Chemical and molecular ecology
of orchid bees (Euglossini)

Inaugural Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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aus Dinslaken

Mai 2010

Aus der WE Biologie,

AG Sinnesökologie

der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der

Mathematisch-Naturwissenschaftlichen Fakultät der

Heinrich-Heine-Universität Düsseldorf

Referent: PD Dr. Thomas Eltz

Korreferent: Prof. Dr. Martin Beye
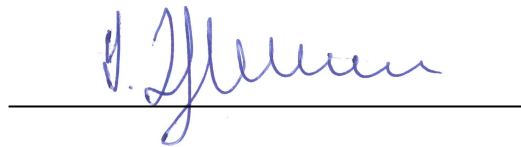
Tag der mündlichen Prüfung: 07. Juli 2010

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Abstract

Chemical signaling in neotropical orchid bees (Hymenoptera: Apidae) is outstanding since males of these bees do not use self-synthesized odors but collect volatile chemicals from flowers and other odoriferous substrates to store them in specialized hind leg pockets. This behavior is the basis of the "euglossine pollination syndrome", where ~700 orchid species are exclusively pollinated by scent-seeking males. Male orchid bees combine the collected odors to form predictable fragrance blends, which are exposed during pre-mating display and believed to function as pheromone analogues. The present dissertation (based on three publications and three manuscripts) focuses on the species-specificity of male fragrance blends, their potential function in courtship behavior and species isolation, as well as on orchid bee conservation genetics. An analysis (gas chromatography/mass spectrometry) of male fragrances of 15 species of sympatric *Euglossa* revealed significant chemical distinctness with the greatest disparity between the most closely related species, which is consistent with a role of the fragrances in mate recognition, ensuring premating isolation. Analyses of two morphotypes of *Euglossa viridissima* Friese in southern Mexico revealed component-specific differences in male fragrances, and also laid open a shift in antennal sensitivity of males of the two forms to the relevant components, suggesting that peripheral olfaction has driven fragrance differentiation and divergence of the two lineages. Further morphological, chemical and genetic characterization led to the description of one of the "morphotypes" as a new species, *E. dilemma* sp. nov., cryptic sibling to *E. viridissima*. Paternity analyses of brood of three *Euglossa* species using microsatellite markers revealed that females of all three species were mated with only a single male, suggesting that monandry is the rule in orchid bees and might have been the ancestral state in corbiculate bees. Monandry is consistent with the idea that orchid bee females choose a single best mate based on its fragrance blend, and that male volatile collection evolves through sexual selection. Concerning conservation genetics I conducted population genetic analyses with microsatellite markers that revealed no or only weak genetic structure among populations of euglossine bees in southern Mexico, suggesting substantial gene flow between them. Habitat fragmentation appears not to have caused loss of allelic diversity within or differentiation between orchid bee populations of eight different *Euglossa* species. Noticeable genetic differentiation was

only found across substantial geographic distances, e.g. between populations on the Yucatán peninsula and those in Veracruz. Finally, a comprehensive analysis of orchid bee males of 27 different species from all over the neotropical region detected extremely low frequencies of (infertile) diploid males, contradicting earlier studies based on allozyme analysis, and rejecting the hypothesis that euglossine bees decline due to the genetic mechanism of Hymenopteran sex determination (haplodiploidy, complementary sex determination).

Contents

General Introduction

Chemical communication of insects

Insects are classified as the most successful group of animals according to the number of species, the biomass they encompass, and their distribution over the planet (Lubbock 2005). This success is also based on their sophisticated ways of communication between individuals, which is thought to increase chances of survival and reproduction. In addition to acoustical and visual modes of communication, chemical signals for transmitting information are universally used by insects. They play a crucial role in most important aspects of insect life (Ma & Krings 2009, Wyatt 2004). There are several advantages of communication via chemical signals which rely on the movement of molecules and therefore have the abilities to cross barriers, cover long distances, and last over certain time-spans. Furthermore, chemical signals show an enormous diversity and are thought to have low production costs partly because generally small amounts are sufficient to elicit a response in recipients (Thornhill & Alcock 1983). As a special advantage for small organisms like insects, chemical signaling and perception do not suffer limitations constituted by distance as mechanical or visual signals might do (Greenfield 2002). The detection of different chemicals is accomplished by the olfactory system of an organism. This is located in a characteristic organ – in the case of insects normally the antennae. Here, the molecular information of chemical signals is first perceived by olfactory receptors to which the odorants bind. These receptors are located in olfactory receptor neurons (ORNs) which forward the information in the form of electrical signals to the insects' brain. The activation of different subsets of ORNs to different degrees is responsible for the ability to discriminate a large number of different molecules (Hildebrand & Shepherd 1997). The ability to distinguish many different odorants is promoted by the large gene family that encodes for the olfactory receptor types, such that the expression of different complements of receptor genes in different ORNs leads to functional distinctness of individual sensory neurons (Vosshall 2001, Vosshall et al. 1999).

Chemical signals that trigger a certain metabolic, developmental, or behavioral response are called semiochemicals or infochemicals. They are mostly self-produced with numerous and highly diverse functions that can be associated for instance with the recognition of resources, the arrangement of social organization, protection, or with sexual advertisement (Law & Regnier 1971). Generally, chemical communication can be classified into two groups: odorant exchanges between individuals of different species (interspecific) called allelochemicals, and between individuals of the same species (intraspecific), which are called pheromones. The latter were originally defined as 'substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process' (Karlson & Luscher 1959). Distinguished by their various mediating actions, pheromones can be subdivided into releaser pheromones, which have an immediate effect and trigger for instance a definite behavior, and primer pheromones, with a delayed effect causing a developmental process. Communication by pheromones has been most extensively studied as well as primarily detected in insects (Law & Regnier 1971).

Specificity of pheromones

There is a great diversity of interactions that are mediated by pheromones. They are often divided by their function, such as aggregation pheromones, alarm pheromones or sex pheromones. However, a clear attribution is usually difficult since functional overlap occurs. There are contexts in which the species specificity of a pheromone is not of great importance, as it has been shown for example in alarm pheromones of ants. Here, 4-methyl-3-heptanone was chemically detected as a generally common alarm pheromone compound, verified by behavioral tests in which this ketone triggered alarm behavior in six different *Atta* and seven different *Pogonomyrmex* species (Hughes et al. 2001, McGurk et al. 1966). Nevertheless, in the majority of situations a species-specific pheromone initializing a defined behavioral response is required. There are two main ways of achieving species specificity in a pheromone. The release of a single unique molecule with a complex structure that is not easily duplicated in nature is one option, but individual compound pheromones are actually rare in insects (Roelofs 1995). Instead, pheromones mainly consist of a composition of several active components in

relative proportions, resulting in an enormous possible diversity (Silverstein & Young 1976). Chemical species specificity enlarges the possibilities for pheromone use: Long distance attraction of conspecific mates, recognition of kin, and territorial marking are examples. Furthermore, the effective social organization of insect colonies, in which numerous individuals perform different tasks, underlies a complex chemical communication system that has been identified to different degrees in virtually all social species, including ants, bees, wasps and termites. Here, specific pheromones ensure the recognition of kin, nest and colony – a central basis to social behavior which relies on the discrimination of nestmates from non-nestmates, and sisters from less-related relatives (Hölldobler 1995, Winston 1992). Nestmate recognition is also a critical aspect of colony defense (Breed et al. 2004). The highly social honeybees have a colony odor that is located on each bees' surface. Cuticular pheromones are secreted by the bee itself and mixed with compounds from comb wax in the nest. These additional compounds include pheromones from other workers as well as floral scents from foraging trips (Breed 1998). However, chemical communication in social insects is highly diverse and can be moreover found as a regulator for colony structure, division of labor, aggressive behavior, gathering resources, and the control of reproduction and queen number (Free 1987). A key role within social organization can be attributed to primer pheromones with a releaser effect, which alter developmental, physiological and neural systems (Vargo 1998, Le Conte & Hefetz 2008).

Relevance of sex pheromones in mating

The use of pheromones is frequently found in a courtship context, thus as sex pheromones. Within sex pheromone, species specificity has surely a major relevance since the location or recruitment of the correct mate is a critical event in sexual reproduction (Andersson 1994). Basic information affecting reproductive success can be expressed through the chemistry of pheromones and therefore minimizes fitness costs for the discriminating sex (Johansson & Jones 2007). Considering pheromones as species recognition signals, it is thought that species-specific pheromones evolved to function as pre-mating reproductive isolation mechanism for closely related species (Cardé & Baker 1984, Lofstedt 1993). This is based on the idea that avoiding costly matings with individuals of the wrong species results in nonviable or less fit hybrids. This is especially

relevant for sympatric species. The use of sex pheromone communication is surely widespread in insects and has been well investigated in the Lepidoptera. An example for the potential role of sex pheromones in their reproductive isolation can be found within nine species of European ermine moths (Yponomeutidae) (Lofstedt et al. 1991). Females of these sympatric moth species secrete species-specific sex pheromones to attract conspecific males for mating. The pheromone chemical composition varies in the ratios of main components and the presence or absence of complementary single compounds. In addition to their pheromone, females of different species also use different host plants and time-frames for their sexual activity, which composes an effective model of niche differentiation involving reproductive isolation.

A further aspect regarding sex pheromones as influence on mate choice is their potential to act as a mate assessment signal. The complex chemical mixture of many sex pheromones provides a potential for considerable inter-individual variation. This can be either expressed by the ratio or abundance of certain compounds, but also in the intensity of its release. Such a variation may somehow be costly and vary qualitatively with the individuals' condition, thus resulting in an "honest" signal (Grafen 1990). Benefits of mate choice can be divided into direct benefits, as higher fertility or access to territories, and indirect genetic benefits, which lead to an increase in fitness of the resulting progeny (good gene theory) (Tregenza & Wedell 2000). In a number of studies mate assessment pheromones have been found to correlate with numerous quality factors, such as fertility, reproductive status, age, parasite load, hierarchal status or maturity (Johansson & Jones 2007). As the sexual selection theory predicts, such a reflection of fitness might result in mate choice and a preference of some pheromone phenotypes over others. An example for a pheromone-based mate choice can be found in the parasitic wasp *Nasonia vitripennis*. Here, females can sense the males' quality of functional fertility by his sex pheromone and are therefore able to discriminate against sperm-limited individuals. Sperm limitation is found in newly emerged as well as multiply mated males and strongly correlates with reduced pheromone concentrations. As it is the case for all haplodiploid insects, only fertilized eggs produce female offspring and the production of daughters therefore depend on the availability of sperm. An offspring sex-ratio towards sons would mean a fitness loss for this parasitic wasp, since the flightless sons would compete among each other for females, and only the daughters

are able to disperse to locate new host patches for reproduction. Behavioral tests showed a strong preference of females towards higher concentrations of pheromones as a sign for a high sperm load, consequently increasing their reproductive success by producing an optimal sex ratio in offspring (Ruther et al. 2009).

Evolution of pheromones

As signals, pheromones are subject to natural and sexual selection and there are two different views of how the observed diversity of pheromones between species has arisen. One option for the evolution of different blends would be a gradual process of small changes in the pheromone composition, such as the loss or gain of single components, or a change of their relative proportion. As a result, closely related species should be observed to have similar or even identical chemical signals (Symonds & Elgar 2008). In mymicine ants for example, aromatic pyrazines have been identified within several genera as trail pheromone, which serves as a recruitment signal to direct nestmates to new food sources (Jackson & Morgan 1993). In the genus *Pogonomyrmex* the recruitment pheromone is not species-specific but appears to be anonymous, with one specific alkylpyrazine as major component in at least five different species, triggering the same behavior (Hölldobler et al. 2001). Opposed to this gradual pheromone evolution is the hypothesis of more saltational, rapid shifts in pheromones with their components changing substantially or completely. Recent studies demonstrate that simple genetic switches are sufficient to change enzymatic pathways that synthesize pheromone components resulting in fundamentally different pheromone compositions, therefore illustrating a possibility to generate new blends (Roelofs et al. 2002). Whenever these compositions are promoted by natural selection – with some individuals of a species favoring the new chemical signal over the other – it might directly lead to species divergence. Several recent analyses indicate rather saltational shifts in pheromone evolution, as for example a study about 34 different bark beetle species from two genera using species-specific aggregation pheromones. Clear differences in the pheromone blends between the two genera were observed, but interestingly, the difference of perfume composition between species within the genera was not related to their phylogeny. Instead, closely related species were found to have the greatest numbers of pheromone component differences, a finding that is at odds

with an origination due to small changes (Symonds & Elgar 2004). Further phylogenetic comparative studies, also including potential chemical receiver influences, will be needed to conclude on the driving forces responsible for pheromone diversity.

Chemical signals in general and pheromones in particular surely play an important role in insect interactions. There are numerous behavioral and developmental actions that rely on chemical communication, leaving room for further investigations. An intriguing example for chemical communication can be found in the studied organism of the present dissertation, the neotropical orchid bees (Apinae: Euglossini). While most pheromones are known to be self-synthesized, there are some rare examples of additional components that are collected or gained in other ways. Males of the solitary mining bee *Anthophora abrupta* (Apoidae: Anthophorini), for example, collect volatiles of parsnip plants in mustache hairs in order to use it for territorial markings during courtship behavior (Norden & Batra 1985). Males of the orchid bees are even more unusual in that they use a complex mixture of only exogenous volatiles for chemical signals. To acquire these "fragrances" or "perfumes", males perform a life-time search for volatiles from numerous natural sources, which get stored in their voluminous hind leg pockets where a complex bouquet of volatiles is accumulated. The exact purpose of the male perfumes remains so far unknown, though it has been suggested that they are used as pheromone analogues. This hypothesis is supported by the active exposure of the perfumes' by males at their mating territories, and by their chemical species specificity that has been found in several species so far (Eltz et al. 2005a, Eltz et al. 1999, Zimmermann et al. 2006). The publication in chapter 2 of this dissertation will report on the existence of chemical structure (distinctness) of male fragrance blends in a community of 15 sympatrically living *Euglossa* species.

Biology of orchid bees

The brightly colored, long-tongued orchid bees (Apidae, tribus Euglossini) are inhabitants of the neotropical region reaching from southern Chile over Panama up to northern Mexico (Roubik & Hanson 2004) and even Florida (USA), with the recent establishment of one *Euglossa* species (Skov & Wiley 2005). Together with the highly eusocial honey bees (Apini), stingless bees (Meliponini) and bumblebees (Bombini),

they form the monophyletic clade of corbiculate bees (Michener 2000). Euglossine bees include >200 species grouped into five genera: *Aglae, Eufriesea, Euglossa, Eulaema* and *Exaerete* (Kimsey 1987) and are generally described as solitary to primitively social (Zucchi et al. 1969). Biologists' attention is drawn to the males' special behavior of collecting volatiles from numerous natural sources – they originate from flowers, fruits, feces, decaying wood or tree wounds (Dressler 1982, Roubik & Hanson 2004, Vogel 1966).

Volatile collection of male orchid bees

The specialized behavior of collecting fragrances and their release is based on a set of morphological as well as physiological adaptations shared by all male orchid bees (Bembé 2004). During the collection process, males apply small amounts of self-produced lipids from the cephalic labial glands on the odorant surface to dissolve the volatiles, and use their foretarsal brushes for absorption (Whitten et al. 1989). During a hover flight, the mixture of lipids and volatiles gets transferred into the enlarged cuticular pockets of their hind tibia (Evoy & Jones 1971, Kimsey 1984, Vogel 1966) where a complex, species-specific blend accumulates (Eltz et al. 1999, Zimmermann et al. 2006). The lipid components are thought to function only as a carrier for volatiles, comparable to the Enfleurage process in the traditional perfume industry. Behavioral tests showed no attractant effect of males' lipid extracts, neither to males nor females, suggesting solely the exogenous fragrance mixture as active part in the bees' chemical communication system (Zimmermann et al. 2006). Since male orchid bees collect volatiles throughout their life-span – up to several months – the proportion of lipids in the fragrance bouquet would be overbalanced, thus pointing towards a lipid reuse (Eltz 1997, Whitten et al. 1989). In fact, Eltz et al. (2007) recently were able to show that chemically labeled lipids that were applied directly into the males' hind-leg pockets were recycled and reused for fragrance uptake. Their results demonstrated a relocation of carrier lipids back to the labial glands, followed by a re-application on a further odorant surface. This procedure decreases the ratio of lipids in the fragrance bouquet and functions as an economical way of lipid use in volatile collection (Eltz et al. 2007).

The composition of the exogenous fragrance mixture was found to be broadly species-specific, usually being comprised of terpenoids and aromatics in certain relative quantities. Species specificity is classified as an important basis for chemical signals in the context of courtship (Andersson 1994). Within the group of self-produced pheromones, specificity is mostly achieved by certain proportions of components in a mixture, controlled by highly conserved biochemical processes (Roelofs et al. 2002). Conversely, in the case of euglossine bees, the males must complete the task of compiling a species-specific mixture actively from a number of exogenous sources in a changing environment (Dressler 1982, Roubik & Hanson 2004). The males' fragrance market is composed of a great variety of sources for volatile chemicals. Orchids and other flowers offer a specific chemical mixture as floral scent (Gerlach & Schill 1991, Whitten et al. 1986, Williams & Whitten 1983). Other known sources like sap from tree wounds, and decaying wood might present single component scents (Eltz et al. 1999, Whitten et al. 1993). Species specificity is supposedly achieved by a combination of innate preferences and a refinement of preferences due to learning in male orchid bees. Innate preferences roughly restrict the spectrum of attractive volatiles, and learning regulates the dosage of components, e.g. avoids over-collection (Eltz et al. 2005a).

It is a great advantage for studies on orchid bees that males can be attracted in high numbers to synthetic, single compounds of volatiles presented as chemical baits. This method of chemical baiting has been often used to study diversity, seasonality and population dynamics of euglossine bees (Ackerman 1983, Armbruster 1993, Dressler 1968a, Eltz et al. 1999, Janzen et al. 1982, Roubik & Ackerman 1987).

Orchid bees' mating behavior

The purpose of the accumulated complex fragrance mixture of males is so far not known but it likely plays a role in the bees' mating behavior. Males of several species have been observed performing a distinct display behavior, which usually takes place around forest clearings. It includes defending a non-resource based territory with a tree trunk as a central perch site, where males perform series of wing buzzes (*Eulaema*) or hover flights (*Euglossa, Exaerete*), alternated by perch standing and patrol flights (Kimsey 1980, Stern 1991). Males actively expose their collected perfume mixture

during this display behavior, using several specific morphological structures (brushes of hairs) as well as stereotype sequences of leg movements (Bembé 2004, Eltz et al. 2005b). As mentioned above, the chemical species specificity strengthens the view that the males' perfume has evolved as an intraspecific communication signal, most likely addressed to females. Analogous to endogenous pheromones it might function as short- or long-range attraction signal to females. Alternatively, an attraction of conspecific males to form facultative leks has been discussed by some authors, since males are sometimes observed to interact with a few to many conspecifics within their territories (Peruquetti 2000). Also, exposed hind leg extracts attract conspecific males to perch sites in some species, as shown in field experiments (Zimmermann et al. 2006). However, the attraction and or intrusion of other males might also be motivated by favorable habitat qualities (light conditions), and a general intention of overtaking a territory. It may also be important for males to regularly assess a conspecifics' competitive strength by its fragrance blend. A classical lek situation – the female is given the opportunity to choose one mate out of several in a courtship arena – seems unlikely within the orchid bees, since all of the rarely observed matings took place on perch sites with only a single male present (Dodson 1966, Kimsey 1980, Zimmermann et al. 2006). However, the chemical attraction of conspecific females remains to be demonstrated in behavioral tests.

Indeed, the observation that matings take place within a males' defended territory – the place of perfume exposure – is somewhat at odds with the absence of attraction of females to hind leg extracts in field experiments. Nevertheless, the most likely primary function of the orchid bees' fragrances is that it acts as a signal to conspecific females (Dodson 1966, Eltz et al. 2005b, Lunau 1992, Roubik & Hanson 2004, Stern 1991, Vogel 1966). The failure of female attraction to hind leg extracts in behavioral tests might be due to the lack of optical or behavioral stimuli that accompany the chemical signals, such as the colorful presence of an actual bee and its wing buzzing. Furthermore, the rarity of matings to occur is actually typically seen in the Hymenoptera, with the majority of females mating only once. This decreases the frequency of receptive females at any point in time, and consequently the likelihood to observe fragrance-guided approaches in behavioral experiments (Boomsma & Ratnieks 1996, Strassmann 2001). However, the species specificity of the collected mixture would

ensure the recognition of mates of the correct species. Since the euglossines are remarkably species-rich, with many species occurring sympatrically, a chemical distinctness appears beneficial since it decreases costly matings with wrong partners which might cause unfit or unfertile offspring (Wyatt 2004). Moreover, the males' fragrance bouquet has the potential to act as a mate assessment signal. Individual differences in the collected mixture within the same species are evident and understandable, since the achievement of specificity in such complex mixture does not seem to be an easy task for a male orchid bee (Eltz et al. 1999). Besides the ability to combine the correct components, male bees spend much of their life time collecting fragrances from numerous sources. This is time- and energy-consuming, as well as risky. Therefore, females might be able to estimate a males' genetic quality by means of his perfume blend, which somehow could reflect individual fitness factors such as longevity, absence of parasites or strong dispersibility. Based on the good genes hypothesis a female chooses a male judging attributes she would like to pass on to her offspring, therefore increasing the progenies' fitness and chance of survival (Andersson 1994).

Experiences with caged orchid bees in our working group show that breeding these bees is no trivial task. The construction of an optimal euglossine cage-habitat is so far successful in terms of feeding, long-term survival, and the initiation of males' display behavior. By contrast, successful matings and the rearing of male as well as female offspring turned out to be far more challenging. Fine tuning of several cage characteristics like light conditions, supply of pollen and nest material was rewarded with nest-building activity of several females and successful rearing of a small number of male progeny developed from unfertilized eggs. The absence of actual matings might be caused by so far unknown missing features in the cage conditions, such as a greater selection of pollen sources or the correct climatic conditions (pers. com. T. Eltz & F. Fritzsch, pers. obs., (Fritzsch 2009)). To offer males the potential to compose an appropriate fragrance mixture is a further difficulty. It seems to be unfeasible to offer an adequate volatile market comparable to their natural one, especially with an imperfect knowledge of all volatile sources. However, it is puzzling that females do not choose one best mate of the available and displaying males but seem to be contented with rearing no offspring or only a small number of sons.

The role of orchid bees as pollinators

Besides the intriguing volatile collection of males and their baffling courtship behavior, orchid bees are also famous for their plant associations and their role as pollinators. Females as well as males are thought to function as long-distance pollinators of several neotropical plant families. A great diversity of flowering plants from different species is used by female orchid bees as food and nest material sources (Arriaga & Hernandez 1998, Ramirez et al. 2002, Roubik & Hanson 2004), and they have been observed to cover great distances while foraging (Janzen 1971). Male orchid bees do not only use flowers for nectar, but also as sources for volatiles. Many neotropical plant species (see Ramirez et al. (2002) for a reviewed species list) produce specific volatile blends – mostly as plain mixtures of 3-10 different components – as attractant to assure the pollination services by male orchid bees (Armbruster et al. 1989, Dodson et al. 1969, Williams & Dodson 1972). This special association between plants and male orchid bees constitutes the "euglossine pollination syndrome" (Dressler 1968b, Williams 1982). A substantial fraction of these plants is comprised by orchids, with nearly 700 different species being exclusively pollinated by volatile-seeking male orchid bees, many with impressive morphological adaptations to manipulate the males to effect their pollination. The species-specific fragrance blend produced by orchids attracts only a single or a small set of euglossine species, which is believed to function as reproductive isolation mechanism for the orchids (Hills & Norris 1972). While the orchids depend entirely on male euglossine bees for pollination, this mutualism appears to be only facultative for the bees. The orchid bee species *E. aff. viridissima*, which has been naturalized recently in southern Florida (USA) (Skov & Wiley 2005) is a case in proof, since it now prospers in an environment without perfume orchids and functions as efficient pollinator of selected native and invasive plant species (Liu & Pemberton 2009, Pemberton & Wheeler 2006). Their impressive role as key pollinators in tropical forests makes euglossine bees an important target for conservation. Fragmentation due to land-use and deforestation is a major threat of neotropical forests these days, resulting in a vegetation-mosaic of forest patches as habitat for euglossine bees. (Mendoza et al. 2005, Myers et al. 2000, Roubik & Hanson 2004, Whitmore 1997). An understanding of how orchid bee populations and communities respond to such changes is of great importance.

Habitat fragmentation and its potential consequences

The fragmentation of habitat is a widespread phenomenon in tropical as well as temperate regions. Since tropical rain forests are the most species-rich of terrestrial ecosystems – underlying a rapid deforestation – these areas count as today's biodiversity hotspots for conservation priorities (Myers et al. 2000). Estimations in the 1990s revealed that one in four tropical rainforest countries had less than 30% of their original lowland rainforest area remaining. Some tropical regions are even more affected, as for example the coastal forests of Brazil that were estimated to occupy only 12 % of their original area 18 years ago (Whitmore & Sayer 1992). Habitat loss has vast negative effects on biodiversity and counts – together with invasive species, over-exploitation of environment and pollution – as one primary factor contributing to species extinction. These factors are caused by humans and can be related to the rapid growth of the human population following intensive land-use. Fragmentation itself can be seen as a process of transforming a once continuous habitat into a patchwork, involving both reduction of total habitat area as well as creation of separate isolated patches from a previously larger continuous distribution (Frankham et al. 2002). Therefore, fragmentation per se is not equal to habitat loss and is believed to have weaker effects, but this notion strongly depends on the exact habitat characteristics (Tscharntke et al. 2002). Studies on the effects of habitat fragmentation not only uncovered a relation to direct measures of biodiversity such as species richness, population abundance and distribution, but also to several indirect measures, like the loss in population growth rate, a decline in breeding success, or an increase of overall genetic diversity (Fahrig 2003). It is clearly of major interest to fully understand the effects of ongoing habitat fragmentation on ecological processes and to maintain a natural equilibrium since loss of species may lead to changes in ecosystem functions (Kruess & Tscharntke 2000).

Effects of fragmentation on populations

Biogeographic factors like fragment size, degree of isolation and proportion of edges are thought to have an impact on population abundance and species diversity (Debinski & Holt 2000, Fahrig 2001, Frankham et al. 2002). Habitat area in general is a

reliable measure of species diversity. Larger areas are more likely to include different habitat types, consequently supporting a greater number of different species (Kruess & Tscharntke 2000, Rosenzweig 1995). In contrast, smaller and more isolated areas are threatened by species loss and remoteness from the nearest species pool, potentially followed by a decrease in colonization rates. Furthermore, the proportion of edge areas is also increased in smaller areas, which is believed to negatively effect the actual species composition due to altered habitat conditions that reduce survival chances (Thies & Tscharntke 1999).

However, the impact of fragmentation depends on both the characteristics of the habitat as well as on the characteristics of the affected species. The dispersal or gap-crossing ability of an organism is an important aspect concerning habitat fragmentation, since habitat connectivity or size is of minor importance in highly motile species. In the highly mobile Eurasian nuthatch (*Sitta europaea*) for example, neither evidence for reduced reproductive success in fragments versus larger forests, nor any relationship towards habitat size or degree of isolation was found (Matthysen & Adriaensen 1998). In general, habitat specialists are more prone to extinction than generalists. Species that depend on a certain blend of resources that is less likely to occur on small patches are at a disadvantage in contrast to species with a broad and unspecific habitat use (With & Crist 1995). Requirements of euglossine bees towards their habitat are thought to be considerably challenging – it includes food plants, nesting sites, nest building materials and volatile sources (Roubik & Hanson 2004). It is questionable if small forest patches are able to meet these requirements. The bees' ability to cross large distances, their longevity, and their ability to adapt to new environments might buffer negative effects of fragmentation (Janzen 1971, Kroodsma 1975, Skov & Wiley 2005, Williams & Dodson 1972). Previous studies on responses of euglossines to forest fragmentation found varying results regarding bee abundance and species diversity (Becker et al. 1991, Brosi 2009, Powell & Powell 1987, Tonhasca Jr et al. 2003). The results of some indicated a positive relationship between forest area and bee abundance, while others reported an affinity of bees towards forest edges and highly disturbed forest sites. However, responses of euglossines towards fragmentation seem to be highly variable between species.

Generally, it is dangerous to underestimate the process of species extinction that follows the destruction of natural habitat. In complex ecosystems like tropical rainforests many species obligatorily depend on one another. Such a system is especially receptive for the process of coextinction, defined as the loss of a species upon the loss of another (Dunn et al. 2009). For example, a strong association between butterfly species and their specific larval hostplants developed on the tropical island of Singapore. In total, there were 382 butterfly species and 908 potential hostplant species recorded, of which at least 56 butterfly and 208 hostplant species had gone extinct over the past two centuries (Turner et al. 1994). A simulation by Koh et al. showed that the number of extinct butterfly species is expected to increase exponentially with that of extinct hostplants, suggesting that coextinction between these two taxa might have occurred (Koh et al. 2004b). The threat of coextinction for affiliates and their hosts affects a wide range of coevolved interspecific systems, like pollinators and their pollinated plants, and basically all parasites and their specific hosts (Biesmeijer et al. 2006, Koh et al. 2004a). Such coextinction might also concern the specialized association between volatile producing plants and their exclusive pollinators, the male orchid bees. A decline in the orchid bee population caused by fragmentation would subsequently negatively affect plant species diversity in the habitat.

Conservation genetics

Effects of tropical forest fragmentation may vary, but biological diversity is normally reduced. An unknown although large number of species already went extinct, while many others have reduced population sizes (Frankham et al. 2002). Within conservation management, genetic analyses to determine the genetic diversity of populations have become widespread in the past decade (Hedrick 2001). Genetic diversity reflects the evolutionary potential of a population and its maintenance is considered essential for populations to respond towards environmental changes (Wright 1969). Populations with low genetic diversity are often associated with reduced fitness of individuals and expected to suffer more seriously from diseases, pests and parasites than those with high genetic diversity (Lande 1988). A frequent consequence of fragmentation is a reduction in population size for most species. Small populations have generally lower levels of genetic diversity, thus are more likely to suffer from

extinctions than large populations. However, the impact of fragmentation on genetic diversity and genetic differentiation critically depends on the gene flow among populations. Migration between fragmented populations would ensure a genetic exchange. Such an exchange of genes homogenizes allele frequencies and prevents the fixation of alleles, therefore counteracting the process of population differentiation and speciation (Barton & Hewitt 1985). Prevalently, gene flow and the consequent genetic differentiation of populations correlates with geographic distance (Balloux & Lugon-Moulin 2002). Dispersal among fragments is reduced with increased degree of isolation, thus distant fragments are thought to receive fewer migrants than more proximate ones.

The loss of genetic diversity also involves the threat of inbreeding, which is proposed to contribute to the decline and eventual extinction of small and isolated populations (Frankham 1995b). Inbreeding is defined as the production of offspring from related individuals. Within small populations, the opportunities for mating are unavoidably restricted. As a consequence, potential mating among relatives increases. Inbreeding is associated with an increase of homozygous individuals and results in a reduction of fitness, termed inbreeding depression. It has been shown to reduce reproduction and survival rate (reproductive fitness). Since such an inbreeding depression is mostly a consequence of little genetic diversity it is equally dependent on effective population sizes and a genetical exchange via constant migration rates. There have been several examples of fitness reduction caused by inbreeding in captive populations (Charlesworth & Charlesworth 1987, Hedrick & Kalinowski 2000) and this has also been detected in wild populations (Keller et al. 1994, Keller & Waller 2002, Madsen et al. 1996). A recent field study on a natural population of Glanville fritillary butterflies (Lepidoptera: Nymphalidae) demonstrated the effect of inbreeding on local extinction for the first time (Saccheri et al. 1998). The authors found a significant increase of extinction risk through lower rates of heterozygosity, which count as strong indicator for inbreeding. Fitness indicators such as larval survival, adult longevity, and egg-hatching rate have been shown to be all adversely affected by inbreeding. Within conservation genetics, inbreeding depression and loss of genetic diversity both account as main threats for the extinction of small and isolated populations (Frankham 1995a).

Genetic characteristics of bees

Bees are major pollinators of wild plants and crops in terrestrial ecosystems (Klein et al. 2007), but lately there has been strong evidence that they are in decline (Biesmeijer et al. 2006, Cane 2001). Habitat fragmentation and loss appears to be a major cause in the decline of bees (Foley et al. 2005), and as mentioned above, genetic factors can play an important role in population decline and extinction (Frankham 2003). Bees are somewhat outstanding in that they have some unusual genetic properties since they belong to the insect order of Hymenoptera, which is characterized by haplodiploidy. Here, haploid males arise from unfertilized eggs receiving only a single set of maternal chromosomes, whereas diploid females arise from fertilized eggs with both maternal and paternal sets of chromosomes. Their presumed sex determination mechanism is the single locus complementary sex determination (sl-CSD) (Bell 1982, Beye et al. 2003, Cook 1993). The sex of the offspring is determined by the genotype on a single locus with several alleles. Offspring resulting from fertilized eggs usually possess a heterozygous genotype at the sex-determination locus and develop into females, whereas hemizygotes from unfertilized eggs develop into males. The sex determination locus has been molecularly identified in *Apis mellifera* (Beye et al. 2003) and genetically mapped in *Bombus terrestris* (Gadau et al. 2001).

Single locus complementary sex determination includes the genetic disadvantage of diploid male production (van Wilgenburg et al. 2006). When females mate with a male that has the sex-determination allele in common, half of the progeny will have a homozygote genotype at this locus. Consequently, they will develop into diploid males (Crozier 1977). Those males are mostly either inviable or sterile which makes them incapable of reproduction – thus they are considered to be a genetic dead end (Stouthamer et al. 1992). Studies on the males' diploidy show several negative aspects concerning their reproductive success: males may fail to produce or transfer sperm (Krieger et al. 1999), or transferred sperm may fail to fertilize eggs (Duchateau & Marien 1995), or only sterile triploid offspring is produced (Naito & Suzuki 1991). Therefore, diploid males have been generally considered as a genetic cost either to their parents because they do not transfer genetic material, or to the females they mate with because these will produce no or fewer fertile offspring (Heimpel & de Boer 2008). In social

species they can decrease colony growth rates and may even increase colony mortality (Plowright & Pallett 1979). However, diploid males do not always imply fitness costs, since some rare exceptions can also be found: In the solitary vespid wasp *Euodynerus forminatus* (Cowan & Stahlhut 2004), for example, fertile diploid males are frequently produced and capable of producing normal offspring with diploid daughters.

In natural populations, diploid males are thought to occur at low frequencies, since allelic diversity at the sex locus should be high but it poses a particular threat for small and isolated populations. Smaller populations with less genetic diversity are expected to maintain fewer sex-determination alleles. Since the number of these alleles indirectly controls the frequency of diploid male production, smaller populations are more affected by diploid male production than larger populations (Zayed 2009). The frequency of diploid males has been proposed to function as a sensitive measure of pollinator decline for bees (Zayed et al. 2004) and the production of effectively sterile diploid males has been reported in several bee species so far (see table 1 in Zayed (2009)). High proportions of diploid males have also been detected by allozyme analysis in different species of euglossine bees (Lopez-Uribe et al. 2007, Roubik et al. 1996, Zayed et al. 2004), but ecological observations on these bees do so far not support the hypothesis of their decline. In a simulation model, Zayed and Packer (2005) compared the probability of extinction caused by inbreeding depression of diploid organisms with that caused by inbreeding/diploid male production in haplodiploid populations. They found the extinction risk in haplodiploids with CSD over an order of magnitude higher than the extinction risk of inbreeding in diploids (Zayed & Packer 2005). Therefore, complementary sex determination and the production of diploid males are a potentially large genetic threat to population viability, making bees especially prone to extinction and a major target for conservation.

Outlook of my dissertation

The results of my dissertation are presented in the form of three articles, published in international peer-reviewed journals, and three manuscripts. Thematically, they can be divided into two main topics, with Chapter 2 – 5 dealing with the chemical distinctness of male fragrances of different species and their potential function in the

bees' mating behavior and species isolation, whereas Chapter 6 and 7 concentrate on orchid bee population genetics and the potential consequences of forest fragmentation.

The exogenous fragrance mixture of male orchid bees is often associated with a use as pheromone analogue (Vogel 1966, Zimmermann et al. 2006). Pheromones – especially sex pheromones – play an important role in species recognition. The recruitment and or localization of a conspecific mate are important targets in an individual's life (Wyatt 2004). Therefore, communication with species-specific volatiles is thought to provide substantial support by counteracting costly hybrid matings (Higgie et al. 2000, McElfresh & Millar 1999). The previously demonstrated species specificity of male fragrances within three *Euglossa* species (Eltz et al. 1999) emphasizes its potential to function as species or even mate recognition signal. Based on this, it was the concern in Chapter 2 to test whether euglossine bees within a community of numerous sympatric species would still be able to acquire distinct chemical blends. The evolution of chemical distinctness during orchid bee phylogenesis and the potential role of male fragrances as premating isolation mechanism are discussed. In Chapter 3 and Chapter 4 we report on the discovery of a closely related sibling species of *Euglossa viridissima* Friese in southern Mexico, whose differentiation seems to be associated with an olfactory shift due to component-specific differences of perfume perception and collection in males. Chemical, morphological as well as genetic data are combined for an integrated characterization of the two lineages. The publication of Chapter 5 is about the orchid bee nest biology and female mating frequency. Information about female orchid bee mating frequency are desirable both in the context of social evolution in corbiculate bees and as well as for a better understanding of the significance of euglossine fragrance collection.

Chapter 6 and Chapter 7 are concerned with euglossine population and conservation genetics. Fragmentation due to deforestation and land-use is a major threat for today's neotropical forests (Myers et al. 2000). Genetic effects on euglossine bee populations were investigated since euglossines count as important pollinators with a positive influence in neotropical plant species diversity (Roubik & Hanson 2004). In Chapter 6 it was our aim to investigate genetic consequences of habitat fragmentation on populations of different *Euglossa* species in the tropical forests of southern Mexico.

We tested for genetic differentiation due to reduced gene flow between populations caused by the distance alone and as a consequence of fragmentation and habitat loss. Chapter 7 focuses on diploid male production within euglossine bees, which is generally thought as an indicator of reduced genetic diversity and inbreeding within populations (Cook 1993). In euglossine bees it is of special interest since previously detected high numbers of diploid males postulated a decline of these important neotropical pollinators (Lopez-Uribe et al. 2007, Roubik et al. 1996, Zayed 2004).

Chemical niche differentiation among sympatric species of orchid bees

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COVER PHOTO: A male orchid bee (*Euglossa purpurea*) collecting fragrance from a crystal of synthetic *p*-dimethoxybenzene placed on a log in Costa Rica. Tarsal brushes are used to absorb the chemical, which is then transferred to pockets on the hind tibiae. Zimmermann et al. examine chemical niche differentiation among sympatric species of orchid bees (see pp. 2994–3008). Photo credit: Bernhard Jacobi.

Chemical niche differentiation among sympatric species of orchid bees

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Abstract. Male Neotropical orchid bees (Euglossini) collect volatile substances (fragrances) from flowers and other sources (e.g., decaying wood) and store them in specialized hind tibial pockets. The accumulated chemicals are later emitted during courtship display, presumably to lure conspecific females for mating. We analyzed tibial fragrances of males of 15 sympatric Panamanian species in the genus *Euglossa* to test whether communities of euglossine bees are chemically structured, and to elucidate whether male fragrance signals evolve to convey premating isolation. Our analysis revealed substantial chemical disparity among all lineages. Disparity was mediated by compounds that were exclusive to certain species but also by differences in relative quantity of shared compounds. We mapped tibial fragrance compounds present in each species on a DNA-based phylogeny (reconstructed using partial sequences of *COI*, *EF1-α*, *ArgK*, and *Pol-II*) and found that most dominant compounds were highly homoplasious. In an analysis of chemical differentiation in relation to phylogenetic divergence through time, disparity was greater than expected from a null model at any point during evolutionary history, suggesting that diversifying selection has shaped fragrance phenotypes. Notably, chemical disparity was greater within recently diverged lineages than among them, suggesting that chemical preferences in orchid bees evolved rapidly in the early stages of species divergence. We postulate communication interference as the possible mechanism behind the observed fragrance differentiation, which may be the product of reproductive character (fragrance) displacement. Our findings are consistent with the hypothesis that male fragrance signals evolve to convey premating isolation.

Key words: Barro Colorado Island, Panama; chemical communication; diversifying selection; Euglossine bees; Euglossini; fragrance; mate recognition; pheromone; phylogeny; reproductive character displacement.

Introduction

Insects frequently use chemical signals to attract conspecifics for sex or resource exploitation. The chemical nature of the molecules emitted by insects is diverse, but the signals must be distinguishable from those used simultaneously by other species and from background noise (Wyatt 2003). In diverse communities, chemical signals may be subject to diversifying selection due to communication interference with sympatric species that use similar chemical signals (Groot et al. 2006). Such interference has been proposed for sex attractants, where the correct chemical signal is wholly or partly responsible for maintaining reproductive isolation (McElfresh and Millar 1999, Higgie et al. 2000, Gries et al. 2001, McElfresh and Millar 2001). The

breakdown of chemically mediated mate recognition systems could impose high fitness costs due to either the waste of gametes or the production of inferior hybrids. Such fitness costs are thought to drive signal divergence in areas of sympatry (i.e., reproductive character displacement) (Butlin 1987, Coyne and Orr 2004).

Chemical distinctness may be achieved in two major ways, which frequently interact and can be considered as two extremes of a continuum. At the one end, a single type of molecule is used to transfer information, but because of its complex structure, it is unlikely to be duplicated by other species. At the other end, simple molecules occur in blends of distinctive quantitative proportions (Bjostad et al. 1987). Among the best-studied examples of the latter are the female sex pheromones of certain moths, which consist of variable blends of common fatty-acid derivatives (Bjostad et al. 1987, Morse and Meighen 1987, Roelofs and Rooney 2003).

Neotropical orchid bees (Apidae, Euglossini; ~250 species) are a group of conspicuous, long-tongued bees that share a unique system of chemical communication that is most likely used in the context of mate recognition (Roubik and Hanson 2004, Zimmermann

et al. 2006). Males of all species of orchid bees possess voluminous pockets in their hind legs, which they use for the collection and accumulation of exogenous volatiles, mostly derived from flowers, fragrant plant exudates, decaying vegetative matter, or feces (Vogel 1966, Whitten et al. 1993, Eltz et al. 2007). This unique behavior has led to the evolution of one of the most intriguing pollination syndromes. Flowers of >700 species of Orchidaceae, among others, each produce scents that specifically attract males of one or a few orchid bee species. As males collect fragrances from these flowers, they act as their exclusive pollinators (Dressler 1982, Ramírez et al. 2002). Male orchid bees accumulate volatiles over much of their lives and thus acquire complex blends consisting of diverse terpenoids and aromatics (Eltz et al. 1999). These blends are later exposed by males during courtship display, which usually takes place in the forest understory where males defend nonresource display territories (Bembé 2004, Eltz et al. 2005b). Females have been observed to approach displaying males, in some cases from downwind, and copulate with males on the perch tree (Kimsey 1980, Stern 1991, Zimmermann et al. 2006). Although the direct proof of female attraction to male fragrances remains elusive, it is likely that fragrance signaling is involved in mate recognition and choice.

In samples of males of the same species, fragrances may vary substantially in both quantity and complexity. However, when we compared three species of Central American *Euglossa*, we found that the composition of individual blends was significantly more similar within species than between species, and that the distinction persisted even when samples from different localities and habitats were included in the analysis (Eltz et al. 2005a). Bioassays with hind-leg extracts confirmed that the information contained in individual chemical blends is sufficient to mediate species-specific attraction (Zimmermann et al. 2006).

Attaining chemical distinctness in male orchid bees seems a nontrivial task for three reasons. First, specific blends are accumulated from sources that vary in availability both spatially and temporally in the bees' habitat. This has been addressed in a previous study (Eltz et al. 2005a), which demonstrated that bees exert experience-dependent choices (negative feedback) to compensate for fluctuating availability. Second, specific blends must accumulate from sources that normally (but not always) produce blends of components themselves (e.g., Gerlach and Schill 1991, Whitten et al. 1993), thus limiting the degrees of freedom that are available for accumulating bee-specific blends. Third, chemical distinctness in orchid bees is made difficult by the elevated species diversity characteristic of Euglossini; a given community may contain up to 50 species, with a substantial fraction of them being active at any one time (Roubik and Hanson 2004).

In the present paper we analyzed male hind-leg fragrances of 15 sympatric species of Panamanian *Euglossa* to determine whether bees within a diverse community can acquire distinct chemical blends, and to elucidate the potential role of exogenous fragrances as premating isolation mechanisms. Specifically we asked (1) whether chemical distinctness between species collapses as more species are included in the analysis, (2) how overall chemical distinctness relates to phylogeny, and (3) to what extent major fragrance components are shared by closely related species. We found that chemical distinctness is substantial between all sympatric species, but most notably so between closely related taxa. Our findings support the hypothesis that male fragrances function as recognition cues and suggest that fragrance phenotypes evolve rapidly in response to chemical interference from congeners.

Materials and Methods

Sampling

From 6 February to 13 March 2006, 176 males of 15 species of *Euglossa* were sampled from an old-growth forest on the island of Barro Colorado (BCI) in the Panama Canal. Bees were netted during standardized baiting assays at the radio tower clearing in the center of the island using the following bait chemicals: 1,8-cineole, methyl salicylate, *p*-dimethoxybenzene, methyl cinnamate, skatole, vanillin, eugenol, benzyl acetate, 2-phenylethyl alcohol, and terpinene-4-ol. On a total of 24 mornings (8:30 to 12:30 hours), these substances were exposed on strips of filter paper housed in plastic "Wiffle" balls. The Wiffle balls were covered by nylon mesh to keep bees from gaining direct access to the chemicals and suspended by rope from branches around the clearing. Arriving bees were captured, cooled on ice in Eppendorf caps (Carl Roth, Karlsruhe, Germany), and later frozen in a laboratory freezer. Fragrance loads were sampled on the same day by extracting individual pairs of hind legs in 0.5 mL of hexane containing 1 mg/mL 2-undecanone as an internal standard. For reference, we also extracted individual heads of all species to gain information on compounds produced by male cephalic labial glands. Labial gland lipids are spat on fragrant surfaces by the males and serve as a carrier during the process of fragrance collection (Eltz et al. 2007). As both lipids and exogenous volatiles are present in hind legs, we used information on head extracts to identify exogenous compounds.

Chemical analysis

Gas chromatography/mass spectrometry (GC/MS) was done at the Department of Neurobiology, Düsseldorf, Germany, using a HP 5890 II GC fitted with a 30-m nonpolar DB-5 column and a HP 5972 mass selective detector (Hewlett Packard, Wilmington, Delaware, USA). Injection was splitless, the oven programmed from 60° to 300°C at 3°C/min with automatic pressure programming.

Mass spectra and associated retention indices of integrated peaks were compared and cross-referenced

TABLE 1. Collection data, taxonomic information, and National Center for Biotechnology Information (NCBI) GenBank accession numbers of samples of *Euglossa* bees used for phylogenetic analyses in the present study.

Taxon	DNA extraction ID	Country	Locality	Province	Collection date
E. allosticta	EU34	Colombia	La Virginia	Risaralda	4 Jan 2003
E. azureoviridis	EU52	Costa Rica	La Selva	Heredia	5 Apr 2003
E. bursigera	EU89	Colombia	Lloro	Chocó	25 Apr 2003
E. cognata	EU27a	Perú	Lagunas	Loreto	5 Mar 2005
E. cognata	EU27	Colombia	Mocoa	Putumayo	10 Jan 2003
E. crassipunctata	EU144b	Colombia	La Virginia	Risaralda	4 Jan 2003
E. deceptrix	EU58	Panamá	Cerro Azul	Panamá	May 2003
E. despecta	EU84	Colombia	Bahia Solano	Chocó	14 Apr 2003
E. dissimula	EU44	Colombia	Mocoa	Putumayo	10 Jan 2003
E. dodsoni	EU13	Costa Rica	La Selva	Heredia	9 Aug 2005
E. hemichlora	EU77	Colombia	Bahia Solano	Chocó	14 Apr 2003
E. heterosticta	EU19	Costa Rica	La Selva	Heredia	11 Aug 2002
E. igniventris	141d	Panamá	BCI	Panamá	Feb/Mar 2006
E. imperialis	EU5	Costa Rica	La Selva	Heredia	10 Aug 2002
E. mixta	EU8	Costa Rica	La Selva	Heredia	16 Aug 2002
E. tridentata	EU9	Costa Rica	La Selva	Heredia	13 Aug 2002
E. villosa	EU70	Guatemala	Chiquimula	Esquipulas	10 Sep 2003

Note: Abbreviations are as follows: BCI, Barro Colorado Island, Panama; *CO1*, cytochrome oxidase; *ArgK*, arginine kinase; *Pol-II*, RNA polymerase II; *EF1-α*, elongation factor 1-alpha.

with entries in the local user library (T. Eltz, *unpublished data*). New components were added over the course of the study. Chemical characterization of components was done by comparison with authentic standards or by matching spectra and retention times with those in the literature (Adams 2001). Mass spectral characteristics alone were not considered sufficient for compound identifications, but were used for an assignment to broader substance classes. Additional identifications were made by R. Kaiser (*personal communication*), who analyzed representative samples of some species. We excluded straight-chain lipids (alkanes, alkenes, alcohols, acetates, diacetates, and wax esters) from the analysis of fragrance composition, because such compounds are typically produced by the bees' labial glands and were prominent in the head extracts of the study species.

Fragrance similarity

Fragrance similarity was analyzed with nonmetric multidimensional scaling (MDS) and associated techniques (Legendre and Legendre 1998, Clarke and Warwick 2001). MDS is flexible concerning the similarity measure employed, which allowed us to use the Bray-Curtis index as a measure of chemical similarity/dissimilarity between samples (individuals). The value that this index takes between any two individuals is affected only by chemical compounds jointly present in the two individuals, but not by those jointly absent. This is desirable because similarities are fixed between pairs of individuals irrespective of the chemical phenotypes of other individuals in the data matrix (Clarke and Warwick 2001). Prior to calculating the index, absolute peak areas (integrated MS ion currents) were standardized to represent relative peak contributions to individual fragrance composition (in percent), and these were

then square-root transformed. From these data we derived a triangular similarity matrix based on the mentioned Bray-Curtis index. Similarities (in percent) were ordinated in two or three dimensions using the nonmetric MDS algorithm in PRIMER v6 (Clarke and Gorley 2001). Ideally, MDS plots have interpoint distances that exactly match the rank order of dissimilarities between samples in the underlying similarity matrix. Deviations from this match are expressed in terms of "stress," with stress values <0.15 indicating a good fit concerning the overall structure of the plot. We tested the null hypothesis that the factor "species" had no effect on the rank order of between-individual similarities using ANOSIM one-way permutation tests (Clarke and Green 1988).

To determine whether chemical distinctness between species was affected by increasing the numbers of congeners present in fragrance space, we randomly assembled bee communities that differed in total species number. Average Bray-Curtis dissimilarity (1 − Bray-Curtis similarity) was calculated between the focal species and each of the other members of the communities, with a total of 15 focal species, 14 different communities per focal species (with one to 14 congeners), and 25 permutations of the order in which species were assigned to these communities. We plotted minimum community-wide dissimilarity to the focal species, averaged across communities, with the same number of coexisting species (Fig. 3).

Search for independent "building blocks" (motifs) in blends

The complexity of individual fragrance loads represents a major obstacle for discerning the more simple "building blocks" that compose male fragrance blends. We based our search on the assumption that compounds

TABLE 1. Extended.

Voucher no.	Collector	CO1	ArgK	Pol-II	EF1-α
SR52	S. Ramírez	EU421517	EU421648	EU421266	EU421389
DM1	D. McKenna	EU421541	EU421672	EU421288	EU421412
JCN.124	J. Neita	EU421573	EU421701	EU421319	EU421446
SR1189	S. Ramírez		EU421640	EU421261	
SR395	S. Ramírez	EU421510			EU421381
SR74	S. Ramírez	EU421494	EU421626	EU421246	EU421365
R1	D. Roubik	EU421546	EU421675	EU421293	EU421418
TA235	T. Arias	EU421568	EU421696	EU421314	EU421441
SR394	S. Ramírez	EU421530	EU421661	EU421277	EU421401
CS11	C. Skov	EU421484	EU421613	EU421234	EU421353
TA258	T. Arias	EU421561	EU421689	EU421306	EU421433
CS80	C. Skov	EU421500	EU421632	EU421252	EU421371
EU141d	T. Eltz	EU421492	EU421623	EU421243	EU421362
CS26	C. Skov	EU421537	EU421668	EU421284	EU421408
CS155	C. Skov			EU421309	EU421436
CS122	C. Skov	EU421574	EU421702	EU421320	EU421447
Yurr912	C. Yurrita	EU421556		EU421301	EU421428

derived from the same source would exhibit a tight positive correlation in quantity across individual males, with the proportion corresponding to that in the source (e.g., a certain species of orchid). However, a tight correlation would only be expected if the compounds in question are not also collected from alternative sources where they occur in different proportions. In the latter case, the composition of the original source is likely obscured. We first analyzed each species separately and selected components that were present in the majority (>60%) of individuals. Pearson R was calculated between square-root transformed peak areas of all pairs of these components across individuals. Only the top 5% of positive correlations per species were further analyzed and subjected to visual inspection by scatterplot. Finally, we analyzed whether convincing correlations would hold in cross-species analyses, suggesting shared fragrance sources. To do so we calculated R and visually inspected scatterplots across the individuals of two or more species.

Phylogenetic inference

The genus *Euglossa* comprises a relatively well-defined group of >100 species, of which 44 have been recorded from Barro Colorado Island, Panama (BCI) (Ackerman 1989). The 15 ingroup taxa here included represent a random (unbiased) sample of those taxa (all species with three or more individuals captured were included). To reconstruct the phylogenetic relationships of those taxa, we sequenced a total of ~4.0 kb from four different loci, including the mitochondrial protein-coding gene cytochrome oxidase (*CO1*, 1.2 kb) and the nuclear protein-coding genes elongation factor 1-alpha (*EF1-α*, ~1.2kb), arginine kinase (*ArgK*, ~0.7kb), and RNA polymerase II (*Pol-II*, 0.8kb). We used the species *Euglossa villosa* as our outgroup based on previous results (Ramírez 2008).

We followed standard protocols of DNA extraction, amplification, and sequencing as indicated in Ramírez (2008). Voucher specimens for all sampled species were deposited in the Entomological Collection of the Museum of Comparative Zoology (Harvard University, Cambridge, Massachusetts, USA); GenBank (National Center for Biotechnology Information [NCBI]) accession numbers are indicated in Table 1.

We used the software ModelTest, Version 3.7 (Posada and Crandall 1998), to determine the best-fit model of sequence evolution for each locus separately. We implemented Bayesian phylogenetic analyses in the software package MrBayes, Version 3.1.1 (Ronquist and Huelsenbeck 2003). Tree searches were performed assuming a single model of sequence evolution for all loci, and two independent Markov chain Monte Carlo (MCMC) searches were made for 10 million generations, sampling every 1000 generations, for a total of 10000 trees. Four different chains per search replicate were used. We estimated model parameters during runs and estimated Bayesian posterior probabilities as the proportion of trees sampled; the trees obtained in the first one million generations were discarded as "burnin," which we determined by plotting the log likelihood values against the number of generations. Additionally, we estimated the phylogenetic relationships among bee lineages with maximum likelihood (ML), as implemented in the software package Garli, Version 0.96 (Zwickl 2006). We determined node support using ML by running 100 nonparametric bootstrap replicates. Equivalent models of sequence evolution and estimated parameters were applied to both Bayesian and ML analyses. We calculated between-species genetic distances (nucleotide substitutions per site) via likelihood, optimized with the GTR + I + Γ (best fit) model of sequence evolution in the software package Paup* version 4.0b (Swofford 2003). In addition,

chemical compounds were coded into a data matrix (presence/absence) to determine fragrance motifs and to calculate character statistics. These analyses were performed with the software package Paup* version 4.0b (Swofford 2003).

To evaluate the evolutionary history of chemical variation among lineages in the genus *Euglossa* and determine how chemical disparity is partitioned within and among bee lineages, we calculated disparity-through-time (DTT) plots based on the method developed by Harmon et al. (2003, 2008). DTT plots allow one to calculate the diversification of multiple traits simultaneously by estimating the dispersion of points in multivariate space across time intervals in a phylogeny. This methodology first estimates trait disparity across the whole phylogenetic tree, and then, for each subclade in the phylogeny, disparities are calculated and standardized relative to the disparity observed in the whole tree. It should be noted that this method avoids the difficulties of inferring ancestral character states on the phylogeny. Instead, it relies on estimating the average relative subclade disparity for each point in time whose lineages were present at that time (Harmon et al. 2003). We used DTT plots to examine the time course of the observed phenotypic (chemical) variation in relation to the chemical disparity expected under a null model. Subclade disparities were calculated by moving up the phylogeny from the root node to the tips. We used pairwise Manhattan distance as a measure of chemical disparity, which we estimated as the average variance at each node.

RESULTS

Fragrance distinctness

Individual fragrances were highly variable in complexity, ranging from five to 75 different components; we found a total of 514 different components in all our samples. There were significant effects of species affiliation on the total amount of fragrances (sums of integrated ion currents; Kruskal-Wallis $H = 91.09$, $P < 0.0001$, df $= 14$, $N = 176$) and the number of components ($H = 71.88$, $P < 0.001$, df $= 14$, $N = 176$; see Fig. 1). There was a significant positive correlation between the total amount and the number of compounds across all individuals (Spearman $R = 0.619$, $P < 0.0001$, $N = 176$), but the slope of that relationship was not the same in all species. E.g., males of *Euglossa allosticta* had large amounts of fragrance with low complexity, whereas males of *E. imperialis* were on the opposite end of the spectrum, having small amounts of comparatively diverse blends (Fig. 1). The chemical components were distributed nonrandomly among individuals, both qualitatively and quantitatively. Across all individuals and species, ANOSIM permutation tests showed highly significant effects of species affiliation on fragrance similarity (Global $R = 0.945$, $P < 0.001$). Fig. 2a shows a two-dimensional multidimensional scaling (MDS) plot in which interpoint distances reflect interindividual chemical dissimilarity. It should be emphasized that the two-dimensional view is only an imperfect (highly constrained; stress = 0.22) representation of the true between-species distinctness in the underlying multidimensional similarity matrix. Between-species overlap is reduced to almost zero by including a third MDS dimension, which can be confirmed by viewing the rotating three-dimensional representation (less constrained; stress = 0.15) provided as Appendix A: Fig. A1. Pairwise tests of species distinctness using ANOSIM were all significant at the 95% level (all R values >0.56) except the test between *E. mixta* and *E. dodsoni*, the latter of which had very low sample size ($N = 3$; $R = 0.25$, not significant). Average Bray-Curtis dissimilarity was high between all pairs of species (88.3% ± 7.86%; range 63.30% to 98.65%). Accordingly, minimum dissimilarity of sympatric species decreased only slightly with species richness in randomly assembled communities, asymptotically approaching ~75% in more speciose communities (Fig. 3).

Fig. 4 shows the quantitative representation of the 80 most abundant components in all the 15 *Euglossa* species (see Appendix A: Fig. A2 for an undivided version of Fig. 4). Restricting the similarity analysis to these 80 compounds provides a similar picture to that shown in Fig. 2 (data not shown). The percentage data are given in Appendix B.

It is evident that many of the abundant fragrance compounds were shared by two or more species, although species tended to differ in relative amount. An outstanding example is the sesquiterpenoid hexahydrofarnesyl acetone (hha), which occurred (at least occasionally) in each of the 15 species and was a major component of eight (see Fig. 2b). In contrast, exclusive major components (e.g., compounds that were abundant in one species but absent from all others) were relatively rare. For many of these, we were unable to make structural assignments. Major compounds that were abundant in a small set of species include Germacrene D-4ol (in *E. bursigera*, *E. crassipunctata*, and *E. igniventris*; see Fig. 2c) and an unknown sesquiterpene ketone, molecular weight 218 (in *E. cognata* and *E. mixta*; see Fig. 2d). Thus it is clear that chemical differentiation was mediated both by possession of rare (exclusive) compounds as well as by possession of specific proportions of common ones.

Fragrance motifs ("building blocks")

Individual amounts (peak areas) of components showed variable degrees of intercorrelation among individuals within the different species, but the average correlation coefficient was positive within each species (Appendix C). The lowest overall association between compounds was found in *E. deceptrix* ($R = 0.09$), where most components seemed to vary almost independently of each other, the highest in *E. dissimula* ($R = 0.71$), where individual profiles were most coherent. Due to these large differences in background intercorrelation,
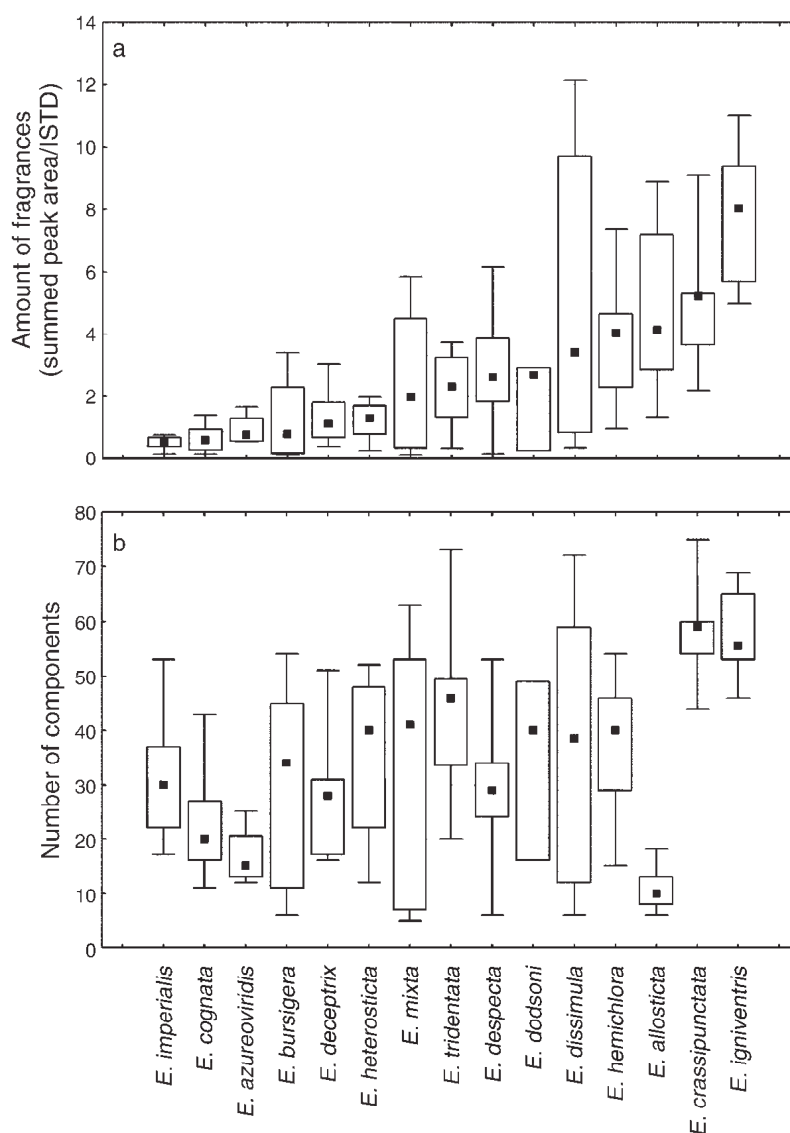
Fig. 1.   (a) Median quantity of fragrances and (b) median number of different fragrance compounds detected in individual hind-leg extracts of males of 15 species of *Euglossa* from Barro Colorado Island (BCI), central Panama. Fragrance quantity is plotted as the ratio of the summed peak areas (integrated ion currents) of all fragrance compounds to the peak area of the internal standard (ISTD). Small black squares represent the median, the boxes extend to the 25% and 75% quartiles, and the whiskers show the minimum and maximum values.

we further analyzed only correlations that were outstanding for the respective species (>95th percentile of the distribution of correlation coefficients). In this way we inferred 22 fragrance motifs (i.e., associated groups of compounds that are likely derived together from specific sources). A list of these motifs and their distribution across species is given in Appendix C. Most motifs consisted of only a few (two to four) compounds, with one of them being quantitatively dominant, and frequently of structurally related compounds (e.g., a relatively widespread association between the (*E*) and (*Z*) isomers of methyl-4-methoxy cinnamate; motif 1). Four correlated stereoisomers of 2-hydroxy-6-nonadienyl benzaldehyde (motif 13) occurred in *E. mixta*. This

motif was also found in males of *E. viridissima* in southern Mexico (not part of the present study) where it attracts *E. viridissima* males in bioassays (Eltz et al. 2008). Most widespread among species was motif 2, consisting of 97% hexahydrofarnesyl acetone (hha) and minor amounts of two structurally related compounds (the respective alcohol and, presumably, an unsaturated derivative of hexahydrofarnesyl acetone). This simple motif seemed to have been collected as such (i.e., without any other associated compounds) by at least eight species. This is confirmed by an individual of *E. despecta* that contained only motif 2 but no other fragrance compound. Most other motifs were not traceable between species.
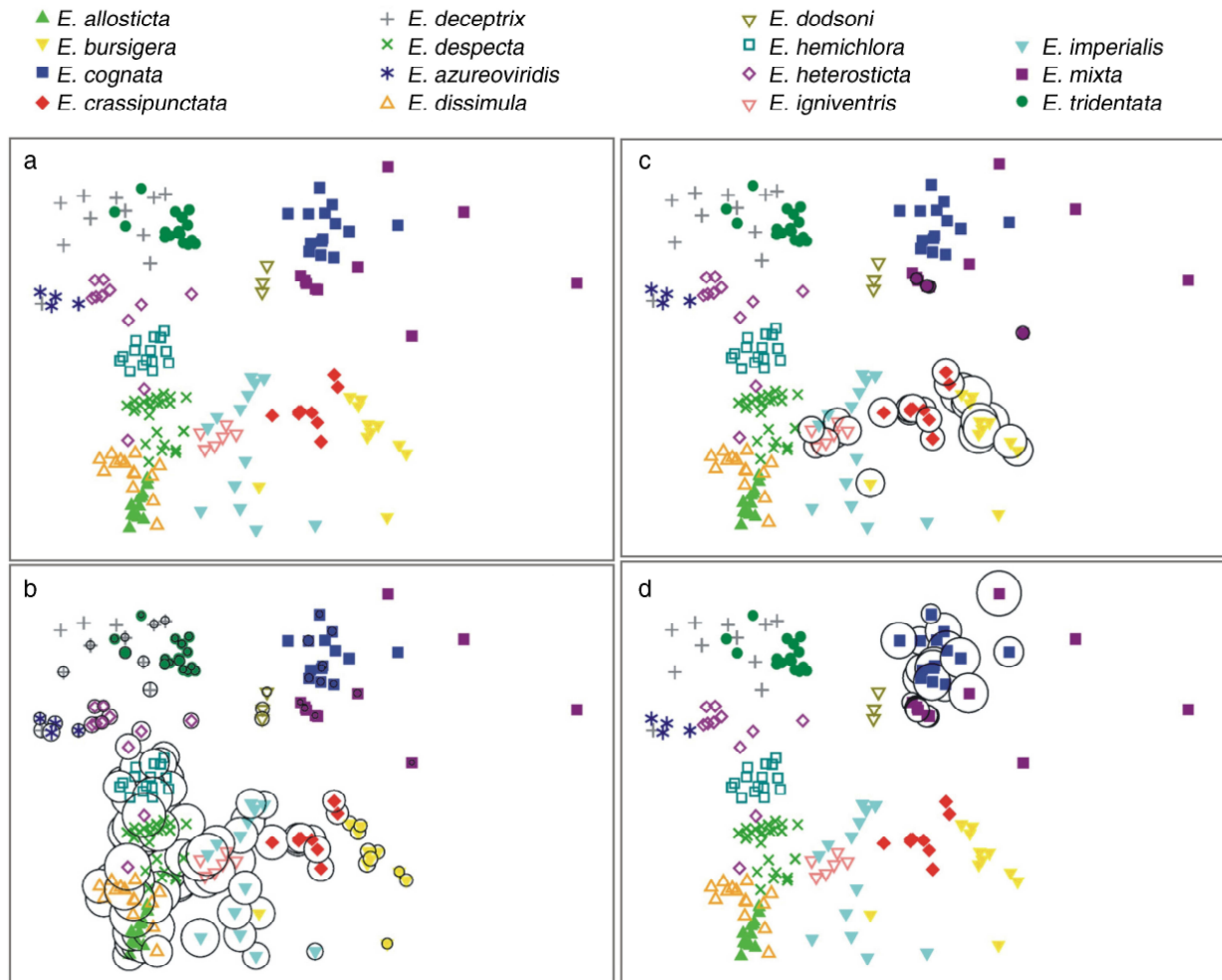
Fig. 2. Two-dimensional representation of the similarity/dissimilarity of fragrance mixtures of 176 individual male *Euglossa* belonging to 15 sympatric species from Barro Colorado Island (BCI), central Panama, and examples of how major compounds contributed to the observed pattern. (a) Multidimensional scaling (MDS) plot based on the Bray-Curtis index (stress = 0.22). Axes are simple distance dimensions without units. The proximity/distance of points represents the chemical similarity/dissimilarity of individual fragrances. Bubble overlays in panels (b)–(d) represent the relative contributions of (b) hexahydrofarnesyl acetone, (c) germacrene D-4-ol, and (d) an unknown sesquiterpene ketone, molecular weight 218, to individual blends. Bubble size reflects relative amounts between 0.1% and 99% (square-root scale).

Fragrance composition and phylogeny

Using the Bayesian DNA-based phylogeny, we estimated character statistics on the chemical traits alone. Of the total 514 chemical characters (fragrance compounds), 219 were variable but parsimony-uninformative, 293 were parsimony-informative, and only two were constant. Based on the tree topology obtained via Bayesian analysis with DNA data, the chemical data had a consistency index (CI) of 0.475, a retention index (RI) of 0.172, and a homoplasy index (HI) of 0.525. Most major fragrance components were present in two or more species, but the co-occurrences were often not congruent with phylogeny (Fig. 4, see also Appendix A: Fig. A2). The only convincing case of a synapomorphy is the unknown sesquiterpene ketone, molecular weight 218, which was present (and a major compound) only in the closely related *E. cognata* and *E.*

mixta. In contrast, many other major compounds were clearly homoplasious, including gemacrene-D-4-ol, which is characteristic of the distantly related *E. bursigera*, *E. crassipunctata*, and *E. igniventris*. Also, the few species that did not possess hexahydrofarnesyl acetone as a major compound were not all close relatives.

Disparity-through-time plots show that chemical niche use in the genus *Euglossa* exhibits high values of average subclade disparity. Overall, the observed chemical disparity was greater than expected under a null model (Brownian motion) throughout the phylogenetic span of the taxa included (Fig. 5). We also observed that the average subclade chemical disparity peaks near the recent (Fig. 5), suggesting that much of the chemical disparity is concentrated among closely related taxa.

DISCUSSION

Chemical niche differentiation

The present analysis corroborates the notion of nonrandom, species-specific accumulation of volatiles in male orchid bees. It confirms previous analyses that had been restricted to a few selected species (Eltz et al. 2005a, Zimmermann et al. 2006) and allows us to extrapolate how orchid bee communities partition their chemical environment. Of 15 species of sympatric *Euglossa* on Barro Colorado Island, all were sufficiently different in volatile composition to allow individuals to be assigned to their own species. The opportunities for between-species differentiation in "fragrance space" seemed unlimited; e.g., successive inclusion of species into "assemblages" only marginally increased the average chemical overlap between taxa. Instead, additional fragrance compounds were introduced along with additional species, and created additional niche space.

Between-species chemical differentiation is consistent with the hypothesis that fragrances are used for mate recognition. Previous studies have shown that male orchid bees expose and ventilate tibial fragrances during lengthy series of courtship display (Bembé 2004, Eltz et al. 2005b). The exposure of fragrances involves a range of cuticular structures shared by all male Euglossini, suggesting that active fragrance signaling is a basal trait of orchid bees (Eltz et al. 2005b). Although the attraction of conspecific females to male fragrances has yet to be demonstrated in bioassays, the high specificity of chemical signals is consistent with a role in mate recognition and premating isolation. Further support for this view comes from the pronounced chemical disparity among the most closely related species, evidenced by the disparity-through-time analysis. This finding suggests that male fragrances diverge rapidly between sibling species, possibly in response to potentially costly hybrid matings. Males of different euglossine species have been observed to display syntopically in the forest understory. For instance, males of the closely related *Eulaema meriana* and *E. bombiformis* display at the same time on the same forested hilltops in Panama (Stern 1991, Zimmermann et al. 2006; see also Kimsey 1980). The males of the two species are almost indistinguishable with respect to size, general morphology, and coloration. However, they differ in the circumference of their perch trees as well as in the composition of tibial fragrances (Stern 1991, Zimmermann et al. 2006). *Euglossa flammea* and *E. imperialis*, both in the Glossura species group (Cameron 2004), have also been observed to display in close proximity in the forest understory of the Azuero peninsula in Panama (Roubik and Hanson 2004:114). However, for most of the species of *Euglossa* analyzed in the present study, male display has never been observed.

Visual cues may assist in mate recognition. Most orchid bees, especially members of *Euglossa*, sport conspicuous structural colors ranging from green over hues of red to deep blue. However, body coloration
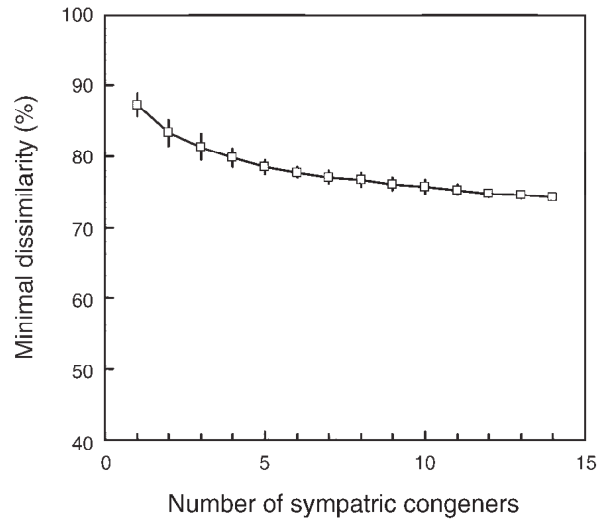

FIG. 3. Average observed minimum dissimilarity (measured as $1 -$ Bray-Curtis index, $\pm$SD) between the fragrance blend of a focal species and that of the most similar species in randomly assembled communities of Panamanian *Euglossa* (with increasing species richness). Note that additional congeners would not further reduce the average dissimilarity in the community.

often fails to distinguish closely related species, which may be difficult to separate for bee taxonomists (see Fig. 6).

The costs of heterospecific matings in sympatry may range from waste of gametes in species that are already isolated by postmating barriers to the production of infertile (or less fit) hybrid offspring in species that are not. Such costs would result in natural selection to favor males with more distinct blends of chemicals. If such differentiation follows initial differentiation in allopatry, it represents "reproductive character displacement" (between already isolated species) or "reinforcement" (with some gene flow) (Coyne and Orr 2004).

As an alternative to chemical interference, fragrance differentiation could result from interspecific competition for limited chemical resources. In this case, natural selection would favor chemical niche differentiation between species because it reduces competition for chemicals among males ("ecological character displacement"), not because it reduces the risk of hybrid matings (Coyne and Orr 2004). Fragrances are likely scarce in the natural habitat, and male bees must allocate considerable time and energy to the acquisition of large quantities (Eltz et al. 1999). In agreement with competition for fragrances, males of some species have been observed to aggressively defend fragrance sources (e.g., flowers; Janzen 1981, Gracie 1993). However, it seems unlikely that interspecific competition alone is sufficient to create the extent of chemical differentiation and specialization found by the present study.

Fragrance sources and foraging

Many of the fragrance compounds found in male extracts are known from published euglossine sources.
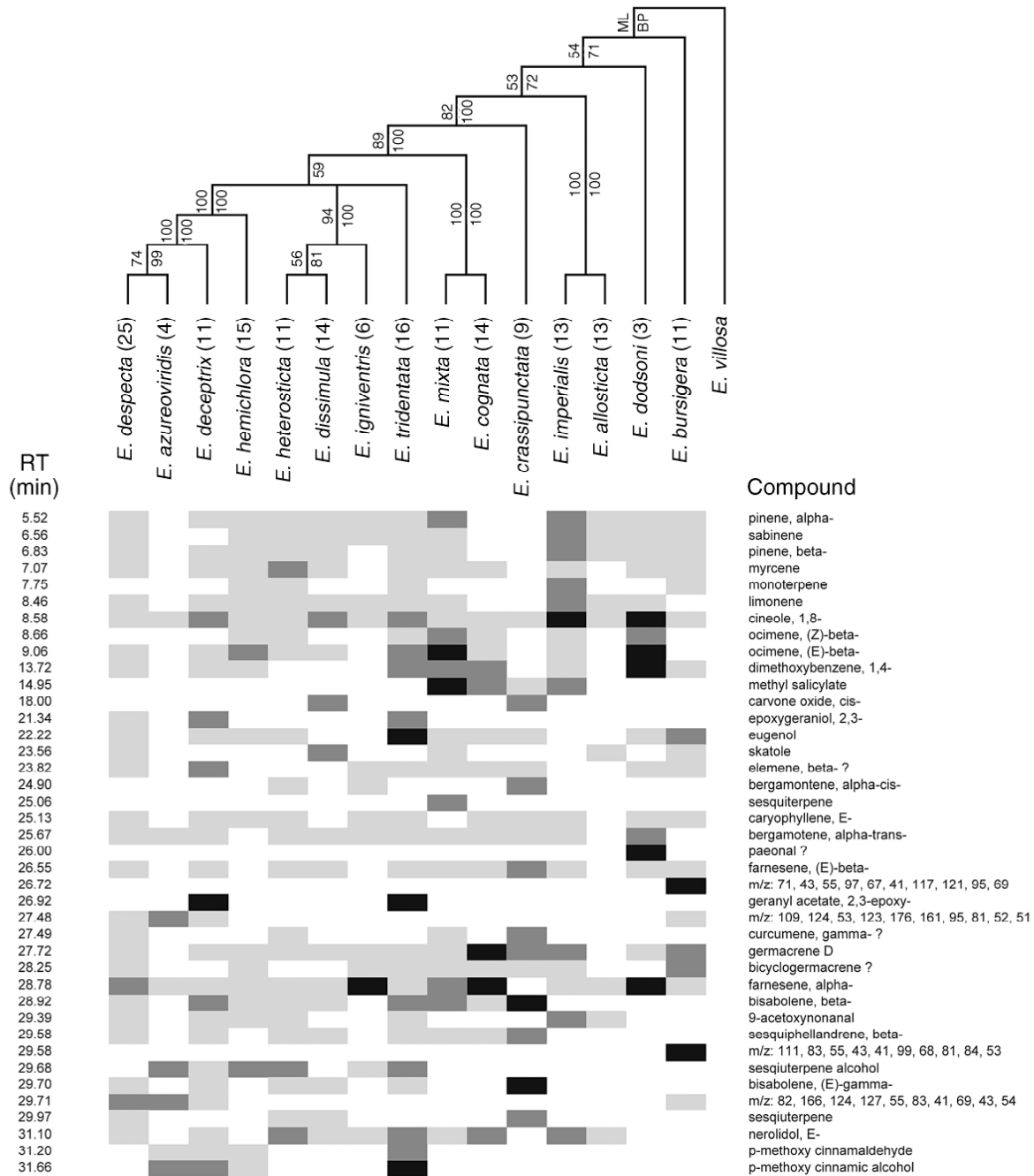
Fig. 4. Presence of fragrance compounds in hind-leg extracts of 15 sympatric species of *Euglossa* grouped by their phylogenetic relationships. The numbers of individual males analyzed per species are given in parentheses, and shades of gray indicate how much a compound contributed on average to the total peak area in a given species. Compounds are categorized as major (black, >5% of total peak area), minor (dark gray, 1–5%), and trace (light gray, <1%) components. The numerical data are given in Appendix B. Only the 80 most abundant compounds (across species) are shown, ranked from top to bottom by their retention time (RT) on a DB-5 nonpolar capillary column (30 m; 60°–300°C at 3°C/min). The 10 most abundant mass fragments (indicated by m/z) are given for unknown compounds. The cladogram corresponds to a 50% majority-rule consensus obtained from a Bayesian tree search; posterior probabilities are shown on the right side of the branches (along with bootstrap values of a maximum-likelihood analysis on the left side). See Appendix A: Fig. A2 for an undivided version of this figure.

The floral scents of euglossophilous flowers are normally chemical mixtures, although frequently of low complexity and characterized by pronounced dominance of one or two components (Williams and Whitten 1983, Gerlach and Schill 1991, Whitten and Williams 1992, Kaiser 1993). Single-component scents occur in decaying wood visited by male euglossines (Whitten et al. 1993, Eltz et al. 1999). The fragrance blends found in male
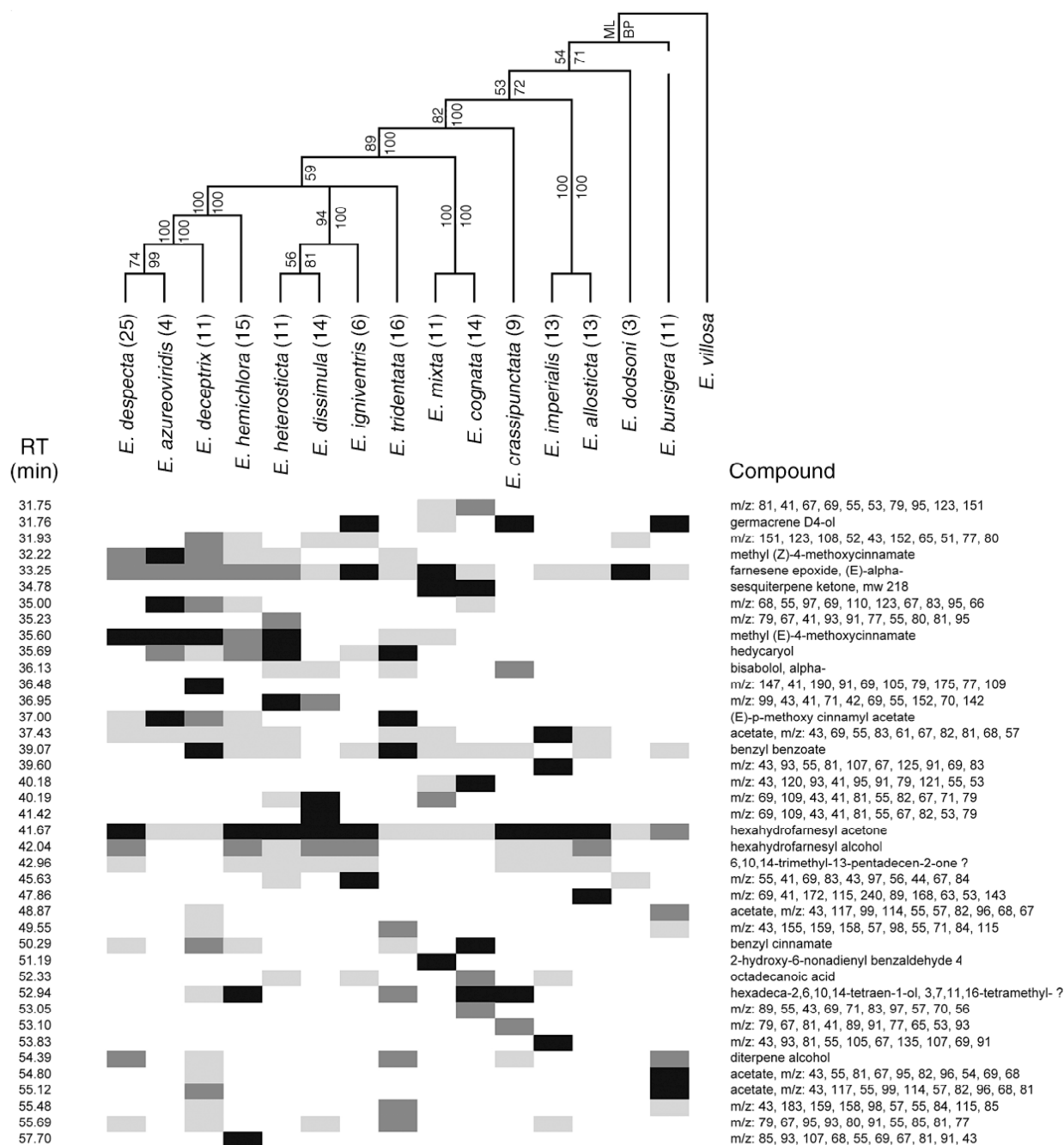
FIG. 4. Continued.

hind legs cannot be attributed to any known single source but are clearly assembled from subsets of compounds derived from different sources. However, the attempt to identify such subsets (i.e., motifs or building blocks) by an analysis of compound intercorrelation has had limited success. Nonetheless, a few simple motifs have been inferred. For instance, hexahydrofarnesyl acetone (hha) was found to be a major compound of eight species, representing 17% to 70% of total fragrances in their respective blends. Together with two structurally related trace compounds, hha represented a well-defined motif that appeared to be derived from the same source by all species. In recent bioassays, synthetic hha proved attractive to males of *E. imperialis* (T. Eltz, J. Andersson, J. Bång, and E. Hedenström, *unpublished data*). Males landed on and performed collecting behavior on the filter papers to which hha had been applied. Thus hha is a behaviorally active component in tibial fragrances of male *Euglossa*. Unfortunately, the source of hha remains elusive. Among the many floral scents and essential oils analyzed by R. Kaiser (*personal communication*), hha is relatively
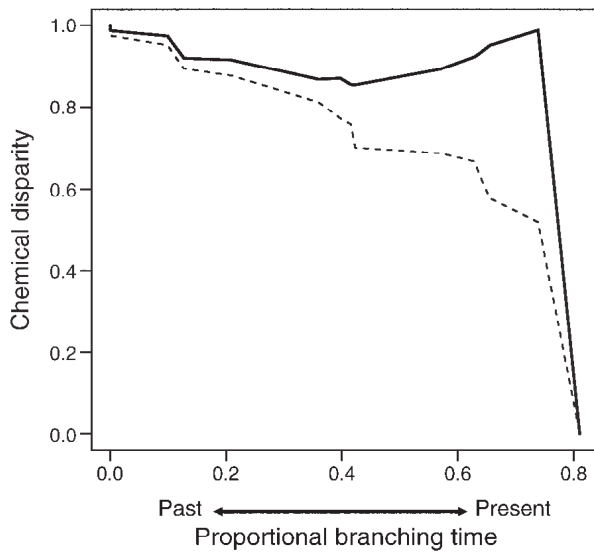
FIG. 5. Disparity-through-time (DTT) plot of chemical characters showing subclade dissimilarity in relation to relative branching times. The solid line corresponds to the observed empirical disparity, and the dashed line to the simulated null (1000 iterations; Brownian motion). Disparity was calculated via average Manhattan distance and is a unitless index.

widespread, but only as a minor or trace component. Notably, it occurs as a major compound in some euglossophilous orchids and an aroid (R. Kaiser and M. Whitten, *personal communication*). However, of these only one orchid species, *Kegeliella kupperi*, occurs in lowland central Panama, but due to its low population density is unlikely to be the source. Rather, the outstanding abundance of hha in male fragrances suggests that the hha source is highly available (i.e., not an orchid).
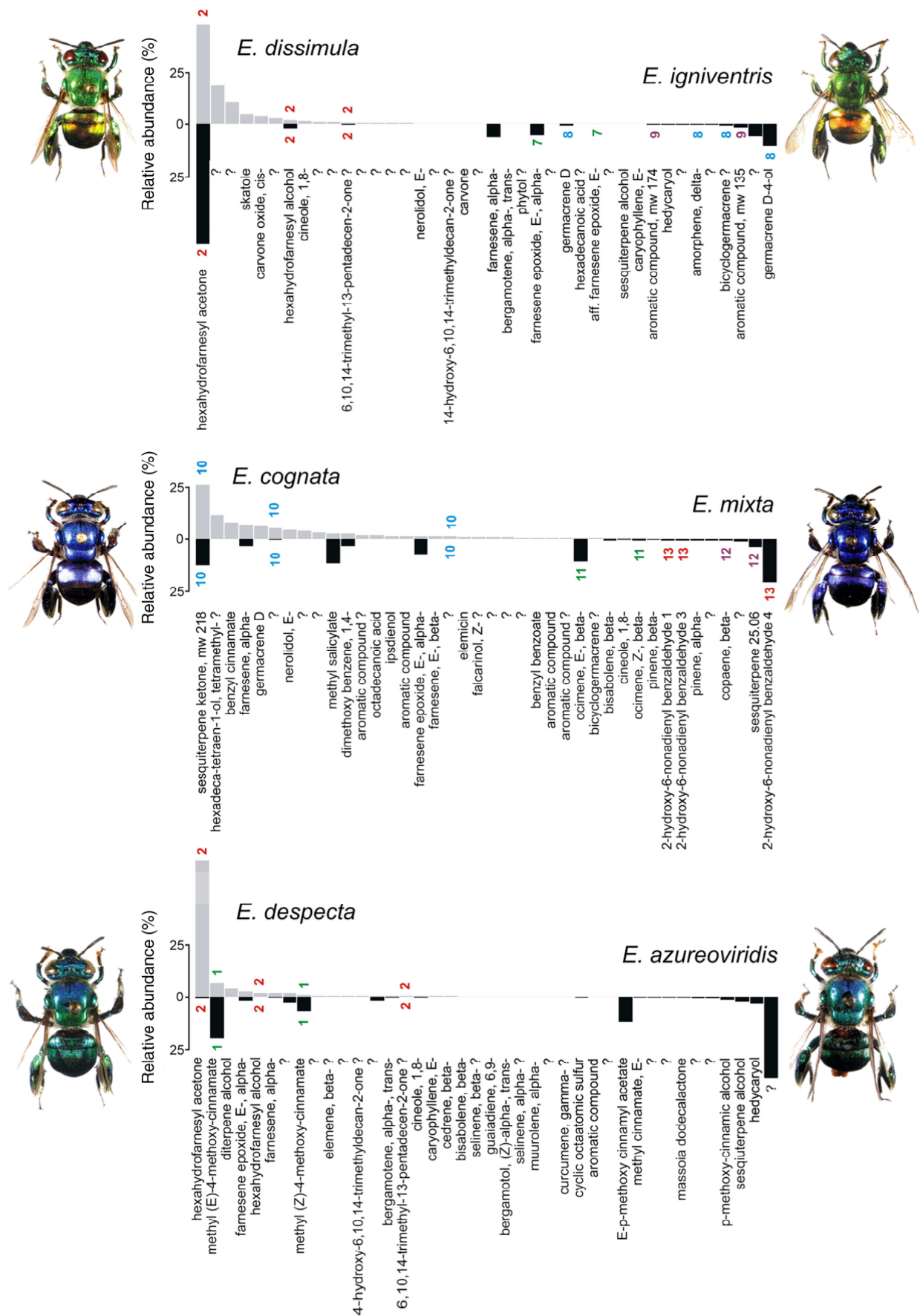
Other inferred fragrance motifs were also simple; i.e., they consisted of one highly dominant compound that appeared to be collected along with a few minor or trace compounds. While the predominance of simple motifs in our analysis may partly be an artifact (more complex motifs are increasingly likely to be obscured in complex blends), it is in general agreement with the prevalence of simple blends produced by most natural fragrance sources of orchid bees.

To accumulate complex fragrance blends, individual male bees must visit a range of different source types, presumably covering extensive areas of forest during their search. To attain specific blends, male bees appear to use innate fragrance preferences in combination with odor learning and experience-dependent choices (Ackerman 1989, Eltz et al. 2005a). Differences in innate

preferences between species are evident from long-term baiting studies that used a range of synthetic chemicals to lure males. For instance, Ackerman (1989) used 16 different chemical attractants (single compounds) during a yearlong baiting program in Panama. Although there was considerable overlap in compound choices between species, each was attracted to a unique set of chemicals. The same author also found variation in choices of most species between geographic areas and in different seasons. In light of evidence from cage experiments (Eltz et al. 2005a), such differences were most likely due to differential availability of natural fragrances at the different localities and times. When caged males of *Euglossa imperialis* had repeated access to a fragrance compound (e.g., 1,8-cineole) in one experimental treatment, they satiated within a few days and finally stopped collecting this compound altogether. When a different compound (e.g., methyl salicylate) was given later, the males resumed their collecting activity. Thus it appears that male orchid bees learn the odor of visited sources and use a negative feedback mechanism to avoid overcollecting from abundant sources. Such modification of innate chemical preferences by experience would increase the species specificity of accumulated blends by compensating for fluctuating availability of fragrance source types (Eltz et al. 2005a).

There is controversy about the size of the foraging areas covered by male euglossines when searching for chemicals. Artificial chemical sources (baits) have been used to lure males over distances of one to several kilometers, even across open water or stretches of nonforest habitat (Ackerman 1981, Raw 1989, Tonhasca et al. 2003). While artificial baits emit volatiles in unnaturally high concentrations, observations at natural sources (orchids) support the notion of long-distance attraction (Janzen 1981). It has been discussed whether orchid bees are in fact vagabonds leading a nomadic life driven by their need for chemicals (Dodson et al. 1969). However, this view has been challenged by the observation that marked individuals revisit patches of nectar-offering food plants on consecutive days (Ackerman et al. 1982), and by perch site fidelity of males during their courtship behavior (Stern 1991). Furthermore, males appear to use spatial memory (not the scent plume) to revisit fragrance sources from which they have already collected on previous occasions. This is suggested by the observation that male bees continued to approach plants of the scent-offering *Dalechampia spathulata*, although these had already dropped their fragrant flowers (Armbruster and Webster 1979). Returning to the same source site by memory would be

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FIG. 6. Pairwise comparison of fragrance composition of closely related species of *Euglossa*. The 40 most abundant compounds of each pair of species are shown, and bars represent untransformed relative abundances (average percentage contribution to total peak area). Gray bars above the axis represent the bee species on the left side; black bars below the axis represent the species on the right side. Colored numerals refer to putative fragrance motifs (see Appendix C); i.e., the tagged compounds are likely co-derived from the same source, which are partly shared between species.

E. dissimula

E. igniventris

Relative abundance (%)

hexahydrofarnesyl acetone
?
skatole
carvone oxide, cis-
?
hexahydrofarnesyl alcohol
cineole, 1,8-
?
6,10,14-trimethyl-13-pentadecen-2-one ?
?
?
?
nerolidol, E-
?
14-hydroxy-6,10,14-trimethyldecan-2-one ?
carvone
farnesene, alpha-
bergamotene, alpha-, trans-
phytol ?
farnesene epoxide, E-, alpha-
?
germacrene D
hexadecanoic acid ?
aff. farnesene epoxide, E-
sesquiterpene alcohol
caryophyllene, E-
aromatic compound, mw 174
hedycaryol
amorphene, delta-
bicyclogermacrene ?
aromatic compound, mw 135
?
germacrene D-4-ol

E. cognata

E. mixta

Relative abundance (%)

sesquiterpene ketone, mw 218
hexadeca-tetraen-1-ol, tetramethyl-?
benzyl cinnamate
farnesene, alpha-
germacrene D
?
nerolidol, E-
?
?
methyl salicylate
dimethoxy benzene, 1,4-
aromatic compound ?
octadecanoic acid
ipsdienol
aromatic compound
farnesene epoxide, E-, alpha-
farnesene, E-, beta-
?
elemicin
falcarinol, Z- ?
?
?
benzyl benzoate
aromatic compound
aromatic compound ?
ocimene, E-, beta-
bicyclogermacrene ?
bisabolene, beta-
cineole, 1,8-
ocimene, Z-, beta-
pinene, beta-
2-hydroxy-6-nonadienyl benzaldehyde 1
2-hydroxy-6-nonadienyl benzaldehyde 3
pinene, alpha-
?
copaene, beta-
?
sesquiterpene 25.06
2-hydroxy-6-nonadienyl benzaldehyde 4

E. despecta

E. azureoviridis

Relative abundance (%)

hexahydrofarnesyl acetone
methyl (E)-4-methoxy-cinnamate
diterpene alcohol
farnesene epoxide, E-, alpha-
hexahydrofarnesyl alcohol
farnesene, alpha-
methyl (Z)-4-methoxy-cinnamate
?
elemene, beta-
?
4-hydroxy-6,10,14-trimethyldecan-2-one ?
bergamotene, alpha-, trans-
6,10,14-trimethyl-13-pentadecen-2-one ?
cineole, 1,8-
caryophyllene, E-
cedrene, beta-
bisabolene, beta-
selinene, beta- ?
guaiadiene, 6,9-
bergamotol, (Z)-alpha-, trans-
selinene, alpha- ?
muurolene, alpha-
?
curcumene, gamma- ?
cyclic octaatomic sulfur
aromatic compound
E-p-methoxy cinnamyl acetate
methyl cinnamate, E-
?
massoia dodecalactone
?
?
p-methoxy-cinnamic alcohol
sesquiterpene alcohol
hedycaryol
?

expected if only small amounts of chemicals can be collected at any one time, or if intermittent collection from other sources renews interest in previously visited ones (see Eltz et al. 2005a). The use of spatial learning and memory during foraging opposes the idea that males are transient vagabonds. Instead, it suggests that males have foraging ranges, albeit large ones, and that communities of orchid bees are spatially structured. Such structuring is also suggested by detectable differences in the species composition of chemical bait samples taken at different sites within a given habitat (Armbruster 1993; but see Tonhasca et al. 2002).

Saltational mode of fragrance evolution?

There are currently two different views concerning the dynamics of chemical evolution in chemical mate recognition systems (Symonds and Elgar 2008). The traditional view emphasizes slow and gradual changes in sex pheromone blends due to the need for optimal recognition. Recently, however, it has been shown in moths that major saltational changes in signal chemistry can occur due to alterations in pheromone biosynthetic pathways, and that such shifts may initiate species divergence (Baker 2002, Roelofs et al. 2002). Studies in moths and bark beetles have revealed substantial differences in pheromones between closely related taxa, which appear to support a saltational mode of signal evolution (Löfstedt et al. 1991, Symonds and Elgar 2004). In orchid bees, we have also found substantial differentiation among closely related taxa. Furthermore, we have found that many major compounds or motifs co-occur in several, often unrelated species. The pattern of compound distribution shown in Fig. 4 (see also Appendix A: Fig. A2) indicates recurrent loss and gain of components in blends over time, consistent with saltational shifts in fragrance collection. Such shifts may be based on changes in behavioral preferences or on alterations in peripheral olfaction. Genes encoding odorant receptors (ORs) or odorant binding proteins (OBPs) are members of large multigene families. Genomic studies in *Drosophila* and *Apis* suggest they evolve in a birth-and-death mode, undergoing frequent changes in copy number due to duplication and pseudogenization (Robertson and Wanner 2006, Vieira et al. 2007). Loss and reactivation of odorant receptors may have induced shifts in fragrance collection in male orchid bees and caused the recurrent (homoplasious) pattern of signal evolution. A recent study on Mexican *Euglossa* has underlined the importance of peripheral olfaction for fragrance choice in males. In a pair of closely related sibling species, the male perfumes differ only in a set of four structurally very similar compounds, which are collected in large quantities by the males of only one lineage. The presence of these compounds in males of that lineage is associated with outstanding sensitivity of male antennae to that compound (Eltz et al. 2008). Future studies have to show whether there is a general congruence between male fragrances and antennal response profiles across the euglossine phylogenetic tree.

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APPENDIX A

Perfume differentiation in orchid bees. Fig. A1 shows a rotating, three-dimensional fragrance space of *Euglossa* spp. on Barro Colorado Island, Panama, in which the multidimensional scaling plot is based on the same data and analysis as the two-dimensional representation in Fig. 2a, but allows one additional dimension and thus accommodates more of the variation in the underlying similarity matrix (stress = 0.15); Fig. A2 is a full version of Fig. 4 (*Ecological Archives* E090-215-A1).

APPENDIX B

Average relative abundance of fragrance compounds in hind-leg extracts of 15 sympatric species of *Euglossa* from Barro Colorado Island, Panama (*Ecological Archives* E090-215-A2).

APPENDIX C

A list of 22 putative fragrance motifs inferred from intercorrelation analysis of male fragrance compounds and their occurrence in different species of *Euglossa* (*Ecological Archives* E090-215-A3).

Appendices are available online:

http://www.esapubs.org/archive/ecol/E090/215/

and on CD, attached to this dissertation

An olfactory shift is associated with male perfume differentiation and species divergence in orchid bees

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Report

An Olfactory Shift Is Associated with Male Perfume Differentiation and Species Divergence in Orchid Bees

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Summary

Saltational changes may underlie the diversification of pheromone communication systems in insects, which are normally under stabilizing selection favoring high specificity in signals and signal perception [1–4]. In orchid bees (Euglossini), the production of male signals depends on the sense of smell: males collect complex blends of volatiles (perfumes) from their environment [5, 6], which are later emitted as pheromone analogs at mating sites [7]. We analyzed the behavioral and antennal response to perfume components in two male morphotypes of *Euglossa* cf. *viridissima* from Mexico, which differ in the number of mandibular teeth. Tridentate males collected 2-hydroxy-6-nona-1,3-dienyl-benzaldehyde (HNDB) as the dominant component of their perfume. In bidentate males, blends were broadly similar but lacked HNDB. Population genetic analysis revealed that tri- and bidentate males belong to two reproductively isolated lineages. Electroantennogram tests (EAG and GC-EAD) showed substantially lower antennal responses to HNDB in bidentate versus tridentate males, revealing for the first time a mechanism by which closely related species acquire different chemical compounds from their habitat. The component-specific differences in perfume perception and collection in males of two sibling species are in agreement with a saltational, olfaction-driven mode of signal perfume evolution. However, the response of females to the diverged signals remains unknown.

Results and Discussion

Orchid bees (Euglossini, Apidae) are solitary to primitively social bees of the Neotropics. The males of all five genera and 200-plus species collect volatile substances from flowers and nonfloral sources; they do not ingest these substances but accumulate them outside their body in hind leg (tibial) pockets [8]. This behavior has given rise to the orchid bee pollination syndrome, where about 700 species of orchids are exclusively pollinated by scent-seeking male orchid bees [9]. Male bees are selective in what they gather, and different species have different preferences for natural and synthetic volatiles. In older males, the accumulated perfume is broadly species specific, with respect to both qualitative composition and relative amounts of compounds [10]. These blends are emitted by the males specifically and exclusively at display sites, which are established in the forest understory for the single purpose of mating [7, 11–14]. Conspecific females have been observed to approach displaying males from downwind, and copulations take place on the male perch [12, 13, 15]. Although female attraction to perfumes has not been demonstrated in bioassays, the perfumes have a likely role in mate recognition.

Communication with exogenous perfumes implies that mutations affecting the olfactory system have the potential to alter male signals, e.g., by increasing or decreasing the males' likelihood of acquiring a certain perfume component. Here we report on component-specific differences in perfume accumulation and antennal perception in two closely related lineages of *Euglossa*.

Morphological and Perfume Variation of *Euglossa viridissima*-like Males in Southern Mexico

E. viridissima Friese [16] is a medium-sized (~12 mm), metallic green orchid bee distributed from Mexico to Costa Rica. Males are characterized by two large tufts of hair on the second sternite and the shape of midtibial hair tufts [17]. Dressler (1978) noted that males are variable with respect to the number (3 or 2) of mandibular teeth [18], with 3 being the typical number. Our investigations broadly confirmed this dichotomy across the Yucatan Peninsula, where the majority of males were tridentate (3D). A variable proportion of males (3% to 70% at different localities) had only two mandibular teeth (2D, Figure 1A). Tests with commercial synthetic attractants carried out at different localities showed that both male morphotypes responded positively to the same compounds; both 3D and 2D males were most strongly attracted to p-dimethoxybenzene, followed by methyl cinnamate and eugenol (Figure 2C).

Notably, there was a conspicuous difference in the composition of volatiles stored in hind leg pockets between 2D and 3D males (Figure 1B). Coupled gas chromatography-mass spectrometry (GC-MS) analyses of hind leg extracts revealed four compounds that were highly characteristic for 3D males but absent in 2D males (HNDB 1 to 4 in Supplemental Data 2 available online). The four compounds furnished almost identical mass spectra, indicating stereoisomers of the same basic structure (see Supplemental Data 1). Column chromatography of a pooled hexane extract of 20 pairs of hind legs yielded HNDB in amounts sufficient for structure elucidation by nuclear magnetic resonance (NMR) spectroscopy, which revealed the target compound to be 2-hydroxy-6-[(1E, 3E)-nona-1,3-dienyl]benzaldehyde (HNDB 4, Supplemental Data 1). The other mentioned 3D-specific compounds are the three stereoisomers of HNDB 4 (HNDB 1 to 3; see Supplemental Data 1 and 2).

HNDB represented on average 67% of the total amount (sum of integrated ion current) of perfume in 3D males, and differences in the amount of HNDB were primarily responsible for

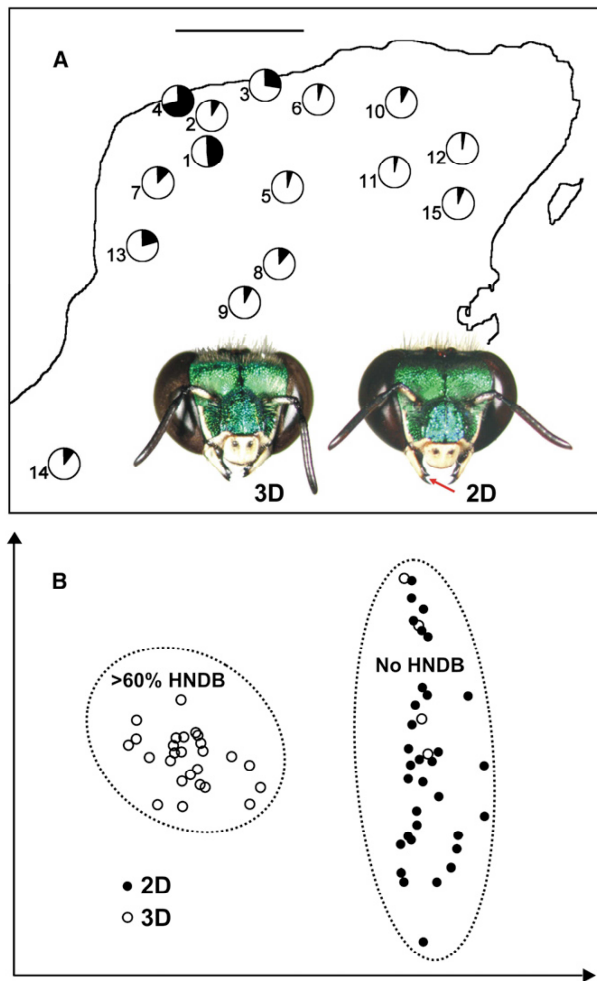*Correspondence: eltz@uni-duesseldorf.de

Figure 1. Morphological and Chemical Variability of *Euglossa viridissima*-like Males on the Yucatán Peninsula

(A) Relative abundance of bidentate (2D) and tridentate (3D) males across the Yucatán peninsula. Black segments represent bidentate males. Localities are (1) Xmatkuil, (2) Chablekal, (3) San Crisanto, (4) Chelem, (5) Sotuta, (6) Yalsihón, (7) Maxcanu, (8) Hobonil, (9) Tigre Grande, (10) Tizimin, (11) Valladolid, (12) Nueva Xcan, (13) Hecelchakan, (14) Escarcega, and (15) Coba. A total of 3491 males were morphotyped (at least 73 per site). The scale bar represents 100 km for the map and 4 mm for the bees.
(B) Differences in tibial perfumes between 2D and 3D males as revealed by a multidimensional scaling (MDS) analysis of perfume composition between 63 individual males. Only 3D males contained HNDB. See text for details on the four exceptional tridentate males.

Figure 2. Male Attraction during Bioassays

(A) Attraction of 2D and 3D males to synthetic and natural (isolated) HNDB, contrasted with attraction to p-dimethoxybenzene and solvent controls. All stimuli were presented on the same days at Xmatkuil, where there is a high relative abundance of 2D males (~50% of males).
(B) Attraction of 2D and 3D males to complete hind leg extracts of individual males (2D and 3D) at Xmatkuil.
(C) Attraction of 2D and 3D males to commercial synthetic compounds, p-dimethoxybenzene, methyl cinnamate, and eugenol, pooled across 12 Yucatecan localities (subset of those in Figure 1A, see Experimental Procedures).

the separation of individuals along the horizontal axis in a two-dimensional perfume space (Figure 1B; Spearman Rs = 0.883; p < 0.0001). Most of the other perfume components, representing monoterpenes, sesquiterpenes, aromatics, and straight-chain aliphatic acetogenins, were present in variable amounts in both types of males.

The source of HNDB is unknown. Interestingly, we have also found the same four HNDB isomers in perfumes of *Euglossa mixta* from central Panama, suggesting that the source is widespread in Central America (Y.Z. and T.E., unpublished data). Structurally similar compounds occur in phytopathological fungi [19, 20], and fungus-infected substrate (e.g., decaying wood) is a likely candidate for a HNDB source. It should be emphasized that male orchid bees are much less dependent on orchid flowers than is widely believed, and nonfloral substrate such as fungi may have been their original source of perfume [21, 22].

Population Differentiation
We used three microsatellite markers to test for population genetic differentiation among bait-captured males: 2D and

Table 1. Differences in Microsatellite Allele Frequency between 2D and 3D Males at Three Localities in Northern Yucatán, Mexico

Locality (Locus)	p	SE	n (3D)	n (2D)
Ixmatkuil				
ann02	<0.001	0	31	36
ann08	0.078	0.005	36	36
Egc17	0.001	0	36	36
Chablekal				
ann02	<0.001	0	30	18
ann08	0.004	0.001	31	18
Egc17	0.003	0.001	31	18
San Crisanto				
ann02	<0.001	0	32	36
ann08	0.191	0.007	36	36
Egc17	0.014	0.003	36	36

Differences as revealed by exact probability tests [37]. The abbreviations refer to three microsatellite loci ([35], R. Paxton, personal communication).

3D males showed significant differences in allele frequencies at most loci at the three localities (Table 1). This suggests that 2D and 3D males represent two lineages that are reproductively isolated on the Yucatán peninsula, i.e., cryptic sibling species. Notably, females of both lineages established nests in wooden boxes (trap nests) placed around buildings in Xmatkuil. All foundresses had three mandibular teeth, and no differences in morphology were apparent between females that produced 2D sons (n = 13 females) and those that produced 3D sons (n = 3).

The microsatellite analysis indicated that the number of mandibular teeth is not completely fixed in males of the 2D genetic lineage. The four exceptional males in Figure 1B, which had 3 teeth but lacked HNDB, had typical 2D microsatellite haplotypes. Thus, there appear to be rare males of the 2D lineage, which express an additional mandibular tooth but retain 2D-typical perfume preferences.

Behavioral and Antennal Response to HNDB
Synthetic HNDB and HNDB isolated from hind leg extracts of 3D males attracted exclusively 3D males during field bioassays, whereas males of both lineages were attracted in equal numbers to p-dimethoxybenzene at the same time (Figure 2A; significant difference in 2D and 3D relative frequencies between synthetic HNDB and p-dimethoxybenzene, Fisher's exact test, n = 143, p < 0.0001). This demonstrates that HNDB is a behaviorally important compound that is actively approached and collected by 3D males only. 2D and 3D males also showed a strong difference in attraction to crude pentane extracts of hind legs of other males that either contained HNDB (3D) or not (2D) (Figure 2B; Fisher's exact test, n = 49, p < 0.0001). Presently, HNDB is the only compound we know of that is exclusively attractive to males of one lineage (the 3D males).

An electroantennogram (EAG) is believed to record the sum of potentials from olfactory receptor neurons (ORNs) located all across the antenna, thus reflecting the overall sensitivity of the antenna to tested compounds [23, 24]. In EAG tests, responses of 2D males to synthetic HNDB were significantly smaller than those of 3D males and did not differ from the solvent control. Responses to other compounds varied but were not different between 2D and 3D males (Figure 3A). Differences in the response to HNDB are in agreement with results obtained with gas chromatography coupled to electroantennography
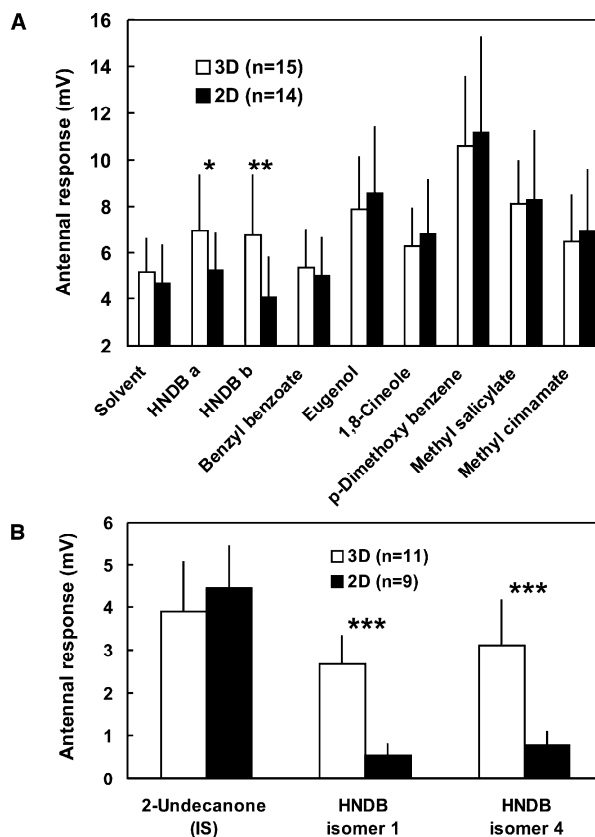

Figure 3. Antennal Response of 2D and 3D Males to HNDB and Reference Stimuli Measured as the Amplitude of Negative Baseline Deflections—Mean and Standard Deviation—during EAG and GC-EAG Tests

(A) EAG: Only HNDB (both stimuli) elicited significantly different responses from antennae of 2D and 3D males (t test: *, p < 0.05; **, p < 0.01).

(B) GC-EAG: GC peaks of HNDB isomers 1 and 4 elicited strong responses in antennae of 3D males. 2D male antennae showed much weaker (t test: *** p < 0.0001) and often marginal responses to the same isomers, but did not differ from 3D male antennae in response to the reference compound, 2-undecanone.

(GC-EAG): antennae of 3D males showed strong baseline deflections in response to eluting peaks of the HNDB isomers 1 and 4, whereas antennal responses of 2D males were marginal (Figure 3B, Supplemental Data 2).

Peripheral Olfaction and Speciation in Orchid Bees
This study has revealed compound-specific differences in antennal perception between males of two closely related lineages of orchid bees. Notably, the observed sensory differences were restricted to the only compound (HNDB) that mediated lineage-specific attraction in bioassays and distinguished male perfume blends in their natural habitat. The component-specific differences in antennal perception reveal for the first time a mechanism by which closely related species acquire different chemical compounds from their habitat. It is tempting to argue that an olfactory shift with respect to HNDB has initiated divergence of 2D and 3D lineages. However, selection or drift after an initial divergence may also have added to the observed olfactory and chemical differentiation.

Lineage-specific differences in antennal sensitivity to HNDB could be based on changes in the binding affinity of a specific

olfactory receptor (OR), or on differential expression of HNDB-receptive ORs or olfactory binding proteins (OBPs) in olfactory receptor neurons. Genomic studies in honeybees and *Drosophila* suggest that families of OR and OBP genes evolve by a birth-and-death mode, with frequent changes in copy numbers as a result of gene duplication, deletion, or pseudogenization [25–27]. Both an increase and a decrease in copy numbers of HNDB-sensitive ORs or OBPs could have mediated differential HNDB collection in 3D and 2D males.

Shifts in signal chemistry as a result of olfactory mutations may also occur in other insects in which pheromone production depends on the sense of smell. For example, male *Bactrocera* fruit flies sequester volatiles from plant sources and release them as courtship signals [28]. In both *Bactrocera* and orchid bees, a shift in male odor acquisition might spread quickly within a population if it is promoted by natural selection, e.g., through reduced search time for males or a reduced risk of falling prey to source-specific predators. Mostly, however, the evolutionary success of an olfactory mutation in males will depend on how females respond to the altered chemical signal. Unfortunately, all previous attemtps to test the behavioral response of females to male perfumes were unsuccessful (see [12, 15]), and no attempt was made here. The lack of data on the female response remains an obstacle for drawing conclusions concerning the mode of signal evolution in orchid bees. However, because male and female perfume preference are probably influenced by overlapping sets of olfactory genes, pleiotropic effects could enhance coevolution between senders and receivers (see [29]). In orchid bees, olfactory pleiotropy in males and females could result in assortative matings within genetic lineages, driving population differentiation by means of divergent sexual selection.

Experimental Procedures

Baiting and Sampling

For assessment of local proportions of 2D and 3D males, baiting with commercially available attractants was done once or twice at 15 localities across the Yucatan peninsula (Figure 1) from October 2006 to April 2008. p-Dimethoxybenzene, eugenol, and methyl cinnamate were used as lures in mesh-covered dispensers that do not allow bees to directly access the bait chemical. The used chemicals are known attractants for *Euglossa viridissima*. In total, 3491 males were captured with hand nets, morphotyped with a hand lens, and released after baiting was finished for the day. In October 2007, males from the localities (1) Xmatkuil, (2) Chablekal, and (3) San Crisanto were collected and processed for perfume analysis (n = 63) and population genetics (n = 193) (see below). For the analysis of chemical preferences (Figure 3C), we used only data from the 12 localities where we had baited twice (excluding localities 2, 14, and 15).

Chemical Analysis of Male Perfumes

Male perfumes were extracted from individual pairs of hind legs in 0.5 ml of hexane containing an internal standard (2-undecanone). GC-MS was carried out with a HP 5890 II GC fitted with a DB-5 column (30 m × 0.25 mm Ø × 0.25 μm film thickness) and a HP 5972 MSD. Injection was splitless, and the oven was programmed from 60°C to 300°C at 10°C/min. A second set of GC-MS analyses was performed with a gas chromatograph series 8000 linked to a Fisons MD800 quadrupole mass spectrometer (Fisons Instruments, Ismaning, Germany) operated at 70 eV. With helium used as the carrier gas, separations were performed with a CP8912 VF-1ms fused silica column (30 m × 0.32 mm Ø × 0.25 μm film thickness) and carried out as follows: after splitless injection at 60°C for 0.5 min, the temperature was kept at 60°C for 5 min and then programmed to 300°C.

Structure assignment of natural compounds was carried out by comparison of analytical data with those of authentic reference samples or by matching spectra and retention indices with those given in the literature [30, 31]. Excluded from the analysis were straight-chain lipids (alkanes, alkenes, alcohols, acetates, diacetates, and wax esters), contained in the bees' labial glands and prominent in head extracts of the studied species

[6]. Differences in perfume composition between individuals were calculated as Bray-Curtis distances based on relative compound contributions (% of total ion current) to individual blends. These distances were visualized in two dimensions by nonparametric multidimensional scaling (MDS) with the software Primer v6 [32, 33].

Isolation, Structure Assignment, and Synthesis of HNDB

The isolation of the 3D-specific compounds from pooled hexane extracts (20 pairs of hind legs of 3D males) was carried out by chromatography on 60 Å, 32-63 mesh silica gel (MP Biomedicals, Eschwege, Germany) with a pentane-ethyl acetate gradient starting with pure pentane and stepwise increase of ethyl acetate (1%, 2%, 5%, 10%). NMR spectra of natural products and synthetic samples were recorded with a Bruker Avance 400 (^{1}H: 400.25 MHz, ^{13}C: 100.65 MHz) and a Bruker DRX 500 (^{1}H: 500.13 MHz) spectrometer (Bruker BioSpin, Rheinstetten, Germany). Samples were dissolved in C_6D_6 with tetramethylsilane (δ = 0) as the internal standard. Mass spectra and details on HNDB synthesis are given in Supplemental Data 1.

Bioassays

Ten milligrams of synthetic HNDB were dissolved in 1 ml of n-pentane (Uvasol, Merck, Germany), and aliquots of 30 μl were applied to filter papers (Whatman 1, 2.5 cm), which were then attached to stems of treelets in Xmatkuil. Repeated presentations were made on three sunny mornings (8:45 to 12:30) in September 2007. Attracted males were captured with hand nets, morphotyped, and retained in vials until the end of the bioassay. On the same days, we also exposed filter papers with HNDB isolated from hind legs (in n-pentane, at the same concentration as synthetic HNDB) and solvent control, as well as mesh-covered dispensers with pure p-dimethoxybenzene to obtain a reference of relative morph abundance at the time in Xmatkuil. In October 2006, we performed similar bioassays with 30 μl aliquots of individual hind leg extracts of 2D and 3D males (n = 6 and 8, respectively) in 1 ml n-pentane. Captures at extracts of 2D and 3D males were pooled for analysis. We later confirmed with GC-MS that all extracts from 3D males contained HNDB, whereas those of 2D males did not.

Electrophysiology

Detailed descriptions of EAG and GC-EAG procedures are given in [34]. For EAG, 5 μg of synthetic HNDB, benzyl benzoate, eugenol, 1,8-cineole, p-dimethoxybenzene, methyl salicylate, and methyl cinnamate (all in n-pentane) were applied to strips of filter paper. The solvent was allowed to evaporate, and air puffs (200 μl) were directed over the strips into air flowing over the antennal preparation. The HNDB stimulus was given twice for each antenna (HNDB a and b in Figure 3A). For comparison of antennal sensitivity in response to single HNDB isomers, GC-EAG was done with HNDB isolated from hind leg extracts of 3D males. In addition to the four HNDB isomers (in relative concentrations similar to those in 3D male hind leg extracts), the test mix also contained 2-undecanone as reference stimulus (Figure 3B). In addition, GC-EAD was carried out with a complete hind leg extract of a 3D male containing all four HNDB isomers plus a variety of perfume components (see Supplemental Data 2). For both EAG and GC-EAG, the antennal response is given as the amplitude (in mV) of the negative baseline deflection upon stimulus onset.

Microsatellites, PCR, and Population Genetics

One hundred and ninety-three males from three localities (1, 2, and 3) were screened for three polymorphic microsatellite markers (Egc17, accession number EF451841 [35]; ann02, BV728898, and ann08, BV728902 [R. Paxton, personal communication]). DNA was extracted from thoraxes of ethanol-preserved specimens via the protocol given in [36]. Multiplex-PCR reactions were conducted with fluorescent-dye-labeled primers (VIC, 6-FAM and NED; Applied Biosystems). Four microliters of DNA template was used with 12.5 μl HotStar Taq Master Mix (QIAGEN), and the reaction volume was filled up to 25 μl in total with RNase-free water (QIAGEN). PCR reactions were performed in an Eppendorf Mastercycler with the following profile: 95°C for 15 min, then 94°C for 30 s, 52°C for 90 s, and 67°C for 90 s for 22 cycles, and then 67°C for 10 min. Fragment analysis of PCR products was carried out with an ABI Prism 310 Sequencer (PE Applied Biosystems) at the Biologisch-Medizinisches Forschungszentrum in Düsseldorf. For visualization and allele calling, we used the software GENEMARKER V1.71. Exact probability tests of linkage disequilibrium between markers were calculated with GENEPOP v 3.4 [37] and were all nonsignificant within 2D and 3D males. Exact probability tests for genetic differentiation between 2D and 3D males were calculated with the same program.

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> **Supplementary data can be found online and on CD, attached to this dissertation.**

Characterisation of the orchid bee *Euglossa viridissima* and its cryptic sibling species, *Euglossa dilemma* sp. nov., by morphological, chemical, and genetic characters

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Manuscript in preparation

>>Disclaimer: All nomenclature relevant acts in this manuscript have to be regarded as unpublished according to Article 8 of the International Code of Zoological Nomenclature, and will become available later, by separate publication<<

Characterisation of the orchid bee *Euglossa viridissima* and its cryptic sibling species, *Euglossa dilemma* sp. nov., by morphological, chemical, and genetic characters

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ABSTRACT

In orchid bees, males signal their availability as mates by fanning "perfumes", i.e. blends of volatiles which are collected from environmental sources and stored in hind leg pouches. Distinctive chemical composition of such perfumes in males with two rather than three mandibular teeth has previously led to the discovery of two sympatric lineages within *Euglossa viridissima* Friese on the Yucatan peninsula, Mexico (Eltz et al. 2008a). Here, we combine chemical, morphological, and genetic data for an integrated characterisation of the two lineages. The valid lectotype of *Euglossa viridissima* Friese in the Museum of Natural History in Vienna has two mandibular teeth, and the species name *viridissima* must thus be assigned to the (predominantly) bidentate lineage, whereas the (completely) tridentate lineage is described as a new species, *Euglossa dilemma* n. sp.. Chemical profiling and microsatellite genotyping revealed that *Euglossa viridissima* males can occasionally (~10 % of individuals) express a third mandibular tooth, but this tooth is not positioned centrally on the mandible as in *E. dilemma*, but displaced towards the tip. Thus, males of the two lineages can be unambiguously diagnosed by mandibular characters alone. 889 bp of COI sequence data confirm *E. viridissima* and *E. dilemma* n. sp. as a monophyletic sister group within the genus *Euglossa*, but failed to separate the lineages due to the lack of parsimony informative base changes. Both species occur in broad sympatry across Central America, but the orchid bees recently introduced to Florida (Skov & Wiley 2005) have three mandibular teeth in males, i.e. are pure *E. dilemma*.

INTRODUCTION

Sibling species can often be distinguished by differences in secondary sexual characters that underlie divergent selection due to their function as recognition signals. However, not all taxa communicate reproductive signals visually and are therefore not easily recognized by taxonomists (Bickford et al. 2007). In insect, chemical signals are frequently involved in mate finding and recognition (Cardé & Baker 1984a, Roelofs 1995, Cardé & Baker 1984b), and chemical dichotomies are sometimes a first cue to differentiate cryptic sibling species (Byers & Struble 1990). Cryptic sibling species have also been discovered in neotropical orchid bees (Euglossini) that use environmental odours for communication (Eltz et al. 2008a, Roubik 2004). Males are attracted to volatiles produced by flowers and certain non-floral sources, e.g. fungus-infected wood, tree wounds, and feces (Dressler 1982, Roubik & Hanson 2004, Vogel 1966). They collect the emitted volatiles and store them in voluminous pockets on their hind tibiae, where complex and species-specific blends of "perfume" accumulate (Eltz et al. 2005a, Eltz et al. 2007). Later, such perfumes are exposed by the males during display behavior, for which they perch on tree trunks in the forest understory (Bembé 2004, Eltz et al. 2005b). Although this has not been demonstrated directly with bioassays, it is believed that the perfume signals target females and serve to communicate availability for mating. A function in the context of mate recognition is indirectly supported by the finding that chemical blends are different between species, especially so between the most closely related taxa (Zimmermann et al. 2009).

Recently, chemical analysis of male perfumes has revealed cryptic diversity in what was previously regarded as a single species, *Euglossa viridissima* Friese, medium-sized, metallic green orchid bees distributed from Mexico to Costa Rica (Eltz et al. 2008a). Males of all *E. viridissima*-like bees are distinguished from other *Euglossa* by two large patches of hair on the second sternite and the characteristic shape of midtibial hair tufts. However, already Dressler (1978)(Dressler 1978b) had noted that males with those characters are variable with respect to the number (3 or 2) of mandibular teeth (Dressler 1978), with 3 being more common (see also the identification key in (Roubik &
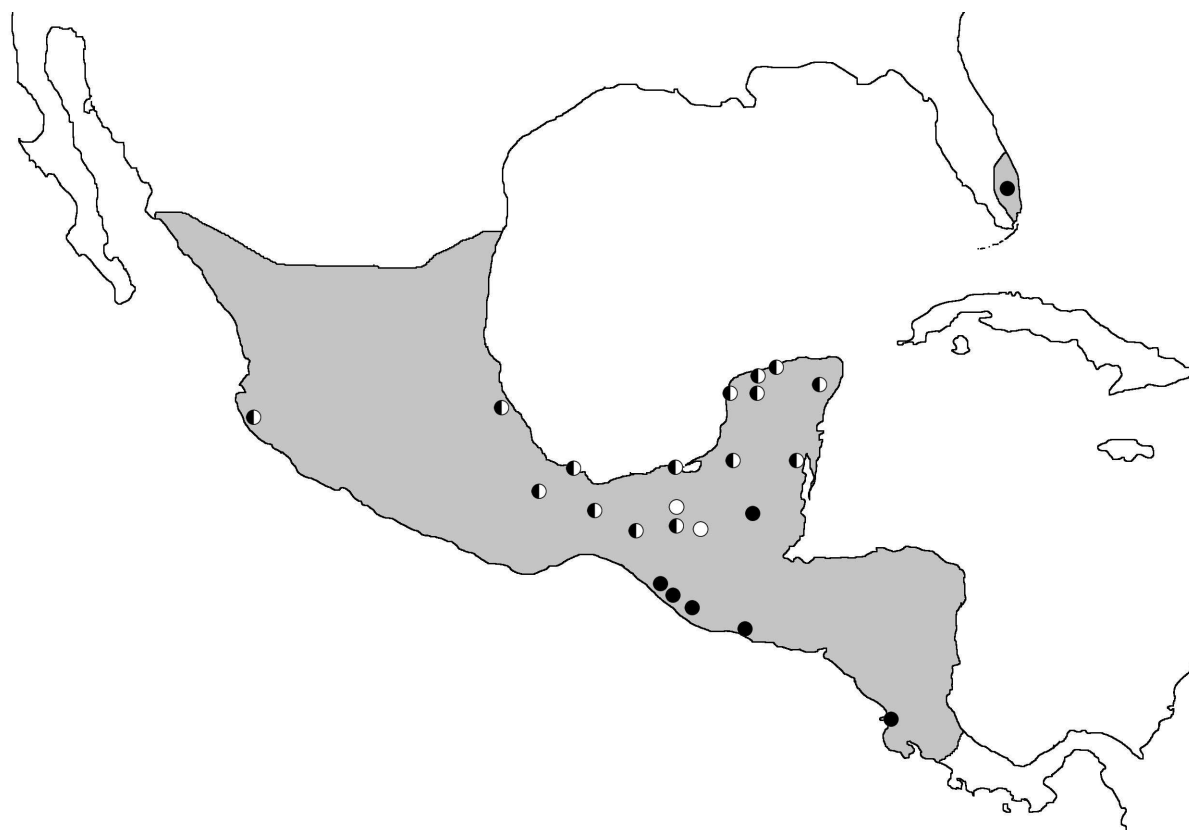
Hanson 2004)). Chemical analysis of male perfumes on the Yucatan peninsula revealed that most tridentate individuals contained a set of highly characteristic compounds (2-hydroxy-6-nona-1,3-dienylbenzaldehyde (HNDB) isomers) as major perfume ingredients, whereas those compounds were completely absent in bidentate males (Eltz et al. 2008a). The distinction in perfume composition was associated with striking differences in olfactory sensitivity towards HNDB isomers, which could explain differential perfume accumulation by males. Finally, allele frequencies of microsatellite loci were different between tridentate and bidentate individuals, suggesting that the two morphotypes belong to two reproductively isolated lineages (Eltz et al. 2008a). However, the assignment of males based on the number of mandibular teeth alone was not perfect, possibly because males of the bidentate lineage occasionally express a third tooth. In the present paper we further explore morphological, chemical, and genetic variation in this sibling species complex, with the aim to identify morphological characters that allow unambiguous diagnosis of males of the two lineages. Based on such characters, and on a comparison with type material, we describe one lineage as *Euglossa dilemma*, sp. nov. Furthermore, we confirm the monophyly of the sibling group within the framework of a recently published molecular phylogeny of the Euglossini, and provide additional data on geographic ranges.

MATERIALS AND METHODS

<u>BAITING AND SAMPLING</u>

To obtain specimens and to assess local proportions of bidentate and tridentate males, baiting with commercially available attractants (p-Dimethoxybenzene, eugenol, and methyl cinnamate) was done at various localities in southern Mexico (**Fig. 1**) from October 2006 to July 2009. Males were captured with hand nets, morphotyped using a hand lens, and partly preserved for later chemical, genetic, and/or morphometric analysis. 198 males from the localities (1) Xmatkuil, (2) Chablekal, (3) San Crisanto, and (4) Coba (see **Fig. 8**) were subjected to detailed perfume analysis and microsatellite genotyping. These 198 individuals include 63 that were already analysed in (Eltz et al. 2008a). A separate set of 245 individuals was used for general morphometrics. These were from Yucatecan localities 1, and 3 to 13 of (Eltz et al. 2008a). Finally, we compiled data on the geographic range of the two lineages, including data from baiting assays in

various localities in southern Mexico and as well as an investigation of dried specimens present in insect collections.

Figures 8 Geographic distribution of tridentate *Euglossa dilemma* n. sp. (black) and predominantly bidentate *Euglossa viridissima* (white) as inferred from recent baiting assays.

CHEMICAL ANALYSIS OF MALE PERFUMES

Male perfumes were extracted from hind legs in 0.5 ml of hexane. Coupled Gas Chromatography/Mass Spectrometry (GC/MS) was carried out with a HP 5890 II GC fitted with a DB-5 column (30 m x 0.25 mm Ø x 0.25 μm film thickness) and a HP 5972 MSD. Injection was splitless, the oven programmed from 60 to 300°C at 10°C/min. Extracts of 176 males from the localities (1) Xmatkuil, (2) Chablekal, (3) San Crisanto, and (4) Coba were subjected to detailed perfume analysis as described in (Eltz et al. 2008). Briefly, structure assignment of extracted compounds was carried out by comparison of mass spectra and retention indices with those of authentic reference

samples or those given in the literature (Adams 2001). Excluded from the analysis were straight chain lipids (alkanes, alkenes, alcohols, acetates, diacetates, and wax esters), contained in the bees' labial glands and prominent in head extracts of the studied species. Only individuals with at least six perfume compounds above the detection threshold (on average 26) were further analysed (N=176), thereby excluding 12 presumably young bees that had almost no perfume. Differences in perfume composition between individuals were calculated as Bray-Curtis distances based on relative compound contributions (% of total ion current) to individual blends. These distances were visualized in three dimensions by non-parametric Multidimensional Scaling (MDS) using the software Primer v6 (Clarke & Gorley 2001).

MICROSATELLITE GENOTYPING

Males subjected to perfume analysis were also genotyped at three polymorphic microsatellite loci, ann02, ann08, and Egc17 (Paxton et al. 2009b, Souza et al. 2007, Paxton et al. 2009a). DNA was extracted from thoraxes of ethanol preserved specimens using the protocol given in (Hunt & Page 1995). Multiplex-PCR reactions were conducted with fluorescent dye labelled primers (VIC, 6-FAM and NED; Applied Biosystems). 4 µl of DNA template was used with 12.5 µl HotStar Taq™ Master Mix (Qiagen), and the reaction volume was filled up to 25 µl in total with RNase-free water (Qiagen). PCR reactions were performed in an Eppendorf Mastercycler with the following profile: 95°C for 15 min, then 94°C for 30 s, 52°C for 90 s, 67°C for 90 s for 22 cycles, and then 67°C for 10 min. Fragment analysis of PCR products was carried out with an ABI Prism 310™ Sequencer (PE Applied Biosystems) at the BMFZ in Düsseldorf. For visualisation and allele calling we used the software GENEMARKER V1.71. We tested for differentiation using exact tests implemented in the web version of GENEPOP (Raymond & Rousset 1995).

QUANTITATIVE MORPHOMETRY

A total of 245 males from 12 localities (all on the Yucatán peninsula) were measured. Individual heads, thoraxes and hind tibiae were gently squeezed into plasticine to keep them fixed during measurements. We took 15 morphometric measures of each individual; LAA (forewing length), AAA (forewing width), LAP (hind wing length), AAP (hind wing width), LTP (hind tibia length), ATP (hind tibia width), LTO (thorax length),

ATO (thorax width), AO (width eye), LO (length eye), LC (length clypeus), AC (width clypeus), LCA (length head), ACA (width head) and DIN (distant between teeth). The morphometric measures were taken following Hartfelder and Engels, (1992), Ken *et al.*, (2003) and Quezada-Euán *et al.*, (2007) methods. The length and width of the fore and hind wings were measured with an inverted microscope (Indumex), a digital table (Summasketc Professional Plus), a cold light (Indumex) and the program Afusda 7 (Rubink, dates not published). Stereoscopy (Motic®) in combination with the software Motic Image (Advanced 1.3.) were used to perform the other measurements. We tested for differences in sizes of the various structures between bidentate and tridentate males using ANOVA (Statgraphics Plus 5.1.). Furthermore, to test for an overall morphometric separation of the two lineages we performed a principal component analysis (PCA), including those morphometric variables that showed significant univariate differences (Ken et al. 2003, Quezada-Euan et al. 2007). The first four components were tested for differences between the two kinds of males using ANOVA, and individuals were plotted in a two dimensional space, with the axis representing the two principal components that showed a statistically significant difference.

PHYLOGENETIC ANALYSIS

We explored the phylogenetic position of *Euglossa viridissima* and *Euglossa dilem*ma n. sp. based on a 889 bp fragment of the mitochondrial cytochrome oxidase 1 gene (CO1). A DNA matrix was assembled using the software package MacClade v4.06 (Maddison & Maddison 2003), and a parsimony analysis was conducted using the software package PAUP* v4.10b (Swofford 2003) by assuming unordered transitions and weighting all characters equally. Heuristic tree searches consisted of 100 random addition sequences, using the TBR swapping algorithm. Support values were obtained by non-parametric bootstrapping, with 1,000 replicates. In addition, we implemented Bayesian analyses using the software package MrBayes v3.1.1 (Ronquist & Huelsenbeck 2003) with models of sequence evolution partitioned by codon positions. Parameters were estimated separately during runs for first, second and third codon positions. Markov Chain Monte Carlo (MCMC) searches were run for 10 million generations, sampling every 1000 generations.

We estimated the time of divergence between *Euglossa viridissima* and *Euglossa dilemma* n. sp. using molecular clock methods. We used Penalized Likelihood (PL) as implemented in the software package r8s v1.71 (Sanderson 2002). A molecular phylogenetic tree of the genus *Euglossa* from a previous study (Ramirez et al. in review) based on both nuclear and mitochondrial loci was used. Based on the fossil-calibrated molecular clock analysis of Ramírez et al., we fixed the age of the most recent common ancestor of *Euglossa* to 15-20 million years before present.

RESULTS

GENETIC, CHEMICAL, AND MORPHOLOGICAL CHARACTERISATION

The MDS analysis of tibial perfume similarity of males produced two distinct clusters of individuals that were characterised by the presence or absence of 2-hydroxy-6-nona-1,3-dienylbenzaldehyde (HNDB, **Fig. 1**). In individuals with HNDB (black circles in **Fig. 1**), the HNDB isomers represented the dominant component of the individual perfume blend (on average 77.8 +/- 9.25 % of the total peak area; minimum 44.5%), whereas all other individuals had no HNDB at all. While individuals with HNDB were all strictly tridentate, the individuals lacking HNDB fell into two groups. Most were bidentate, but seven individuals had three mandibular teeth (red circles in **Fig. 1**). This confirms Eltz *et al.* (2008), and, given the extended sample size, we are now able to further characterise these aberrant individuals (tridentate males with HNDB).
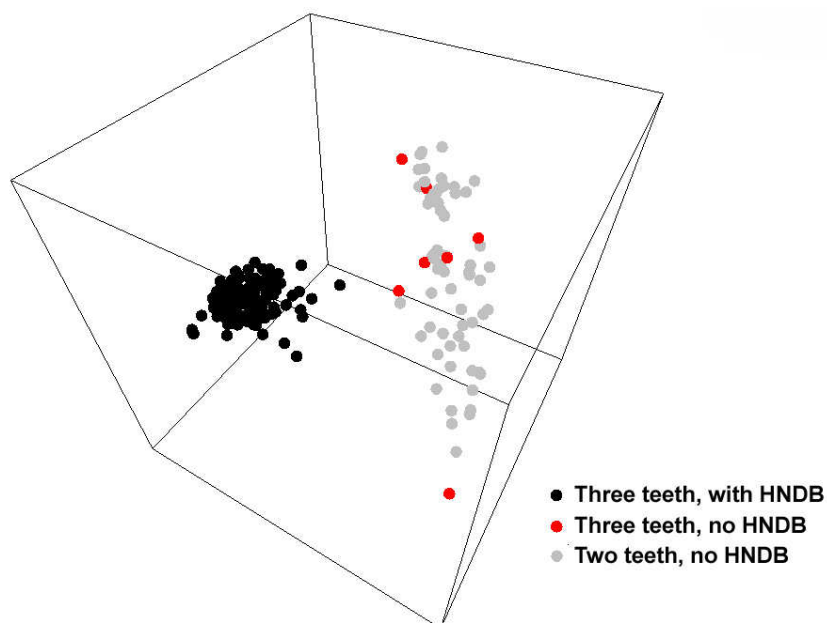
Figure 1 Differences in the chemical composition of tibial perfumes between tridentate (black circles) and bidentate (grey circles) *Euglossa-viridissima*-like males as revealed by a Multidimensional Scaling (MDS) analysis. Only tridentate males contained HNDB. Tridentate males without HNDB are highlighted (red symbols).

Allele frequencies were significantly different between bidentate and tridentate males at each of the three screened loci (Exact probability test, N=176; p<0.001 for ann02 and Egc17, p<0.05 for ann08). However, only one of the markers, ann02, showed sufficiently little overlap in fragment sizes so as to allow genetic assignment of individuals to lineages. This is shown in Fig. 2, were the tridentate males without HNDB are again highlighted as red circles. Unambiguously, these individuals all have small fragment sizes (179 to 183 bp) characteristic for the bidentate lineage. In contrast, tridentate males with HNDB, with a single exception, have fragment sizes above 199 bp. Thus, it is evident that males of the bidentate lineage occasionally express a third mandibular tooth. Based on this finding we examined the form of the male mandible, in particular the position of the middle tooth. The seven tridentate individuals lacking HNDB had interdental distance ratios consistently and significantly lower than tridentate males with HNDB (t-test, t=5.1, p<0.01, N=49), i.e. their middle tooth was displaced towards the tip of the mandible (**Fig. 3b**). The seven aberrant individuals represented 10.3 % of bait-captured males of the predominantly bidentate lineage on the Yucatán peninsula.
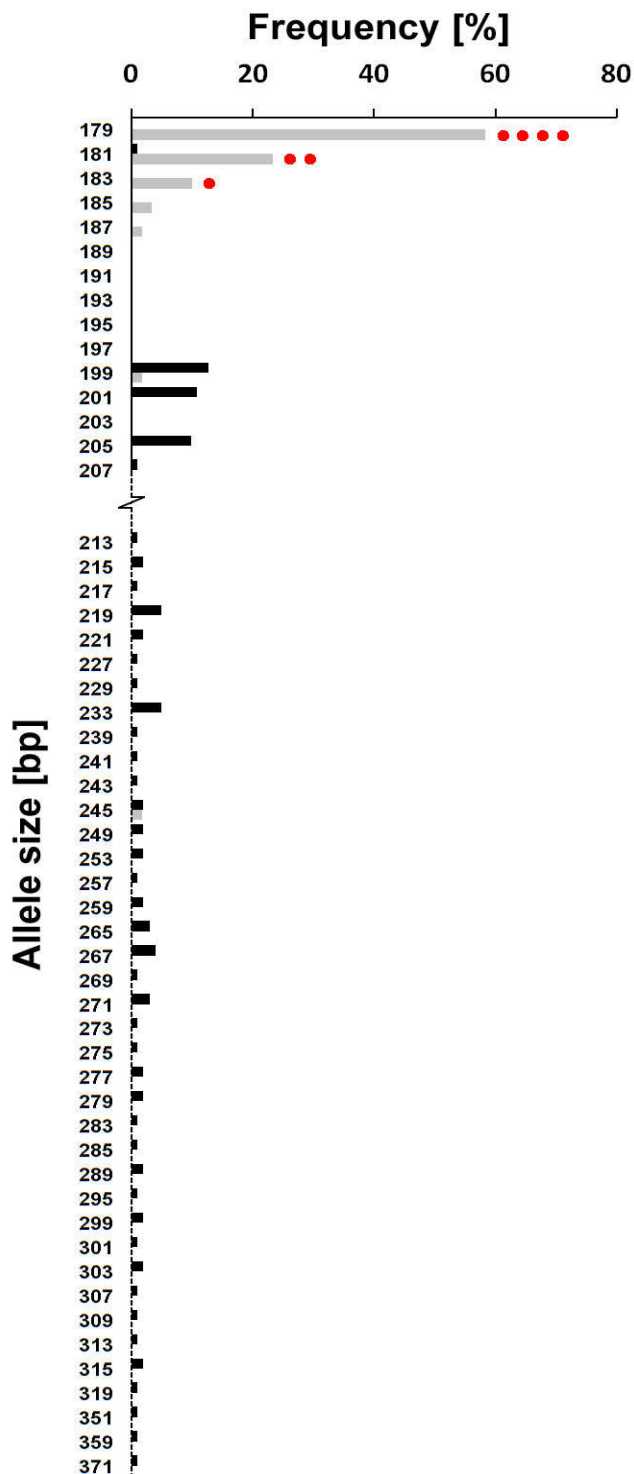
Figure 2 Allele size distributions of *Euglossa-viridissima*-like males from the Yucatán peninsula at the microsatellite locus ann02. Overall, bidentate males (grey bars) had significantly smaller allele sizes than tridentate individuals (black bars), and there was little overlap in allele size. The seven individuals indicated as red circles were also tridentate, but had been clustered with bidentate males in the analysis of perfume similarity (see Fig. 1), lacking HNDB. These seven individuals had the third (central) mandibular tooth significantly displaced towards the tip of the mandible (nearer to the distal tooth, see Fig. 3b), unlike in other tridentate males. Their ann02 allele size suggests that they in fact belong to the bidentate lineage.

Of the 245 males examined for quantitative morphometrics, 152 had three mandibular teeth and 93 had two. However, among the 152 tridentate males were six that had their middle tooth displaced towards the tip of the mandible, and according to the findings reported above these six males were grouped with the bidentate individuals. There were significant differences between the two groups of males in the size of six of the 15 measured characters (**Table 1**). All these characters were smaller in males of the tridentate lineage, but in each case there was considerable overlap between the two groups of males. The clearest size differences were found in the width and length of the hind tibia (ATP and LTP), reflecting a subtle but consistent difference in hind tibial shape (**Fig.5**). In the PCA analysis component 1, explaining 58 % of the variability, and component 3, explaining 9 % of the variability, were significantly different between lineages (ANOVA; $p < 0.01$), but again there was substantial overlap between them (**Fig.4**).

Table 1 Morphological measurement taken from males of the two lineages, with means and standard deviations in [mm]. Results of an ANOVA are also given and significant differences are highlighted in bold letters and numerals.

Measurement [mm]	E.viridissima n=99	E. dilemma n.sp. n=146	F	p
Forewing lenght	9.082 ± 0.043 a	9.038 ± 0.035 a	0.63	0.429
Ferewing width	2.833 ± 0.014 a	2.824 ± 0.011 a	0.22	0.639
Hind wing length	4.414 ± 0.022 a	4.415 ± 0.018 a	0.00	0.973
Hind wing width	1.789 ± 0.009 a	1.712 ± 0.008 b	37.67	0.000 *
Hind tibia length	4.107 ± 0.025 a	3.978 ± 0.020 b	15.74	0.000 *
Hind tibia width	2.730 ± 0.016 a	2.641 ± 0.013 b	17.70	0.000 *
Length clypeus	1.102 ± 0.008 a	1.122 ± 0.007 a	3.47	0.063
Width clypeus	1.007 ± 0.006 a	0.982 ± 0.005 b	8.32	0.004 *
Thorax width	3.395 ± 0.013 a	3.422 ± 0.011 a	2.20	0.139
Thorax length	3.013 ± 0.015 a	3.011 ± 0.012 a	0.02	0.891
Width eye	1.451 ± 0.008 a	1.452 ± 0.007 a	0.02	0.876
Length eye	2.867 ± 0.012 a	2.897 ± 0.010 a	3.45	0.064
Length head	2.754 ± 0.016 a	2.692 ± 0.013 b	8.25	0.004 *
Width head	5.083 ± 0.020 a	5.090 ± 0.016 a	0.08	0.774
Distance mandibular base	2.242 ± 0.012 a	2.185 ± 0.010 a	12.09	0.000 *

* ANOVA p <0.01 *, g. 1=1, 243. Different letters indicate a statistic difference and the bold numbers represents higher means

Figure 3 a) *Euglossa-viridissima*-like males attracted to a bait dish at Xmatkuil, Yucatán. **b)** Mandibular morphology of males of tridentate *E. dilemma* nov. spec., and tridentate and bidentate males of *E. viridissima*. The position of the central mandibular tooth in tridentate individuals is expressed as the ratio of the distance between the distal and the central tooth to the distance between the central and the basal tooth. Means and standard deviations are given.
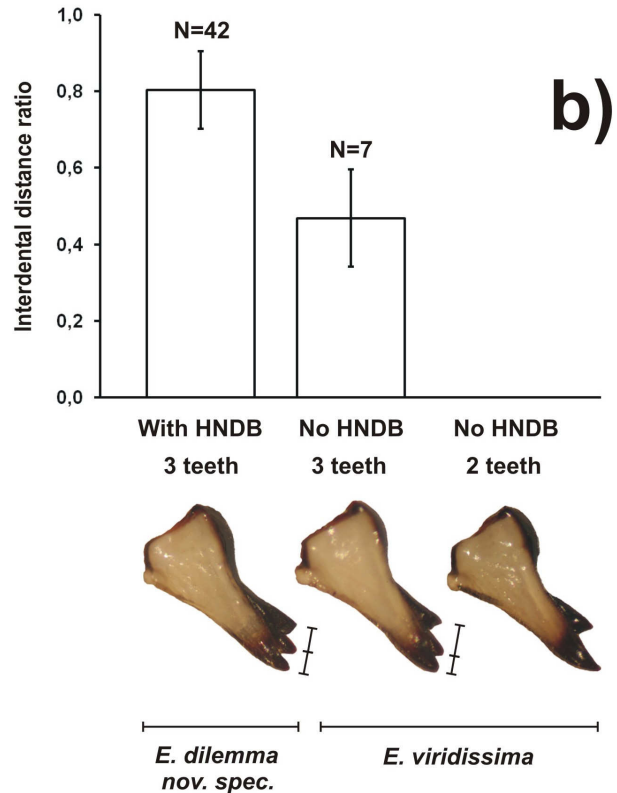
DESCRIPTION OF *EUGLOSSA* (EUGLOSSA) *DILEMMA* SP. NOV.

The presented chemical, molecular, and morphological dichotomies require a new evaluation of the taxonomic status of what was previously considered *Euglossa viridissima* Friese. The male lectotype of that taxon at the Natural History Museum in Vienna presents two mandibular teeth (see below) and, unambiguously, belongs to the less common (predominantly) bidentate lineage. As a consequence this lineage has priority over the name *Euglossa viridissima* Friese, and we describe the more common tridentate lineage as a new species.

Material and Methods. The studied specimens belong to the entomological collections of the Zoologische Staatssammlung München (ZSM), Germany, the Smithsonian Institution (SI) in Washington, USA, the Collection of Thomas Eltz (CTE), Düsseldorf, Germany, and to the Collections of G. Gerlach (CGG) and B. Bembé (CBB), both Munich, Germany. The type series is comprised of a number of male specimens. *E. dilemma* sp. nov. has been compared to the male lectotype of *E. viridissima* from the Naturhistorisches Museum Wien, Austria. The lectotype is dirty, missing the right antenna above the scape, left

hindleg, and left forewing. Its right mandible, which bears two pointed teeth, is clearly visible. It has tree labels: "Bilimek Mexico 1871 // Euglossa § viridissima det. Friese 1898 // LECTOTYPE viridissima Friese, J. S. Moure 1958". In the following, terga and sterna are referred to as T1, T2, T3, etc., and S1, S2, S3, etc. Integument and setae coloration were described by eye using a Leica MZ 6 microscope.

Diagnosis. Males of *E. dilemma* sp. nov. can be distinguished from those of *E. viridissima* by two morphological characters: They have three mandibular teeth, with the intermediate tooth placed approximately at half distance between its neighbours (**Fig. 3**), and their hind tibia is less inflated, and its distal/posterior edge is more ending in a point; in *E. viridissima* the hind tibia is subtly more obtuse and more rounded (**Fig. 5**).
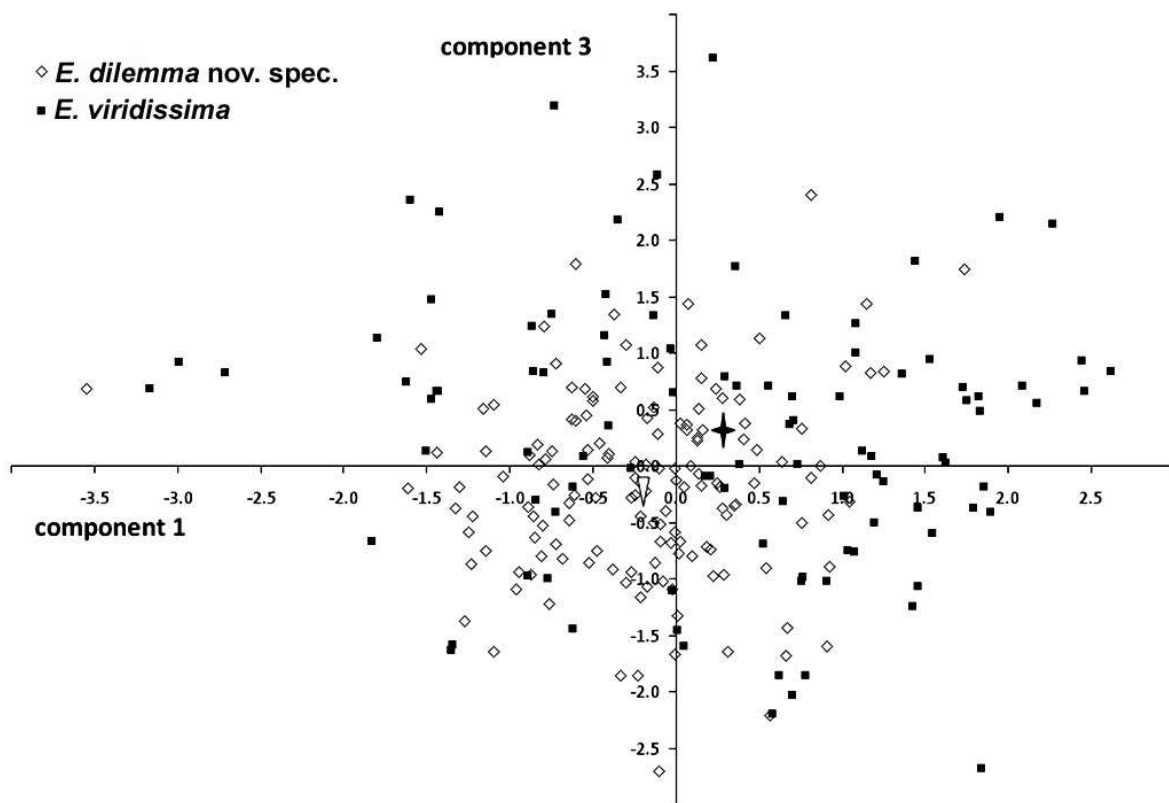
Figure 4 Results of a Principal Component Analysis of 15 morphological variables measured in male *Euglossa viridissima* and *E. dilemma* n. sp.. Component 1 and 3 showed significant differences between the species are used for this two-dimensional representation. Note that *E. dilemma* shows slightly less variability and is essentially nested within *E. viridissima* morphospace. Centroids of distributions are shown.

Color and vestiture (male). Whole bee metallic green, darkest green on front of clypeus (seldom with bluish hue) and hind legs, lighter green on face near antennal scape and behind the ocelli, on propodeum, pleurae, and on T7. Violet stripe in front of velvet area on mid tibia. Clypeus and front of head with white setae, on top of head mixed with black setae; scutum and scutellum dorsally covered with a mix of many white and some black setae, end of scutellum only white; Terga with tiny white setae, on top of T2-T5 some black setae, Sterna with white setae, center of S2 with two large joining cushions of dense brownish hairs; wings transparent, covered with little black setae, wing venation dark brown. Jugal comb at base of hind wing with 16 blades.

Head (male). Width 4.6 mm, height from labrum to front 2.6 mm. Mandibles with three teeth, with the intermediate tooth placed approximately at half distance between its neighbours (Fig. 3b). Tongue in repose reaching S2. Front of clypeus densely punctuated, with a complete medial rigde; ivory paraocular marks well developed, reaching the malar area; forward side of antennal scape with white stripe occupying two third of its length. Labrum white with a medial rigde and two oval transparent wind.

Thorax and abdomen (male). Total body length 10.5 to 11.5 mm; anterior wing 9.2 mm; scutum intertegular distance 3.7 mm and 2.7 mm long; scutellum 2.7 mm wide and 1.3 mm long. Punctuation on scutum dense and regular, all points have nearly the same size. Scutellum rounded on posterior margin. Punctuation on scutellum not as dense as on scutum. Anterior rim of scutellum with small points, at the center and towards the posterior margin with larger points as scutum, nearly as many micropoints as points. Median depression covering about two thirds of scutellum, depression without points or with small points only.

Punctuation on abdomen (male). T1 front half sparse with big points, behind half dense with very small points; T2 – T4 dense with smaller points as scutum; from T5 – T7 points become larger and more sparse.

Legs (male). Mid tibia, anterior tuft smaller than posterior tuft, triangular or comma-like. Posterior tuft oval or in form of a drop, very near on anterior tuft. Velvet area dense on anterior side, sparse and incomplete on posterior side. Hind tibia triangular, 3.3 mm long and 2.6 mm wide. Basal third densely punctuated. The two distal thirds sparsely punctuated; distance between points one or two diameters of points; points long, as many micropoints as points. Hind tibia flat and not inflated, distal/posterior edge ending

in a point (not as obtuse and rounded as in *E. viridissima*) (**Fig. 5**). Post-glandular area fringed with medium-sized hairs.

Type locality. Holotype collected at Mérida (Xmatkuil), Yucatán, Mexico (20°52'11.47"N, 89°37'10.03"W).

Distribution. From Mexico to Costa Rica. Recently introduced into southern Florida, USA (see also Geographic distribution below)

Female. >>detailed female description follows<<

Etymology. The species epithet refers to the dilemma of having to describe a taxon as a new species although this taxon had been widely considered as an existing species (dilemma is a noun in apposition).

Type material. HOLOTYPE. male, with the following label data: "Mérida (Xmatkuil) Yucatán, Mexico April 2006, coll. Thomas Eltz // Holotypus § Euglossa dilemma, Autoren 2010" (ZSM). PARATYPE. a number of males, with the following labels data: "" (ZSM), (SI), (CTE), (CGG), (CBB)

Figure 5 Series of right hind legs of males of the two species. Male *E. viridissima* (top line) have slightly larger, more obtuse, and distally less pointed hind tibiae than male *E. dilemma* n. sp. (bottom line). The asterisk indicates an exceptional tridentate individual of *E. viridissima* exhibiting typical *E. viridissima* hind tibial shape.

PHYLOGENETIC ANALYSIS

Both parsimony and Bayesian analyses supported the monophyly of *E. viridissima* + *E. dilemma* with high bootstrap and posterior Bayesian support. However, neither lineage was recovered as monophyletic due to the lack of parsimony-informative characters within lineages. Only eight characters (from a total of 889) were variable between the

two lineages, but parsimony-uninformative. The phylogenetic position of *E. viridissima* + *E. dilemma* within the genus *Euglossa* was congruent with the placement recently proposed based on several nuclear and mitochondrial genes (Ramirez et al. in review).

Our molecular clock analysis suggests that the lineages *Euglossa viridissima* and *Euglossa dilemma* n. sp. shared a most recent common ancestor between 110.000 and 150.000 years ago.

GEOGRAPHIC DISTRIBUTION

Since *E. viridissima* and *E. dilemma* n. sp. have been lumped by previous authors and collectors, we reassess distributional ranges by compiling information from recent baiting assays (Fig. Xx).

DISCUSSION

Dressler ((Dressler 1978a) page 189) stated the following about *E. viridissima*: "This species appears to be polymorphic in the number of teeth. It is possible, of course, that there are two sibling species, and the number of teeth is the only distinction we have found between them." It turns out that both alternatives are actually true. *Euglossa viridissima* is indeed polymorphic in the number of male mandibular teeth (rare males expresses a third tooth), and it coexists with a widespread sibling species, *E. dilemma spec. nov.*, that has the fixed number of three mandibular teeth. Thus, one cryptic sibling has been obscured by a morphological variation (polymorphism or plasticity) in the other. It is evident from our microsatellite data, and also supported by morphological dichotomies, that the two lineages are reproductively isolated species, warranting the establishment of *E. dilemma* as a new species. It is intriguing that the cryptic diversity was revealed by chemical dichotomies in male perfumes, which probably play an important role in mate/species recognition in orchid bees (Zimmermann et al. 2009). Secondary sexual characters involved in mate recognition are expected to be most divergent in closely related lineages due to diversifying selection in sympatry (Andersson 1994). Such diversifying selection can occur either before postcygotic barriers are established between lineages due to lower fitness of hybrids, and is then

equivalent to "reinforcement" (e.g. (Butlin 1987)). Alternatively, it can occur after the process of speciation is essentially complete. In this case, recognition errors could be selected against due to costs measured in loss of time, energy, and gametes, as well as the predation risk involved in spurious mate searching (Coyne & Orr 2004). In both cases selection is expected to favour signals that allow unambiguous recognition of mates in sympatry. Although direct evidence for the male perfumes to affect mate localization/choice in orchid bees is still lacking, there is evidence for diversifying selection on perfumes (Zimmermann et al. 2009). Among 15 sympatric species of the genus *Euglossa* in central Panama, the chemical composition of male perfumes was found to be non-random. All species had predictable blends, both in terms of diagnostic compounds as well as in relative compound proportions. Furthermore, the difference in blends between species was larger than expected from a neutral null model of chemical evolution, and especially conspicuous amongst the most recently diverged lineages. This suggests that the perfumes have diverged quickly during or after the speciation process, as expected for recognition characters involved in mate finding (Zimmermann et al. 2009). The chemical dichotomy found between *E. viridissima* and *E. dilemma* n. sp. in southern Mexico may also have resulted from such diversifying selection. However, in this particular case the chemical dichotomy is heavily based on a single compound, HNDB, present only in perfumes of male *E. dilemma* n.sp., and is associated with a sensory adeptness to detect this compound in male *E. dilemma* n. sp.. It remains unclear whether this sensory shift was a cause or a consequence of the speciation process.

>>This discussion is incomplete, because data on divergence times and geographic ranges from museum specimens (to be provided by co-authors) were yet lacking at the time of submission of this thesis. . <<

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Single mating in orchid bees (*Euglossa*, Apinae): implications for mate choice and social evolution

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RESEARCH ARTICLE

Single mating in orchid bees (*Euglossa*, Apinae): implications for mate choice and social evolution

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Abstract Neotropical orchid bees (Euglossini) are conspicuously different from other corbiculate bees (Apinae) in their lack of advanced sociality and in male use of acquired odors (fragrances) as pheromone-analogues. In both contexts, orchid bee mating systems, in particular the number of males a female mates with, are of great interest but are currently unknown. To assess female mating frequency in the genus *Euglossa*, we obtained nests from three species in Mexico and Panama and genotyped mothers and their brood at microsatellite DNA loci. In 26 out of 29 nests, genotypes of female brood were fully consistent with being descended from a singly mated mother. In nests with more than one adult female present, those adult females were frequently related, with genotypes being consistent with full sister–sister ($r = 0.75$) or mother–daughter ($r = 0.5$) relationships. Thus, our genetic data support the notions of female philopatry and nest-reuse in the genus *Euglossa*. Theoretically, single mating should promote the evolution of eusociality by maximizing the relatedness among individuals in a nest. However, in Euglossini this genetic incentive has not led to the formation of eusocial colonies as in other corbiculate bees, presumably due to differing ecological or physiological selective regimes. Finally, monandry in orchid bees is in agreement with the theory that females select a single best mate based on the male fragrance phenotype, which may contain information on male age, cognitive ability, and competitive strength.

Keywords Euglossini · Mating frequency · Mate choice · Microsatellites · Sociality · Corbiculate bees

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Introduction

Hymenoptera are a favored group for studies on mating systems because of the presumed effect of female mating frequency on the evolution of sociality (Hamilton, 1964; Boomsma, 2007; Hughes et al., 2008). The majority of female Hymenoptera are thought to mate only once or have an effective mate number very close to one (Boomsma and Ratnieks, 1996; Strassmann, 2001). Well known exceptions are the highly eusocial honey bees (Apini), attine ants and vespine wasps, some of which are highly polyandrous (Foster and Ratnieks, 2001; Villesen et al. 2002; Tarpy et al., 2004). Queens of the European honey bee (*Apis mellifera*) have an effective mate number of 12 on average (Strassmann, 2001). Honeybees belong to the monophyletic clade of corbiculate bees (Apinae) that also includes the eusocial stingless bees (Meliponini) and bumblebees (Bombini) (Michener, 2000), both of which generally exhibit monandry (Strassmann, 2001), and the strictly neotropical orchid bees (Euglossini). Information on the mating frequency of female orchid bees is lacking.

Orchid bees are generally described as solitary (Zucchi et al., 1969), but in some cases two or several females share a nest or nest cavity. For some species there are reports of reproductive dominance of one female (Garofalo, 1985), providing a potential opportunity for the evolution of more complex sociality (Augusto and Garofalo, 2004; Cameron, 2004; Cocom Pech et al., 2008; Otero et al., 2008). However, why eusociality as an obligate condition has not evolved in orchid bees is an open question (Roubik et al., 1996).

Kin selection theory predicts that female mating frequency affects the likelihood that eusociality evolves because it elevates relatedness to 0.75 among female hymenopteran offspring, increasing their inclusive fitness when helping their mother or sisters to reproduce (Queller and Strassmann, 1998; Boomsma, 2007). Female multiple mating, on the other hand, decreases relatedness among a mothers' daughters (to nearly 0.25), and their theoretical incentive to cooperate. Thus, polyandry may reduce the likelihood of advanced sociality to evolve. Its existence in honey bees, attine ants and vespine wasps is therefore considered to be derived and to postdate the evolution of sociality and permanent worker castes (Boomsma, 2007).

Other evolutionary factors may favor polyandry over monandry. For example, in social species polyandry provides an increase in intracolonial genetic variation (Crozier and Fjerdingstad, 2001) that has been shown to result in a better colony phenotype that, for example, minimizes the adverse effects of parasitism (Baer and Schmid-Hempel, 1999; Tarpy and Seeley, 2006; Seeley and Tarpy, 2007). Furthermore, polyandry in social and non-social insects is thought to provide compensation for difficulties in identifying the single best mate. The latter hypothesis predicts that multiple mating occurs in species where male quality is difficult to assess, thus constraining the evolution of choosiness in females (Strassmann, 2001).

Orchid bees have a highly unusual mating biology, aspects of which are still poorly understood. Males forage for volatile chemicals (fragrances) in a time-consuming and risky manner (Eltz et al., 1999). Flowers of orchids and other plants, as well as decaying wood or fruits, serve as natural sources of such fragrances, which consist mostly of terpenoids and aromatics. For a great number of neotropical orchid species, male orchid bees act as specific pollinators, giving rise to the Euglossine pollination syndrome (Dodson et al., 1969; Williams, 1982; Cameron, 2004). Collected volatiles are stored by euglossine males in voluminous leg pockets (Vogel, 1966), where a species-specific blend of chemicals accumulates (Eltz et al., 2005a; Zimmermann et al., 2006). During courtship display these blends are actively released and ventilated at courtship territories (Bembé, 2004; Eltz et al., 2005b), where females have been observed to mate (Kimsey, 1980; Stern, 1991;

Eltz et al., 2003). Although the attraction of females to these odors has yet to be demonstrated in behavioral experiments, the male perfume is likely to function as a species specific chemical signal analogous to endogenous sex pheromones (Vogel, 1966; Zimmermann et al., 2006). In addition, the individual perfume of a male could represent a fitness indicator, giving an approaching female the possibility to evaluate a male's quality and to choose her best mate, making potentially costly multiple mating unnecessary.

Thus, information on orchid bee female mating frequency is desirable both in the context of social evolution in corbiculate bees as well as for a better understanding of the significance of euglossine fragrance collection. The genus *Euglossa* represents the largest and most widely distributed genus of orchid bees (more than 100 described species) (Roubik and Hanson, 2004), with females of some species accepting artificial trap nests for their brood rearing. To analyze female mating frequency in orchid bees we genetically analyzed brood from nests of three species of *Euglossa*—*E. hemichlora*, *E. viridissima* and *E. sp.* '2dentate'—using polymorphic microsatellites and calculated relatedness among brood, among cohabiting females and between brood and adults.

Materials and methods

Sample collection

We studied *Euglossa hemichlora* from Panama, *Euglossa viridissima* from Mexico and another so far undescribed species of *Euglossa* from Mexico, which is morphologically almost identical to *E. viridissima*, but males have two instead of three mandibular teeth. Population genetic analysis demonstrated that the lineage is reproductively isolated from *E. viridissima* (Eltz et al., 2008). For the purpose of this study, we refer to this species as *Euglossa sp.* '2dentate'.

We used wooden boxes ($10 \times 3 \times 6$ cm) as trap nests, which were placed around buildings in a private forest preserve in Santa Rita, Colon, Panama (8.39°N, 82.34°W) in October 2007 and at the campus of the Universidad Autónoma de Yucatán in Xmatkuil, Mexico (20.52°N, 87.37°W) in October 2006 and October 2007. The nest boxes were monitored over several months at each locality, and the number and condition of brood cells therein were recorded. Nests were collected when the boxes were populated with at least five (preferentially more) brood cells and at least one adult female was present (which was normally the case during afternoons). For microsatellite DNA analysis, adult females present in the nest boxes were immediately preserved in 99% ethanol and stored at +8°C.

The brood cells were kept in a cabinet at 26°C and 70% humidity until the offspring emerged. We recorded the dates of eclosion of progeny, immediately preserved individuals in 99% ethanol and stored them at +8°C.

To obtain more precise estimates of allele frequencies, we also sampled males of all three species up to 3 km radius from the nest boxes. Males were attracted with fragrance baits (p-dimethoxy benzene for *E. hemichlora*, p-dimethoxy benzene, methyl cinnamate and eugenol for *E. viridissima* and *E. sp.* '2dentate'), caught and immediately preserved in 99% ethanol at +8°C.

DNA extraction and microsatellite DNA analysis

Microsatellite DNA analysis was conducted by using seven different microsatellite loci: ann02 (GenBank accession no. BV728898), ann04 (BV728900), ann08 (BV728902), ann24 (BV728906) (Paxton et al., 2009), Egc17 (EF451841), Egc18 (EF451842), and Egc37 (EF451846) (Souza et al., 2007). Not all of the samples were typed at all seven loci, but at the most polymorphic loci for the given species, with each individual being typed at an average of 3.36 loci (range 2–6). DNA was extracted from tissue from half of the thorax of each specimen using the method of Hunt and Page (Hunt and Page, 1995). After phenol/chloroform extraction, DNA pellets were dried at 37°C, resuspended in 40 µl of distilled water and stored at −20°C. All PCRs were performed as multiplex reactions with three loci 5′labeled with fluorescent dye (VIC, 6-FAM or NED; Applied Biosystems). Four µl of DNA template was used with 12.5 µl HotStar Taq^{TM} Master Mix (Qiagen) in a final reaction volume of 25 µl, made up with RNase-free water (Qiagen). PCR reactions were performed in a Mastercycler gradient (Eppendorf), with the profile of an initial 95°C for 15 min (HotStar Taq Polymerase), followed by 22 cycles of 94°C for 30 s, 52°C for 90 s, 67°C for 90 s, and then a final extension step of 67°C for 10 min. Fragment analysis of PCR products was carried out with an ABI Prism 310^{TM} Sequencer (PE Applied Biosystems) at the University of Düsseldorf (BMFZ). Allele lengths were assigned with the software GENEMARKER V1.71, using an internal standard run in every lane. Allele sizes were rounded to the nearest integer.

Data analysis

We tested for linkage disequilibrium between loci within each species with the program GENEPOP (Raymond and Rousset, 1995) (web version at http://genepop.curtin.edu.au/), using only the haploid male data because GENEPOP does not allow for a joint analysis of haploid and diploid data.

A standard suite of descriptive statistics was then calculated for each locus using Microsatellite Analyzer (MSA) version 4.05 (Dieringer and Schlotterer, 2003), which supports the option of jointly analyzing haploid and diploid data. To avoid pseudoreplication, only unrelated females were included in the calculation of population allele frequencies, which means one female per nest with one exception where a further unrelated female was present.

For each population of the three species we calculated the genetic non-detection error of the mating frequency, defined as the probability of an undetected second father among a female's progeny, which occurs when two males have identical genotypes at all investigated loci (Boomsma and Ratnieks, 1996).

Estimation of mating frequency

Assignments of adult females as mothers of brood were first made by visual inspection of genotypes; all daughters of a mother had to carry one single maternal allele at all loci, and all sons had to carry one of the two maternal alleles at each locus. Under monandry, all female offspring of a mother should carry the same paternal allele at a locus. To support pedigree determination based on visual inspection of genotypes, we examined intra-nest relationships using the likelihood function of KINSHIP 1.3.1 (Goodnight and Queller, 1999), (http://www.gsoftnet.us/GSoft.html#Kinanchor), using the same data set as we used for the descriptive statistics. Female genotypes of each nest were analyzed separately in a group. In the first step we analyzed, whether one or more of the adult females present in a nest at the time of sampling were the mother of the brood. Specifically we tested whether her genotype allowed the rejection of the null hypothesis (no relationship to female brood; $r = 0$) in favor of the alternative hypothesis of a mother–daughter relationship ($r = 0.5$). In the second step, we tested whether the genotypes of female offspring in a nest allowed us to reject the null hypothesis that they were half sisters ($r = 0.25$ as expected with multiple mating of the mother) in favor of the alternative hypothesis that they were full sisters ($r = 0.75$ as expected by single mating). These tests were performed as pairwise comparisons calculated with 1,000 simulations between each female progeny pair within each nest.

With the relatedness function of KINSHIP 1.3.1 we performed pairwise relatedness (R) calculations between all female individuals to generate the mean value of relatedness between a mother and its female offspring and among all female offspring.

Results

In total, we collected 13 populated nest boxes from *E. hemichlora* (all in the year 2007), five from *E. viridissima* (two from 2006 and three from 2007) and 14 from *Euglossa sp.* '2dentate' (eight from 2006 and six from 2007). Two types of nests were distinguished: newly founded nests (NFN) were in artificial boxes that had been colonized for the first time by a single female foundress, building one homogenous cell cluster. We found six NFNs for *E. hemichlora*, three for *E. viridissima* and 11 for *E. sp.* '2dentate'. The other nest type, older or re-used nests (RN), contained remains of previous brood (enclosed brood cells) and had obviously been re-used by other females, potentially by progeny of the original foundress. In RNs there was frequently more than one adult female and/or cell cluster, which made the assignment of mothers to progeny more difficult. For *E. hemichlora* we found four RNs, and for *E. viridissima* and *E. sp.* '2dentate' we found two RNs each.

Three additional nests of *E. hemichlora* and one nest of *E. sp.* '2dentate' were excluded from our analysis of mating frequency because they had both a low number of female progeny ($n < 3$) as well as more than one adult female present.

Microsatellite data analysis

No significant linkage disequilibrium between loci was detected in any of the three analysed species ($n = 43$ for *E. hemichlora*, $n = 69$ for *E. sp.* '2dentate' and $n = 73$ for *E. viridissima*).

For the analysis of allelic variation at loci we genotyped males of each population (*E. hemichlora* $n = 43$, *E. sp.* '2dentate' $n = 69$, *E. viridissima* $n = 73$) and pooled these data with the genotype data of unrelated females (see above; *E. hemichlora* $n = 13$, *E. sp.* '2dentate' $n = 15$, *E. viridissima* $n = 6$). Genetic diversity estimates are given in Table 1. The expected heterozygosity (H_e) of our markers ranged from 0.41 to 0.98 and we found between five and 49 alleles per locus. In every species H_e was 0.8 at a minimum of 2 loci.

The extremely high variability of ann02 ($H_e = 0.98$) in *E. viridissima* as well as the large mean allele size of 238 bp (minimum 150 bp and maximum 387 bp) led us to suspect non-specific PCR products. However, repeated independent processing (including DNA extraction) of the same samples confirmed fragment sizes in all cases ($n = 14$). Also, the marker was clearly inherited in a strictly Mendelian manner within families of bees.

As a result of the high variability of our markers, the population-wide probability of genetic non-detection of a second fathering male's offspring among progeny genotypes was very small within each of the three species; the non-detection error (d_p) varied from 0.002 to 0.00007.

Estimation of mating frequency

In 17 of the 20 analyzed NFNs, the single adult female present was clearly identified as mother of the whole nests female progeny, both by visual inspection of genotypes as well as by KINSHIP (Table 2). One exception was NFN 6

Table 1 Allelic diversity for each species, with the number of genotyped male and female individuals per locus

	n genotyped individuals		Expected heterozygosity	Mean allele size (bp)	*n* observed alleles	Allele frequency range
	Males	Females				
E. hemichlora						
ann02	42	10	0.807	189	20	0.435–0.016
ann04	21	0	0.847	134	6	0.333–0.048
ann08	43	4	0.906	151	14	0.255–0.020
ann24	42	4	0.698	174	9	0.500–0.020
Egc17	9	13	0.861	236	8	0.257–0.029
Egc37	9	13	0.795	162	8	0.343–0.029
E. sp. '2dentate'						
ann02	69	14	0.671	186	11	0.505–0.010
ann08	69	14	0.923	154	22	0.186–0.010
ann24	33	9	0.706	171	9	0.453–0.012
Egc17	36	6	0.812	226	7	0.313–0.063
E. viridissima						
ann02	68	6	0.985	244	46	0.088–0.013
ann08	73	6	0.934	157	23	0.153–0.012
ann24	37	3	0.405	170	5	0.767–0.023
Egc17	36	4	0.780	225	8	0.422–0.022

Table 2 Summary of the KINSHIP likelihood analyses of mother-daughter and full-sister relationships among *Euglossa* females in newly founded nests (NFNs)

	Likelihood of a mother–daughter relationship			Likelihood of a full-sister relationship			*n* male progeny
	n female progeny	*n* significance level	Mean R (±SE)	*n* pairwise comparisons of female progeny	*n* significance level	Mean *R* (±SE)	
E. hemichlora							
NFN 3	6	6***	0.64 (±0.09)	15	10*** 5**	0.71 (±0.11)	1
NFN 6	5	NA		10	6*** 4**	0.73 (±0.17)	0
NFN 7	6	6***	0.79 (±0.17)	15	13*** 2**	0.80 (±0.15)	2
NFN 9	6	6***	0.49 (±0.09)	15	15***	0.64 (±0.20)	3
NFN 108	4	4***	0.54 (±0.06)	6	6***	0.89 (±0.12)	0
NFN 111	4	4***	0.50 (±0.00)	6	6***	1.00 (±0.00)	0
E. sp. '2dentate'							
NFN 1	3	3***	0.46 (± 0.05)	3	1*** 2**	0.65 (±0.30)	4
NFN 2	3	3***	0.78 (± 0.19)	3	3***	0.69 (±0.04)	4
NFN 3	7	5*** 2**	0.55 (± 0.15)	21	9*** 12**	0.72 (±0.20)	3
NFN 4	2	1*** 1**	0.35 (± 0.01)	1	1***	0.63	4
NFN 8	2	2***	0.67 (± 0.00)	1	1***	1.00	2
NFN 10	8	7*** 1**	0.78 (± 0.06)	28	13*** 15**	0.91 (±0.09)	6
NFN 13	7	4*** 1**	0.34 (±0.14)	21	11*** 10**	0.61 (±0.28)	3
NFN 22	2	2***	0.25 (±0.00)	1	1***	1.00	2
NFN 23	7	7***	0.72 (±0.10)	21	6*** 15**	0.83 (±0.13)	5
NFN 24	7	NA	–	21	1*** 20**	0.59 (±0.20)	3
NFN 25 a	4	4***	0.45 (±0.06)	6	2*** 4**	0.64 (±0.24)	1
NFN 25 b	4	NA	–	6	6***	0.91 (±0.10)	0
E. viridissima							
NFN 5	5	5***	0.47 (±0.22)	10	10***	0.92 (±0.07)	0
NFN 9	7	7***	0.61 (±0.25)	21	21***	0.91 (±0.08)	0
NFN 14	7	7***	0.58 (±0.08)	21	21***	0.87 (±0.15)	0

Nests generally contained a single adult female, and we tested the null hypothesis that female brood in the nest is unrelated to the adult female ($r = 0$) versus the alternative hypothesis that the female brood is descended from this mother ($r = 0.5$). Monandry was evaluated by testing the null hypothesis that female descendants were in a half-sister relationship ($r = 0.25$) versus the alternative hypothesis of a full-sister relationship ($r = 0.75$). Significance values are given as a flag (**$P < 0.01$; ***$P < 0.001$; *NA* not analyzed, because the adult female could not be the progeny's mother based on visual inspection of genotypes). Integers next to the significance flags refer to the number of female progeny that tested accordingly. Mean relatedness (R) between mother and daughters as well as between sisters are given (SE values apply to individuals); number of male progeny per nest is also given

(*E. hemichlora*), where the adult female was unrelated to the offspring ($r = -0.03 \pm 0.09$), but the female progeny still consisted solely of full-sisters (see below). In the second exception in NFN 24 (*E. sp.* '2dentate'), the single adult female was likely a daughter of the deceased foundress as its genotype was fully congruent with a full-sister

relationship with all other female progeny (brood) of the nest. In the third exceptional case (NFN 25 in *E. sp.* '2dentate') the adult female was identified as the mother of four of the emerging females, whereas the remaining four females (that emerged >21 days earlier) were unrelated to this mother.

Genotypes of female progeny were consistent with a full-sister relationship in 19 out of 20 NFNs, and this finding was confirmed by KINSHIP tests (Table 2). The single exception was NFN 25 (*E. sp.* '2dentate'). Here, the first four emerged females formed a group of full-sisters that was likely descended from the adult female present in the nest at the time of sampling. The remaining four females that emerged later were not related to them ($r = -0.09 \pm 0.12$), but were in a full-sister relationship amongst each other. For reasons of clarity the results of KINSHIP tests are given separately for these two groups of offspring (NFN 25a and NFN 25b in Table 2).

The haploid genotypes of male progeny of the NFNs were consistent with being derived from unfertilized eggs laid by the identified nest mothers. Numbers of emerged males per nest are given in Table 2. We did not find a single male offspring that was unrelated to the mother of the diploid brood.

Within the RNs we always found more than one adult female per nest, but only in RN 15 (*E. viridissima*) was an adult female present which was unrelated to the brood ($r = 0.009 \pm 0.17$) in addition to the clearly identified nest mother. It had no offspring. In RN 20 (*E. hemichlora*) we found two unrelated pairs of full sisters present ($r = 0.08 \pm 0.22$). In all other cases, relatedness estimates were consistent with adults being either sisters or in a mother-sister relationship (Table 3). The close relatedness

between the adult females made an assignment of the offspring more difficult. Nonetheless, full-sister relationships among female offspring, as an indication for single mating, were found in five RNs (RN 1 in *E. hemichlora*, RN 6 and 26 in *E. sp.* '2dentate' and RN 15 and 20 in *E. viridissima*). In these cases one adult female was clearly identified as the mother of the entire female progeny. The other related female(s) had definitely produced no female offspring, but definite exclusion of their contribution to male progeny was impossible based on the available data. One exception was RN 1 (*E. hemichlora*), where a likely sister of the major reproductive female appeared to be responsible for at least three of the nine male offspring. RN 18 (*E. hemichlora*) consisted only of female progeny in a full-sister relationship (including four adult females), but the mother was apparently no longer present. The female offspring of RN 4, RN 20 (*E. hemichlora*), and RN 21 (*E. sp.* '2dentate') were not explainable with one single mother (even taking into account multiple mating), and a full-sister relationship between the female progeny was not detected. The genetically mixed brood must have been the offspring of more than one reproductive female. In RN 4 and RN 21 we could identify one main reproductive female; these two nests are explainable as derived from multiple, closely related mothers. In RN 20 we were not able to explain the origin of the progeny with the adult females present, which were two pairs of full sisters. This "nest" was located in an old stingless bee nest box (larger than the artificial trap-nests we employed for *Euglossa*) and contained three cell clusters. Two of them belonged to *E. viridissima* (RN 20) and one outlying cell cluster to *E. sp.* '2dentate' (RN 26).

Altogether, we found strong evidence for single mating in 26 out of 29 nests. Even if multiple mating occurred

Table 3 Number of adult females and cell clusters in reused artificial trap-nests (RNs)

	n present females	Likely relationship of adult females	Mean R ($\pm$ SE)	n cell cluster	n female progeny	n male progeny
E. hemichlora						
RN 1	2	Mother + sister	0.76	2	3	9
RN 4	2	Mother + daughter	0.50	2	8	16
RN 18	2	Two daughters	1.00	2	3	2
RN 20	4	Two pairs of sisters	0.85 ($\pm$0.77)	2	3	3
E. sp. '2dentate'						
RN 6	2	Mother + daughter	0.79	2	2	8
RN 21	5	Three sisters + two daughters	0.71 ($\pm$0.14) + 0.56	1	8	2
RN 26	2	Mother + sister	0.89	1	3	6
E. viridissima						
RN 15	2	Mother + unrelated female	−0.04	2	6	3
RN 20	4	One mother + three daughters	0.66 ($\pm$0.12)	2	4	11

With one exception (RN 15), all adult females in a nest had genotypes consistent with a mother–daughter or a full-sister relationship. Values of mean relatedness (*R*) between adult females are given (SE values apply to individuals). Genotypes of male and female progeny were compared with those of adult females in order to clarify family relationships

among females that produced the three problematic nests, the average number of effective mates of the three *Euglossa* species would be close to one. Within the nine nests that were populated by multiple adult females, we found four cases with progeny that were definitely derived from more than one female. These contributing females were close relatives in three cases, most likely in a full sister relationship ($N = 1$) or in a mother-daughter relationship ($N = 2$).

Discussion

Our results represent strong evidence for a predominance of single mating in three *Euglossa* species and suggest that single mating is the rule in this genus and perhaps in the Euglossini as a whole. Single mating (monandry) within the Euglossini is consistent with the idea that females select a single best mate based on male fragrance phenotypes. However, as single mating is common among bees (Boomsma and Ratnieks, 1996; Roubik, 2006; Soro et al., 2009), and probably ancestral for corbiculate bees (Hughes et al., 2008), monandry in orchid bees is unlikely to be an adaptation resulting from fragrance-based mate selection by females. In any case, single mating is fully consistent with the rarity with which matings are observed at euglossine display sites. Males of most if not all species of orchid bees establish non-resource based display sites for fragrance signaling (Eltz et al., 2005b). These display sites are usually centered around perches (trunks of trees or tree lets, often in the forest understory), where males perform series of hovering flights during which they release their fragrances. Extensive studies of displaying males in the field have resulted in only a handful of observed matings (Kimsey, 1980; Stern, 1991). In most cases the female suddenly appeared and quickly landed on the perch, where it was mounted by the resident male. Such rare events may seem to be lucky strikes in the long lives of males, which otherwise appear to display unsuccessfully for weeks or more. Given single mating in females, the rarity of observed matings is understandable. Orchid bee males devote much of their life to fragrance collection, a behavior that requires specialized morphological features (Bembé, 2004), an intricate metabolic recycling mechanism (Eltz et al., 2007), and certainly a lot of energy and risk-taking in order to create their perfume. Although the composition of the male's fragrance mixture is broadly species specific, there is substantial individual variation in quantity and complexity of the blends (Eltz et al., 1999; Zimmermann et al., 2006). It is, therefore, conceivable that females evaluate male fragrance phenotypes to obtain information on male suitability as a mate. If so, females may not need to mate more than once to obtain good genes for their offspring.

Our results fuel the discussion on how bee mating systems evolved (Paxton, 2005) and how they might affect the evolution of advanced sociality. The Euglossini were the last group of corbiculate bees for which information on female mating frequency was lacking. Our study confirms the prevalence of single mating among corbiculate bees: with the exception of honeybees, all other corbiculate clades (bumblebees, stingless bees, and orchid bees) have been demonstrated to be predominantly monandrous. Although the exact phylogenetic relationship among corbiculate bees is still controversial (Ascher et al., 2001; Kawakita et al., 2008), monandry in orchid bees confirms the view that single mating was the ancestral state in this clade. Correspondingly, polyandry in honeybees is likely derived (Hughes et al., 2008).

Kin selection theory predicts that single mating promotes the evolution of eusociality, because it increases the genetic relatedness among offspring and, accordingly, their incentive to cooperate in the care of brood (Trivers and Hare, 1975; Cole, 1983; Boomsma, 2007). This is suggested because non-reproductive individuals can gain greater inclusive fitness by functioning as helpers of close relatives (Hamilton, 1964; Queller and Strassmann, 1998). As orchid bees seem to be mostly singly mated, the mating system cannot account for the conspicuous lack of advanced sociality in this group. Female offspring were full sisters in most of the analyzed nests of this study, and adult females present in a given nest were almost always close relatives. This would seem to represent the ideal genetic background for reproductive division of labor and eusocial behavior to evolve. Indeed, some early stages of sociality and reproductive division of labor exist in orchid bees. Previous studies (Dressler, 1982; Santos and Garofalo, 1994) as well as our own observations here have provided evidence for the frequent occurrence of multi-female nests, which seem to result predominantly from nest re-use by bees of the next generation (Garofalo, 1985; Soucy et al., 2003; Augusto and Garofalo, 2004). In some species of *Euglossa* there is evidence for individual females gaining reproductive dominance over their nest mates that allocate more time to foraging (Michener, 1974; Garofalo, 1985; Cocom Pech et al., 2008; Otero et al., 2008). There is also a suggestion of reproductive conflict, e.g. evidenced by the frequent occurrence of oophagy, usually by the reproductively dominant female (Garofalo, 1985; Roubik and Hanson, 2004; Cocom Pech et al., 2008; Otero et al., 2008). In natural nest cavities, nest sharing may even be more common than in artificial trap-nests, because natural cavities are potentially larger and alternative nest sites perhaps more difficult to find. The benefits of nest sharing may include the avoidance of mortality risks associated with searching for new nest cavities and nest construction material. Furthermore, multi-female nests may be better

protected against nest parasites, which regularly enter the nest while the female is out foraging (Soucy et al., 2003; Cocom Pech et al., 2008). However, while the presence of host females in the nest has been observed to deter attacking cleptoparasitic bees, such effects may not necessarily translate into better protection of multiple-female nests (Garofalo and Rozen, 2000).

Ecological factors such as limited nest sites and economy of nest material have already been hypothesized to favor nest sharing in studies about communal bees (Paxton et al., 1996), where females tolerate unrelated conspecifics as well. Nest reuse by succeeding generations would avoid the search for a suitable nesting place and allow the recycling of nest material from hatched brood cells (Dressler, 1982). Thus, given the right ecological conditions, females might benefit from cooperation with conspecifics or even heterospecifics. However, there is no evidence for highly eusocial behavior in orchid bees, or for the formation of long-lived colonies with highly skewed reproduction among individual females. This evolutionary avenue may be barred by several constraining factors: First, euglossine bees have relatively long generation times, with development from egg to adult taking at least 6 weeks and often longer (Roubik and Hanson, 2004). This probably reduces the amount of time that mother and offspring overlap in their adult lives and reduces the opportunities for daughters to help their mothers (but see (Cocom Pech et al., 2008). Second, female reproduction may be limited by physiological factors, even when help from related individuals is available. Possible physiological constraints include the number of sperm that can be stored in the female spermatheca (euglossine spermatozoa are exceptionally large, see (Zama et al., 2005)) or, more likely, the availability of protein for egg production.

In our study, we found little evidence for communal nesting of unrelated females. There were only three cases where an unrelated conspecific female was present in a nest box, and in two cases these had no own progeny. It is conceivable that the unrelated females were in search of nest cavities or even for nest building material, since for example *E. viridissima* females are known to glean resin from populated nests of stingless bees (J. Quezada-Euan, unpubl. data). Detailed behavioral observations of individually marked females coupled to genetic analysis of offspring will help resolve the uncertainty over the degree to which Euglossines exhibit social behavior.

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Population genetic structure of orchid bees (Euglossini) in anthropogenically altered landscapes

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Population genetic structure of orchid bees (Euglossini) in anthropogenically altered landscapes

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Keywords: *Euglossa*, long-distance gene flow, fragmentation, dispersal, conservation

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Running title: Population genetic structure of orchid bees

Abstract

Habitat degradation and fragmentation are widespread phenomena in tropical regions. Negative effects on the biota are numerous, ranging from interruption of gene flow among populations, to the loss of genetic diversity within populations, to a decline in species richness over time. Orchid bees (Hymenoptera: Apidae: Euglossini) are of major conservation interest since they act as exclusive pollinators for a large number of orchid species and other tropical plants. Here, we used microsatellite markers to investigate the effects of geographic distance and habitat fragmentation on gene flow among populations of euglossine bees. Populations of *Euglossa aff. viridissima* in three geographic regions – the Yucatán peninsula (Mexico), Veracruz (Mexico), and Florida (USA) – were genetically structured predominantly across the three regions, with the strength of differentiation among populations being positively correlated with

geographic distance. Within geographic regions only little substructure was found, suggesting that dispersal is substantial in the absence of geographic or ecological barriers. Habitat fragmentation following deforestation appeared to have little influence on gene flow among populations of eight species of *Euglossa* in southern Mexico (Veracruz, Mexico). Specifically, most bee populations in the 9800 ha forest fragment of Los Tuxtlas (Volcano San Martin) were neither differentiated from, nor had less genetic diversity than, populations in near-continuous forest separated from Los Tuxtlas by ~130 km of agricultural land. It appears that orchid bees may be genetically more resilient to habitat fragmentation than previously thought. Nevertheless, we cannot exclude that the forest fragmentations in southern Mexico were too recent events to cause measurable genetic effects at the population level in orchid bees.

Introduction

Gene flow among populations of a given species is important for the preservation of genetic diversity and determines the relative effects of selection and genetic drift (Roderick 1996). The exchange of genes between populations homogenizes allele frequencies and prevents the fixation of alleles, thus hindering the process of population differentiation and speciation (Barton & Hewitt 1985). Since the geographic distribution of a species is usually more extended than an individuals' dispersal potential, populations often show genetic sub-differentiation just through isolation by distance (Balloux & Lugon-Moulin 2002). Furthermore, the ongoing fragmentation of natural habitat due to human activities alters the landscape and forms isolated habitat patches. This can change the ecological context of a population and may disturb its dispersal dynamics (Cane 2001). Characteristics of habitat fragments such as size, degree of isolation, proportion of edges, and habitat quality influence the abundance of individuals in populations as well as their genetic connectivity (Tscharntke et al. 2002). Populations which are reduced in size and isolated from others are likely to experience a reduction of genetic diversity over time. This can have a negative influence on the populations' likelihood of persistence since it lowers the preparedness to respond to environmental change and may lead to inbreeding depression (Darvill et al. 2006, Frankham et al. 2002, Keller & Waller 2002).

Orchid bees (Euglossini, Apidae; ~200 species) are native to lowland forests of the Neotropical region (Roubik & Hanson 2004) which contains several biodiversity hotspots of prime conservation priority due to alarming rates of deforestation (Turner & Corlett 1996, Myers et al. 2000). In this habitat the solitary to primitively social orchid bees represent an important group of pollinators. Female orchid bees search for nectar and pollen in notable foraging ranges (up to 23 km in a single foraging trip, Janzen 1971) and are known to pollinate members of a great number of plant families (e. g. Rubiaceae, Fabaceae, Costaceae, Lecythidaceae) (Ramirez et al. 2002). Male orchid bees are – besides their foraging need for nectar – on a life time search for volatile chemicals, which they store in their voluminous hind leg pockets (Dressler 1982). The accumulated fragrance mixture is species-specific and believed to function as a pheromone analogue (Eltz et al. 2005, Zimmermann et al. 2006). Male orchid bees use a great variety of sources of volatile chemicals, including resin and sap from tree wounds, fruits, feces, decaying wood, and flowers. The use of floral volatiles has shaped the euglossine pollination syndrome in which male orchid bees are responsible for the exclusive pollination of ~ 700 orchid species (Dressler 1968a, Vogel 1966, Whitten et al. 1993), and other plants. Among orchids, many Stanhopeinae and Catasetinae possess impressive morphological adaptations for male euglossine pollination, and their flowers produce unique fragrance blends to lure only a few (1 to 3) out of a larger diversity of local species (Armbruster et al. 1989, Dodson et al. 1969, Williams & Dodson 1972). While the orchids depend entirely on male euglossine bees for pollination this mutualism appears to be only facultative for the bees. The orchid bee species *Euglossa aff. viridissima*, which has been recently naturalized in southern Florida (USA) (Skov & Wiley 2005) now thrives in an environment without perfume orchids (Pemberton & Wheeler 2006).

Since orchid bees act as key pollinators in tropical forests, it is of major interest to know how populations and communities respond to land-use and deforestation (Roubik & Hanson 2004). An adequate habitat for orchid bees has to contain food plants, nesting sites, nest building materials and volatile sources. It is questionable whether the bees' large flight range (Janzen 1971, Kroodsma 1975, Williams & Dodson 1972), their potential to adapt to new environments (Skov & Wiley 2005), and their occurrence in forest edges (Brosi 2009) are sufficient to counteract all negative effects of

fragmentation. Previous studies about the response of euglossines to forest fragmentation focused on the numerical abundance of individuals and species diversity (Becker et al. 1991, Brosi 2009, Powell & Powell 1987, Tonhasca Jr et al. 2002). Brosi (2009) and Powell & Powell (1987) were able to find a positive relationship between forest area and euglossine bee abundance, but the second study was conducted shortly after a major disturbance, which might have influenced the results (Becker et al. 1991, Cane 2001). Male orchid bees have been observed to enter disturbed forests and even severely degraded farmland in their search for volatiles, but this propensity appears to be highly variable between different species (Brosi 2009, Milet-Pinheiro & Schlindwein 2005, Otero & Sandin 2003, Rincon et al. 1999). In the present study we examined for the first time the consequences of fragmentation on the genetic structure and population differentiation of orchid bee populations by using polymorphic microsatellite markers. In a genetic distance study we measured population differentiation of *Euglossa aff. viridissima* within and among three different geographic regions: the Yucatán peninsula (Mexico), southeastern Veracruz (Mexico), and southeastern Florida (USA). In a fragmentation study on eight different *Euglossa* species we compared genetic diversity of populations in three forest localities in southern Mexico (states of Veracruz and Oaxaca). All three localities were approximately equidistant from each other, but only two localities were connected with each other by forest, whereas the third locality, the Los Tuxtlas reserve, is an isolated forest fragment separated from the other two localities by at least 130 km of agricultural land. Our intention was to distinguish between the effects of deforestation and distance on population genetic differentiation.

Material and Methods

Male orchid bees can be attracted to synthetic, single compound chemical baits. We used this method of chemical baiting to collect samples of specimens as in previous studies on the diversity, seasonality and population dynamics of euglossine bees (Ackerman 1983, Armbruster 1993, Eltz et al. 2007, Roubik & Ackerman 1987, Zimmermann et al. 2009a).

(a)

Florida

FL

Mexico

Cuba

Ch SC
XM Co
EC
ER

Yucatán

Veracruz

MP

Belize

Guatemala   Honduras

(b)

A

B

C
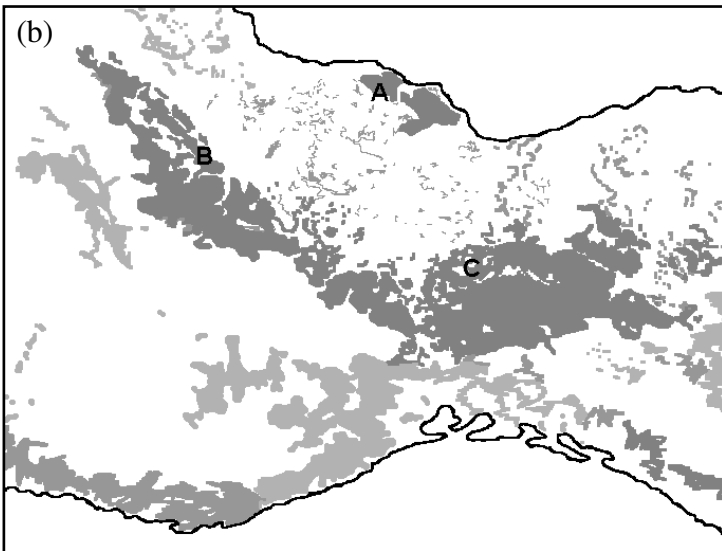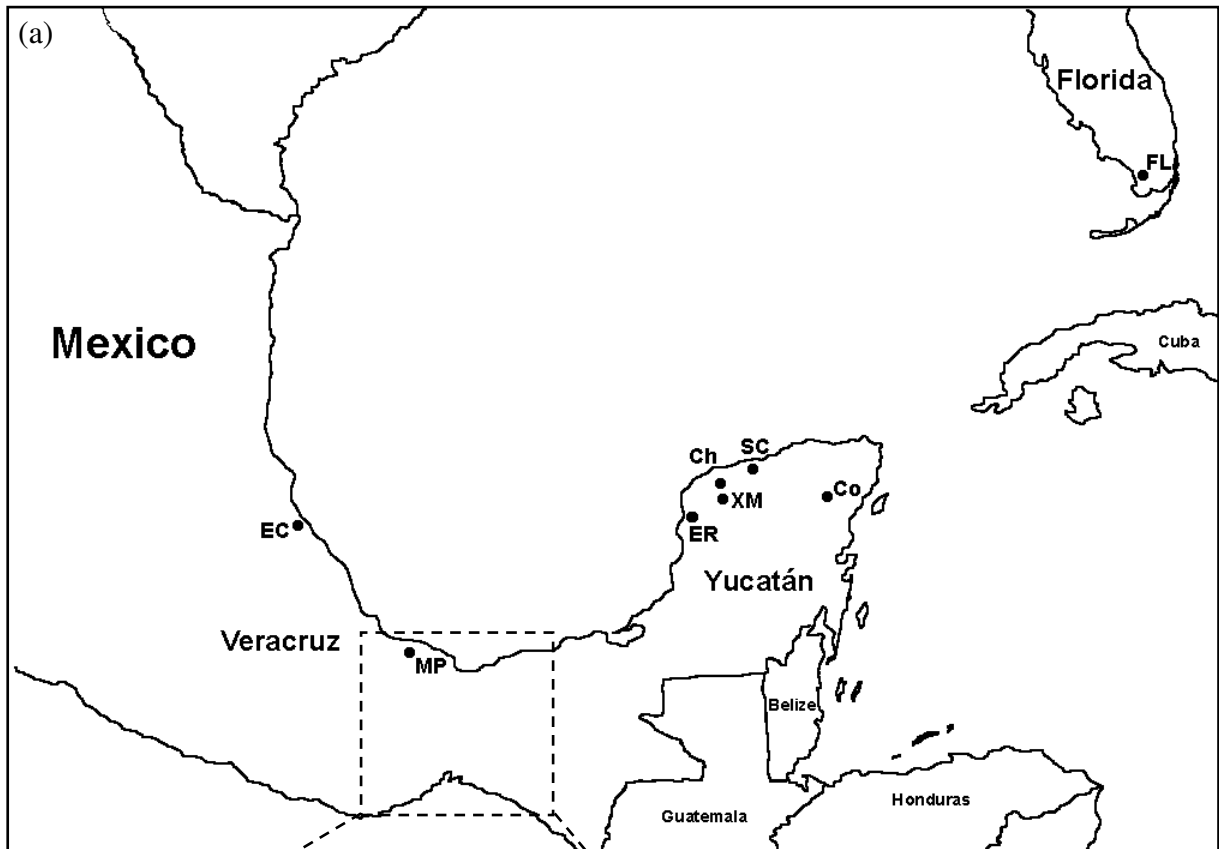
Figure 1 (a) Map of the study area highlighting the eight sampling localities of the genetic distance study on *Euglossa aff. viridissima*. Co = Coba, Ch = Chablekal, EC = El Chote, ER = El Remate, Fl = Florida, MP = Monte Pio, SC = San Chrisanto, Xm = Xmatkuil. (b) Detailed map of southern Mexico with the sampling Localities A, B and C of the fragmentation study. The area highlighted in grey is forested, with the darkest grey representing tall evergreen forest and the lighter shades of grey representing less dense/secondary forest vegetation (modified from Instituto Nacional de Estadistica Geografia e Informatica; 1990-2000).

Genetic distance study

The genetic distance study focused on *Euglossa aff. viridissima,* a yet undescribed sibling species of *E. viridissima* occurring in central America from Mexico, including the Yucatán peninsula, to Costa Rica. Recently (~2003) it was naturalized in southern Florida. Male *E. aff. viridissima* have three mandibular teeth (instead of two as in the lectotype of *E. viridissima* (Eltz et al. in prep.)), and population genetic analysis revealed that the two forms are reproductively isolated species (Eltz et al. 2008). Males of *E. aff. viridissima* were caught in April to June of the years 2007 and 2008 with chemical baits (p-dimethoxybenzene, methyl cinnamate and eugenol) at eight different localities (**Figure 1a**): El Remate, Xmatkuil, Chablekal, San Chrisanto and Coba located on the peninsula of Yucatán, Monte Pio and El Chote situated in the state of Veracruz in southern Mexico, and Fort Lauderdale in Florida (USA). The Florida samples were collected at eugenol baits only. Geographical distances between populations were measured as terrestrial distances, except the distances between Florida and all other localities, which were measured as airline distances.

Fragmentation study

For an assessment of genetic differences in a fragmented landscape we collected male *Euglossa spp.* at three different Localities (A, B and C, see **Figure 1b**) in southern Mexico. Tropical Mexico experienced a reduction of ~28% of its total forest area only between 1977 and 1992, leaving a highly disturbed landscape that includes cultivated land, secondary forest, grazing lands, and disturbed forests Cairns et al. (2000). Locality A was near the Biological Station "Los Tuxtlas" situated in the northeastern lowland part (18°30' N, 95°8' W) of the biosphere reserve Los Tuxtlas (Volcán San Martín) in the state of Veracruz. It is part of a ~9800 ha area of forest remnants which includes the only substantial lowland forests that remain in coastal Veracruz (http://www.catemaco.info/biosphere/areas.html). This forest area is isolated from other lowland tropical forests by a broad belt (>130 km) of intensively used land dominated by pastures and cash crops. The ecological isolation of Los Tuxtlas forests on this scale has existed for at least 35 years (see maps in (Cairns et al. 2000)) but most likely since the 1950's, when extensive transformations of natural habitats to mostly cattle ranches began (Mendoza et al. 2005). Localities B and C are in an area of larger forest remains beyond that belt of farmland, flanking the northeastern slopes of the

central cordillera in western Veracruz/eastern Oaxaca. Baiting sites were at 17°48'N, 96°6' W (B) and 17°23' N, 94°11'W (C) and situated on ridges within the least disturbed forests that we could find. Although the area between B and C is no longer covered by continuous forest, there are no large scale interruptions in the forest patchwork. Thus, all three localities are at approximately equal distance to each other (130-200 km), but locality B and C are connected by forest habitat. This setup was chosen to be able to separate the effects of distance and lack of forest habitat on genetic structuring of orchid bee populations. Baiting took place in May and June 2009 and was conducted in the mornings with a standard set of chemical baits (1,8-cineole, eugenol, ipsdienol, methyl salicilate, p-dimethoxybenzene, methyl cinnamate and vanillin) that are known attractants of males of numerous species of orchid bees. Attracted males were caught with a net and identified with a hand lens in the field or under a microscope at the Los Tuxtlas Biological Field Station. The eight most common species were preserved in 99% ethanol. These eight species were *Euglossa cyanura*, *E. imperialis*, *E. mixta*, *E. obtusa*, *E. tridentata*, *E. variabilis*, *E. viridissima* and *E. aff. viridissima.* Released males were marked with permanent color dots on the notum (thorax) of the bees or by wing notching, enabling us to collect data on bee community composition without pseudoreplication. For an assessment of euglossine abundance we additionally baited on six days at three sites in the intermittent belt of farmland in between locality A and C. In addition to *Euglossa*, the estimates of species diversity also included species from the other euglossine genera in southern Mexico, *Eulaema*, *Eufriesea* and *Exaerete*.

DNA extraction and microsatellite analysis. All individuals were preserved in 99% ethanol and stored at +8°C. DNA was extracted from one leg of each male using the method of Hunt and Page (Hunt & Page 1995). The PCRs were performed as multiplex reactions with 5' fluorescent labeled primers (HEX, TET or 6-FAM) (see (Zimmermann et al. 2009b) for PCR conditions). The *E. aff. viridissima* individuals for the genetic distance study were genotyped at three different microsatellite loci: ann02 (GenBank accession no. BV728898), ann08 (BV728902) (Paxton et al. 2009) and Egc37 (EF451846) (Souza et al. 2007). Fragment analyses of PCR products were carried out with an ABI Prism 310™ Sequencer (PE Applied Biosystems) at the University of Düsseldorf (BMFZ). For the Fragmentation study on eight different *Euglossa* species we used three additional markers: Egc18 (EF451842), Egc24 (EF451843) and Egc26

(EF451844) (Souza et al. 2007). PCR products were analyzed in a MegaBACE 1000 capillary sequencer at the Molecular Ecology Research Group at the University of Halle-Wittenberg. In all cases allele lengths were assigned with the software GENEMARKER V1.71 whereby allele sizes were rounded to their nearest integer.

Data analysis. We tested for linkage disequilibrium among loci over all populations of a given species using GENEPOP 4.0.10 (Raymond & Rousset 1995). Significance values were adjusted following standard Bonferroni corrections (Rice 1989). We used the program Microsatellite Analyzer (MSA 4.05) (Dieringer & Schlotterer 2003) to calculate genetic diversity characteristics and applied the special option for inbred lines to determine results for 200 randomly discarded data sets. For each marker we calculated the expected Heterozygosity (H_{exp}) and the observed number of alleles for each locus (A). Genetic variability within populations was estimated as Shannon diversity index (H) (with the variable p_i as the proportion of a given allele relative to the total number of alleles) and allelic richness (N_a), adjusted for different sample sizes. Wilcoxon matched-pairs tests were used to test for differences in H and N_a between localities for each species (data paired with regard to locus, N=6). To examine genetic differentiation we calculated the unbiased estimator of F_{st} (Weir & Cockerham 1984) between population pairs. Significance levels were determined by permuting genotypes 100 000 times among all population pairs. This conservative procedure does not assume Hardy-Weinberg equilibrium, for which we could not test since our data set included only haploid (male) genotypes. In each of the performed tests all significances were obtained after Bonferroni correction for multiple tests.

In addition, for the genetic distance study with *E. aff. viridissima,* we calculated a linear regression of $F_{st}/(1-F_{st})$ estimates for pairs of populations on their geographical distances. Rousset (1997) suggests this as an estimation of gene flow under isolation by distance when differentiation should be independent of the details of the mutation process (Rousset 1997).

In the fragmentation study we also attempted to obtain simple measures of species diversity to test whether ecological diversity shows trends congruent with genetic diversity. Therefore, we calculated species accumulation curves with the program

Estimate S 8.2 (Colwell 2009) for each of the three forest localities A, B and C, and for the farmland area in between A and C. All captures of a given species on a given sampling day were pooled. Thus, the curves visualize the likelihood that another baiting day would yield an additional species in a given locality. To assess species composition quantitatively at the different localities we calculated the percentage of individuals that each *Euglossa* species contributed to the entire sample. Differences in species abundances between localities were tested with a Chi-square Test for Independence.

Results

Linkage disequilibrium was not detected for any pair of loci after a Bonferroni correction for multiple comparisons, neither across the *E. aff. viridissima* populations of the genetic distance study nor for any *Euglossa* species of the fragmentation study.

Table 1 Genetic diversity among samples of male *E. aff. viridissima* from eight localities of the genetic distance study. Number of genotyped males, maximum number of observed alleles (A) and expected Heterozygosities (H_{exp}) are given. Genetic diversity within populations is given as Shannon Index of diversity (H) and allelic richness (N_a) with their standard errors (±SE).

Area	Population	N males	N alleles (A) ann02	ann08	Egc17	H_{exp} ann02	ann08	Egc17	Shannon Index (H) (± SE)		Allelic richness (N_a) (± SE)	
Yucatán												
	Xmatkuil	67	38	20	10	0.97	0.93	0.72	2.57	± 0.87	15.49	± 7.62
	Chablekal	31	18	15	7	0.96	0.92	0.68	2.21	± 0.69	12.43	± 5.11
	Coba	30	24	14	7	0.98	0.93	0.76	2.36	± 0.76	13.78	± 7.34
	San Chrisanto	30	18	14	7	0.94	0.92	0.74	2.19	± 0.60	12.03	± 4.89
	El Remate	20	13	13	5	0.91	0.94	0.74	2.04	± 0.56	10.33	± 4.62
Veracruz												
	El Chote	31	6	12	8	0.82	0.92	0.81	1.92	± 0.36	8.42	± 2.87
	Monte Pio	29	8	15	7	0.79	0.94	0.79	1.95	± 0.51	9.58	± 4.12
Florida												
	Ft. Lauderdale	30	6	6	2	0.75	0.76	0.13	1.08	± 0.73	4.58	± 2.24

<u>Genetic distance study</u>

In total, we genotyped 268 *E. aff. viridissima* males at three microsatellite loci and found the greatest within-population genetic diversity on the peninsula of Yucatán (**Table 1a**). Here, expected heterozygosities (H_{exp}) were generally high and, averaged over all three markers, it varied from 0.89 (± 0.12) in Coba to 0.85 (± 0.15) in Chablekal. We detected the highest number of different alleles and a maximum value of the Shannon Index in the

population of Xmatkuil (total A = 68, H = 2.57±0.87). Relatively low values of these diversity measures were found in coastal El Remate (total A = 31, H = 2.04±0.56). In comparison to Yucatecan populations the two populations in Veracruz had similarly high H_{exp}, but were slightly less diverse, with a Shannon Index of 1.92 (±0.36) for El Chote and 1.95 (±0.51) for Monte Pio. By far the lowest genetic diversity was detected in the population in Florida, with a H_{exp} of only 0.55 (±0.36) and a Shannon Index of only 1.08 (±0.73).

Table 2 Estimates of genetic differentiation as F_{st} (above diagonal) in pair wise comparisons among the eight populations of *E. aff. viridissima* and their Bonferroni corrected significances (below diagonal). Populations are sorted by geographic area. Significance values: * < 0.05, ** < 0.01, *** < 0.001, n.s. >0.05.

		Yucatán					Veracruz		Florida
		Xm	Ch	Co	SC	ER	EC	MP	FL
Yucatán	Xm	-	0.009	0.001	-0.003	0.013	0.037	0.052	0.122
	Ch	n.s.	-	0.010	0.020	0.019	0.047	0.065	0.127
	Co	n.s.	n.s.	-	-0.006	-0.004	0.030	0.045	0.135
	SC	n.s.	n.s.	n.s.	-	0.004	0.039	0.049	0.149
	ER	n.s.	n.s.	n.s.	n.s.	-	0.055	0.056	0.153
Veracruz	EC	**	*	n.s.	*	*	-	-0.003	0.182
	MP	***	***	*	*	*	n.s.	-	0.202
Florida	FL	***	***	***	***	***	***	***	-

Overall, the populations of *E. aff. viridissima* showed moderate genetic differentiation (global F_{st}=0.051, p=0.0001). Pairwise estimates of F_{st} among populations on the peninsula of Yucatán showed no significant differences (-0.006 > F_{st} < 0.020, see **Table 2**). Equally, the two populations in Veracruz showed no differentiation (pairwise F_{st} = -0.003). When comparing between regions, however, 9 out of 10 pairs of populations had significantly different allele frequencies, with the exception of the comparison between Coba and El Chote. Between regions, F_{st} values were generally higher, indicating moderate genetic differentiation (0.030 > F_{st} < 0.065). The most pronounced genetic differences were found between Florida and all other populations (7 pair wise comparisons, 0.12 > F_{st} < 0.20, p<0.001).

To test for isolation by distance we plotted multilocus estimates of pairwise differentiation ($F_{st}/1-F_{st}$) against the logarithm of geographical distances (**Figure 2**). The best linear fit was obtained for $y = 0.049\,x - 0.227$, and there was a positive effect of distance on genetic differentiation across all populations ($R^2=0.52$, $p<0.0001$).
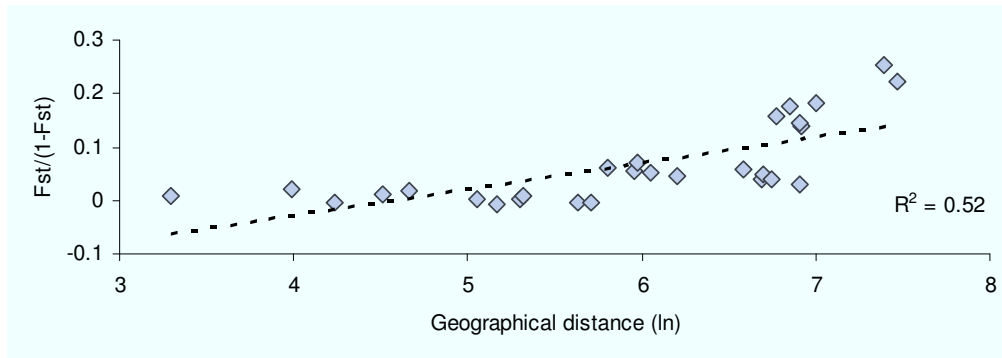
Figure 2 Relationship between geographical distance and genetic differentiation (measured as $F_{st}/(1-F_{st})$) across eight populations of *E. aff. viridissima* in pairwise comparisons.

Fragmentation study

The eight species of *Euglossa* varied substantially in the degree of genetic variability at the six microsatellite loci, with the majority of species being highly polymorphic at several loci (**Table 3**). Variability was very low in *E. cyanura* with only two reasonably informative markers. *E. imperialis* and *E. mixta* were of intermediate variability with four informative markers each, whereas the remaining five species showed substantial polymorphism at all six microsatellite loci. Ann08 was found to be the most variable marker across species, ranging from $H_{exp}=0.496$ in *E. obtusa* (A=6) to $H_{exp}=0.922$ in *E. aff. viridissima* (A=22). The least variable marker was Egc24, which still had a H_{exp} range of 0.433 in *E. viridissima* (A=3) to 0.697 in *E. variabilis* (A=7).

Table 3 Genetic variability at six microsatellite loci of eight *Euglossa* species in the fragmentation study. Expected heterozygosity (H_{exp}) and the number of observed alleles (A) are given for each locus.

	E. cyanura		*E. imperialis*		*E. mixta*		*E. obtusa*		*E. tridentata*		*E. variabilis*		*E. viridissima*		*E. aff. viridissima*	
	H_{exp}	A	H_{exp}	A	H_{exp}	A	H_{exp}	A	H_{exp}	A	H_{exp}	A	H_{exp}	A	H_{exp}	A
ann02	0.000	1	0.773	8	0.486	3	0.652	6	0.563	8	0.783	10	0.612	7	0.797	13
ann08	0.507	2	0.575	3	0.784	8	0.496	6	0.894	25	0.841	11	0.914	17	0.922	22
Egc26	0.000	1	0.000	1	0.038	3	0.113	4	0.745	9	0.776	12	0.769	8	0.797	12
Egc17	0.243	4	0.164	3	0.588	7	0.853	10	0.709	10	0.805	11	0.820	10	0.762	13
Egc18	0.685	7	0.677	5	0.514	3	0.724	8	0.669	11	0.797	10	0.798	11	0.828	12
Egc24	0.027	2	0.000	1	0.000	1	0.498	3	0.529	4	0.697	7	0.433	3	0.670	5

To compare genetic diversity between the three localities A, B and C we calculated the Shannon index of average allelic diversity across loci as well as the average allelic richness across loci for each locality and species (whenever sample size was above 10 individuals per locality). Values for the two diversity measures varied greatly between species and were found to be the lowest in *E. cyanura* and the highest in *E. aff. viridissima*, which corresponds closely to general marker variability (**Table 4**). We found no tendency for any locality being generally more or less diverse than another. Wilcoxon matched-pairs tests between localities (matched with regard to locus) were non-significant for allelic richness in all species. For the Shannon index we found a marginally significant difference (p=0.048) between locality A and locality C in *E. aff. viridissima*.

Table 4 Genetic variability of *Euglossa spp.*, measured as average allelic diversity across loci (H = Shannon index) and average allelic richness across loci (N_a) at three localities in southern Mexico (A, B & C). Number of males per locality is given. Values of F_{st} between pairs of localities are computed, significance values are Bonferroni corrected (* < 0.05, ** < 0.01, n.a. = not analyzed because of a sample size <10 within one locality).

|	N males	Shannon Index (H) [± SE]		Allelic richness N_a [± SE]		Pair wise F_{st}	
E. cyanura							
Locality A	39	0.40	± 0.56	2.29	± 2.24	A - B	n.a.
Locality B	5	n.a.		n.a.		A - C	0.009
Locality C	29	0.44	± 0.49	2.33	± 1.51	B - C	n.a.
E. imperialis							
Locality A	32	0.66	± 0.70	3.17	± 2.40	A - B	0.008
Locality B	46	0.70	± 0.66	3.30	± 2.28	A - C	n.a.
Locality C	3	n.a.		n.a.		B - C	n.a.
E. mixta							
Locality A	37	0.71	± 0.57	3.13	± 2.01	A - B	0.003
Locality B	28	0.70	± 0.60	2.83	± 1.83	A - C	0.046 *
Locality C	40	0.67	± 0.62	3.11	± 2.17	B - C	0.016
E. obtusa							
Locality A	23	1.08	± 0.70	4.26	± 2.61	A - B	-0.002
Locality B	68	1.11	± 0.54	4.37	± 1.77	A - C	-0.004
Locality C	13	0.98	± 0.61	3.83	± 1.94	B - C	-0.015
E. tridentata							
Locality A	31	1.30	± 0.40	6.17	± 2.56	A - B	0.008
Locality B	47	1.36	± 0.66	6.70	± 5.31	A - C	-0.002
Locality C	59	1.53	± 0.63	8.51	± 5.33	B - C	-0.004
E. variabilis							
Locality A	26	1.73	± 0.23	7.67	± 1.75	A - B	n.a.
Locality B	9	n.a.		n.a.		A - C	0.015
Locality C	25	1.54	± 0.18	6.83	± 1.17	B - C	n.a.
E. viridissima							
Locality A	34	1.59	± 0.66	8.00	± 4.20	A - B	n.a.
Locality B	8	n.a.		n.a.		A - C	0.016
Locality C	36	1.55	± 0.51	7.49	± 3.14	B - C	n.a.
E. aff. viridissima							
Locality A	36	1.79	± 0.47	8.48	± 2.81	A - B	0.014
Locality B	43	1.80	± 0.47	8.60	± 3.75	A - C	0.008
Locality C	20	1.50	± 0.44	6.50	± 2.66	B - C	0.049 **

Pairwise F_{st} values between localities in each of the eight species were generally low (-0.015 > F_{st} < 0.016) but were found to be significant in two out of 16 comparisons (again, we excluded localities in which a species had a sample size below 10). The *E. aff. viridissima* population of locality B was found to be significantly different from the population of locality C (F_{st}=0.049, p=0.0013), and the *E. mixta* population of locality A was different from the population at locality C (F_{st}=0.046, p=0.017).
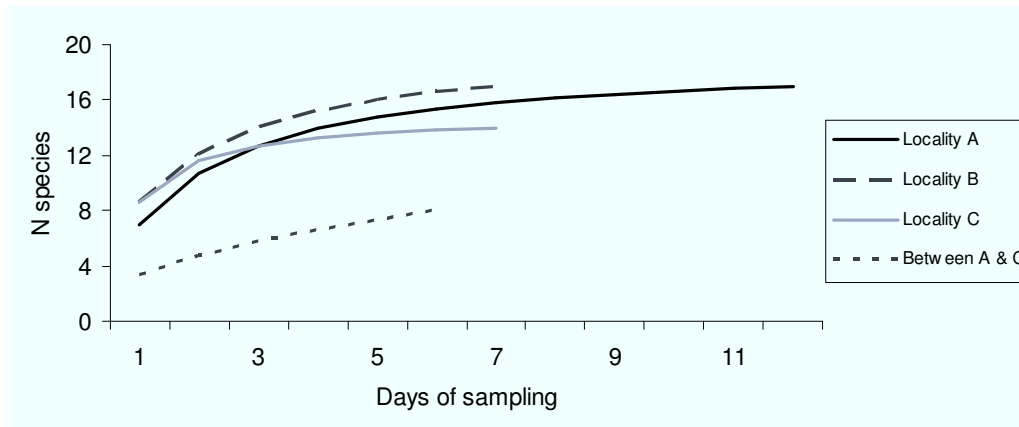
Figure 3 Species accumulation curves illustrate the expected number of species (species richness) in a forest locality (A, B & C), respectively the farmland area between A and C per days of sampling.

Whereas there was little indication of population genetic substructure, the species communities of the different localities were clearly not identical. *Euglossa* was the most common genus in the three forest localities (A, B and C) as well as in the farmland area in between locality A and C (12 of 20 attracted species). The remaining species belonged to the genera *Eulaema* (2 sp.), *Eufriesea* (4 sp.), and *Exaerete* (2 sp.). Species accumulation curves (**Figure 3**) showed little difference in the species diversity of the three forest localities, saturating after approximately seven days of sampling at 14 to17 species. In contrast, baiting in the farmland area between A and C yielded a total of eight different species in six sampling days. Remarkably, the relative species contribution to the local community varied strongly among forest localities, and even more so between forests and farmland. Across all four localities/areas there was a significant difference in relative *Euglossa* species frequency (Chi-square Test for Independence: N=1639, Chi^2=1402.9, p<0.0001; see **Figure 4**). Whereas *E. cyanura* was highly represented in locality A, it was much less abundant in locality C and nearly absent in locality B.

E. tridentata clearly dominated in locality C, whereas in locality B the species *E. imperialis*, *E. obtusa* and *E. tridentata* had an equally high abundance. In the farmland region between A and C bait captures were heavily dominated by males of the two sibling species *E. aff. viridissima* and *E. viridissima*, and only two other *Euglossa* species (*E. tridentata and E. obtusa*) appeared there as well, but in very low numbers.
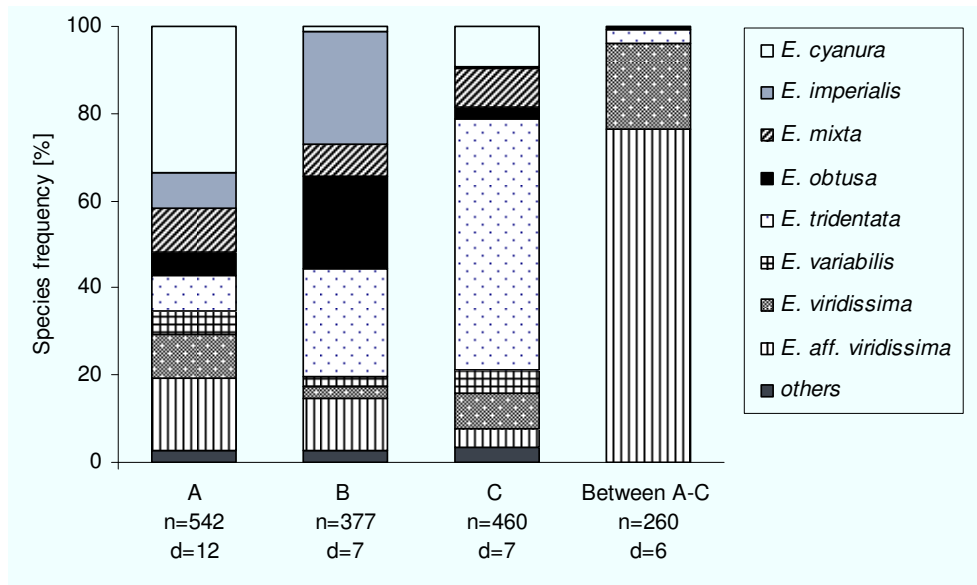
Figure 4 Species composition of male *Euglossa* bait captures in percent of individuals in the three forest localities (A, B & C) and the farmland area in between A and C. Numbers of individuals (n) and days of sampling (d) are given for each locality.

Discussion

Genetic distance study

The original vegetation of tropical dry forest of the Yucatán peninsula was heavily degraded or cleared during the Sisal era in the late 1960s, leaving mostly secondary forests today (Turner et al. 2001). The actual landscape is a mosaic of patches that alternate between traditional agriculture and deciduous secondary forests of low stature (Hartter et al. 2008), but generally lacks large areas without forested vegetation. This landscape seems to represent a suitable habitat for *E. aff. viridissima*, supporting high population densities and providing substantial opportunity for dispersal and gene

flow. High population densities of *E. aff. viridissima* on the Yucatan peninsula are also evident from broad scale baiting assays, which sometimes yield more than three hundred males in a single locality on a single morning (J. Ramirez-Pech pers. com., T. Eltz pers. obs). Such high numbers for a single species are unusual for euglossine bees, even in Panamanian or Costa Rican lowlands which are well known for their rich euglossine communities. Large Yucatecan population sizes are also suggested by our genetic data, especially by the high allelic diversity and the substantial genetic variability within localities combined with a general lack of genetic differentiation within the Yucatán peninsula. These genetic patterns suggest that *E. aff. viridissima* occurs in a large, highly interconnected metapopulation on the Yucatan peninsula, with substantial gene flow across distances of several hundreds of kilometers. The moderate genetic differentiation between populations of the Yucatán peninsula and those in Veracruz is in agreement with this view. Some of that differentiation, might be due to isolation by distance (Hardy & Vekemans 1999). However, gene flow between Yucatán and Veracruz is probably also restricted by habitat barriers south of the Mexican gulf. This area is covered by mangrove forest along the coast (southwestern Campeche) and moist seasonal tropical forest in the south, possibly forming a belt of suboptimal habitat contrasting with the drier north of the Yucatán peninsula (Vester et al. 2007). Baiting assays in coastal southwestern Campeche yielded substantially lower numbers of male *E. aff. viridissima* compared to the rest of the Yucatán peninsula, and baiting in moist forests of northern Chiapas failed to lure *E. aff. viridissima* at all (T. Eltz pers. obs.). Abundances increased markedly only further to the west, in the state of Veracruz, where this species was found to be common around settlements and in forest/farmland mosaic. The two analyzed populations of Veracruz showed high genetic variability and only marginal genetic differentiation despite their relatively large distance from each other, probably due to a continuous availability of suitable habitat in between them.

The genetic make-up of the *E. aff. viridissima* population in Florida bears signs of a recent bottle neck, as would be expected from a population that was established only very recently, and possibly from only a single nest. Such an establishment of a new population from a small number of individuals that are reproductively isolated from the main population can result in a loss of alleles and reduced genetic diversity (Frankham et al. 2002). The microsatellite genotypes of Floridan *E. aff. viridissima* are of lower

genetic variability, but also composed of rather different alleles than those of all Mexican populations. This indicates that the founding individuals came from a geographic area that was not included in our study. Further sampling and genotyping of bees from more southern localities, such as Honduras and Costa Rica (the southern most part of the native range), is necessary to resolve this issue.

Along with data from other studies (Liu & Pemberton 2009, Pemberton 2007, Pemberton & Liu 2008, Pemberton & Wheeler 2006, Skov & Wiley 2005), our findings confirm *E. aff. viridissima* as a species that is well adapted to anthropogenically altered habitats and largely independent of old-growth forest. This it has in common with its sibling species, *E. viridissima*, which is the only other *Euglossa* species common on the Yucatán peninsula (Eltz et al 2008). Nests of both species can be found in human-made cavities around houses and gardens in suburban and even urban settings (Cocom Pech et al. 2008)(T. Eltz & Y. Zimmermann & R. Pemberton pers. obs.). In their tolerance of, or even preference for, anthropogenically disturbed habitat the two species are unusual amongst euglossine species, many of which are believed to be highly dependent on mature forest (Dressler 1982, Roubik & Hanson 2004). It is for such differences among species in ecological requirements that our fragmentation study aimed to include a range of different species

Fragmentation study

Different species of *Euglossa* are thought to have different habitat requirements, possibly based on differences in their choice of nectar and pollen plants, volatile sources or nesting sites (Dressler 1982, Ramirez et al. 2002, Roubik & Hanson 2004). Previous baiting studies in Brazil revealed that species differ in their tendency of traveling out of forest habitat (Milet-Pinheiro & Schlindwein 2005), suggesting different dispersal potential in fragmented landscapes. Accordingly, we expected differential fragmentation impact on genetic population structure in the eight *Euglossa* species that we had chosen for our study. Specifically, we expected no or weak genetic structuring in *E. viridissima* and *E. aff. viridissima*, which commonly occur in disturbed habitats (see above), and more pronounced structure in some of the rarer, presumably more forest dependent species. The baiting results from the intermittent farmland area confirmed the expected ecological dichotomy. Only *E. aff. viridissima* and *E. viridissima* were attracted   in

substantial numbers in open farmland, whereas captures of other species, if they occurred at all, were rare exceptions. However, patterns of genetic differentiation did not correspond to these baiting results. First of all, population genetic structure between localities was very low or absent in all eight study species. F_{st} values were generally below 0.05, and only two out of 16 pairwise comparisons yielded significant population differences in allele frequencies.

In our set up, the Los Tuxtlas Reserve (locality A) represented an isolated forest fragment which is surrounded by >100 km of farmland with only tiny patches of interspersed forest-like vegetation. Two rather different causes might explain the overall absence of genetic differentiation of the Los Tuxtlas bees. First, it is possible that euglossine bees, even true forest species, are occasionally capable of crossing large areas of inhospitable landscape, leading to occasional gene flow between widely separated forest islands. Long-distance dispersal seems particularly probable in euglossine bees given that they are strong fliers with already very large foraging ranges (Dressler 1968b, Janzen 1971) and, at least in males, a tendency to invade non-habitat areas in search of chemical resources (Milet-Pinheiro & Schlindwein 2005). It remains unclear to what extent small interspersed patches of woody vegetation can be used by bees as stepping stones between larger forest areas. Such patches, which are likely insufficient in size and habitat quality to support bee populations on their own, could provide shelter or nectar food for dispersing individuals. In summary, it is possible that even the large area of non-forest habitat between Los Tuxtlas and the other two localities did not function as an efficient barrier for gene flow.

An alternative explanation for the absence of population structure is the limited amount of time that was available for isolation to take genetic effect. The separation of the Los Tuxtlas fragment from other remaining forests was not an immediate event, but progressed continuously over the 20th century, with substantial isolation beginning approximately in the 1960ies. Thus, fragmentation had only $\sim$ 50 years time, possibly less, to affect gene flow among orchid bee populations. This may have been insufficient time to result in a reduction of allelic diversity or shift in allelic composition among populations, even if no long-distance dispersal occurred (Saunders et al. 1991). Individual orchid bee life time spans of up to six months coupled with long generation

times and relatively stable population sizes (Roubik & Ackerman 1987) might considerably delay population genetic effects in isolated populations of orchid bees. At present we cannot distinguish between the two alternative explanations. We can conclude, however, that forest fragmentation on substantial spatial and temporal scales did not result in measurable population genetic effects in the genus *Euglossa*. A replication of this study in other fragments and covering even longer time-spans of forest fragmentation is surely of interest and might yield different results. Overall, our results are in agreement with earlier findings that some euglossine bees fare reasonably well in disturbed habitat and forest edges (Brosi 2009, Otero et al. 2008, Rincon et al. 1999). Furthermore, our results agree with previous observations that orchid bees have weak population structure (Dick et al. 2004) and low production of diploid males (Souza et al. in press). The last issue is particularly noteworthy since high numbers of diploid males have been claimed by some allozyme-based studies, interpreting this as a sign for inbreeding and genetic degeneration of euglossine populations (Lopez-Uribe et al. 2007, Roubik et al. 1996, Zayed et al. 2004). In the present study we genotyped 1005 individual males with highly variable markers and found only one diploid (heterozygous) male. This is clearly within the range normally expected for non-inbred haplodiploid hymenopterans (Cook 1993).

The remarkable differences of relative species contribution to bait captures in the three forest localities are puzzling, especially given the lack of genetic differentiation between populations. It might be attributed to spatiotemporal differences in food, chemical, or nesting resource availability, which might lead to differences in abundance of species at the different localities, at least temporarily. Shifts in the numerical composition of euglossine species in baiting studies is commonly found also on smaller spatial scales and across seasons (Ackerman 1989, Armbruster 1993, Janzen et al. 1982, Pearson & Dressler 1985), suggesting that our baiting results might represent snap shots of dynamic communities.

Male orchid bees deserve special attention with regard to the discussion of gene flow. In their search for volatiles they are believed to cover large areas of habitat and may even lead a nomadic lifestyle. While the latter is perhaps unlikely (Ackerman & Montalvo 1985), long-distance travel of males is suggested by direct and indirect evidence. Most

importantly, males can be lured to artificial chemicals over long stretches of uninhabitable area, including open water and non forest areas (Ackerman 1981, Raw 1989, Tonhasca Jr et al. 2003). This suggests that their function as long distance pollinators (Ackerman et al. 1982, Janzen 1971, Kroodsma 1975) is not necessarily precluded by fragmentation and that some gene flow of euglossophilous plants can be expected between forest fragments (Tonhasca Jr et al. 2003). Furthermore, volatile-driven male dispersal might be of particular importance for long-distance gene flow. So far, there is no evidence for male-biased gene flow in orchid bees, but this is clearly a promising avenue for future research. In contrast to males, females are nest-based central-place foragers and seem often philopatric with respect to their natal nest. E.g., the adult females of *E. viridissima* that were found in a given nest box in Yucatan were almost always close relatives (Cocom Pech et al. 2008, Zimmermann et al. 2009b), suggesting that hatched females remain in and reactivate the nest from which they have hatched. Thus, it is quite likely that individual females are less apt to long-distance dispersal than males.

While not providing evidence for, our results are in overall agreement with the idea that biodiversity benefits from the conservation of even small fragments of tropical rainforest (Turner & Corlett 1996). Fragmentation as a breaking apart from formerly continuous forest may not be equalized to habitat loss, since negative effects for populations still depend on the fragments' quality, the degree of isolation, and the edge area ratio (Fahrig 2003, Tscharntke et al. 2002). Forest fragments can act as last refugees for plant and animal species (Turner & Corlett 1996), and the preservation of orchid bee populations acting as effective pollinators would surely have a positive influence on the plant species diversity.

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Conservation genetics of Neotropical pollinators revisited: microsatellite analysis demonstrates that diploid males are rare in orchid bees

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Conservation genetics of Neotropical pollinators revisited: microsatellite analysis demonstrates that diploid males are rare in orchid bees

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Running Title: Low diploid male production in orchid bees

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ABSTRACT

Allozyme analyses have suggested that Neotropical orchid bee (Euglossini) pollinators are in decline because of putative high frequencies of diploid males, a result of loss of sex allele diversity in small Hymenopteran populations with single locus complementary sex determination. Our analysis of 1010 males from 27 species of euglossine bees sampled across the Neotropics at 2-11 polymorphic microsatellite loci revealed only 5 diploid males at an overall frequency of 0.005 (95% CIs 0.002-0.010); errors through genetic non-detection of diploid males were likely small. In contrast to allozyme-based studies, we detected very weak or insignificant population genetic structure, even for a pair of

populations >500 km apart, possibly accounting for low diploid male frequencies. Technical flaws in previous allozyme-based analyses have probably led to considerable overestimation of diploid male production in orchid bees. Habitat destruction and other environmental factors may have a more immediate impact on population persistence than the genetic load imposed by diploid males on these important Neotropical pollinators.

INTRODUCTION

Single locus complementary sex determination (slCSD), in which homozygosity at the sex locus leads to the production of effectively sterile diploid (2N) males, is thought to be ancestral to the haplodiploid Hymenoptera and widespread within the order (van Wilgenburg et al. 2006; cf. Cowan & Stahlhut 2004; de Boer et al. 2007, 2008; Heimpel & de Boer 2008). The frequency of 2N males theoretically increases with inbreeding, small population size and reduced gene flow due to lack of allelic diversity at the sex locus (Cook 1993; Cook & Crozier 1995; van Wilgenburg et al. 2006). slCSD may itself lead to lower effective population size (N_e) compared to diploidy (Zayed 2004).

All bees appear to be slCSD haplodiploids (van Wilgenburg et al. 2006; Zayed 2009) and there is growing evidence for their widespread decline (Brown & Paxton 2009); unequivocal evidence is seen in solitary bees in England and the Netherlands (Biesmeijer et al. 2006), bumble bees in Ireland (Fitzpatrick et al. 2007) and honey bees (*Apis mellifera*) in the USA (Oldroyd 2007; vanEngelsdorp et al. 2009). This is cause for concern because bees are important pollinators in natural and agro-ecosystems (Klein et al. 2007). Pollination is an important ecosystem service that is being degraded by anthropogenic changes (Kremen et al. 2002; Steffan-Dewenter et al. 2005), including habitat destruction, pollution and facilitation of invasive species (Mooney et al. 2005). Degradation of habitat may result in a loss of genetic diversity, so the frequency of 2N males has been proposed to be a sensitive measure of pollinator decline for bees (Zayed et al. 2004). From a study of 2N male frequencies in Euglossine bee populations, Zayed and Packer (2005) concluded that diploid males exert a high genetic load on populations, which could potentially drive a genetic extinction vortex in slCSD haplodiploids.

The Euglossini comprise ca. 200 species of Neotropical bees that are the sole pollinators of around 700 orchid species (Dressler 1982; Cameron 2004; Roubik & Hanson 2004). Males collect perfumes from orchid blossoms and other sources in their hind tibiae and later release them at mating sites, possibly to attract females (Eltz et al. 2005, 2007). To date the conservation genetics of orchid bees has relied on the use of allozymes as genetic markers to study 2N male frequency and determine ploidy (a male heterozygous at one or more loci is a 2N male). An early study of seven Panamanian orchid bee species suggested that 2N males comprised 12-100% of males per species (Roubik et al. 1996). In contrast, Takahashi et al. (2001) found very low (mean 0-2% per species) frequencies of 2N males in 14 Brazilian species. Zayed et al. (2004) subsequently detected 13-56% (across populations) of Panamanian *Euglossa imperialis* males to be diploid and inferred extremely limited gene flow and low N_e in the species, supporting Roubik et al.'s (1996) view that orchid bees suffered a heavy genetic load due to high 2N male frequencies and low diversity at the sex locus. More recently, López-Uribe et al. (2007) also found high 2N male frequencies in five Colombian orchid bee species; across species, 8-32% of males were estimated to be diploid. Though all these studies employed substantial sample sizes (n = 142-695 males per study), confidence intervals of 2N male frequencies were large due to the low variability of allozymes, the only polymorphic markers then available for orchid bee population genetics.

The notion that orchid bees suffer high 2N male production is at odds with other aspects of the taxon's biology. For example, males of many species are common at chemical baits and hence are employed in Neotropical biodiversity inventorying (e.g. Brosi 2009) whilst both sexes are thought to be extremely mobile (Jansen 1971, 1981; Dressler 1982; Cameron 2004). This contradiction between biological observations and allozyme-based genetic analysis prompted our re-assessment of 2N male frequency and gene flow in orchid bees. Using three suites of recently developed microsatellite markers, we genotyped 1010 males from 27 species of euglossine bees, each at 2-11 polymorphic loci, sampled from across the Neotropics and including *Eg. imperialis* from Panama, to reveal extremely low (0.5%) frequencies of 2N males and very weak population genetic structure even across 500 km.

Figure 1 Map of the Neotropics with the 22 sampling sites highlighted as dots (five adjacent localities in Panama are given one dot).

MATERIAL AND METHODS

In Brazil and Colombia, 483 males from 23 species were collected across multiple years at odor baits (1,8-cineole, skatole and vanillin) at 14 sites in seven Brazilian states and one site in Colombia (Table 1, Fig. 1). These included 143 males already genotyped using allozymes and reported by Takahashi et al. (2001). In Panama, 257 males from three species were collected at odor baits; *Eg. imperialis* was collected from three sites across March-May 2005, *Eg. tridentata* from two sites across 16 days in March-April 2006 (both at 1,8-cineole baits) and *Euglossa hemichlora* from one site in September 2007 (at p-dimethoxybenzene baits, Fig. 1). In Mexico, 73 *Euglossa aff. viridissima* males (the lineage with three mandibular teeth, 3D, to be described as a new species; Eltz et al. unpublished manuscript) and 57 *Euglossa viridissima* males (the lineage with two mandibular teeth, 2D; see Eltz et al. 2008) were collected at odor baits (p-dimethoxybenzene) from one site in March 2006 and May 2007. Finally, in Costa Rica, 140 *Eulaema bombiformis* males were collected from 19 forest fragments around Las Cruces Biological Station (maximum site separation 13.5 km) in June-September 2004, as described in Brosi (2009). Insects were stored in ethanol at -20^0C or were dried and stored at room temperature.

DNA was extracted from legs or thoraxes using a high salt protocol (Paxton et al. 1996) or a Qiagen DNeasy Blood & Tissue Kit following manufacturer's recommendations. Individuals were genotyped at 2 – 11 polymorphic microsatellite loci (male haplotypes/genotypes in Supplementary Table S1), developed for *Euglossa cordata*, *Eulaema nigrita* (Souza et al. 2007) and *Euglossa annectans* (Paxton et al. 2009); these are unlinked loci that are in Hardy Weinberg equilibrium (HWE) in the species for which they were developed (Souza et al. 2007; Paxton et al. 2009). Genotyping and scoring were performed using autosequencers in three different laboratories (Megabace 750, ABI 310 or ABI 3100) and Genotyper or GeneMarker V1.71 software with internal size standards. All trace files were inspected by eye to check for potential allele mis-calling due, for example, to stutter. Approximately 5% of individuals were re-amplified and alleles scored using the same autosequencer or they were genotyped in a fourth laboratory by radio-labeling and resolving on manual sequencing gels (methods in Paxton et al. 1996). Allele calling across these duplicate analyses of the same individual-

locus combination were identical. We therefore estimate extremely low genotyping error rates.

Non-detection of 2N males may arise if genetic markers exhibit low allelic diversity (low heterozygosity). To compensate for genetic non-detection, we calculated the resolving power of our markers, namely the probability that a diploid individual was heterozygous at one or more loci, P_{het}, as:

$$1 - \prod_{j=1}^{L} \sum_{i=1}^{N} \left(x_i^2 \right),$$

where summation is across the N alleles at a locus and multiplication is across L loci. This assumes Hardy-Weinberg equilibrium (HWE), though moderate levels of inbreeding have only a slight effect on P_{het} (e.g. see Paxton et al. 2000). In estimating allelic frequencies, males carrying only one allele at all loci were considered haploid, which is a close approximation given the high allelic diversity of the loci and therefore the high probability that a diploid male is heterozygous at one or more loci (Supplementary Tables S1 and S2). In addition, microsatellite analysis of four of our study species has not revealed any deviation from HWE (*Eg. annectans* in Paxton et al. 2009; *Eg. cordata* and *El. nigrita* in Souza et al. 2007; and *Eg. viridissima* in Zimmermann et al. 2009), suggesting random mating in orchid bees.

Null alleles can nevertheless cause difficulties in microsatellite allele scoring and lead to an overestimation of P_{het}. To account for putative null alleles, we assumed that a male lacking an allele at a locus was caused by a null allele, and we reduced allelic diversity (H_{ina}) and P_{het} at that locus accordingly (Supplementary Table S1). We also analyzed females from 7 of the 27 species at the same loci as males of the respective species (Supplementary Table S2). As female euglossines are not attracted to odor baits and are therefore far more difficult to sample than males, we did not have access to females of the other 20 species. Of the 7 species with females, n >20 females for 5 species. Their

genotypes were tested for the presence of null alleles using MICRO-CHECKER (van Oosterhout et al. 2004) and we reduced allelic diversity (H_{ina} or expected heterozygosity accounting for null alleles) and P_{het} for the three loci showing evidence of null alleles using equation (4) of Brookfield (1996; see Supplementary Table S2). For the other loci, we calculated expected allelic diversity (H_{ina} or expected heterozygosity) from female genotypes using GENEPOP (Raymond & Rousset 1995). We conservatively used the lowest estimates of H_{ina} and P_{het} derived from males or females for each species-locus combination. Binomial 95% confidence intervals (2-tailed) of the proportion of diploid males were calculated using J.C. Pezzullo's Interactive Stats javascript (http://statpages.org/confint.html).

Four species were collected at two or more sites spanning 4 – 538 km: *Eg. cordata* (2 sites), *Eg. imperialis* (3 sites), *Eg. tridentata* (2 sites) and *Eufriesea violacea* (2 sites; see Table 2) and genotyped in the same lab. For each population pair, we computed estimates of genetic differentiation to infer population connectivity. Both F_{ST} and Hedrick's (2005) unbiased estimator of population differentiation, G_{ST}', were calculated with MSA v. 4.05 (Dieringer & Schlötterer 2003) using the male data set as MSA can simultaneously handle both haploid and diploid data. The significance of differentiation measures was determined using an exact test with 1000 permutations in MSA.

RESULTS

Allelic diversity accounting for null alleles (expected heterozygosity) of our loci, H_{ina}, ranged from 0.02 – 0.96 (Table 1). It was generally above 0.5 for most loci in most species (Supplementary Tables S1 and S2) and averaged 0.62 (Table 1). H_{ina} differed little between males and females; it was greater by 0.044 in males versus females (n=5 species and n=26 locus-species combinations), suggesting that our estimates of P_{het} in species for which we did not sample females are only slightly inflated. Using 2 – 11 loci per species gave an average P_{het} of 0.991 (range 0.948 – >0.999), sufficient resolving power to detect the majority of diploid males as heterozygotes at one or more loci.

Table 1 Species name, collection site, number of males sampled (n males), number of polymorphic loci used (n loci), range of expected intralocus allelic diversity (H_{ina}, adjusted for putative null alleles; see Supplementary Tables S1 and S2), mean allelic diversity across loci (H_{exp}, adjusted for putative null alleles), probability of detecting a heterozygous male if diploid (P_{het}), observed number of diploid (2N) males and 95% binomial confidence intervals of the observed frequency of 2N males in 27 orchid bee species from Brazil, Colombia, Costa Rica, Mexico and Panama. See Fig. 1 for sampling locations; Brazilian state codes are: Amazonas – AM; Espírito Santo – ES; Minas Gerais – MG; Mato Grosso – MT; Paraíba – PB; Rio de Janeiro – RJ; and São Paulo – SP.

Species	Collection Site	n males	n loci	H_{ina}	H_{exp}	P_{het}	2N males	95% CIs of 2N frequency
Euglossa annectans	São Carlos - SP, Brazil	17 *	6	0.17 – 0.75	0.48	0.988	1 *	0.002 – 0.288
Eg. chalybeata	Manaus - AM, Brazil	19	6	0.28 – 0.72	0.6	0.998	0	
Eg. cognata	Villavicencio, Colombia	1	9 [b]				0	
Eg. cordata	Caraguatatuba - SP, Brazil	37 *	8	0.30 – 0.88	0.63	>0.999	0	
	São Carlos - SP, Brazil	30 *						
Eg. fimbriata	São Carlos - SP, Brazil	7 *	8	0.25 – 0.86	0.56	>0.999	0	
Eg. hemichlora	Santa Rita, Panama	43	3	0.62 – 0.84	0.75	0.987	0	
Eg. imperialis	Manaus - AM, Brazil	30	6	0.44 – 0.81	0.67	>0.999	0	
	Barro Colorado, Panama	47					0	
	Fort Clayton, Panama	23	5	0.02 – 0.83	0.45	0.983	0	
	Gigante Peninsula, Panama	28						
Eg. intersecta	Manaus - AM, Brazil	1	6 [b]				0	
Eg. mandibularis	Viçosa - MG, Brazil	95 * [a]	8	0.08 – 0.87	0.56	>0.999	1 *	0 – 0.057
Eg. melanotricha	Analândia - SP, Brazil	8 *	9	0.38 – 0.88	0.66	>0.999	0	
Eg. mixta	Villavicencio, Colombia	3	5	0.44 – 0.67	0.49	0.968	0	
Eg. moure	Manaus - AM, Brazil	1	7 [b]				0	
Eg. pleosticta	São Carlos - SP, Brazil	4 *	9	0.38 – 0.75	0.63	>0.999	0	
Eg. securigera	Camburí - SP, Brazil	2	9	0.22 – 0.78	0.57	>0.999	0	
	Rifaina - SP, Brazil	3						
	São Carlos - SP, Brazil	3 *						
Eg. townsendi	Araras - SP, Brazil	3	8	0.38 – 0.75	0.56	0.999	0	
	Rifaina - SP, Brazil	1						
Eg. tridentata	Barro Colorado, Panama	60	2	0.67 – 0.89	0.78	0.964	1	0 – 0.049
	Parque Natur. Metro., Panama	56						
Eg. truncata	São Carlos - SP, Brazil	10 *	7	0.42 – 0.78	0.65	>0.999	0	
Eg. viridis	Villavicencio, Colombia	1	9 [b]				0	
Eg. aff viridissima 3D [c]	Xmatkuil, Mexico	73	2	0.85 – 0.89	0.87	0.984	0	
Eg. viridissima 2D [d]	Xmatkuil, Mexico	57	2	0.59 – 0.87	0.73	0.948	0	
Eulaema bombiformis	Manaus - AM, Brazil	21	11	0.58 – 0.89	0.79	>0.999	0	
	Las Cruces, Costa Rica	140	9	0.16 – 0.61	0.34	0.981	2	0 – 0.051
El cingulata	Manaus - AM, Brazil	8	7	0.47 – 0.81	0.63	>0.999	0	
El meriana	Manaus - AM, Brazil	26	10	0.27 – 0.89	0.69	>0.999	0	
El nigrita	Cuiabá - MT, Brazil	4	11	0.61 – 0.91	0.77	>0.999	0	
	Manaus - AM, Brazil	4						
	Marliéria - ES, Brazil	5						
	Mimoso - MG, Brazil	4						
	Poconé - MT, Brazil	3 *						
	Rifaina - SP, Brazil	5						
	S. J. Campos - SP, Brazil	5						
	São Carlos - SP, Brazil	5 *						
	Viçosa - MG, Brazil	5						
Eufriesea violacea	São Carlos - SP, Brazil	16	10	0.37 – 0.85	0.59	>0.999	0	
	Viçosa - MG, Brazil	37						
Exaerete frontalis	João Pessoa - PB, Brazil	8	3	0.66 – 0.78	0.74	0.983	0	
Ex. smaragdina	João Pessoa - PB, Brazil	50	3	0.79 – 0.83	0.81	0.993	0	
	São Carlos - SP, Brazil	1						
GRAND TOTAL		**1010**		**0.02 – 0.91**	**0.62**	**0.991**	**5**	**0.002 – 0.010**

* the same samples as analyzed by Takahashi et al. (2001);
[a] n = 76 new samples added in addition to those of Takahashi et al. (2001);
[b] for n = 1 male analyzed, n loci = number of loci employed (see Supplementary Table S1);
[c] all males from the species with three mandibular teeth, 3D (see Eltz et al. 2008), to be described as a new species (Eltz et al. Unpublished manuscript)
[d] all males from the species with two mandibular teeth, 2D (see Eltz et al. 2008).

We detected five heterozygotes among the 1010 males that we genotyped, one each in *Eg. annectans*, *Eg. mandibularis*, and *Eg. tridentata*, and two in *El. bombiformis* (Table 1). The *Eg. mandibularis* male heterozygous at microsatellite locus Egc24 (Supplementary Table S1) was the same individual that Takahashi et al. (2001) also detected by allozyme analysis as a heterozygote. We additionally detected one heterozygous *Eg. annectans* male (heterozygous at loci Egc18 and Egc24; see Supplementary Table S1) that Takahashi et al. (2001) found to be homozygous by allozyme analysis. Over all males, and accounting for genetic non-detection errors (i.e. where P_{het} <1), diploid male frequency averaged 0.005 (95% CI's 0.002-0.010).

Population differentiation in orchid bees was generally small and non-significant (Table 2), suggesting considerable gene flow. For the *Eg. imperialis* dataset comprising three Panamanian populations 4 – 34 km apart, global F_{ST} = 0.001 (P = 0.384) and G_{ST}' = 0.034 (P = 0.786). Pair-wise measures of *Eg. imperialis* population differentiation were similarly not significantly different from zero (Table 2). The two *Eg. tridentata* populations separated by 36 km were also not significantly differentiated (Table 2). The two *Eg. cordata* populations separated by 310 km showed low, though significant, estimates of F_{ST} and G_{ST}' (Table 2). In contrast, the two *Ef. violacea* populations separated by 538 km were not significantly differentiated (Table 2), suggesting considerable gene flow between them.

DISCUSSION

We found strong evidence for extremely low frequencies of diploid males among common and widespread orchid bees of the Neotropics. Our broad taxonomic sampling from across a wide geographic area lends weight to our analyses, while consistency in genotyping at four independent laboratories and low estimated frequencies of null alleles mean that the low 2N male frequencies we detected are unlikely to be a technical artifact. We found little or no population genetic structure over 10's – 100's km; these results imply high gene flow, which could explain the apparently adequate sex allele diversity in orchid bees. Both low 2N male frequency and weak population genetic structure suggest that many orchid bees have both high gene flow and high N_e, and that they do not suffer from inbreeding through genetic drift and loss of *csd* diversity.

Why is there a discrepancy between our microsatellite-based study and all but one of the earlier allozyme-based studies demonstrating high 2N male frequencies, high population viscosity and low N_e (Roubik et al. 1996; Zayed et al. 2004; López-Uribe et al. 2007)? We offer two explanations.

Firstly, high frequencies of diploid males might be site or species-specific, and our sampling may not have captured sites or orchid bee species with high 2N males revealed by earlier allozyme-based studies. However, we analyzed males from four of the seven Panamanian species reported by Roubik et al. (1996) that exhibited high 2N male frequencies (Roubik et al. 2006, their Table 1), and we included two species (*Eg. imperialis* and *Eg. tridentata*) from the same sampling sites as Roubik et al. (1996). Furthermore, we did not detect any 2N males among the 98 *Eg. imperialis* males that we analyzed (95% CI's 0-3.8%) from the same three sampling sites at which Zayed et al. (2004) found 37.7% of *Eg. imperialis* males to be 2N. It is therefore unlikely that our sampling scheme was responsible for the discrepancies between our results and those of previous studies. A caveat of our interpretation is that diploid males may be produced during a specific season of the year, a period when Roubik et al. (1996) and Zayed et al. (2004) sampled but we did not.

Table 2 Geographic distances between pairs of populations of orchid bees (males) and genetic differentiation measured as F_{ST} and Hedrick's (2005) G_{ST}', with exact *P* values (1000 permutations) from MSA (Dieringer & Schlötterer 2003). For locations, see Figure 1.

Pair of populations	n (males)	n (loci)	Distance (km)	F_{ST} (P)	G_{ST}' (P)
Euglossa cordata (Brazil) Caraguatatuba *versus* São Carlos	37 30	8	310	0.024 (0.005)	0.175 (0.037)
Euglossa imperialis (Panama) Barro Colorado *versus* Fort Clayton	47 23	5	34	0.012 (0.176)	0.014 (0.827)
Euglossa imperialis (Panama) Barro Colorado *versus* Gigante Penninsula	47 28	5	4	-0.011 (0.767)	0.096 (0.422)
Euglossa imperialis (Panama) Fort Clayton *versus* Gigante Penninsula	23 28	5	32	0.004 (0.308)	0.038 (0.710)
Euglossa tridentata (Panama) Barro Colorado *versus* Parque Natural Metropolitano	60 56	2	36	0.001 (0.354)	0.072 (0.379)
Eufriesea violacea (Brazil) São Carlos *versus* Viçosa	16 37	10	538	-0.025 (0.991)	0.074 (0.598)

Secondly, allozyme-based genotyping can suffer from allele mis-scoring, possibly due to protein instability, whereas DNA is more stable and therefore microsatellite genotyping more robust (Schlötterer 2004). This may have resulted in an artificial excess of male heterozygotes in allozyme studies; positive controls (diploid females) were generally lacking in allozyme-based studies. Our microsatellite loci detected high heterozygosity in females whenever they were available for analysis (*Eg. annectans* in Paxton et al. 2009; *Eg. cordata* and *El. nigrita* in Souza et al. 2007; *Euglossa igniventris*, T. Eltz unpublished data; *Euglossa hemichlora*, *Eg. townsendi*, *Eg. viridissima* and *Exaerete smaragdina* in Supplementary Table S2) and yet frequencies of putative null alleles, a potential cause of microsatellite allele mis-calling that may lead to an underestimate of 2N male frequency, were low. As we sampled females from only 5 of the 27 study species in sufficient number to test statistically for null alleles, we urge caution in the interpretation of our results pending analysis of females from additional species. We nevertheless conclude that allozyme-based studies of orchid bees are probably methodologically flawed due to allele mis-scoring, and that this flaw accounts for the differences between allozyme-based studies and our microsatellite-based study. More direct methods of assessing diploid male frequencies and including analysis of females, for example by karyotype analysis (Eltz et al. 1998) or genome size estimation by flow cytometry (Aron et al. 2005; Cournault & Aron 2009), are needed to support our microsatellite-based conclusions.

Our interpretation of orchid bee population genetics, that they have low 2N male production, very weak population structure, high gene flow and high N_e, fits with many independent observation of the taxon. For example, individual orchid bees have been reported to travel long distances (> 20 km; Janzen 1971). Also, other orchid bee species are common faunal elements in natural and disturbed habitats (Brosi 2009) and even in urban centers (López-Uribe et al. 2008). Census data suggest that orchid bee abundance and diversity appear to have been maintained (Roubik 2001), even within the highly fragmented Atlantic rainforest of Brazil (Tonhasca et al. 2002). These behavioral and genetic lines of evidence support the view that orchid bee populations are large, weakly structured and unlikely to suffer from inbreeding through loss of sex allele diversity.

Clearly, orchid bees may not be an informative test case of the idea that 2N male frequencies are a sensitive measure of bee pollinator decline (Zayed et al. 2004) as they seem to exhibit high mobility and high allelic diversity at the sex locus. For other bees, inbreeding is not necessarily associated with high frequencies of 2N males as detected by microsatellites (Paxton et al. 2000). Also, severely bottlenecked populations of the sweat bee *Lasioglossum leucozonium* with high 2N male frequencies detected by microsatellite genotyping have recently expanded across Eastern USA (Zayed et al. 2007), suggesting that high 2N male frequencies are not necessarily correlated with population decline in this invasive species. Yet for the honey bee (*Apis mellifera*) with a well-characterized system of sex determination based on slCSD (Beye et al. 2003), high frequencies of 2N males have a catastrophic effect on colony survival (Woyke 1980), as in other social bees (Plowright & Pallett 1979; Carvalho 2001) and ants (Ross & Fletcher 1986). An appropriate test of the diploid male extinction vortex (Zayed & Packer 2005) and the idea that the frequency of 2N males is a sensitive measure of pollinator decline (Zayed et al. 2004) awaits analysis of slCSD populations at their range margins or of those that have been anthropogenically compromised. Eusocial Hymenoptera such as bumble bees (e.g. Takahashi et al. 2008) may be more suitable subjects for such a test than the largely solitary and subsocial orchid bees (cf. Cocom Pech et al. 2008) because hymenopteran eusociality is associated with reduced genetic diversity and low N_e (Pamilo et al. 1978, 1997; Graur 1985; Hedrick & Parker 1997; Chapman & Bourke 2001; Packer & Owen 2001).

Though bees are thought to possess slCSD (van Wilgenburg et al. 2006), another hymenopteran has recently been shown to possess multilocus CSD (mlCSD; de Boer et al. 2008) and diploid males in hymenopterans with regular inbreeding produce fertile diploid males (de Boer et al. 2007; Cournault & Aron 2009); in one wasp with regular inbreeding, diploid males may themselves produce haploid sperm (Cowan & Stahlhut 2004). The presence of occasional diploid males in otherwise haploid-male orchid bees indicates that the taxon possesses CSD. The low frequency of 2N males that we observed may be a consequence of mlCSD.

Our sampling of 26 orchid bee species from across a wide geographic range and habitat types (coastal Atlantic forest, cerrado open woodland, Amazonian tropical forest),

including sites with old-growth vegetation (Barro Colorado Island) and others with highly disturbed vegetation (e.g. São Carlos; Soares *et al.* 2003), allow us to draw conclusions concerning the conservation genetics of this taxon. Firstly, orchid bees currently appear to have extremely low frequencies of 2N males, suggesting that continental populations are probably not prone to the diploid male extinction vortex (Zayed & Packer 2005), possibly because of high gene flow maintaining adequate allelic diversity at the sex locus. Secondly, they appear to be highly mobile, again increasing N_e beyond those predicted from estimates of census size at one point in time and space. Nevertheless, we urge caution in the generalization of our results. Morphological similarity among orchid bees (Roubik & Hanson 2004; Eltz et al. 2008) may hide cryptic species diversity, and rare species or isolated populations at range margins may yet be found to suffer the genetic load of high diploid male production. For common species, however, ongoing habitat destruction is likely to have a much larger impact on population demography and persistence than the genetic load imposed by diploid males.

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Supplementary data is available on CD, attached to this dissertation

The authors' contribution

Chapter 2

Chemical niche differentiation among sympatric species of orchid bees

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Ecology (2009) 90: 2994-3008

- Sampling and data collection in the field
- Chemical analysis by gas chromatography/mass spectrometry
- Chemical data processing and multivariate analysis of fragrance similarity

Chapter 3

An olfactory shift is associated with male perfume differentiation and species divergence in orchid bees

T. Eltz, Y. Zimmermann, C. Pfeiffer, J. Ramirez Pech, R. Twele, W. Francke, J. J. G. Quezada-Euan & K. Lunau

Current Biology (2008) 18: 1844-1848

- Sampling and data collection in the field together with T. Eltz and J. Ramirez Pech
- Chemical analysis by gas chromatography/mass spectrometry
- Microsatellite genotyping and statistics on genetic differentiation

Chapter 4

Characterisation of the orchid bee *Euglossa viridissima* and its cryptic sibling species, *Euglossa dilemma* sp. nov., by morphological, chemical, and genetic characters

T. Eltz, F. Fritzsch, J. Ramirez Pech, Y. Zimmermann, S. R. Ramírez, J. J. G. Quezada-Euan & B. Bembé

Manuscript in preperation

- Microsatellite genotyping
- Chemical analysis by gas chromatography/mass spectrometry
- DNA sequencing and phylogenetic analysis together with S. R. Ramírez

Chapter 5

Single mating in orchid bees (*Euglossa*, Apinae): implications for mate choice and social evolution

Y. Zimmermann, D. W. Roubik, J. J. G. Quezada-Euan, R. J. Paxton & T. Eltz

Insectes Sociaux (2009) 56: 241-249

- Sampling and data collection in the field
- Microsatellite genotyping
- Paternity and kinship statistics
- Writing the manuscript

Chapter 6

Population genetic structure of orchid bees (Euglossini) in anthropogenically altered landscapes

Y. Zimmermann, D. L. P. Schorkopf, R. F. A. Moritz & T. Eltz

Manuscript submitted to *Molecular Ecology*

- Sampling and data collection in the field together with T. Eltz and D. L. P. Schorkopf
- Microsatellite genotyping
- statistical analysis
- Writing the manuscript

Chapter 7

Conservation genetics of Neotropical pollinators revisited: microsatellite analysis demonstrates that diploid males are rare in orchid bees

R. O. Souza, M. A. Del Lama, M. Cervini, N. Mortari, T. Eltz, Y. Zimmermann, C. Bach, B. J. Brosi, S. Suni, J. J. G. Quezada-Euan, R. J. Paxton

Manuscript under review in *Evolution*

- Microsatellite genotyping of three of the included *Euglossa* species

9

Synthesis

The orchid bees have a highly unusual mating biology of which several aspects are still poorly understood. Males collect exogenous volatiles from numerous natural sources in an ambitious and exhausting manner (Dressler 1982, Vogel 1966). The collected volatiles accumulate to a species-specific fragrance blend in the males' hind leg pockets and have been shown to get actively released during display behavior at courtship territories (Eltz et al. 2005b, Eltz et al. 1999). Such territories also represent the locality where the seldom observed matings took place (Eltz et al. 2003, Kimsey 1980, Stern 1991). Although an ultimate proof of female attraction towards male perfume in behavioral tests is still missing, the general hypothesis for the fragrance purpose is to function as a chemical signal analogous to sex pheromones.

Several aspects of my results are in agreement with the idea that males' volatiles constitute an important part in the bees' complex mating behavior. Earlier studies with three *Euglossa* species indicated a broad species-specific character of male perfumes (Eltz et al. 1999), and behavioral tests with hind leg extracts of two *Eulaema* species demonstrated reliable attraction of conspecific males (Zimmermann et al. 2006). In my results, the hind leg perfumes of 15 sympatric *Euglossa* species were found to be sufficiently different in their volatile composition to allow individuals to be assigned to their own species (Chapter 2). Such chemical differentiation is consistent with the idea that they play a role in mate recognition and premating isolation. The pronounced chemical disparity among the most closely related species suggests that male fragrance blends diverge more rapidly between sibling species and serve to avoid potentially costly hybrid matings. The substantial differentiation among closely related taxa disagrees with a slow and gradual change in the males' perfume, but supports a more rapid mode of evolution. Changes in behavioral preferences or modification in the bees' odorant receptors might trigger such saltational shifts in fragrance perception and collection. In the two closely related sibling species of *Euglossa* cf. *viridissima,* we were able to find an example for component-specific differences in perfume accumulation and

antennal perception (Chapter 3). Here, the male perfumes differ only in a set of four structurally very similar isomeric compounds, which are collected by males of one lineage in large quantities but are totally absent in the other. Hypothetically, a heritable olfactory shift might have resulted in the acquirement of different chemical compounds by males of these sibling species within the same habitat. A following promotion by natural or sexual selection, with mutant females favoring the altered chemical signal, might have lead to differentiation of the two lineages of *E. viridissima* and its sibling. Genetic analysis revealed the existence of two reproductive isolated lineages, leading to the characterization of the sibling *E. dilemma* as new species (Chapter 4). A predominance of single mating in female orchid bees (Chapter 5) is consistent with generally low mating frequencies in Hymenoptera, but also suggests that females could benefit strongly from choosiness with regard to males. An assessment of a males' fragrance phenotype as fitness indicator might make potentially costly multiple mating unnecessary for female orchid bees. However, data on female responses towards the male perfumes is still lacking. A demonstration of female attraction toward males' chemical signals in future behavioral tests is urgently needed for demonstrating that the fragrances function as sex pheromone analogues, to draw further conclusions about the mode of signal evolution in euglossine bees.

Ecologically, orchid bees play a significant role as pollinators of neotropical plants and are thought to have a positive influence on plant diversity within their habitat. The ongoing fragmentation due to deforestation in neotropical forests raises concerns of how orchid bee populations react towards environmental changes. Earlier studies on fragmentation effects on euglossine bees' species richness and diversity turned out to be ambiguous (Becker et al. 1991, Brosi 2009, Powell & Powell 1987, Tonhasca Jr et al. 2003). Allegedly high frequencies of diploid males (Lopez-Uribe et al. 2007, Roubik et al. 1996, Zayed et al. 2004), detected by allozyme-based analyses, suggested that orchid bee pollinators are in severe genetically-driven decline. An updated microsatellite-based study with numerous individuals from several species and localities refutes this possibility by showing that diploid males are rare in most if not all euglossine populations (Chapter 7). Furthermore, orchid bee populations were found to have generally only weak population genetic structure and, presumably, high levels of gene flow (Chapter 6 and 7). This confirms earlier reports of long distance gene flow

(Dick et al. 2004) and long distance travel (Janzen 1971). Population genetics analysis of *Euglossa aff. viridissima* on the Yucatán peninsula (Chapter 6) further relates that some orchid bee species are highly adaptable towards anthropogenically altered habitats. This, again, is in agreement with previous studies (Pemberton & Wheeler 2006). High genetic variability within the species we analyzed for our population genetic studies in the fragmented forests of southern Mexico (Chapter 6) supports the idea of substantial gene flow within orchid bee populations. The general lack of genetic differentiation among fragments/populations suggests that even large areas of non-forest habitat do not function as an efficient barrier for gene flow. This might rely on both the orchid bees' capability of crossing large areas of unsuitable habitat as well as on their use of small interspersed forest patches as stepping stones.

Detailed Summary

Chemical signals are frequently used within insects to communicate with conspecifics. Mostly self-produced, volatiles in the form of pheromones often facilitate mate recognition and may function as mate assessment signal. The subject of this dissertation is the ecology of neotropical orchid bees that caught the attention of scientists due to the males' specialized behavior of collecting volatiles from numerous natural sources, such as orchids and other flowers, resin, decaying wood and feces. The exogenous volatile mixture gets stored in the males' hind leg pockets, where a species-specific blend accumulates. Male euglossine expose their fragrance blend within mating territories and it is thought to function as a pheromone analogue. While seeking for various volatiles, male orchid bees function as important pollinators in neotropical forests since they are exclusively responsible of the pollination of >700 orchid species. My dissertation gives a further insight into orchid bee ecology by adopting behavioral, chemical and molecular approaches. Comprised of three publications and three manuscripts, it is concerned with the bees' mating biology, with special emphasis on the potential purpose of male fragrances in species recognition and reproductive isolation. Furthermore, it deals with orchid bee population genetics and genetic consequences of fragmentation. In the following, my results are summarized in individual chapters.

Chapter 2

Chemical niche differentiation among sympatric species of orchid bees. Male hind leg fragrances of 15 sympatric Panamanian *Euglossa* species were analyzed with gas chromatography/mass spectrometry and revealed a nonrandom, species-specific accumulation of volatiles in their perfume compositions. Species specificity is manifested by distinct compounds as well as by certain relative compound proportions. Such a chemical distinctness supports the hypothesis that the extensive collected fragrance bouquet functions as mate recognition signal. Chemical differentiation in relation to phylogenetic divergence through time, based on DNA sequences, revealed the greatest chemical disparity among the most closely related species. Potentially costly

hybrid matings might have caused a more rapid fragrance differentiation between sibling species, involving changes in chemical preferences of orchid bees in the early stages of species divergences. The results are consistent with a saltational evolution of males perfume blends and with the assumption that they play a major role in premating isolation.

<div align="right">**Chapter 3**</div>

An olfactory shift is associated with male perfume differentiation and species divergence in orchid bees. Here, we report on two male morphotypes of *Euglossa cf. viridissima* from the Yucatán peninsula in Mexico that exhibit a different number of mandibular teeth. Morphologically, they could be divided into tridentate (3D) and bidentate (2D) males, and population genetic analysis with microsatellite markers identified them as two reproductively isolated lineages. Chemical analysis revealed conspicuous differences in the composition of stored volatiles between 2D and 3D males, which were predominantly based on the presence or absence of one main compound (HNDB) and its three stereoisomers. Electroantennogram tests showed substantially lower antennal responses to HNDB in 2D males, which also represent the lineage that lacks this component in the perfume blends. The component specific differences in antennal perception reveal a mechanism by which closely related species acquire different chemical compounds from their habitat. Supposedly such an olfactory shift could have initiated the divergence of the two *Euglossa* lineages.

<div align="right">**Chapter 4**</div>

Characterization of the orchid bee *Euglossa viridissima* and its cryptic sibling species, *Euglossa dilemma* sp. nov., by morphological, chemical, and genetic characters. This manuscript is based upon the previous publication (Chapter 3) and explores further morphological, chemical and genetic variation in the two sibling species. Two morphological characters exist to distinguish males of the newly established species *E. dilemma* sp. nov. from those of its sibling *E. viridissima*: they develop three instead of two mandibular teeth in a certain distance to each other and their hind tibia has a less inflated and edgier shape. Data on their geographic ranges show generally a sympatric occurrence across Central America, although the recently

introduced orchid bee in Florida can be clearly identified as *E. dilemma*. COI sequence data confirm *E. viridissima* and *E. dilemma* as a monophyletic sister group within the genus *Euglossa* but failed to separate the two lineages due to parsimony uninformative base changes.

Chapter 5

Single mating in orchid bees (*Euglossa*, Apinae): implications for mate choice and social evolution. To assess female mating frequency, we genotyped mothers and their brood of three *Euglossa* species at microsatellite DNA loci. Information on orchid bee female mating frequency is desirable both in the context of social evolution in corbiculate bees as well as for a better understanding of the significance of euglossine fragrance collection. The results represent strong evidence for a prevalence of single mating in the genus *Euglossa.* This strengthens the view that single mating was the ancestral state in corbiculate bees, with honeybees as sole exception. According to the kin selection theory, such a mating system might promote the evolution of advanced sociality, although the latter is generally lacking in euglossine bees. Here, we find only early stages of sociality in terms of frequent occurrence of multi-female nests. However, single mating is consistent with the idea that female orchid bees select a single best mate based on male fragrance phenotypes.

Chapter 6

Population genetic structure of orchid bees (Euglossini) in anthropogenically altered landscapes. A set of polymorphic microsatellite markers was used to investigate the effects of geographic distance and habitat fragmentation on gene flow among populations of euglossine bees. In the first part – a genetic distance study – we analyzed populations of *E. aff. viridissima* from three geographic regions: the Yucatán peninsula (Mexico), Veracruz (Mexico) and Florida (USA). In general, they showed only little genetic structure within the three regions. The populations within the Yucatán peninsula and within the region of Veracruz revealed high genetic variability and a general lack of differentiation, suggesting substantial gene flow between populations of this well adapted *Euglossa* species. The reduced genetic diversity within the Florida population reflects a recent establishment of a small number of individuals.

Across regions, genetic differentiation gets more noticeable, with a positive correlation to geographic distance. Secondly, a fragmentation study was conducted to estimate the influences of habitat fragmentation on gene flow among populations of eight different *Euglossa* species in three localities in southern Mexico (Veracruz). Even though we expected a varying fragmentation impact with regard to differences in the species' habitat requirements, population genetic structure between fragmented localities was only very low or absent in all species. Large areas of non forest habitat might not be able to function as a barrier for gene flow. Nevertheless, the time span since the forest fragmentation took place might have also been insufficient to affect genetic differentiation.

Chapter 7

Conservation genetics of neotropical pollinators revisited: microsatellite analysis demonstrates that diploid males are rare in orchid bees. Early allozyme analyses have suggested that orchid bees are in a decline because of the reportedly high frequencies of diploid males, which is generally interpreted as a result of low genetic diversity. On the contrary, our comprehensive analyses of a total of 1010 males from 27 euglossine species with polymorphic microsatellite markers revealed extremely low frequencies of diploid males and very weak population genetic structure. These results are in accordance with other ecological aspects of orchid bees, which characterize them as highly mobile, widespread and well adapted to natural as well as disturbed habitat. Orchid bee populations seem to be large, weakly structured and unlikely to suffer from inbreeding that is often quoted as an effect of anthropogenic changes, such as habitat destruction and fragmentation.

Detaillierte Zusammenfassung

Chemische Signale dienen Insekten häufig zur intraspezifischen Kommunikation. Duftstoffe, in Form von zumeist selbstproduzierten Pheromonen, erleichtern die Erkennung eines Artgenossen und fungieren teilweise sogar als Beurteilungskriterium eines potentiellen Paarungspartners. Thematisch befasst sich die vorliegende Dissertation mit neotropischen Prachtbienen (Euglossini), die die Aufmerksamkeit der Wissenschaftler dem außergewöhnlichen Duftstoffsammel-Verhalten ihrer Männchen verdanken. Diese sammeln an einer Vielzahl natürlicher Quellen – wie z.B. an Orchideen und Blüten anderer Pflanzenarten, Harz, faulendem Holz und Kot – flüchtige Substanzen, um diese in Taschen in ihren vergrößerten Hinterbeinen zu speichern. Die exogenen Duftstoffe werden dort zu einem artspezifischen Gemisch kombiniert, das von den Männchen innerhalb ihrer Balzterritorien wieder freigesetzt wird und vielfach als Pheromon-Analogon interpretiert wird. Auf ihrer Suche nach verschiedenen Duftstoffen nehmen männliche Prachtbienen eine wichtige Bestäuberfunktion in neotropischen Wäldern ein, da sie exklusiv für die Bestäubung von >700 Orchideenarten verantwortlich sind. Die vorliegende Dissertation soll einen tieferen Einblick in die Ökologie der Prachtbienen gewähren, indem sie verhaltensbiologische, chemische und molekulare Ansätze vereint. Die folgenden drei Publikationen und drei Manuskripte befassen sich mit der Paarungsbiologie von Prachtbienen. Thematischer Schwerpunkt dabei ist die mögliche Funktion der männlichen Parfüme in Bezug auf Arterkennung und reproduktiver Isolation. Des Weiteren wird die Populationsgenetik von Prachtbienen untersucht und mögliche Konsequenzen von Habitatfragmentierung werden erforscht. Im Folgenden sind die vorliegenden Ergebnisse ihren jeweiligen Kapiteln entsprechend zusammengefasst.

Kapitel 2

Chemische Nischendifferenzierung sympatrischer Prachtbienenarten. Die Analyse männlicher Hinterbeinextrakte von 15 sympatrischen panamesischen Prachtbienenarten mit Hilfe von Gaschromatographie/Massenspektrometrie hat eine

nicht zufällige, artspezifische Akkumulation von Duftstoffen innerhalb ihrer Parfüme ergeben. Diese Artspezifität wird sowohl durch exklusive Komponenten als auch durch bestimmte relative Mengenverhältnisse der Komponenten erreicht. Solch eine chemische Spezifität unterstützt die Hypothese, dass die komplex zusammengestellten Duftstoffgemische zur Signalisierung der Artzugehörigkeit eines Paarungspartners eingesetzt werden. Ein Abgleich der chemischen Unterschiede mit einer DNA-basierten Phylogenie zeigte, dass die stärkste Diskrepanz zwischen den am nächsten verwandten Arten liegt. Daher könnte eine raschere Parfüm-Differenzierung bei sympatrischer Artbildung der Vermeidung kostspieliger Fehlpaarungen dienen und für die Veränderungen chemischer Präferenzen von Prachtbienenmännchen in den frühen Stadien der Artaufspaltung verantwortlich sein. Unsere Ergebnisse sind im Einklang mit einer sprunghaften (saltatorischen) Evolution männlicher Duftstoffparfüme und unterstützen die Vermutung einer Funktion als präzygotischer Isolationsmechanismus.

Kapitel 3

Eine olfaktorische Verschiebung als Auslöser männlicher Parfümdifferenzierung und Artaufspaltung in Prachtbienen. Diese Publikation berichtet über kryptische Diversität bei der Art *Euglossa cf. viridissima* von der Halbinsel Yucatán in Mexico, deren Männchen eine unterschiedliche Anzahl von Mandibelzähnen ausbilden. Morphologisch können dreizähnige (3Z) und zweizähnige (2Z) Männchen unterschieden werden. Durch populationsgenetische Analysen mit Mikrosatelliten-Markern konnten diese als zwei reproduktiv isolierte Populationen identifiziert werden. Chemische Analysen zeigten einen deutlichen Unterschiede in der Zusammenstellung der gespeicherten Duftstoffbouquets von 2Z- und 3Z-Männchen, der auf dem Vorhandensein oder Fehlen einer 4-Komponentengruppe basiert, welche aus einer Hauptkomponente (HNDB) und ihren drei Isomeren besteht. Die Auswertung der Elektroantennogramme offenbarte eine substantiell geringere antennale Antwort auf HNDB bei 2Z Männchen, denen diese Komponente auch in ihren Parfümen fehlt. Die komponenten-spezifischen Unterschiede in der antennalen Wahrnehmung scheinen der Grund dafür zu sein, dass sich diese beiden nah verwandten Arten unterschiedliche chemische Substanzen aus ihrem Habitat verschaffen. Weiter wird angenommen, dass die Aufspaltung in zwei *Euglossa* Schwesterarten ebenfalls durch die olfaktorische Verschiebung eingeleitet worden ist.

Die Charakterisierung der Prachtbiene *Euglossa viridissima* und ihrer kryptische Schwesterart *Euglossa dilemma* sp. nov. durch morphologische, chemische und genetische Merkmale. Dieses Manuskript basiert auf der vorherigen Publikation (Kapitel 3) und deckt weitere morphologische, chemische und genetische Unterschiede zwischen den beiden Schwesterarten auf. Zwei morphologische Merkmale unterscheiden die Männchen der neueingeführten Art *E. dilemma* sp. nov. von denen ihres Zwillings *E. viridissima*: Die Ausbildung von drei Mandibelzähnen (an Stelle von zwei) in einer bestimmten Distanz zu einander sowie einer Hintertibie, die eine weniger aufgepumpte, dafür aber eckigere Taschenform ausbildet. Die geographische Verteilung der beiden Arten zeigt generell ein sympatrisches Vorkommen in Mittelamerika, wenngleich die erst kürzlich eingeführte Prachtbienenart in Florida eindeutig als *E. dilemma* identifiziert werden kann. Sequenzierungen im COI-Gen bestätigen die Einordnung von *E. viridissima* und *E. dilemma* in eine monophyletische Schwesterngruppe innerhalb der Gattung *Euglossa.* Bemühungen, die Trennung der beiden Schwesternarten durch Unterschiede im COI-Gen zu begründen, scheiterten aber aufgrund der zu geringen und uninformativen Basenpaaränderungen. Analysen zur Bestimmung der Evolutionsdauer (Molekulare Uhr) deuten auf einen noch vor nur sehr kurzer Zeit lebenden, gemeinsamen Vorfahren hin.

Einfachverpaarung bei Prachtbienen (*Euglossa*, Apinae): Mögliche Konsequenzen für die Partnerwahl und die Evolution von Sozialität. Um die Paarungshäufigkeit weiblicher Prachtbienen zu ermitteln, wurden nistende Weibchen und ihre Nachkommen dreier *Euglossa* Arten mit Hilfe von Mikrosatelliten genotypisiert. Informationen zur weiblichen Paarungsfrequenz sind bei Prachtbienen von großem Interesse, da diese zum einen eine Rolle bei der sozialen Evolution innerhalb der corbiculaten Bienen spielt und zum anderen einen Anhaltspunkt für die Bedeutung des Duftstoffsammelns der Prachtbienenmännchen liefern kann. Die Ergebnisse weisen stark darauf hin, dass in der Gattung *Euglossa* Einfachverpaarung vorherrscht. Hierdurch wird der Eindruck verstärkt, dass Einfachverpaarung der ursprüngliche Zustand bei corbiculaten Bienen ist und Honigbienen die alleinige Ausnahme darstellen.

Der Verwandtenselektionstheorie nach zu urteilen, würde solch ein Paarungsverhalten die Evolution von höher entwickelter Sozialität fördern. Diese kann gewöhnlich nicht bei Prachtbienen beobachtet werden. Bisher konnten höchstens frühe Stadien von sozialem Verhalten beobachten werden, die sich im Vorhandensein von Nesterteilung unter Weibchen ausdrücken. Einfachverpaarung bei weiblichen Prachtbienen ist mit der Hypothese vereinbar, dass die Wahl eines einzigen Paarungspartners auf der Zusammenstellung des männlichen Duftstoffgemisches begründet sein kann.

Kapitel 6

Genetische Populationsstruktur bei Prachtbienen (Euglossini) in anthropogen veränderten Landschaften. In dieser Untersuchung wurden polymorphe Mikrosatelliten angewendet, um die Folgen geographischer Distanz und Habitatfragmentierung auf den Genfluss zwischen Prachtbienenpopulationen zu ermitteln. Im ersten Abschnitt – einer Studie über die genetische Distanz – wurden *E. aff. viridissima* Populationen von drei geographischen Regionen analysiert: von der Yucatán Halbinsel (Mexiko), aus der Region Veracruz (Mexiko) und aus Florida (USA). Insgesamt konnten nur geringe genetische Populationsstrukturen innerhalb der drei Regionen festgestellt werden. Die Populationen von *E. aff. viridissima* innerhalb der Yucatán Halbinsel und innerhalb der Region Veracruz zeichneten sich durch hohe genetische Variabilität und geringe genetische Differenzierung aus, was wiederum auf einen substantiellen Genfluss zwischen den Populationen dieser gut an anthropogen veränderte Landschaften angepassten *Euglossa* Art hindeutet. Die verringerte genetische Variabilität der Population aus Florida spiegelt ein kürzlich zurückliegendes Besiedlungsereignis durch eine geringe Anzahl an Individuen wider. Die genetische Differenzierung nimmt bei einem überregionalen Vergleich mit der geographischen Distanz zwischen Populationen zu. Der zweite Abschnitt des Manuskripts (Fragmentierungsstudie) befasst sich mit möglichen Konsequenzen der Habitatfragmentierung im Süden Mexikos (Veracruz) auf die populationsgenetische Struktur von acht verschiedenen *Euglossa* Arten. Die drei Lokalitäten weisen ungefähr die gleiche Distanz zueinander auf, aber eine der Lokalitäten ist durch unbewaldetes Umland isoliert. Entgegen der Erwartung, dass artverschiedene Habitatsanforderungen auch in einen unterschiedlichen Grad an genetischer Struktur resultieren würden, waren die populationsgenetischen Differenzen zwischen den Lokalitäten über alle Arten

hinweg gering. Größere Gebiete unbewaldeten Habitats scheinen bei Prachtbienen nicht auszureichen, um den Genfluss zwischen den Populationen zu unterbrechen. Auszuschließen ist allerdings nicht, dass die Zeitspanne seit der Waldfragmentierung zu gering gewesen ist, um eine genetische Differenzierung zu bewirken.

Kapitel 7

Resümee zur genetischen Vielfalt neotropischer Bestäuber: Mikrosatellitenanalysen zeigen ein seltenes Vorkommen diploider Männchen bei Prachtbienen. In vorherigen Studien, basierend auf Allozym-Analysen, wurden neotropische Prachtbienen als gefährdet eingestuft, da hohe Frequenzen diploider Männchen detektiert wurden, die generell als Zeichen geringer genetischer Diversität gedeutet werden. Im Gegensatz dazu steht die vorliegende, aktuelle und umfangreiche Analyse von insgesamt 1010 Männchen von 27 Euglossinenarten mit polymorphen Mikrosatelliten-Markern, die nur eine sehr geringe Anzahl diploider Männchen und generell sehr schwache populationsgenetische Strukturierung zeigt. Dieses Ergebnis steht im Einklang mit bisherigen ökologischen Untersuchungen, in denen Prachtbienen als mobil, weitverbreitet und gut angepasst charakterisiert werden – sowohl an natürliche, als auch an vom Menschen besiedelte Habitate. Populationen von Prachtbienen stellen sich sehr individuenstark und genetisch nur schwach strukturiert dar. Eine Bedrohung durch Inzucht, die als Konsequenz anthropogener Veränderungen wie Habitatzerstörung und Fragmentierung beobachtet wird, erscheint bei neotropischen Prachtbienen unwahrscheinlich.

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Acknowledgements

I kindly thank Dr. Thomas Eltz for his ambitious supervision of my thesis. He introduced me to the fascinating world of orchid bees and made it possible to arrange my thesis as most diversified, by combining field work in stunning neotropical environments with the execution of instructive analyses back at the University of Düsseldorf. I am most thankful for his time, inventive ideas, valuable advices and the companionate relationship.

I owe sincere gratitude to Prof. Dr. Klaus Lunau, head of the Sensory Ecology Group, for sparking my interest in behavioral and evolutionary biology. I will always remember the Mathon excursion that made me change my mind. I could not have asked for a friendlier and more inspiring atmosphere in his working group through all the years.

Furthermore, I would like to thank all members of the Sensory Ecology Group for the creation of a constructive and enjoyable surrounding. Even if I can't name all, I am thankful for all the support, criticism and delight. Particular thanks go to Monika Haardt and Ellen Poggel for their kindly help with administrative and technical issues. I surely have to thank Sarah Papiorek for numberless little breaks and her generosity in offering a helping hand. I call myself lucky for sharing the lab with Olaf Diestelhorst, whom I owe useful advices as well as a great spirit in a well organized room. It surely wouldn't have been the same without Sebastian Witjes. My dearest colleague, it has been a pleasure to be your twin for all those years and I owe you numerous good memories.

I would like to extend my gratitude to Prof. Dr. Martin Beye for being second examiner of my thesis and offering access to his laboratory at any time. He and the members of his working group were always kind and had an open ear to any of my experimental questions. Special thanks go to Sarah Lechner for her time and Dr. Martin Hasselmann for his unlimited patience.

I am glad for all the support and intellectual or practical input from my co-authors and other scientists I had the chance to work with through my dissertation. In no specific order, I would like to thank the members of the BMFZ (Biologisch-Medizinisches Forschungszentrum) in Düsseldorf for all of their ideas and procedure advices. The staff of the Smithsonian Tropical Research Institute in Panama provided useful assistance that made the execution of my projects abroad so much easier. I am highly appreciative for the scientific support and kindness of Dr. Dave W. Roubik in Panama and his hospitality at his house in Santa Rita. Many thanks go to Prof. Javier J. G. Quezada-Euán for offering time and several practical advices during our field trip in Yucatán, Mexico. I am very thankful for the cooperation with Santiago R. Ramirez and I will keep our meeting in Düsseldorf in good memory. I am much obliged to Dirk-Louis P. Schorkopf for his ambitious collection effort in Mexico and his instructive input during his stay in Düsseldorf. Finally, I want to thank Dr. Robert J. Paxton for letting me include the manuscript and beyond this, for his numerous useful and kind email advices, although we never had the chance to meet in person.

A circle of dearest friends gave me strength, hope and necessary distraction through all the time. I am deeply grateful for them as such a reliable support system and look forward to many more years of friendship. I surely benefited from Manuel Breuers' (err sorry, Dr. Manuel Breuers') experience and patient listening. Thanks for always being there without being near. Special thanks go to my love Thomas Martha, for his patience and encouragement through all the busy time. Giving my best is so much more effortless with him around. My family has provided support and assistance in every way imaginable. I thank my parents, Isolde & Erhard, and my brother Marc, for their faith in me and for walking this path together.