for the proposal that euglossine orchids produce specific recipes and proportions of chemical odors to (1) increase chances of successful pollen dispersal and thus outcrossing and (2) serve as a reproductive isolating mechanism to prevent hybridization. A fragrance complex is designed to attract a limited subset of euglossines that are both effective as pollinators for that species and specialized enough that visits would not increase risk of hybridization. Further studies should be done in testing the variability of fragrance modification among chemical “types.” For instance, this study used chemicals that by themselves act as general attractants. However, modifier chemicals such as alpha-pinene may be more important to specifying the attractive ability of an orchid. The identification and classification of orchid fragrance composition is also lacking. However, once chemicals are identified, it would be interesting to investigate the stereochemistry and test whether different isomers of chiral compounds attract different species. Finally, it would also be useful to test intraspecific variability of fragrance composition in orchids in relation to attraction of the optimal pollinator species set.

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LITERATURE CITED


TABLES AND FIGURES

### TABLE 1. Abundances of male euglossine bee species visiting each chemical bait in the San Luis Valley, Monteverde, Costa Rica.

<table>
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<tr>
<th>Species</th>
<th>Cineole</th>
<th>Benzyl acetate</th>
<th>Cineole/ benzyl acetate</th>
<th>Methyl salicylate</th>
<th>Cineole/ methyl salicylate/methyl benzoate</th>
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<td><em>Eufriesea ornata</em></td>
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<td>0</td>
<td>1</td>
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<td>4</td>
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<td>3</td>
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**FIGURE 1.** Total euglossine bee species richness attracted to each chemical fragrance. SE bars are shown. The letters above the bars denote significant difference in Shannon-Weiner index of the chemical. A total of 191 bees were observed, comprising 15 different species.
FIGURE 2. Euglossine bee species richness from 19.3 °C to 26.6 °C. Trials were run from 8 AM to 12 from October 27th to November 16th in the San Luis Valley, Costa Rica.

FIGURE 3. Euglossine bee abundance from 19.3 °C to 26.6 °C. Trials were run from 8 AM to 12 from October 27th to November 16th in the San Luis Valley, Costa Rica.