FLORAL SCENT IN A WHOLE-PLANT CONTEXT
Integrating floral scent, pollination ecology and population genetics

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Summary

1. Floral scent is a key factor in the attraction of pollinators. Despite this, the role of floral scent in angiosperm speciation and evolution remains poorly understood. Modern population genetic approaches when combined with pollination ecology can open new opportunities for studying the evolutionary role of floral scent.

2. A framework of six hypotheses for the application of population genetic tools to questions about the evolutionary role of floral scent is presented. When floral volatile chemistry is linked to pollinator attraction we can analyse questions such as: Does floral volatile composition reflect plant species boundaries? Can floral scent facilitate or suppress hybridization between taxa? Can the attraction of different pollinators influence plant mating systems and pollen-mediated gene flow? How is population genetic structure indirectly influenced by floral scent variation?

3. The application of molecular tools in sexually deceptive orchids has confirmed that volatile composition reflects species boundaries, revealed the role of shared floral odour in enabling hybridization, confirmed that the sexual attraction mediated by floral odour has implications for pollen flow and population genetic structure and provided examples of pollinator-mediated selection on floral scent variation. Interdisciplinary studies to explore links between floral volatile variation, ecology and population genetics are rare in other plant groups.

4. Ideal study systems for future floral scent research that incorporate population genetics will include closely related taxa that are morphologically similar, sympatric and co-flowering as well as groups that display wide variation in pollination mechanisms and floral volatiles.

Key-words: floral scent, fragrance, genetics, gene flow, odour, pollination, population, volatiles

Introduction

OVERVIEW

It has been hypothesized that the immense diversity of the angiosperms can be attributed in greater part to biotic pollination (Grant 1994; Galen 1999; Johnson 2006). When floral volatiles play a key role in governing the attraction of pollinators they, like other floral traits, are likely to be subject to pollinator-mediated selection. It follows therefore that studying floral volatile variation promises to greatly improve understanding of the evolution of flowering plants. Despite this importance, as noted by various authors, floral scent as a trait is poorly understood and has been studied less thoroughly than other floral characters, such as visual cues like colour or morphology (Azuma, Thien & Kawano 1999; Ervik, Tollsten & Knudsen 1999; Dufa, Hossaert-Mckey & Anstett 2004; Raguso 2008b; Waelti et al. 2008). The relative lack of studies of natural variation in plant volatiles is likely due to several factors. These may include on one hand a bias by chemists who may not realize the relevance of chemical variation and a bias by pollination biologists to more obvious visual characters (Raguso 2008c). It may also simply be that studying floral volatile variation requires a rare combination of inter-disciplinary skills in chemistry and biology and specialized equipment not routinely found in an ecology laboratory.

Another notable gap in the literature is the lack of studies that integrate volatile chemistry with pollination biology and genetics. The literature on floral scent is dominated by a focus on biosynthetic mechanisms and descriptive studies of variation across taxa. Yet, the ever expanding toolkit of molecular methods for investigating population genetic variation, plant mating systems and gene flow remains to be applied to
volatiles. Taking advantage of these tools and technology will allow floral volatile research to be linked to plant reproductive biology and the selective forces of population-level differentiation and speciation. Questions about the consequences of specialized pollination on pollen flow within and among populations, the influence of different guilds of pollinators on pollen dispersal and mating system, and the influence of floral scent on reproductive isolation are all open to inquiry by employing these molecular tools.

The overarching goal of this review is to highlight the benefits of, and provide a framework for, combining volatile, ecological and genetic knowledge to illuminate the evolutionary role of floral volatiles as pollinator attractants. First we provide a brief background on the study of floral volatiles as specific pollinator attractants. We then explore six hypotheses that integrate floral volatiles, genetics and pollination ecology. We highlight studies that address components of these hypotheses and we point to relevant gaps in knowledge. Finally, we consider ‘ideal study systems’ for interdisciplinary research. We believe that exciting new insights in the functioning of floral scent in pollination systems await future investigations that combine research in plant volatile chemistry, pollination ecology and population genetics.

A FOCUS ON THE LINK BETWEEN VOLATILE VARIATION AND FUNCTIONAL SPECIALIZATION

While selection imposed by pollinators has likely played a key role in influencing the patterns of floral scent compound variation across the angiosperms (Knudsen & Tollsten 1993), floral scent is not limited in its role to pollinator attraction. Floral volatiles may fulfil other adaptive functions (Pichulla & Pott 2003; Raguso 2008c), for example as repellents (Omura, Honda & Hayashi 2000; Kessler, Gase & Baldwin 2008) or physiological protection against abiotic stresses (Dudareva et al. 2006; Knudsen et al. 2006). As well as these roles outside pollination, qualitative and quantitative variation in floral volatile profile can be driven by both phylogeny and environment.

Phylogeny can constrain a flower’s biosynthetic repertoire in that the potential for evolution of floral scent profiles is contingent on the variation already present within a lineage. Furthermore, phylogenetic inertia may conserve volatile compound production in the absence of an apparent, present biological function so that floral odour compound variation may be inherited regardless of pollinator preference. The potential for plant secondary compounds to carry phylogenetic information has long been recognized and once was perceived as a promising source of data for botanical systematists (Alston, Turner & Mabry 1963). Floral volatile variation is now being shown to be too labile an evolutionary character to be useful for constructing phylogeny. Nonetheless, some phylogenetic patterns have been recognized in Nicotiana (Raguso et al. 2006), Cypripedium (Barkman 2001), and the Nyctaginaceae (Levin, Mcdade & Raguso 2003) and a recent study on Ophrys orchids has demonstrated utility of chemotaxonomic analysis of non-pollinator-attractive floral volatile compounds in reconstructing phylogeny in that genus (Gögler et al. 2009).

Intraspecific variation in floral odour blends may arise through genetic drift under relaxed selection, introgression of traits through hybridization, pleiotropic effects of biosynthetic pathways, or environmentally driven phenotypic plasticity (Raguso 2008c). However, identifying the basis of intraspecific floral volatile variation for specific cases in nature remains difficult (Ackerman, Melendez-Ackerman & Salguero-Faria 1997; Azuma, Toyota & Asakawa 2001; Knudsen 2002; Schlumberger & Raguso 2008).

In light of this complex odour variation, Raguso (2001) concluded that the evolution of floral scent is a ‘mosaic product of biosynthetic pathway dynamics, phylogenetic constraints, and balancing selection due to pollinator and florivore attraction’. Because of this, pollination biologists studying fragrance face the challenge of sorting ‘signal’ – bioactive compounds potentially subject to pollinator mediated selection – from ‘noise’ of other types of chemical variation (Raguso 2008c). Approaches for addressing this challenge are discussed in more detail later.

While floral odour chemical composition has been documented for hundreds of species (Knudsen et al. 2006), the links between distinct signals and receivers – odour and pollinator – are known for only a very small subset of these studied species, most of which involve specialized relationships. Notwithstanding the predominance of generalized over specialized plant-pollinator systems and much discussion of their relative importance to floral evolution (see Waser et al. 1996; Waser 1998, 2001; Johnson 2006; Ollerton, Armbuster & Vazquez 2006; Ollerton et al. 2009) this limited background knowledge necessitates an intentional restriction of this review to ‘functionally specialized’ systems where the links between odour and pollinator are (at present) more likely to be meaningfully investigated.

The ‘functional group’ concept classifies pollinators into groups that behave similarly with respect to the flowers they visit (Fenster et al. 2004). Plants that are pollinated by a functional group comprised of a single pollinator species occupy the highly specialized end of the specialist-generalist pollination spectrum, while a plant species pollinated exclusively by a functional group such as nocturnal moths, while still classed as functionally specialized, would occupy a less extreme position on the spectrum. Fenster et al. (2004) hypothesize that the selection pressure exerted by a functional group of pollinators is responsible for ‘pollination syndromes’, the occurrence of suites of traits adapted to particular modes of pollination, e.g. white, fragrant flowers of nocturnal moth-pollinated plants.

The literature on floral-fragrance mediated plant-pollinator interactions is dominated by functionally specialized cases and therefore our review necessarily reflects this bias.

Population genetics in the context of floral volatiles

In specialized pollination systems the distinct ecology and behaviour of a plant’s functional group of pollinators will
influence pollination rates, mating system and the extent and distribution of pollen movement. Thus when pollinator specificity is due (at least in part) to floral fragrance (Waelti et al. 2008) and specific pollinators influence these fundamental elements of plant reproductive ecology, floral volatile variation may be expected to influence the genetic composition and evolution of plant populations. For example, changes in floral volatiles that lead to pollinator switching may result in changing patterns of gene flow which in turn may influence the population genetic structure of the species. Presently, these potentially important links between plant volatiles and population genetic structure are poorly understood for any pollination system.

Perhaps the most thorough application of molecular tools in a floral-volatile context has been in the study of sexually-deceptive terrestrial orchid pollination systems in Europe and Australia. Sexual deceit pollination relies on the mimicry of sex-pheromones to attract pollinators (Schiestl 2005) and is characterized by highly specific plant-pollinator relationships which have long been proposed to act as an ethological isolating mechanism between sympatric taxa (Paulus & Gack 1990; Grant 1994). Because this system has benefited from significant collaborative effort between ecologists, chemists and geneticists, a number of examples throughout this review will be drawn from this system. Where possible we also provide examples from other systems, although in many cases complete data are lacking. For example, some cases of well characterized volatile variation have limited supporting pollination ecology and no genetic data. In other cases, genetic patterns and pollination biology are well characterized, but knowledge of volatile composition and variation is lacking. It should be stressed that the bridges between floral scent chemistry, pollination biology and genetics can be built from any direction. Genetics can be revealing when applied to systems for which volatiles and pollinators have been well studied, while establishing population genetic patterns for a poorly studied species may provide evidence of interesting pollination phenomena.

Below we discuss in turn six basic hypotheses we believe to have utility in the study of floral scent variation within taxa (Table 1). At the most basic level the hypotheses could apply to a study-system of sister taxa for which floral odour plays a key role in the attraction of contrasting functionally specialized pollinator guilds (Fig. 1). Under the scenario in Fig. 1, the interaction between floral volatiles, pollination ecology and population genetics can operate in different ways, and at different levels (species, populations, individuals). Combining knowledge of volatile variation with ecological and genetic knowledge is essential to fully address these hypotheses. Below we consider the six hypotheses in turn.

## Hypothesis 1: Volatiles and Specific Pollination

The first hypothesis: ‘Pollinator specificity is due to the distinct volatile composition of the floral fragrance blend’ is perhaps the most critical and most difficult to satisfactorily test. The aims here are threefold: to determine pollinator specificity, to confirm distinct volatile composition and to obtain evidence for the activity of floral volatile components as pollinator attractants. In most cases to date, even for this seemingly straight-forward hypothesis, there are few studies that have investigated all three lines of evidence.

Despite the modest but expanding bank of information on floral volatile variation within and among species (Knudsen et al. 2006), definitive proof of the links between such variation and associated pollinators remain scarce. Demonstrating the link between specific volatiles and specific pollinators

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<thead>
<tr>
<th>Hypothesis</th>
<th>Volatile knowledge</th>
<th>Ecological knowledge</th>
<th>Genetic knowledge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pollinator specificity is due to a distinct volatile composition of the</td>
<td>Confirmation of distinct volatile composition as attractant</td>
<td>Ecological evidence for pollinator specificity associated</td>
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<td>floral blend</td>
<td>Distinct volatile composition among species</td>
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<td>2. Distinct volatile composition reflects species boundaries</td>
<td>Shared volatile components</td>
<td>Ecological evidence for reproductive isolation</td>
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<td>3. Hybridization is due to sharing of key volatile components of the floral</td>
<td>Confirmation that distinct volatile components attract different pollinators</td>
<td>Genetic confirmation of hybridization</td>
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<td>blend</td>
<td>Confirming that distinct volatile components attract different pollinators</td>
<td>Genetic evidence for differences in plant mating system</td>
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<td>4. Different pollinators will influence the plant mating system (selfing vs.</td>
<td>Confirmation that distinct volatile components attract different pollinators</td>
<td>Paternity analysis or other genetic evidence for differences in the extent of pollen flow</td>
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<td>outcrossing)</td>
<td>Ecological evidence for different patterns of pollen movement by different pollinators</td>
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<td>5. Different specific pollinators distinctly influence the extent of pollen</td>
<td>Ecological evidence for different mating systems and/or patterns of pollen movement by different pollinators</td>
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<td>flow</td>
<td>Fertility analysis or other genetic evidence for differences in the extent of pollen flow</td>
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| 6. Different pollinators will influence male contribution to population    | Genetic confirmation of genetically distinct species                               | Measures of genetic variation and differentiation within and among populations (ideally for sDNA and cpDNA)
| genetic structure                                                          | Genetic confirmation of genetically distinct species                               | Genetic confirmation of genetically distinct species       |
normally requires multiple lines of evidence. Ideally this will include field observations or experiments demonstrating a role for floral odour in pollinator attraction followed by evaluation of physiologically active constituents, characterization and synthesis of those compounds and behavioural testing via bioassay to confirm activity in pollinators (Schiestl & Marion-Poll 2002b; Schiestl & Peakall 2005; Franke et al. 2009). Genetic confirmation that pollinator specificity is associated with distinct plant entities can be important, particularly for morphologically similar taxa.

Even before identification of active floral volatile constituents, behavioural experiments should be the first step in examining the importance of scent in pollinator attraction. For example, Okamoto, Kawakita & Kato (2007) confirmed the role of floral scent in the obligate nursery-pollinated Glochidion using choice experiments. When the pollinating Epicephala moths were exposed to air flowing from a pair of bags via a Y-tube they responded only to the air from bags containing flowers of their host Glochidion species. There was no response to flowers of a co-occurring non-host Glochidion species or to empty control bags. In this way, behavioural bioassays provide elegant and powerful tools for confirming that volatiles play a key role in pollinator attraction.

Once a role for floral odour in pollinator attraction has been confirmed, gas chromatography with electroantennographic detection (GC-EAD) offers a powerful tool for identifying the compounds detected by pollinators from the myriad of volatile variation that may be produced by a flower. This method combines gas chromatography (to separate the blend into single constituents) with electroantennographic detection that allows the physiologically active compounds detected by insects to be determined. GC-EAD active compounds are usually subsequently identified by GC-MS and other diagnostic procedures (Schiestl & Marion-Poll 2002b). Complementary to the chemical analysis approach of GC-EAD are field or lab-based bioassays to determine the biological activity of putative floral signals. These behavioural experiments typically use synthetic versions of identified floral volatiles in order to show pollinator attraction in vivo. As well as demonstrating the role of fragrance in pollinator attraction, experiments for confirming attractant activity are crucial for distinguishing signals of attraction from the background chemical mosaic which may include chemical signals with other functions, for example those involved in repelling herbivores or plant defensive signalling (Raguso 2008c).

The well-studied sexually deceptive orchids of Australia and Europe represent two systems in which GC-EAD, GC-MS and other analytical procedures have identified volatile organic compounds whose activity as attractant in the field has been subsequently confirmed through multiple lines of experimental evidence. The attraction of pollinators to specific odour bouquets in the sexually deceptive orchid genus Ophrys has been explored for several species and more than 50 pollinator-active compounds have been discovered (Schiestl et al. 1999; Ayasse et al. 2000; Stökl et al. 2005; Ayasse 2006; Paulus 2006). Some of these compounds have been identified as commonly occurring molecules including esters, aldehydes, alkanes or alkenes, with specificity determined by ratios of the various compounds that mimic sexual signals of female Andrena bees (Ayasse et al. 2000; Schiestl & Ayasse 2002a; Stökl et al. 2005). The important role of specific components for controlling pollinator specificity has been demonstrated by behavioural experiments utilizing synthetic components of the floral bouquet (Ayasse et al. 2000, 2003). In Australian sexually deceptive Chiloglottis orchids pollinated by male thynine wasps, a previously undiscovered class of natural products (rather than blends of common compounds) provides the chemical basis for pollinator specificity (Schiestl et al. 2003; Franke et al. 2009). The first of these compounds to be described, ‘chiloglottone 1’ (2-ethyl-5-propylcyclohexan-1,3-dione), was confirmed as both the female thynine wasp sex pheromone and the orchid pollinator attractant (Schiestl et al. 2003). Subsequent study has revealed other chemical variants of this new class of com-

pounds are involved as specific attractants in other orchid pollinator interactions (Franke et al., 2009).

The euglossine bee-pollinated neotropical orchids have probably the longest history of study into odour-mediated pollinator specificity. These orchids lack nectar and attract male euglossine bee pollinators by floral odour. The bees accumulate volatile compounds from the orchids and other floral and non-floral sources to build complex and often species-specific bouquets presumably to attract mates (Kimsey 1980; Eltz, Roubik & Lunau 2005; Eltz, Ayasse & Lunau 2006). In a landmark paper, Dressler (1968) explored hypotheses on the role of odour in pollination, specificity, reproductive isolation and speciation in euglossine bee-pollinated orchids. Surprisingly, despite the early start, much remains to be learnt about this system. A number of euglossine-pollinated orchid species have been the subject of floral volatile analysis and these studies indicate that specific pollinator attraction is probably conferred by a complex cocktail of chemical compounds. Not to be neglected in future studies are behavioural tests with synthetic chemicals that have demonstrated the attractiveness of individual components of the floral blend and the influence of volatile mixtures on attractiveness (Williams & Dodson 1972; Ackerman 1983). These findings have only recently been augmented by GC-EAD and electroantennography (EAG) which provide evidence that both antennal and central nervous system processes play a role in the specific attraction of bees to odours (Schiestl & Roubik 2003; Eltz & Lunau 2005; Eltz, Ayasse & Lunau 2006). It is apparent we are only beginning to understand the role of floral volatiles in this complex and diverse tropical orchid-pollinator interaction.

The number of plant-pollinator relationships for which floral volatiles have been identified and demonstrated as attractants is small (Table 2) and dominated by deceptive and obligate nursery-pollination systems. Outside sexual-deception, perhaps one of the best examples of an integrated approach to linking volatiles to pollinator is provided by Brodmann et al. (2008), who showed volatiles emitted by the flowers of the orchid Epipactis helleborine to elicit the attraction of social Vespuia wasps in a study that combined tests of natural and synthetic compounds in behavioural assays with GC-EAD confirmation and identification of active floral volatile compounds. Not to be neglected in future studies are those pollination systems outside of these close plant-pollinator relationships where specialization is less extreme. For example, pollination by oligolectic bees (which collect pollen from only one plant species or genus) is common in

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<thead>
<tr>
<th>System</th>
<th>Characterization</th>
<th>Experimental confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figs – fig-wasp mutualism</td>
<td>(Ware et al. 1993; Grison, Edwards, &amp; Hossaert-McKey 1999; Grison-Pige, Bessiere &amp; Hossaert-McKey 2002a; Grison-Pige et al. 2002b) among others</td>
<td>(Gibernau et al. 1998) (Grison-Pige, Bessiere &amp; Hossaert-McKey 2002a) among others</td>
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<td>Euglossine bees and neotropical orchids</td>
<td>(Hills, Williams &amp; Dodson 1972; Whitten &amp; Williams 1992; Cancino &amp; Damon 2007)</td>
<td>(Hills, Williams &amp; Dodson 1972; Eltz &amp; Lunau 2005) (Williams &amp; Dodson 1972; Ackerman 1983)</td>
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<td>Yucca – yucca moth mutualism</td>
<td>(Svensson et al. 2005)</td>
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<td>Dead-horse arum Willows and oligolectic bees</td>
<td>(Stensmyr et al. 2002; Dotterl et al. 2005; Fussel et al. 2007)</td>
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<td>Epipactis helleborine and Vespuia social wasps</td>
<td>(Brodmann et al. 2008)</td>
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some parts of the world (Proctor, Yeo & Lack 1996; Dotterl et al. 2005) yet the floral cues involved in these relationships have barely been studied.

**HYPOTHESIS 2: VOLATILES AND SPECIES BOUNDARIES**

Despite the early discovery that different floral odour composition reflected the delineation of species and their respective pollinators in *Catasetum* orchids (Hills, Williams & Dodson 1972), there appear to be few studies that evaluate the role of distinct volatile composition in maintaining species boundaries. We therefore draw on our own ongoing studies to provide an example. An interesting feature of the Australian sexually deceptive *Chiloglottis* orchids is the high frequency of temporally and spatially co-flowering congeneric species (Bower 1996; Peakall et al. 1997; Mant, Peakall & Weston 2005c; Mant et al. 2005a). One pair of species that can be found co-flowering is *C. valida* and an undescribed species morphologically similar to *C. jeansii* (hereafter *C. aff jeansii*). *Chiloglottis valida* is known to attract its male thynnine pollinator *Neozeloboria monticola* with the single volatile compound, chiloglottone 1 (2-ethyl-5-methylcyclohexan-1,3-dione) (Schiestl & Peakall 2005). Similarly, evidence including GC-EAD, GC-MS and bioassays with synthetic compound has revealed that *C. aff jeansii* attracts its undescribed pollinator (a *Neozeloboria* species in the *inpatiens* species complex) by a structural isomer of chiloglottone 1, called chiloglottone 3 (2-butyl-5-methylcyclohexan-1,3-dione) (Franke et al. 2009; R. Peakall unpublished). Field studies have shown no cross-attraction between the two compounds and their respective pollinators.

To test whether chemical composition corresponds with species boundaries we applied GC-MS with selective ion monitoring (SIM, reduces the detection threshold several orders of magnitude and provides the most sensitive measurement of a compound’s presence or absence) of single orchid labella to identify the active compound in flowers from mixed populations of the two taxa. The results of the floral chemistry indicated that our own diagnosis of species based on morphology and conducted in the field was frequently incorrect (R. Peakall unpublished). Subsequently, chloroplast DNA analysis with the taxa defined only by their chemical composition revealed extensive genetic differentiation between these chemically-defined taxa (Ebert, Hayes & Peakall 2009a). Thus, this study confirms the hypothesis that distinct floral volatile composition should reflect taxonomic boundaries between morphologically similar species when volatiles function as specific pollinator attractants.

The combination of floral volatile analysis and population genetic analysis can sometimes provide unexpected insights into the nature of species boundaries. Mant, Peakall & Schiestl (2005b) investigated the patterns of odour and genetic variation among several species of sexually deceptive *Ophrys*. The odour analysis indicated a previously unknown or cryptic taxon that was characterized by distinct odour composition. Remarkably, this entity was not distinct genetically, at least at the level of the nuclear micosatellite loci investigated, nor were its non-active odour compounds distinct from those of related *Ophrys* species. This discovery in *Ophrys* may well represent an incipient taxon in the early phases of pollinator mediated speciation with as yet little or no accumulated differentiation evolving at neutral traits not under selection. It now remains to be experimentally confirmed in the field that the odour differences are directly linked to specific pollinators and that hybridization is minimal or absent as a consequence. This example thus represents a case where floral volatile and genetic knowledge is in hand, but now requires further integration with pollination ecology.

Exploring the role of floral scent in taxonomic boundaries (Hypothesis 2) may be approached from different directions. Volatile studies indicating strong odour differences among taxa should seek to integrate ecological and genetic data. Similarly, genetic studies revealing unexpected taxonomic boundaries, particularly among closely related sympatric taxa, should consider whether floral odour variation could be linked to specific pollination and reproductive isolation.

**HYPOTHESIS 3: VOLATILES, HYBRIDIZATION AND POPULATION GENETICS**

When floral odour is the major determinant of pollinator specificity, changes or variation in floral odour could break down specificity and increase the frequency of interspecific pollen transfer, thereby promoting hybridization and introgression. Alternatively, when hybridization is detected in otherwise highly specific systems, it is of interest to investigate the role floral odour may or may not play in enabling hybridization.

A direct but unexpected link between floral odour and hybridization can be found in *Chiloglottis* orchids. Extreme pollinator specificity is the norm in these orchids with putative hybrids rarely reported (Peakall et al. 1997). One exception is *Chiloglottis X pescottiana* which, when described, was hypothesized to be a hybrid between *C. trapeziformis* and *C. valida*. Allozyme based genetic analysis subsequently confirmed the hybrid status of this taxon (Peakall et al. 1997). GC-EAD analysis and field bioassays further confirmed that both orchid parents employ the same single volatile compound, chiloglottone, to attract their respective and phylogenetically distinct pollinators (Schiestl et al. 2003; Schiestl & Peakall 2005). By virtue of this shared compound, hybridization between the two taxa (due to pollinator sharing) can occur when their usual geographically and altitudinally separate ranges occasionally overlap (Peakall et al. 2002). This case provides an example of the power of combining floral volatile analysis with ecological and genetic methods to better understand the role of floral volatiles in hybridization.

There are more documented cases of hybridization and introgression in the sexually deceptive *Ophrys* orchids of Europe compared to Australian sexually deceptive orchids (Soliva & Widmer 2003; Mant, Peakall & Schiestl 2005b). This may be due, at least in part, to the differences associated with the chemical basis for pollinator attraction in *Ophrys*.
The volatile blend of Australian sexually deceptive orchids is typically characterized by one, two or three unique active compounds (Mant et al. 2002; Schiestl et al. 2003; Schiestl & Peakall 2005; Franke et al. 2009) while in some of the better-studied Ophrys taxa, specific pollinator attraction is based not on a single chemical odour compound but on emission of distinct blends or ratios of several commonly occurring hydrocarbon compounds (Schiestl et al. 1999; Stökl et al. 2005). Hybridization due to a breakdown of pollinator specificity may occur between some Ophrys more frequently because variation in blends and ratios could result in a floral bouquet more closely resembling that of a sympatric species (Stökl et al. 2008).

An interesting example of marked scent differences among species is found in the genus Silene (Caryophyllaceae). Waelti et al. (2008) investigated floral odour in white and red campions (Silene latifolia and Silene dioica respectively) which are known to be interfertile and to co-occur in parts of their range. GC-MS of floral headspace samples showed distinct odour differences in the relative amounts of biologically active volatile compounds. In a field experiment the biologically active benzenoid phenylacetaldehyde (which dominated the scent of S. dioica and contributed strongly to the odour difference between species) was applied to inflorescences of both species to make floral fragrance more similar. Transfer of fluorescent dye (a pollen analogue) was higher in plots containing scent-manipulated flowers than control plots of un-manipulated inflorescences. Thus, odour differences reduce the potential for gene flow between these species demonstrating the importance of odour for reproductive isolation. This work on volatile variation among species was further supported by an in-depth genetic study of natural hybrid zones among the same two Silene species providing new and detailed insights into the evolutionary role of introgression and hybridization more generally (Minder, Rothenbucher & Widmer 2007; Minder & Widmer 2008).

The extensive experimental inter-disciplinary work on the Silene system illustrates the interpretive power of integrating floral fragrance analysis, population genetics and pollination ecology. Furthermore, while pollination in this system appears to be functionally specialized, it does not represent a case of extreme specialization like sexual deception or nursery pollination. It is therefore apparent that many other less specialized systems that involve related co-flowering taxa may be candidates for exploring the links between volatiles, hybridisation and population genetics. Such systems may offer the opportunity to investigate whether hybridization is more or less common in species with distinct floral volatile blends and whether attraction due to floral fragrance is reduced or maintained in F$_1$ hybrids between taxa with different floral scents.

**HYPOTHESIS 4: VOLATILES, POLLINATION AND PLANT MATING SYSTEMS**

Differences among distinct pollinators in behaviour and abundance can be expected to influence the way pollen is moved within and among individual plants and populations. Therefore, in those systems where floral scent governs pollination specificity, either through innate attraction, floral constancy (see Wright and Schiestl in this feature) or filtering visitor composition, it is likely that floral scent variation will indirectly play a key role in moderating plant mating systems (the degree of selfing vs. outcrossing). To our knowledge there are no studies that have directly linked variation in floral volatile composition to plant mating systems.

A study by Brunet & Sweet (2006), although not directly linked to plant volatiles, provides a rare example of the application of genetic methods for testing the effects pollinators have on the populations they service. This study investigated the effect of different insect pollinators on outcrossing rates in the Rocky Mountain columbine, Aquilegia coerulea, a proliﬁc, self-compatible herb. Many hours of pollinator observations at eight natural populations over 3 years revealed considerable variation in the relative proportions of different pollinator species among populations. Outcrossing rate estimates, achieved by analysis of seed at ﬁve allozyme loci, showed an increase in outcrossing rate with hawkmoth abundance. No effect on mating system was detected for any other pollinator group. This appears to be one of the first studies directly linking different pollinators to outcrossing rates. One explanation for the high outcrossing rates achieved by hawkmoth pollination was that hawkmoths reduced geitonogamous selfing (self-pollination between flowers on the same plant) by preferring to visit female-phase flowers before male-phase flowers. Alternatively, hawkmoths may simply be more effective pollinators. Consequently, A. coerulea populations with low hawkmoth abundance might experience pollen transfer limitation and higher rates of autogamous selfing as reproductive assurance.

In one of the few other examples of studies explicitly investigating the influence of distinct pollinators on plant mating systems, Whelan, Ayre, and Beynon (2009) examined pollination by birds and honey bees in an Australian shrub, Grevillea macleayana. In their experiment they caged some inflorescences to exclude vertebrate pollinators and included in the study one population known to have a high rate of outcrossing. Birds were found to not only deposit more pollen per visit than bees in the high outcrossing population, they also moved longer distances between plants and visited fewer inflorescences on a single plant.

Other relevant insights into how pollinators affect mating system have emerged from research on floral specialization in bees. Pollinator effectiveness, ‘the single-visit contribution by a flower-visitor to the reproductive fitness of a plant’, was compared among specialist bees and generalist pollinators of Knautia arvensis by Larsson (2005). While specialist bees deposited more pollen per visit (higher pollinator effectiveness), their impact on overall pollination success was moderated by their lower abundance relative to generalist pollinators. Thus, higher reproductive success and outcrossing might result when specialist bees are in high abundance. By contrast, when generalist pollinators are in high abundance pollen limitation through wasted interspecific pollen transfer may occur. Pollinators may therefore have an impact on plant
mating systems, and floral scent may indirectly influence plant mating system through its interaction with pollinator fauna.

Floral scent chemistry may influence plant mating systems by influencing pollinator behaviour. The study of Kessler, Gase & Baldwin (2008) on the floral fragrance attractant, benzyl acetone, and the nectar-borne repellent nicotine present in the flowers of self-compatible Nicotiana attenuata offers a novel example. Field experiments with transgenic plants deficient for benzyl acetone synthesis, nicotine synthesis or both demonstrated that outcrossing rates were highest in wild-type plants. This may be due to moderation of the attraction by benzyl acetone by the repellent nicotine that limited the time pollinators spent at any one flower and maximized total number of flower visits.

Even low rates of outcrossing can provide benefits to plants with life-histories such as those with low rates of recruitment (Raguso 2008a). As such, a plant’s mating system can be an important factor in the evolution of plant populations. Determining the role pollinators and their behaviour play in moderating plant mating systems and the extent to which this is mediated by floral volatile variation will no doubt provide interesting insights into the evolution of floral scent.

**HYPOTHESIS 5: VOLATILES, POLLINATORS AND POLLEN FLOW**

The spatial patterns of pollen movement determine neighbourhood size and inbreeding rates (Mitchell *et al.* 2009) and are critical in understanding important evolutionary processes such as population differentiation and speciation. It is well established that pollinator behaviour can control the pattern and extent of pollen dispersal (Richards 1986). For example, nocturnal moth pollinators of Silene alba transport a fluorescent dye pollen analogue further on average than bees (Young 2002). Floral volatiles therefore, through their attraction of different pollinators and influence on pollinator behaviour, could exert an indirect influence on pollen movement within and between plant populations.

In sexually deceptive orchids (where the strong relationship between floral odour and specific pollinator has been repeatedly demonstrated) pollinator behaviour and movements may be controlled by optimal mate seeking strategies potentially leading to quite different patterns of pollen flow compared with other pollination systems (Peakall & Beattie 1996). Two ecological approaches have been taken to investigate pollen flow in sexually deceptive orchids: mark-recapture of pollinators to infer potential pollen movement, and direct measurements of pollen flow by tracking the movements of coloured pollen. In the Australian Caladenia tentaculara, longer distance pollen flow is promoted by the male thynine pollinator’s avoidance of visits to more than one flower in a patch. Pollen movements approximate a linear rather than a leptokurtic distribution (mean distance – 17 m; maximum: 58 m) and mirror movements detected by mark-recapture of the pollinator (Peakall 1990). In Drakaea glyptodon mark-recapture of male thynine pollinators suggests pollen flow could exceed 130 m (Peakall 1990). While near-neighbour pollination may be avoided in male thynnine pollination systems, pollen flow distances will be bounded by the mate search area. Therefore, the type of pollinator exploited by a sexually deceptive orchid may constrain the maximum pollen flow distance. A mark-recapture study of Colletes cunicularius, a bee pollinator of Ophrys, revealed that individual male bees patrol a specific and restricted portion of the total nesting area in search for mates (mean-recapture distances of 5 m, max 50 m). This behaviour may be expected to limit rather than promote long-distance pollen flow in Ophrys orchids (Peakall & Schiestl 2004). By contrast, while presently unknown, longer distance pollen movements may well occur in those plant species visited by foraging female Colletes bees.

It appears intuitively reasonable that in the classic euglossine trapline pollination (Janzen 1971) and perhaps in some fig-wasp pollination systems (Nason, Herre & Hamrick 1998), long range volatile mediated attraction of pollinators will result in long-range pollinator movement and likely long distance pollen flow. If so, such cases will demonstrate a clear link between volatiles and pollen flow. There is little enough research examining and comparing landscape-level gene flow for different pollinators (Mitchell *et al.* 2009) let alone drawing the link to floral volatiles. Such research, while technically challenging, is now very achievable and will be best realized by combining volatile knowledge, pollination ecology (e.g. pollinator mark-recapture and genetics (e.g. paternity analysis).

**HYPOTHESIS 6: VOLATILES AND POPULATION GENETIC STRUCTURE**

If different distinct pollinators can be expected to influence both plant mating systems (Hypothesis 4) and the patterns and extent of pollen flow at the population scale (Hypotheses 5), it follows that any differences in plant mating system and pollen flow may in turn influence population genetic structure – the patterns and extent of genetic variation within and among populations. In this way, floral volatiles may have indirect interactions on population genetic structure through their interaction with pollinator fauna.

Hughes *et al.* (2007) have explored the potential impact of bird vs. fly pollinators on the population genetic structure of two South African species of Streptocarpus. Lower levels of genetic differentiation (based on both nuclear and chloroplast DNA analysis) were detected in the sunbird pollinated S. dunii compared to its long-tongued fly pollinated congener S. primulifolius. This was attributed to the greater vagility and wider distribution of the sunbird that likely facilitates greater population connectivity than that possible by fly pollination. While it was recognized that this conclusion may be confounded by differences in habitat between the two study species, this study highlights a potential impact of pollinator behaviour on population genetic structure. Although no information on floral volatile differences was reported it has been noted by others that ornithophilous flowers often have little odour in comparison to other biotic pollination systems.

(Knudsen & Tollsten 1993; Levin, Raguso & McDade 2001; Raguso et al. 2003). Thus floral volatile differences may indirectly contribute to the population genetic differences between the species.

Population genetic structure in plants is determined by the interaction of multiple factors including mating system, gene flow (both contemporary and historic) by pollen and seed, as well as past population events such as bottlenecks, local extinction and range expansions. A major challenge in linking plant volatile variation to population genetic structure is the need to disentangle these multiple factors. This potential for population genetic structure to be driven by multiple factors can also cloud determination of cause and effect when studying its links to phenotype (e.g. floral scent) and gene flow. The closely related species Clarkia breweri and C. concinna partially overlap in range and conform to the parent-offspring style of rapid speciation due to extreme selection in ecologically marginal populations well characterized in the genus (Lewis 1962). Furthermore, the derivative species, C. breweri, in contrast to its unscented progenitor, C. concinna shows a recent evolution of floral scent production and moth pollination in a largely unscented genus (Raguso & Pichersky 1995). Given that strong selection can dramatically reduce effective population size, with the accompanying founder effects it is conceivable that traits such as floral scent might experience rapid change, elevating rare alleles for floral phenotypes to high frequency by chance within the same genomes as traits under strong selection for fitness (for example drought tolerance).

Given this complexity, careful study design is required in order to be able to definitively identify pollinator-mediated selection as a driver of between-species floral volatile differences. Mant, Peacock & Schiestl (2005b) compared floral odour variation in both putatively selected pollinator-active compounds and non-pollinator-active floral volatile compounds within and among Ophrys species. Population genetic data for neutral markers was also obtained for the same set of samples. In order to enable a meaningful and comparable contrast between odour (both active and non-active components) and genetic data, (Mant, Peacock & Schiestl 2005b) adapted the Analysis of Molecular Variance (AMOVA) framework for the analysis of odour. Although initially developed for molecular data, this procedure can be applied to the hierarchical analysis of variance for any data set that can be input as a pairwise individual by individual distance matrix. The study found significant floral odour differentiation among allopatric populations within species, among allopatric species and among sympatric species. Active odour compounds were more strongly differentiated among allopatric conspecific populations than non-active compounds. In marked contrast, there was limited population or species level population genetic differentiation. It was concluded that the strong odour differentiation but lack of genetic differentiation among sympatric taxa indicated selection imposed by the distinct odour preferences of different pollinating species. This conclusion was reinforced by the low genetic differentiation observed within species that suggested large effective population sizes and therefore little opportunity for genetic drift to account for the observed patterns (Mant, Peacock & Schiestl 2005b). The methods developed and executed in this study may serve as a model for future studies that seek to explore the direct or indirect links between floral odour variation and population genetic structure.

Future directions

In this review we have explored six hypotheses that directly or indirectly link floral volatile variation, pollinator ecology and population genetics. We have highlighted gaps in knowledge and demonstrated that new evolutionary insights can be achieved by combining these often separate fields of research. In this final section we briefly explore ‘ideal study systems’ for multidisciplinary study of the evolutionary role of floral scent. We recommend that targeting these ideal study systems will greatly accelerate discovery and understanding.

A major hurdle in the application of genetic techniques to studying wild populations is the availability of suitable genetic markers (Ebert & Peacock 2009b). The development of genetic markers is costly and time-consuming thus plant groups for which genetic methods have already been developed will offer more tractable systems for population genetic analysis (see Table S2 in Supporting Information). For example, the Solanaceae and Rosaceae as well as having a good record of floral volatile study have undergone much genetic research effort (equivalent to other economically important plant families) and as such provide good targets for identifying ideal study systems.

Ideal systems for studying the pollination consequences of floral fragrance variation must contain controls for other confounding abiotic and biotic variables (Table 3). Abiotic factors that could influence gene flow between taxa (and potentially contribute to reproductive isolation) include geographic distance or vicariant barriers, microhabitat selection

Table 3. Potential abiotic and biotic factors that may confound studies of the consequences of floral fragrance variation. Targeting ideal study systems that minimize these confounding factors will improve our understanding of the links between floral volatiles, pollination ecology and population genetics

<table>
<thead>
<tr>
<th>Confounding factor</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geography (allopatry)</td>
<td>Sympathy</td>
</tr>
<tr>
<td>Flowering time (temporal isolation)</td>
<td>Co-flowering</td>
</tr>
<tr>
<td>Visual cues (ethological isolation)</td>
<td>Morphologically cryptic/similar taxa</td>
</tr>
<tr>
<td>Mechanical isolation</td>
<td>Morphologically cryptic/similar taxa</td>
</tr>
<tr>
<td>Phylogenetic constraint</td>
<td>Closely related/sister taxa</td>
</tr>
<tr>
<td>Post-zygotic isolation</td>
<td>Closely related/sister taxa</td>
</tr>
</tbody>
</table>

and phenology of flowering. The effects of geographical and temporal isolating mechanisms can be eliminated by studying sympatric, co-flowering taxa which must by their nature share a common pollinator pool.

Biotic factors that may confound the detection of floral scent mediated processes include all forms of floral phenotypic variation other than floral volatiles. Grant (1994) identified various forms of mechanical and ethological isolation that rely on floral morphology. As isolating mechanisms the differential placement of pollen on a pollinator’s body, a flower’s physical exclusion of a class of pollinator and the enhancement of foraging efficiency through a pollinator’s behavioural tendency to flower constancy all rely on distinct floral morphologies whether it be colour or floral anatomy. Therefore, studying morphologically similar taxa or experimentally inducing morphological similarity will be desirable. Furthermore, cases may exist where floral volatile fragrance could operate synergistically with visual signals to determine functional specialization (Raguso 2004; Knudsen et al. 2006). Disentangling the respective effects of visual and fragrance cues in synergistic attraction will be a major experimental challenge, therefore studying systems of morphologically similar taxa removes a layer of complexity by limiting variation in floral advertisement to volatile variation.

Sympatric, co-flowering, morphologically similar (ideally cryptic) taxa provide the best chance of studying the biological and evolutionary significance of floral volatile variation and distinguishing adaptive processes from neutral ones. With the exception of extreme cases of convergent evolution, taxa that conform to this description will tend to be closely related. Studying closely related taxa will also provide the corollary benefit of controlling for phylogenetic constraint (limitations to present phenotypic variation imposed by a lineage’s phylogenetic history). Furthermore, eliminating the potential isolating effects of divergent flowering times, geography and morphology focuses on those systems in which pollinator-mediated selection upon floral volatiles is at its strongest (Knudsen 1999; Knudsen et al. 2006). Indeed, closely related animal pollinated plants that co-flower are likely candidates for divergent floral odour.

These characteristics of the ideal study system may appear to be difficult to find, but examples of such systems that meet or approximate these criteria already exist in the literature (see Table S1). While these studies have tended to focus on sexually deceptive or obligate nursery pollination other examples from outside these specialized relationships are well known. One promising example is illustrated by Knudsen (1999) who analyzed the floral volatile composition of eight co-flowering, sympatric species of Geonoma palms with similar floral morphologies and found that the species could be separated on the basis of their distinctive floral fragrance chemistry. Geonoma is described as ‘taxonomically difficult’ and little is known about their pollinators (Knudsen 1999). Clearly, this potential case for floral volatile driven reproductive isolation represents an excellent candidate for application of genetic tools to delineating species boundaries and assessing the occurrence of hybridization.

Although we have narrowed our focus to specialized pollination systems in this review, and indeed recommend such systems for future studies, we expect there are many other cases outside extreme specialization where volatiles are the key to the attraction of different pollinators. Descriptive floral volatile assays across related species have demonstrated that certain volatile profiles or constituents are associated with certain pollinators. For example, Dobson (1997) and coworkers analyzed headspace collections by GC-MS for nine species of Narcissus and found two groupings of fragrance chemistry: one group that emit typical moth-attracting compounds and are pollinated by lepidopteran insects and another group that lack these compounds and are pollinated exclusively by insects other than Lepidoptera. Similar findings have been found among nine species of Nicotiana in which hawkmoth-pollinated species emit ‘nitrogenous compounds, benzenoid esters and/or terpenoid alcohols’ while hummingbird-pollinated species lacked these compounds (Raguso et al. 2003). An extensive survey of floral odour composition across 20 species in three genera of Nyctaginaceae also found evidence for compounds characteristic of hawkmoth-pollination (Levin, Raguso & Mcdade 2001). These types of studies that employ wide-ranging assays of plant volatiles across related species with contrasting pollination mechanisms, when coupled with genetic investigations, will undoubtedly enable new insights into the evolutionary implications of fragrance.

It is apparent that there is much to learn about the way in which floral scent interacts directly and indirectly within complex biological systems. Integrating modern population genetic tools with traditional pollination ecology will open up new possibilities for understanding the operation and evolution of these systems. We have shown here that this integrative approach has already proved valuable in enabling new insights into plant-pollinator interactions. Without doubt both a challenge and a key to future progress will be the effective execution of interdisciplinary collaboration in strategically targeted study systems.

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