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The evolution of mating cues in a beetle hybrid zone: causes of geographic variation in cuticular hydrocarbon profiles

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THE EVOLUTION OF MATING CUES IN A BEETLE HYBRID ZONE: CAUSES OF GEOGRAPHIC VARIATION IN CUTICULAR HYDROCARBON PROFILES

By
Rachel M.S. Zack

Accepted in Partial Completion
of the Requirements for the Degree
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MASTER'S THESIS

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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Rachel M.S. Zack
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Abstract

The reinforcement of pre-mating barriers in a hybrid zone often leads to reproductive character displacement. However, it can be difficult to link evidence for reproductive character displacement with specific traits important to mate choice. I analyzed the cuticular hydrocarbon (CHC) profiles of *Chrysochus cobaltinus* and *C. auratus* to assess whether these pheromones are responsible for the previously observed pattern of reproductive character displacement in the *Chrysochus* hybrid zone. I found significant CHC divergence between the species, but overall CHC divergence was not higher among hybrid zone populations. However, CHC profiles of sympatric *C. cobaltinus* were more homogenous and were significantly different from CHC profiles of conspecific allopatric populations near the hybrid zone. Allozyme based analyses of population structure indicated that genetic relatedness is *not* a likely explanation for *C. cobaltinus* CHC variation. When controlling for genetic distance, there was greater CHC divergence between sympatric populations and allopatric populations near the hybrid zone, compared to divergence between sympatric populations and allopatric populations far from the hybrid zone. This result indicates that populations in the vicinity of the hybrid zone may be responding to unique selection pressures. In addition, species-specific differences in CHC variation support the hypothesis that reinforcing selection on females is species-specific. Overall results provide partial support for the hypothesis that previously observed reproductive character displacement is due to changes in *Chrysochus* CHC profiles. Further research on the compounds governing mate choice will provide more information on the evolution of reproductive barriers in this system.

Acknowledgments

This thesis would not have been possible without the assistance of many people. I would like to thank my advisor, Merrill Peterson, for his valuable guidance, support, and good humor throughout this process. My committee members, James Vyvyan and David Hooper, generously shared their knowledge and contributed thoughtful questions and insights regarding many aspects of this thesis. I am grateful to the several people, unknown to me, who took the time to collect and ship samples of *C. auratus* from afar. These include: Steven Schwartz (University of Nebraska), Lisa McDonald (University of Tennessee), Jonathan Brown (Grinnell College), and Deanne Bowers (University of Colorado). I was also helped by many friends, family, and strangers, who directed me towards possible *C. cobaltinus* on my collection trip. The gas chromatography/mass spectroscopy work was greatly assisted by Erin Macri and Devon Cancilla of Scientific and Technical Services at Western Washington University (WWU). Additional guidance and assistance in the lab was provided by Erica Larson and Andrew Schweitzer. Both lab and field research were facilitated by funding from the office of Research and Sponsored Programs at WWU, as well as the WWU Biology Department. And final thanks must go to Patrick Migas, who has gone above and beyond the call of duty in his support for this project.

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Introduction

Understanding the evolution of new species is a central topic in evolutionary biology. The diversity of life on earth is directly linked to the emergence of multiple species from an ancestral form. However, this important topic can be complicated by the many definitions of what constitutes a species. The differences among these definitions have important implications for how scientists design speciation studies. For example, the phylogenetic species concept emphasizes the phylogenetic history of organisms and defines a species as the smallest monophyletic group of common ancestry (de Queiroz and Donoghue 1988). Using the phylogenetic species concept, speciation research is limited to molecular data, morphological studies, and other tools used to make inferences about phylogenetic history. The evolutionary species concept emphasizes evolutionary cohesion and defines a species as a single lineage of ancestor-descendant populations or organisms that maintain an identity separate from other such lineages and which has its own evolutionary tendencies and historical fate (Wiley 1978). Like the phylogenetic species concept, the evolutionary species concept emphasizes the historical aspect of species formation. Speciation research using the evolutionary species concept can be hampered by the subjective definition of what constitutes maintenance of a "separate identity" between groups.

One commonly accepted definition of a species is the biological species concept, which defines a species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr 1963). An advantage of the biological species concept is that it allows us to study the process of speciation by studying the emergence of reproductive barriers between current populations. Thus, most researchers who study speciation have focused their efforts on understanding the

evolutionary processes influencing reproductive barriers, as well as the relative importance of different types of reproductive barriers in the isolation of species (Coyne 1992).

There are several general models to explain how reproductive barriers between species can potentially evolve. These include sympatric, parapatric, and allopatric speciation models, which form a continuum of gene exchange between diverging groups. Sympatric speciation involves biological barriers to gene exchange arising without any spatial segregation of the incipient species. This species model has been historically controversial (Coyne and Orr 2004), due mainly to the criticism that even low rates of mating and recombination will break down linkage disequilibrium, preventing the formation of genetically distinct subgroups (Mayr 1963). However, evidence for sympatric speciation has increased in recent years, partly due to the advent of molecular phylogenies (Bolnick and Fitzpatrick 2007). Many studies of sympatric speciation deal with co-occurring sister species in isolated environments, where secondary (post-speciation) contact is unlikely to explain their distribution. An example is the cichlid fishes of crater Lake Apoyo in Nicaragua. Barluenga et al. (2006) used molecular phylogenies and geographic information to provide strong support for the sympatric divergence of the endemic Arrow cichlid (*Amphilophus zaliosus*) from the Midas cichlid (*A. citrinellus*). In several cases, such sympatric speciation has been linked to disruptive selection for higher fitness on different resources. A well-known example is the apple maggot fly, *Rhagoletis pomonella*, which diverged into two genetically distinct groups due to a change in host preference that occurred in sympatry (Feder 1998). A review of speciation studies indicates that while sympatric speciation can occur in nature, it appears to be less common than parapatric or allopatric speciation (Bolnick and Fitzpatrick 2007). However, determining relative frequencies of different speciation

models is difficult due to ascertainment bias, taxonomic bias, and the limits of historical biogeographic knowledge.

Parapatric speciation is a model that describes neighboring populations (between which there is modest gene flow) which diverge and become reproductively isolated (Endler 1977). The grass species *Anthoxanthum odoratum* provides an example of parapatric evolution of reproductive isolation. Populations of *A. odoratum* have evolved heavy metal tolerance in areas polluted by mining. These heavy metal tolerant populations have diverged from neighboring non-tolerant populations in traits that are linked to reproductive isolation, such as flowering time (McNeilly and Antonovics 1968). Models indicate that parapatric speciation can occur under a variety of conditions (Gavrilets et al. 2000), but there are relatively few well documented examples. This could be due to the fact that, like sympatric speciation, parapatric speciation requires divergence to happen despite gene flow among the diverging populations.

A third general model of speciation, in which divergence occurs with little or no gene flow among populations, is allopatric speciation. Allopatric speciation is the evolution of genetic reproductive barriers between populations that are geographically separated. This separation can occur through the emergence of a geographic barrier that splits a species into two relatively large populations (vicariance). An example is the emergence of the Isthmus of Panama, which divided many marine organisms into separate Caribbean and Pacific species (Lessios 1998). Alternatively, allopatric speciation may occur via peripatric speciation (also called founder effect speciation), in which a localized colony diverges from a widely distributed ancestral form (Mayr 1982).

During allopatric speciation, natural selection can lead to reproductive isolation in different ways. One possibility is that as populations adapt to their separate environments, reproductive isolation can evolve as a byproduct of other ecological adaptations (i.e. MacNair and Christie 1983, Funk 1998). Through range expansion, species that diverged via allopatric speciation might come back into secondary contact. Such species could then co-occur without interbreeding, as speciation was already completed in allopatry.

Although numerous examples of speciation completed in allopatry are known (Coyne and Orr 2004), it is also known that in many cases, divergent populations are not completely isolated upon secondary contact. In such circumstances, these populations form hybrid zones, locations where genetically distinct groups of individuals meet and mate, resulting in at least some offspring of mixed ancestry (Harrison 1993). Such situations are useful for studying the genetic differences and selection forces which create barriers to gene flow between taxa (Barton and Hewitt 1985). Hybrid zones also provide abundant information on the possible states and degrees of divergence between populations that may be incipient species. Such information can lead to inferences about how reproductive barriers evolved in fully isolated species.

With the formation of a hybrid zone, it is still possible for speciation to be completed via a process known as reinforcement (Dobzhansky 1940). Under this hypothesis, if hybrids have reduced fitness, individuals who mate only with their own group will have a fitness advantage. Under these circumstances, natural selection is expected to favor the evolution of enhanced pre-mating barriers in the area of sympatry (overlap) (Coyne and Orr 2004, but see Lemmon et al. 2004).

Acceptance of reinforcement has varied over time. There was a large amount of initial enthusiasm for the idea, but this enthusiasm was later tempered by many theoretical objections. Some objections were based on models of gene flow into and out of a hybrid zone, and disagreement about the effects of introgression and recombination of genes. These objections, their support, and refutations have been summarized by Howard (1993). Acceptance of reinforcement has generally increased over the last 15 years, as several studies have found that a pattern of increased prezygotic isolation in sympatry compared with allopatry is reasonably common in nature, and that the conditions under which it can occur are not as restrictive as was previously thought (Howard 1993, Coyne and Orr 1997, Noor 1999, Servedio and Noor 2003).

This pattern of increased prezygotic isolation in sympatry compared with allopatry is called reproductive character displacement (Howard 1993). Reproductive character displacement occurs when two species diverge in character traits important to mate choice and reproduction in regions where the species overlap. However, not all apparent cases of reproductive character displacement are due to reinforcement. For example, field observations of reproductive character displacement can be complicated by the existence of any environmental gradients that cause the optimum phenotype to change over space. If two species have a clinal variation in characters related to reproduction that is based on environmental factors, they may appear more divergent in sympatry. However, this divergence would be due to environmental adaptation, and not selection for increased prezygotic isolation. Conversely, two species may be experiencing reinforcing selection, but its effect can be masked by a steep environmental gradient (Goldberg and Lande 2006). Studies of character displacement can address these challenges by including spatial data and

information on the trait under study, so as to better recognize clines based on environmental gradients (Losos 2000, Goldberg and Lande 2006).

Because processes other than reinforcement can cause a pattern mimicking reproductive character displacement, to determine if reinforcement is responsible for an observed pattern, researchers must demonstrate the following: (1) hybridization occurs or probably did occur in nature; (2) there is selection against hybridization in the field; (3) the displacement is perceptible to the opposite sex (when relevant); (4) variation is heritable and thus capable of responding to selection; and (5) displacement has not occurred for other reasons (Howard 1993).

These requirements indicate the many challenges to reinforcement research. Several recent studies have stepped up to the challenge and documented evidence for reinforcement and/or reproductive character displacement in a variety of systems, ranging from *Heliconius* butterflies (Kronforst et al. 2007) to fungi (Le Gac and Giraud 2008). One well known example is the threespine stickleback fish in British Columbia (Rundle and Schluter 1998). Threespine sticklebacks are distributed throughout coastal British Columbia, and a few small lakes contain sympatric species pairs (termed "benthics" and "limnetics"). Past research has shown that threespine sticklebacks do hybridize, and hybrids are at a disadvantage in the wild (McPhail 1992, Schluter 1995). Rundle and Schluter (1998) looked for reproductive character displacement of female mate preference while controlling for possible ecological character displacement (related to size and morphology). They compared mate preference of sympatric benthic females with mate preference of the most "benthic-like" allopatric females and found that sympatric females strongly discriminated between benthic and limnectic males, while allopatric females did not. While a strong pattern of reproductive character

displacement was observed, it is unknown what character trait was the basis for mate discrimination. Several studies in other systems have also documented patterns of reproductive character displacement without looking into specific mate choice cues (e.g. Rundle and Schluter 1998, Kronforst et al. 2007, Le Gac and Giraud 2008, Urbanelli and Porretta 2008). In such cases, although reproductive character displacement is clearly demonstrated, it is not clear what trait has evolved to limit hybridization. Not knowing which trait(s) is under selection limits our ability to understand the evolution of reproductive barriers during speciation.

One study that did focus on characters known to be involved in mate recognition is Höbel and Gerhardt's (2003) work with the green tree frog, *Hyla cinerea*. Some *H. cinerea* populations occur in sympatry with its sister species, *H. gratiosa,* in the southeastern United States. Hybridization occurs in this area, and there is evidence of selection against hybrids (Höbel and Gerhardt 2003 and references therein). Höbel and Gerhardt analyzed three reproductive traits or behaviors: acoustic properties of the male advertisement call; female phonotactic selectivity; and male calling perches. Sympatric *H. cinerea* had diverged from allopatric *H. cinerea*, and these reproductive traits used for mate recognition were more accentuated when *H. cinerea* lived in sympatry with its sister species *H. gratiosa*. Like many studies of reproductive character displacement, Höbel and Gerhardt were able to eliminate some, but not all, possible explanations for this pattern. Because they did not have genetic data for their study populations, one cannot rule out the possibility that observed reproductive character displacement was due to a clinal variation in genetic relatedness, and not reinforcing selection.

Controlling for genetic relatedness and determining what trait selection is acting on are just two difficulties of such studies. To study reinforcement in the field, researchers must also be able to observe mate choices, determine the genetic ancestry of individuals, and demonstrate reduced hybrid fitness. Many of the challenges related to reinforcement research can be overcome by using an appropriate research system. One such system is *Chrysochus* (Coleoptera: Chrysomelidae) beetles, whose natural history makes them quite well suited for reinforcement research (Peterson et al. 2005a).

Study Species

The chrysomelid beetles *Chrysochus auratus* (F.) (Dogbane Leaf Beetle) and *C. cobaltinus* LeConte (Blue Milkweed Beetle) are the only members of their genus found in North America. Mitochondrial DNA sequences suggest these two species diverged 2-3 million years ago (Dobler and Farrell 1999). They average 6.8 – 11.3 mm in length (Hatch 1971), and can be distinguished by color and antennal morphology (Figure 1). The two species also differ in diet. While they both feed on plants in the Apocynaceae, *C. auratus* feeds exclusively on dogbane (subfamily Apocynoideae) plants such as *Apocynum cannabinum* L. and *Apocynum androsaemifolium* L. (Dobler and Farrell 1999). In contrast, *Chrysochus cobaltinus* feeds on dogbane as well as plants from the milkweed (Asclepiadoideae) subfamily, such as *Asclepias speciosa* Torr. and *Asclepias eriocarpa* Benth (Sady 1994, Dickinson 1995, Dobler and Farrell 1999).

The host plant is an integral part of the *Chrysochus* life cycle. Adults emerge in early summer and feed on the host plant leaves for 6-8 weeks (mid-June to late July in central

(a) **(b)**

Figure 1: (a) *Chrysochus auratus* and (b) *C. cobaltinus*. Photos courtesy of M.A. Peterson.

Washington). They average one mating per day during that time. Males seek out females and spend over 1.5 hours in post-copulatory mate guarding, which represents a significant time investment (Dickinson 1995). Females lay egg masses on the host plant, and first instar larvae hatch in mid to late summer. They fall to the ground, burrow into the soil, and feed on tuberous rhizomes of the host plant. Larvae pupate in a chamber in the soil and burrow to the surface by early summer the following year (Weiss and West 1921, Peterson et al. 2005b).

Chrysochus beetles are distributed across much of North America (Figure 2). *Chrysochus auratus* is found mainly in eastern and central North America, while *C. cobaltinus* is restricted to western North America. The two species come into close proximity in Utah, western Montana, central Washington, and northwest Washington-southwest British Columbia (Dobler and Farrell 1999, Peterson et al. 2001).

Chrysochus cobaltinus and *C. auratus* form a 75 km wide hybrid zone in the Yakima River valley of Central Washington. This is a zone of secondary contact that almost certainly arose within the last 13,000 years (Peterson et al. 2005a). Here *Chrysochus* beetles frequently interbreed and produce hybrids which are essentially sterile (Peterson et al. 2005b). The extremely low fitness of hybrids, coupled with the significant amount of time invested in mating, suggests that selection should favor the evolution of enhanced premating barriers between *C. auratus* and *C. cobaltinus* in the hybrid zone.

Previous research has found evidence of reproductive character displacement in the hybrid zone (Peterson et al. 2005a). In lab experiments, *C. cobaltinus* males from a sympatric population are significantly more likely to choose the conspecific (same species) female than *C. cobaltinus* males from an allopatric population. This pattern is strongest if the two female choices (*C. auratus* and *C. cobaltinus*) are from sympatric populations as

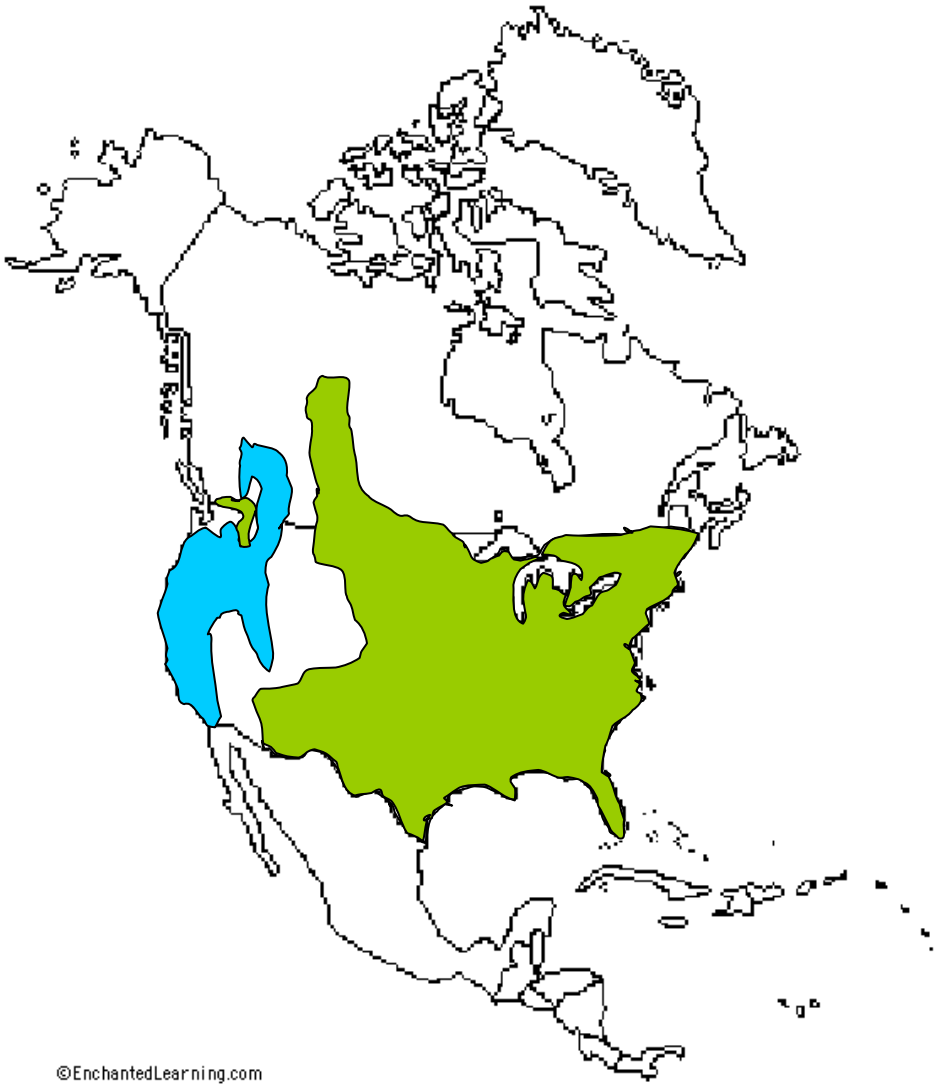


Figure 2: Approximate distribution of *Chrysochus cobaltinus* and *C. auratus* in North America (Peterson et al. 2001). The range of *C. cobaltinus* is indicated by blue and the range of *C. auratus* is indicated by green.

opposed to allopatric populations. Reproductive character displacement was not observed in *C. auratus* males, for whom allopatric and sympatric individuals were equally likely to choose a conspecific mate (Peterson et al. 2005a). These results are unlikely to be an artifact of the lab environment, because patterns of positive assortative mating in the field are similar to what has been observed in the lab (Peterson et al. 2005a).

Cuticular Hydrocarbons

The apparently greater distinction between sympatric females is potentially due to variation in cuticular hydrocarbon (CHC) profiles, and/or the possibility that hybrid zone males are more sensitive to differences in CHC profiles. Peterson et al. (2007) presented *C. cobaltinus* males with dead *Chrysochus* females that had their cuticular chemistry intact, removed, or replaced. Male mating effort varied depending on the source of cuticular chemistry. The results indicate that the response of *C. cobaltinus* males to prospective mates is determined by sex- and species-specific cuticular hydrocarbon profiles, and that male choice plays an important role in sexual isolation. The cuticular hydrocarbon profiles were further analyzed using gas chromatography/mass spectroscopy (GC/MS). Twenty-seven long-chain hydrocarbons were identified in the cuticles of male and female *Chrysochus* individuals. These CHC profiles can be used to differentiate between sexes and species, but it is unknown which specific compounds are important for *Chrysochus* mate choice (Peterson et al. 2007). The results of this study are intriguing when combined with evidence that selection has increased isolation between *C. auratus* and *C. cobaltinus* in the hybrid zone (Peterson et al. 2005a).

In many insect systems different species, sexes, and colonies have distinct CHC profiles, and these contact sex pheromones are frequently important for mate recognition and sexual isolation (Singer 1998, Howard and Blomquist 2005). Given the importance of chemical signaling among insects and other taxa, understanding how chemosensory traits evolve is a major challenge for biologists. Several studies have characterized pheromone based behaviors at the intraspecific level, but fewer studies have investigated the evolution of chemically based behaviors at the interspecific level and its implications for speciation (Smadja and Butlin 2008). Among such studies, several have found evidence of a link between CHCs and increased interspecific sexual isolation between insect populations (Coyne et al. 1994, Higgie et al. 2000, Mullen et al. 2007).

The importance of CHCs leads them to be targets of selection in many systems, but it can be difficult to determine which types of selection are causing changes. Sexual selection is known to influence CHCs, due to the important role they play in mate choice (Singer 1998, Howard and Blomquist 2005). Several *Drosophila* studies have documented the effects of sexual selection on CHC profiles and the consequent isolation between populations within a species (Ferveur 2005 and references therein). CHCs can also be influenced by natural selection due to environmental factors, because CHCs in the insect cuticle often serve important waterproofing functions (Singer 1998). For example, in a study of the grasshopper *Chorthippus parallelus*, Buckley et al. (2003) analyzed vegetation associated with moist habitats as an indicator of environmental conditions. They found a significant correlation between vegetation and CHCs, which was interpreted to mean that natural selection due to environmental pressures was interacting with mating signals. CHCs can also be influenced by reinforcing selection, leading to reproductive character displacement of CHC profiles.

Reinforcing selection was studied by Higgie et al. (2000), who exposed sympatric and allopatric populations of *Drosophila serrata* to experimental sympatry with its sister species, *D. birchii*. They found that after nine generations, the CHCs of allopatric *D. serrata* evolved to resemble sympatric *D. serrata* (which has CHC profiles that are divergent from those of *D. birchii*), while the original sympatric populations of *D. serrata* remained unchanged after experimental sympatry.

Along with selection, neutral changes in allele frequency can be expected to alter CHC profiles (Coyne and Orr 2004). For example, cuticular profiles of the ant, *Petalomyrmex phylax,* are influenced by selective and environmental pressures, as well as genetic bottlenecks during range expansion (Dalecky et al. 2007). Work with the Hawaiian Swordtail Crickets (genus *Laupala*) has also found evidence that CHCs were influenced by a founder effect as well as genetic drift and selection following island colonization (Mullen et al. 2008). However, few studies of this sort exist, and there is a lack of research on the relative importance of neutral versus selective processes in shaping CHC profiles (Howard and Blomquist 2005, Dalecky et al. 2007).

The multiple factors influencing CHC profiles might lead to clinal variation associated with the genetic similarity of neighboring populations. In such cases, the apparent reproductive character displacement of CHCs could actually be due to process other than reinforcement, such as changing environmental pressures or biogeographic history. To know if this is the case, one must examine evidence for clinal variation directly, which few studies have done. If reproductive character displacement is *not* caused by reinforcing selection, then genetic similarity, rather than position relative to a hybrid zone, could be a better predictor of the CHC similarity of conspecific populations. However, few reinforcement

studies have analyzed genetic similarity in conjunction with reproductive character displacement.

In this thesis, I adopt such a population-genetic approach to understanding patterns of reproductive character displacement, by combining analyses of population genetic markers (allozymes) with the analysis of CHC profiles for populations both inside and outside of the *Chrysochus* hybrid zone. Using this novel approach, I addressed a series of key questions regarding speciation in this system, which has emerged as an important system for studying reinforcement.

Study Questions

For this thesis, I have compared female CHC profiles of multiple populations to assess whether geographic patterns of CHC variation are consistent with the pattern of reproductive character displacement observed in the hybrid zone. More specifically, I analyzed divergence of CHC profiles to test the hypotheses that: (1) divergence between *C. cobaltinus* and *C. auratus* CHCs is greater in the hybrid zone compared to outside the hybrid zone and (2) within a single species, CHC profiles of hybrid zone populations have diverged from CHC profiles of allopatric populations. I combined CHC data, allozyme data, and geographic information for *C. cobaltinus* to test the contrasting hypotheses that: (1) geographic variability in CHC profiles is best explained by population genetic structure or (2) geographic variability of CHC profiles is best explained by proximity to the hybrid zone. Finally, these results provide insight into which CHCs are responsible for geographic variation in CHC profiles, helping guide future research on this system.

Methods

Sample Collection

I collected *Chrysochus cobaltinus* and *C. auratus* individuals from populations that fall into three geographic groups: (1) sympatric populations within the hybrid zone; (2) allopatric populations near the hybrid zone (collected in central Washington state); and (3) allopatric populations far from the hybrid zone (outside Washington state). Populations were distributed throughout the geographic range of both species, to the extent that was feasible (Figure 3; Appendix Table A1). To minimize CHC variability due to different diets (Stennett and Etges 1997), I collected all beetles used for CHC analysis from *Apocynum* host plants. When possible, they were collected from *Apocynum cannabinum*, the shared host of both species in the hybrid zone.

I collected the majority of beetles during a single collection trip from June 19th to July 6th 2006. Throughout the trip I kept beetles alive in a cooler, with each population in a separate container holding *A. cannabinum* cuttings and a paper towel to collect condensation. The *A. cannabinum* cuttings were kept fresh by placing them in a capped microcentrifuge tube filled with water. I cleaned containers and replaced the food supply regularly. Upon returning to the lab, I froze each individual at – 80 ºC in a 1.7 mL microcentrifuge tube.

Other members of the Peterson lab collected a subset of beetles prior to 2006 using similar methods. In addition, collaborators in eastern states collected beetles from four *C. auratus* populations and shipped them live on plant cuttings to Western Washington University, where they were frozen in the lab.

Figure 3 (a): Washington state collection sites. Sites were designated as sympatric with both species (●), *Chrysochus cobaltinus* allopatric (▲) or *C. auratus* allopatric (X). Location details are in Appendix Table A1.

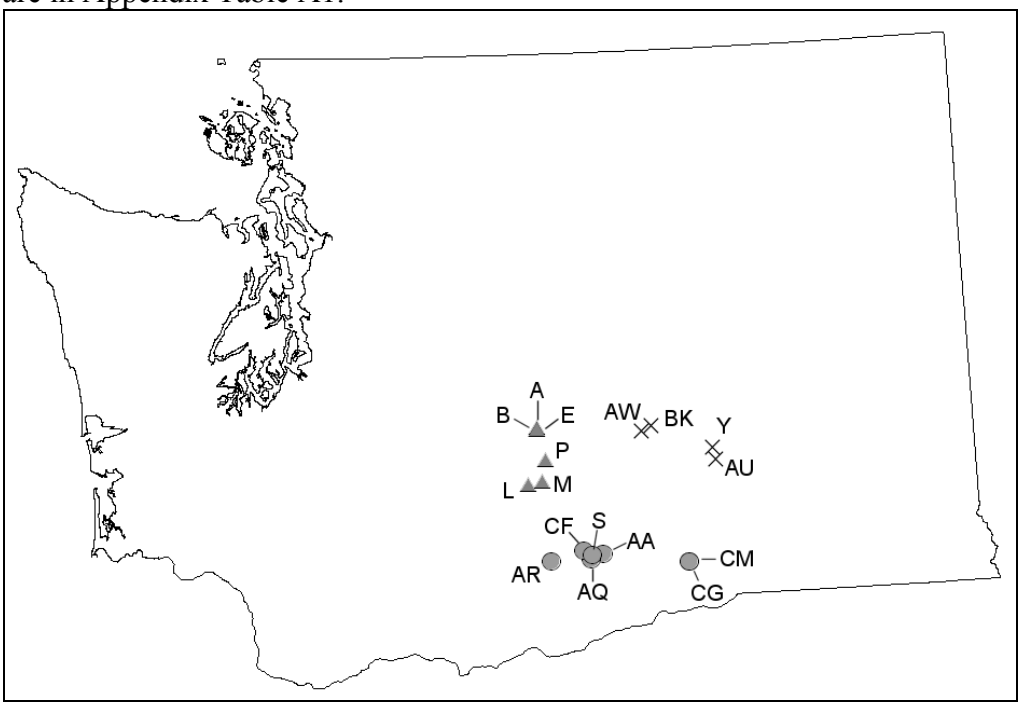
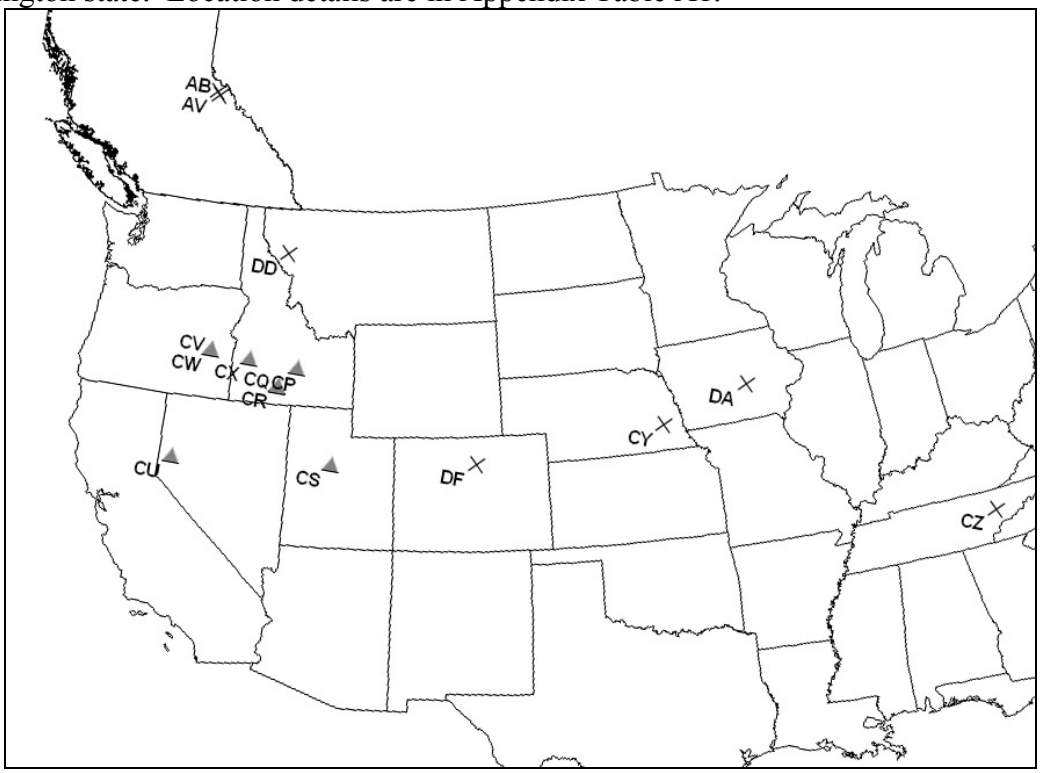


Figure 3 (b): *Chrysochus cobaltinus* (▲) and *C. auratus* (X) populations collected outside Washington state. Location details are in Appendix Table A1.



Chemical Analysis

I collected cuticular hydrocarbons from 8-11 females per population to test the hypothesis that female CHC profiles had diverged in the hybrid zone. Females were identified based on examination of the genetic aperture under magnification (Peterson et al. 2001). I removed hydrocarbons from beetles using a hexane wash. Each beetle was dipped in a 2 mL tube containing 1 mL of 95% n-hexane (99.5% saturated C6 isomers) and the tube was swirled for 10 seconds (Peterson et al. 2007). The hexane and hydrocarbon extract solution was evaporated down to a crust using a vacuum centrifuge and then stored at – 20 ºC until being reconstituted for gas chromatography. My methods departed from Peterson et al. (2007) in that I did not filter samples through a silica column. This was because I found no difference in the gas chromatography spectra for filtered versus unfiltered samples during trial runs, and thus omitted the silica column to save time.

I analyzed hydrocarbon extracts with coupled gas chromatography/mass spectroscopy (GC/MS). The extracts were re-suspended in 1 mL of hexane and 1 μL was automatically injected into a Varian Saturn 2000 GC/MS (with a Varian CP-3800 Gas Chromatograph) (Palo Alto, CA). I used a splitless injection to increase the sample concentration on the column. Prior to injection a split ratio of 50 was maintained, which indicates that 50 units of gaseous sample go out the split vent for every one unit that goes onto the column. On injection, the split vent was closed for 0.5 minutes to maximize sample deposition on the head of the column. The split was then turned on to a split ratio of 100 to clear the injection port of any remaining sample. The split ratio was decreased to 20 one and a half minutes after injection. I used helium as a carrier gas flowing at 2 mL/min. The gas chromatograph contained an Agilent J & W Scientific DB-5MS GC column that was 30 m long, had an inner

diameter of 0.250 mm, and a film thickness of 0.25 μm. Following previous methods for this system (Peterson et al. 2007), the column oven was initially set at 80 ºC and held there for the first 3 minutes of the sample run. It was then increased to 150 ºC at a rate of 20 ºC per minute. This was followed by an increase to 300 ºC at a rate of 10 ºC per minute. The column was then held at 300 ºC for the final 8.5 minutes of the 30 minute run time. The MS detector used electron impact ionization set to impact at 70 eV.

Because GC/MS sensitivity could decrease due to internal hydrocarbon buildup, I took steps to maintain instrument sensitivity throughout the experiment. Between every two samples the column was baked out with a hexane injection for 15 minutes total, during which time the temperature was increased from 80 ºC to 310 ºC at a rate of 20 ºC per minute and then held at 310 ºC. I did an extended column bake-out every night using the same temperature profile as the short bake-out but lasting for 56.5 minutes total. In addition, I changed the septum between every 50-60 samples and the inlet liner between every 100-150 samples.

Gas chromatography peaks were identified with the help of one internal standard (18 ppm caffeine) that was added to the hexane before re-suspension of the CHC crusts. I used caffeine because it is a non-toxic, economical, and soluble compound that could be easily distinguished from *Chrysochus* CHCs. Caffeine eluted at 12.02 minutes (immediately prior to *Chrysochus* CHCs of interest) and had a MS spectrum that was quite different from hydrocarbons. In addition, I injected a mixture of external standards between every 6 samples to help identify GC peaks and track any changes in retention time. The external standards mixture consisted of 20 ppm each of *n*-eicosane, *n*-docosane, *n*-tricosane, *n*-tetracosane, *n*-pentacosane, *n*-hexacosane, *n*-octacosane, *n*-triacontane, *n*-dotriacontane, *n*-

tetratriacontane, and caffeine. I chose these hydrocarbons based on price, their previous identification as a *Chrysochus* CHC (Peterson et al. 2007), and the fact that they spanned the majority of the range of retention times for *Chrysochus* CHCs. The caffeine was used to align the external standards spectra with sample spectra.

I identified hydrocarbon compounds on each beetle using information from both the gas chromatography and mass spectroscopy spectra. First I used the Varian MS Workstation v. 6.5 software to align *Chrysochus* gas chromatography spectra with external standard spectra. This easily identified any un-branched alkane whose retention time matched the retention time of an external standard. I identified additional compounds by combining information from previous identification of *C. cobaltinus* and *C. auratus* hydrocarbons (Peterson et al. 2007), retention time relative to external standards, and MS spectra. Once compounds of interest were identified, I used the Varian MS Workstation data processing software to recognize and integrate the area under each peak in *Chrysochus* samples.

Genetic Analysis

I used allozyme electrophoresis to test the hypothesis that variation in CHC profiles could be explained by population genetic structure. This analysis was limited to *C. cobaltinus* populations, because variable, consistently-scorable allozyme loci were not available for *C. auratus* at the time of this study. Tools for population genetic analysis were limited because previous work found low variation in both mitochondrial DNA of *C. auratus* (Dobler and Farrell 1999, Monsen et al. 2007) and microsatellites of *C. cobaltinus* (Monsen et al. unpublished data). It would be inappropriate to compare population structure between species using different marker types for each species, as different markers yield different

estimates of population structure (e.g. Estoup et al. 1998). This concern, combined with technical, financial, and time constraints, led me to use allozymes to measure population genetic structure.

I used three loci (out of 33 previously screened) that were consistently scorable and polymorphic in *C. cobaltinus.* The loci phosphoglucomutase (PGM, EC 5.4.2.2), glycyl-leucine peptidase (PepGL, EC 3.4.11 or 3.4.13), and glucose-6-phosphate isomerase (PGI, EC 5.3.1.9) were scored for 38-50 *C. cobaltinus* individuals in each of 16 populations (Appendix Table A1). All loci were run for 40 minutes at 180 volts using the following buffers: (1) 0.02 M phosphate (pH 7.0) for PGM; (2) 0.02 M tris-glycine (pH 8.5) for PepGL; and (3) 0.01 M citrate phosphate (pH 6.4) for PGI. I followed the cellulose-acetate gel electrophoresis protocols of Hebert and Beaton (1993) modified for *Chrysochus* as in Peterson et al. (2001).

Statistical Analysis

I analyzed CHC profiles using only those peaks that appeared in all individuals of at least one population. Because these hydrocarbons coat a beetle's cuticle, they are expected to vary based on an individual's size. I avoided this problem by measuring the relative amounts of each hydrocarbon, and not absolute concentrations. Thus all analyses were performed on the relative abundance value for each peak (the peak area ÷ sum of peak areas meeting the above criteria for each individual).

I estimated the similarity between hydrocarbon profiles of individuals and groups using a Bray-Curtis similarity index and other tests based on Bray-Curtis values. These types of analysis have been used successfully in similar studies of insect cuticular hydrocarbons

(Elmes et al. 2002, Schlick-Steiner et al. 2004, Schonrogge et al. 2004, Youngsteadt and DeVries 2005), and were performed in PRIMER version 5.2.9 (PRIMER-E Ltd., Plymouth, U.K.). The Bray-Curtis coefficient (S_{il}) compares two samples, *i* and *l*, using the equation:

$$S_{il} = 100\left\{1 - \frac{\sum_{j=1}^{n}\left|X_{ij} - X_{lj}\right|}{\sum_{j=1}^{n}\left|X_{ij} + X_{lj}\right|}\right\}$$

X_{ij} is the relative abundance of hydrocarbon *j* in sample *i*. S_{il} values will range from 0 (when they have no hydrocarbons in common) to 100 (when the hydrocarbon profiles are identical). I did not know which hydrocarbons were most important for mate choice, and it is known that compounds with relatively low abundances can play a key role in insect communication (Singer 1998). For that reason, I calculated the Bray-Curtis similarity index using X_{ij} values equal to the fourth root of each hydrocarbon, a transformation that prevents relatively low concentration hydrocarbons from being overshadowed by high concentration compounds (Elmes et al. 2002).

Pairwise similarity values within groups and between groups were analyzed using an ANOSIM (analysis of similarities) test in which population was nested in geographic group (sympatric, allopatric near the hybrid zone, or allopatric far from the hybrid zone) or species (Clarke 1993). I used ANOSIM to test the hypotheses that: 1) CHC profiles of *C. cobaltinus* and *C. auratus* are significantly different from each other, and 2) CHC profiles of hybrid zone beetles have significantly diverged from CHC profiles of non-hybrid zone beetles of the same species. ANOSIM tests are based on the *R* statistic, which can range from -1 to 1. R = 1 if *all* samples within a group are more similar to each other than *any* samples from different groups. $R = 0$ if similarities within and between groups are the same, and an *R* value

substantially less than zero indicates that similarities between groups are greater than similarities within a group (Clarke and Warwick 2001).

I used non-metric multi-dimensional scaling (MDS) to look for patterns of CHC divergence between species and population groups, and to test the hypothesis that there is greater divergence between *C. cobaltinus* and *C. auratus* in the hybrid zone. This method uses Bray-Curtis similarity values calculated from the mean relative abundance for each peak in a population. The MDS algorithm then uses iterative plotting to find the position for all points that best represents the similarity between populations. The agreement of the final plot with the similarity matrix is measured using the *STRESS* (STandardized Residual Sum of Squares) statistic, in which a lower stress value indicates a better fit with the original matrix. This equation is defined as:

$$STRESS = \sqrt{\sum_j \sum_k \left(d_{jk} - \hat{d}_{jk}\right)^2 / \sum_j \sum_k d_{jk}^2}$$

where $\hat{d}_{jk}$ is the distance predicted between 2 points based on their dissimilarity. If the plot distance (d_{jk}) is equal to predicted distance for all points, than *STRESS* equals zero (Clarke and Warwick 2001).

The SIMPER analysis in PRIMER analyzed dissimilarity between different species or different geographic groups of samples (Clarke and Warwick 2001). The dissimilarity is equal to 100 minus the Bray-Curtis similarity value. The SIMPER procedure measures the contribution of each hydrocarbon to the mean Bray-Curtis dissimilarity between groups of samples. It also determines the contribution of each hydrocarbon to similarity within a group (Clarke and Warwick 2001).

I used principal components analysis (PCA) in conjunction with SIMPER to determine which compounds contributed to differentiation between species or geographic groups of samples. PCA was done using population means of the fourth root of the relative area of each hydrocarbon. I included a varimax rotation and Kaiser normalization of the data so that variation would be spread more evenly across the principal components to aid in interpretation. Varimax rotation is a standard approach and was done to facilitate comparisons with previous PCA results for this system (Manly 1994, Peterson et al. 2007). I considered a hydrocarbon to be a significant contributor to a principal component if its correlation coefficient exceeded 70% of the largest coefficient for that principal component (Mardia et al. 1979).

I measured genetic divergence between *C. cobaltinus* populations to test the hypothesis that these populations exhibit genetic isolation by distance. Genetic divergence was measured with Wright's standardized variance in allelic frequencies (F_{ST}) (Wright 1951) using Isolation by Distance Web Service v. 3.15 (Jensen et al. 2005). F_{ST} values range from 0, when allele frequencies in two populations are identical, to 1, when two populations are fixed for different alleles. I used Mantel tests to assess the correlation between genetic divergence (F_{ST}) and geographic distance, following a log transformation of both axes. I then used AMOVA (Analysis of MOlecular Variance) to determine how much genetic variation was due to differences among geographic groups, among populations within geographic groups, or within populations (in Arlequin v. 3.11).

Next I compared F_{ST} to CHC divergence between populations to test the hypothesis that variation in CHC profiles is best explained by population genetic structure. To do this I plotted the genetic distance (F_{ST}) between each population pair against the Bray-Curtis

dissimilarity value for CHC profiles. I then used a Mantel test (following a log transformation of genetic distance) to assess the correlation between F_{ST} and Bray-Curtis dissimilarity values of CHC profiles.

To test the hypothesis that CHC profile variation was best explained by proximity to the hybrid zone, I plotted the log of (1 + the geographic distance) between each population pair against the Bray-Curtis dissimilarity value between those populations. This was done to see if population pairs within or near the hybrid zone had a large divergence in CHC profiles despite the small geographic distance between them.

Results

Chemical analysis

The GC/MS analysis found 43 suspected hydrocarbon peaks in *Chrysochus* cuticles. These included 25 confirmed hydrocarbons occurring in all individuals of at least one population. These 25 hydrocarbons were included in the analysis of CHC profiles, and of these, 21 compounds were positively identified, two were tentatively identified, and two remain unknown (Table 1, Figure 4). ANOSIM tests indicated that *C. cobaltinus* and *C. auratus* have significantly different CHC profiles ($R = 0.794$, $p = 0.001$). The mean Bray-Curtis dissimilarity between all possible *C. cobaltinus* and *C. auratus* pairs was 19.99 and was influenced by several compounds (Table 2). The top two contributors to this dissimilarity between species were unknown 14 (tentatively identified as 15,19-dimethyltritriacontane) and *n*-tritriacontane, which together contributed 21% of the dissimilarity. Compounds contributing to dissimilarity between *C. cobaltinus* and *C. auratus* from different geographic categories (sympatric, allopatric near, and allopatric far) were qualitatively similar to the results for overall dissimilarity between species (Appendix, Table A2). The MDS plot, which represents the similarity of population groups with a good amount of accuracy (STRESS = 0.07), clearly shows that the CHC profiles of the two species are distinct from each other (Figure 5).

When comparing *C. cobaltinus* and *C. auratus* populations, there was not a strong relationship between the geographic distance separating two populations and the dissimilarity of their CHC profiles (Figure 6). Furthermore, *C. cobaltinus* and *C. auratus* populations found at the same site in the hybrid zone were not more divergent than allopatric populations of the two species (Figure 6). Collectively, these results indicate that overall CHC

Table 1: Identity of gas chromatography peaks in *Chrysochus* cuticular hydrocarbon profiles. All peaks were found in every individual of at least one population of at least one species, and were identified as hydrocarbons using mass spectroscopy.

GC Peak	Compound	Retention Time (min.)
a	2-methyldocosane	15.851
b	9-tricosene	15.950
c	*n*-tricosane*	16.168
d	*n*-tetracosane*	16.999
e	2-methyltetracosane	17.512
f	9-pentacosene	17.592
g	*n*-pentacosane*	17.801
h	*n*-hexacosane*	18.574
i	2-methylhexacosane	19.050
j	*n*-heptacosane	19.325
k	2-methylheptacosane	19.794
l	*n*-octacosane*	20.046
m	2-methyloctacosane	20.493
n	9-nonacosene	20.595
o	*n*-nonacosane	20.753
p	2-methylnonacosane	20.958
q	*n*-triacontane*	21.432
r	2-methyltriacontane	21.866
s	9-hentriacontene	21.983
t	*n*-hentriacontane	22.143
u	unknown 12	22.370
v	unknown 33 (2-methyldotriacontane**)	23.567
w	unknown 29	23.735
x	*n*-tritriacontane	24.229
y	unknown 14 (15,19-dimethyltritriacontane**)	24.461

* Compound included in external standards mixture
** Tentatively identified based on MS spectra and information from Peterson et al. 2007

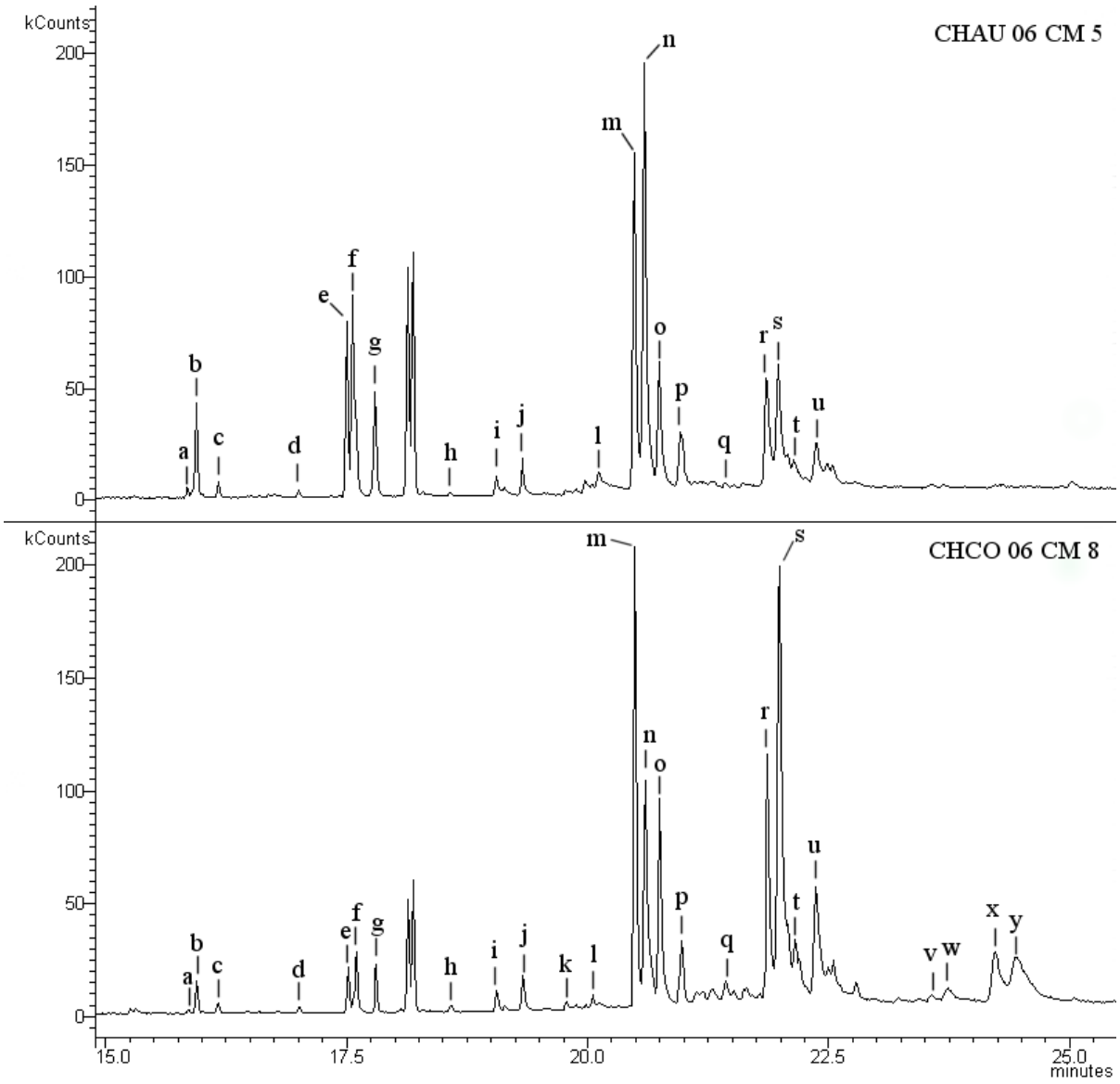


Figure 4: Representative gas chromatogram spectra for female *C. auratus* (top) and *C. cobaltinus* (bottom). Both individuals came from site CM within the hybrid zone. Peak identities are listed in Table 1.

Table 2: Compounds contributing to dissimilarity between *C. cobaltinus* and *C. auratus*. Total average Bray-Curtis dissimilarity between species was 19.99, and can be partitioned as seen below. Average abundance refers to relative abundance values after a fourth root transformation.

Compound	CHCO average abundance	CHAU average abundance	Average dissimilarity (±SD)	% Contribution	Cumulative % contribution
Unknown 14 (15,19-dimethyltritriacontane*)	0.02	0.00	2.17 (0.63)	10.87	10.87
n-tritriacontane	0.03	0.00	2.05 (0.77)	10.27	21.14
2-methylnonacosane	0.02	0.09	1.50 (1.00)	7.50	28.64
9-pentacosene	0.02	0.02	1.08 (0.89)	5.39	34.03
2-methyltetracosane	0.03	0.03	1.03 (0.85)	5.17	39.20
n-triacontane	0.01	0.00	0.99 (0.84)	4.96	44.16
9-nonacosene	0.05	0.15	0.90 (0.40)	4.51	48.67
unknown 29	0.00	0.00	0.89 (0.78)	4.44	53.12
2-methylhexacosane	0.01	0.01	0.78 (0.78)	3.93	57.04
Unknown 33 (2-methyldotriacontane*)	0.00	0.00	0.78 (0.73)	3.89	60.94
n-tetracosane	0.00	0.00	0.77 (0.69)	3.87	64.81
n-pentacosane	0.03	0.04	0.76 (0.61)	3.81	68.62
n-hexacosane	0.00	0.00	0.72 (0.67)	3.60	72.22
2-methylheptacosane	0.00	0.00	0.71 (0.70)	3.57	75.78
2-methyldocosane	0.01	0.00	0.67 (0.72)	3.37	79.15
9-hentriacontene	0.13	0.06	0.66 (0.38)	3.30	82.46
9-tricosene	0.01	0.02	0.65 (0.74)	3.24	85.70
2-methyltriacontane	0.17	0.12	0.46 (0.32)	2.32	88.02
unknown 12	0.08	0.08	0.42 (0.32)	2.09	90.12

* Tentatively identified based on MS spectra and information from Peterson et al. 2007

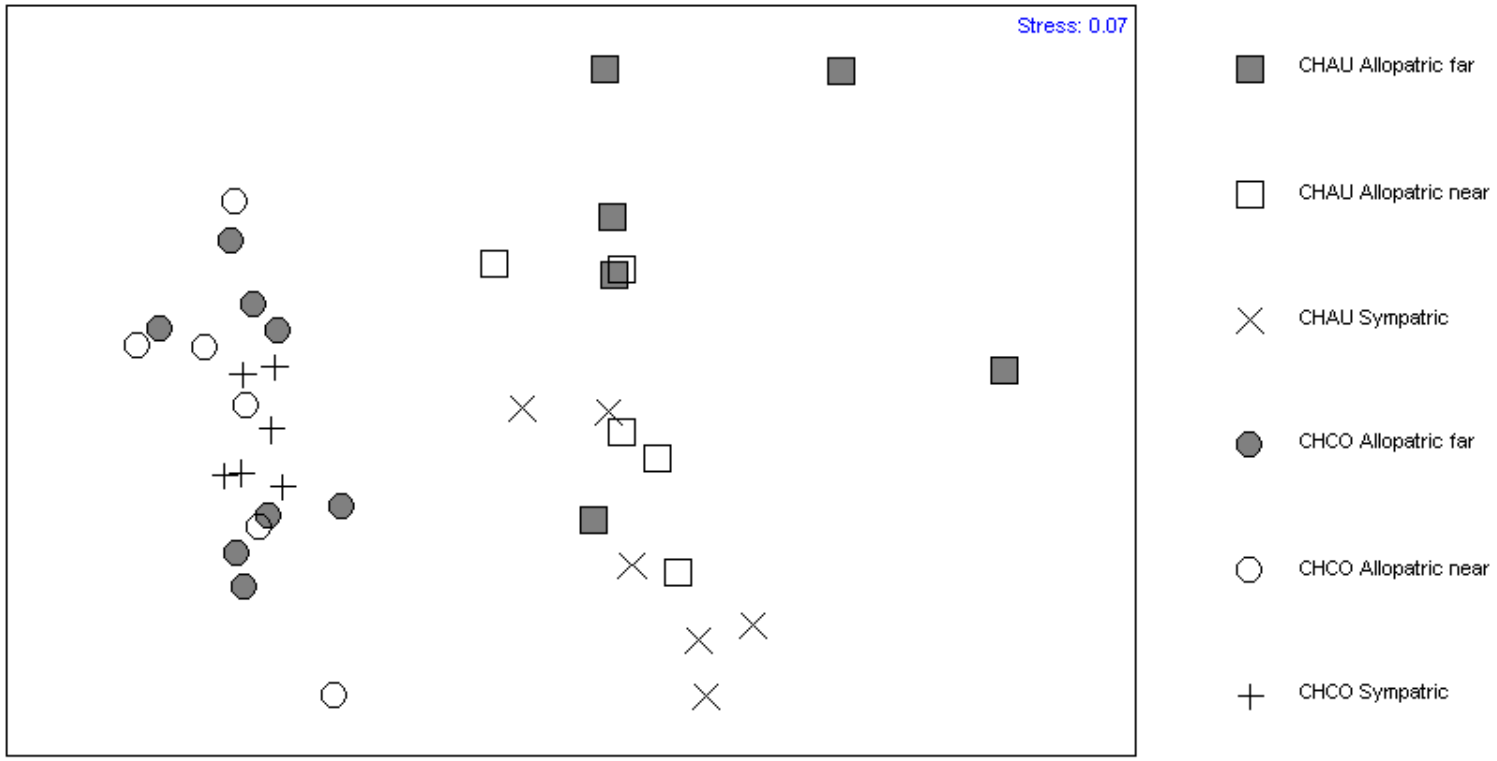


Figure 5: Two-dimensional non-metric multidimensional scaling (MDS) ordination of populations, based on Bray-Curtis similarities of CHC relative abundance (see methods). The top three contributors to differentiation between species were unknown 14 (tentatively identified as 15,19-dimethyltritriacontane), *n*-tritriacontane, and 2-methylnonacosane. "Allopatric far" and "Allopatric near" indicate the proximity of populations to the hybrid zone. CHAU = *Chrysochus auratus*, CHCO = *C. cobaltinus*.

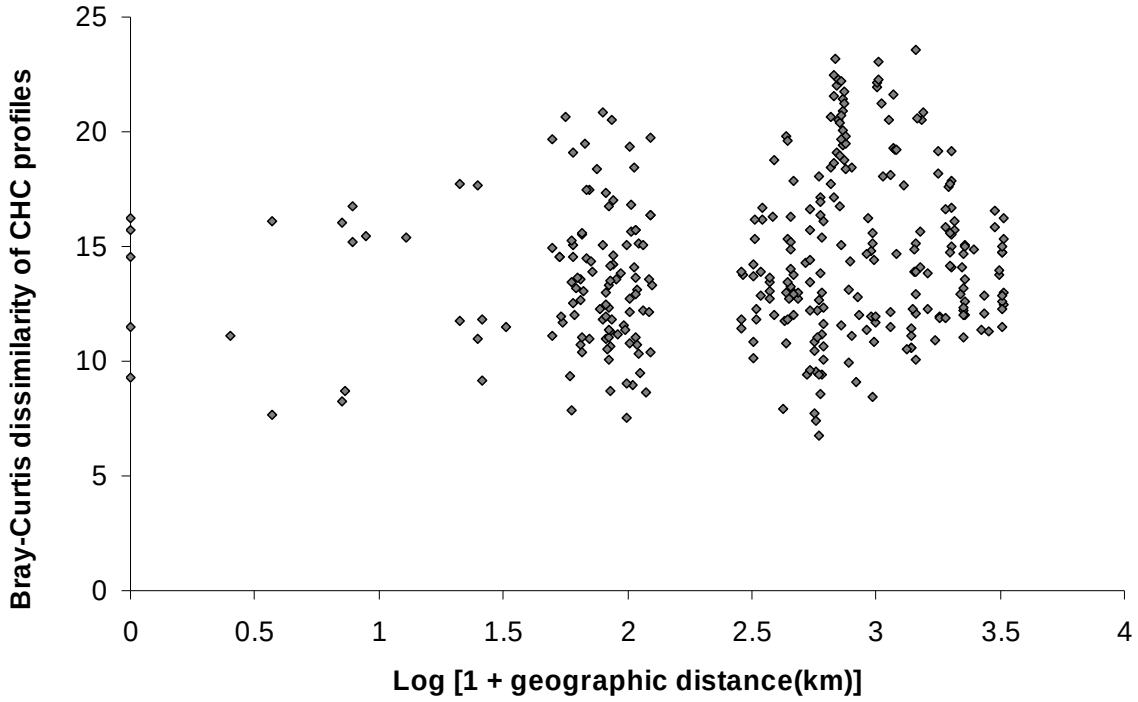


Figure 6: Pairwise comparisons between *C. cobaltinus* and *C. auratus* populations as a function of distance between sampling locations. The five points on the y-axis represent truly sympatric *C. cobaltinus* and *C. auratus* found at the same site within the hybrid zone.

divergence is not higher among *Chrysochus* hybrid zone populations compared to populations outside the hybrid zone. These data were further analyzed by geographic groups, but patterns of CHC divergence between sympatric populations of opposite species were not significantly different from patterns of divergence between allopatric vs. allopatric or sympatric vs. allopatric population pairs (Appendix Figure A1).

Although there was no evidence of greater CHC profile divergence between species in sympatry, I did find evidence that sympatric beetles of each species have more restricted CHC profiles compared to conspecific allopatric beetles. For *C. cobaltinus*, the CHC profiles of sympatric populations appear more condensed in the 2-dimensional space of the MDS plot (Figure 5), relative to allopatric populations. This relatively high resemblance is also observed in a mean Bray-Curtis similarity value of 96.01 (± 0.84) for sympatric *C. cobaltinus* populations (Table 3). Among *C. auratus* populations, CHC profiles of different geographic groups separate out along the MDS axes, such that sympatric populations overlap somewhat with allopatric near populations, but not with allopatric far populations (Figure 5). Geographic patterns observed in the MDS plot were tested with ANOSIM to assess the significance of the apparent divergence of CHC profiles within each species in the hybrid zone. Among *C. cobaltinus*, sympatric populations and allopatric populations near the hybrid zone had significantly different CHC profiles ($R = 0.222$, $p = 0.024$). *Chrysochus cobaltinus* populations far from the hybrid zone were not significantly different from allopatric populations near the hybrid zone ($R = -0.021$, $p = 0.558$) nor sympatric populations ($R = -0.201$, $p = 0.995$). Among *C. auratus*, sympatric populations and allopatric populations far from the hybrid zone were significantly different from each other ($R=0.304$, $p = 0.017$). Populations of *C. auratus* near the hybrid zone were not significantly different

Table 3: Similarity in CHC profiles among populations in different geographic regions (sympatric, allopatric near the hybrid zone, and allopatric far from the hybrid zone). Mean Bray-Curtis similarity values ± standard deviation are shown (number of population pairs in parentheses). Values are based on a similarity matrix between all possible population pairs within a species.

	Between sympatric populations	**Between allopatric near populations**	**Between allopatric far populations**
C. cobaltinus	96.01 ± 0.84 (15)	92.75 ± 3.01 (21)	93.18 ± 2.24 (28)
C. auratus	93.21 ± 2.43 (15)	92.87 ± 2.55 (10)	88.69 ± 3.90 (15)

from sympatric *C. auratus* (R = 0.149, p = 0.117) nor allopatric *C. auratus* far from the hybrid zone (R = 0.083, p = 0.188).

Principal components analysis of the 25 confirmed hydrocarbons resulted in 6 principal components (PCs) which together explained 91.2% of the variance among populations. PCs 1-6 accounted for 32.9, 25.8, 14.5, 8.2, 5.4, and 4.4% of the variance respectively. PC 1 differentiates between species and was significantly influenced by 9-nonacosene, 2-methylnonacosane, *n*-triacontane, 9-hentriacontene, unknown 33 (tentatively 2-methyldotriacontane), unknown 29, *n*-tritriacontane, and unknown 14 (tentatively 15,19-dimethyltritriacontane) (Figure 7; Table 4). These compounds include the three top contributors to dissimilarity between species based on Bray-Curtis values (Table 2). PC 2 is associated with geographic patterns within each species, and is significantly influenced by *n*-tetracosane, 2-methyltetracosane, 9-pentacosene, *n*-pentacosane, 2-methylhexacosane, *n*-heptacosane, and unknown 12 (Figure 7; Table 4). In corroboration with the MDS results, sympatric *C. cobaltinus* populations are tightly clustered compared to other allopatric populations. This indicates that sympatric populations of this species show less variability in those compounds which are significant contributors to PC2. Among *C. auratus*, allopatric populations far from the hybrid zone are associated with low PC 2 values, allopatric populations near the hybrid zone are associated with mid-range PC 2 values, and sympatric *C. auratus* populations are associated with high PC 2 values. This indicates that sympatric *C. auratus* individuals have high concentrations of those compounds which are positively correlated with PC 2 and low concentrations of those compounds which are negatively correlated with PC 2 (Table 4).

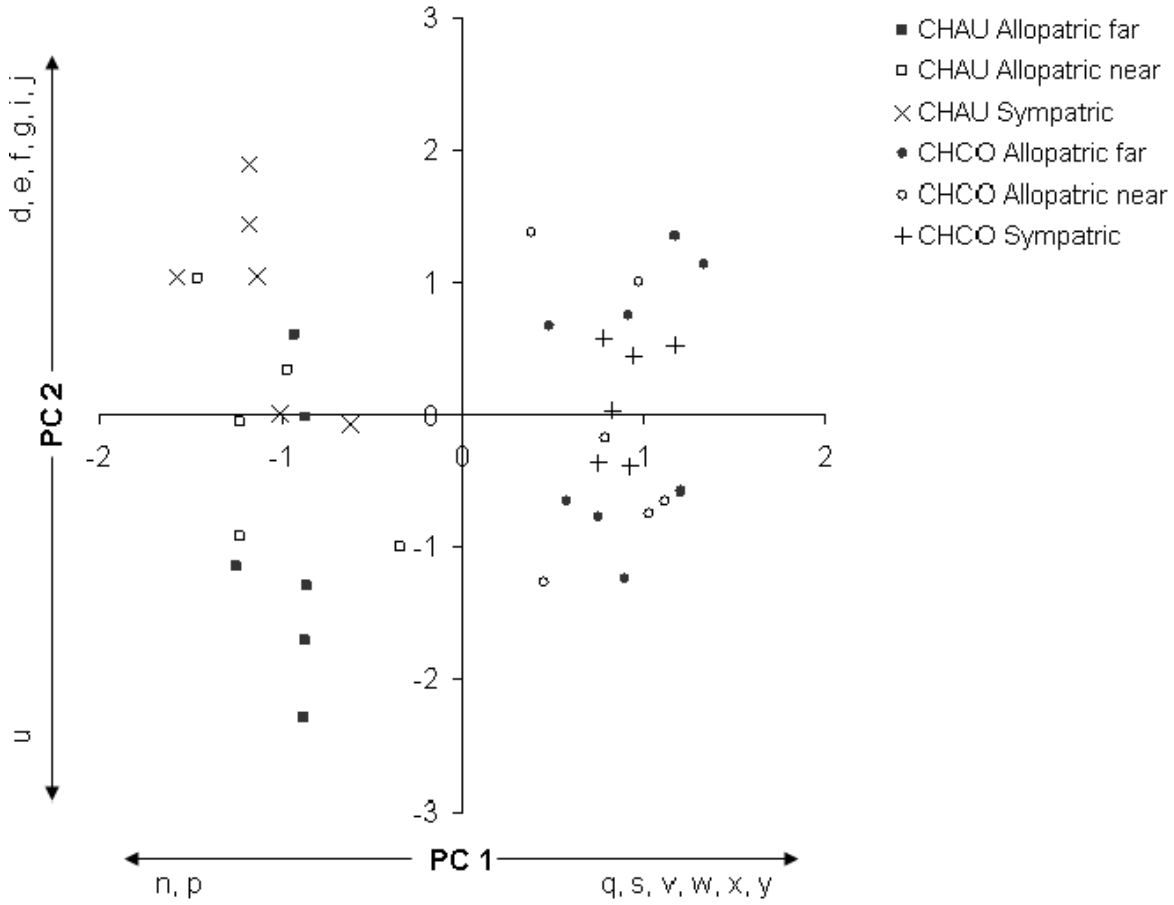


Figure 7: Principal components plot with PC scores for PC 1 and PC 2. The letters along each axis indicate which CHC compounds are positively or negatively correlated with that PC, and correspond with peak identities listed in Table 1. Each data point represents one population and is based on mean relative abundance values of CHCs. "Allopatric far" and "Allopatric near" indicate the proximity of populations to the hybrid zone. CHAU = *Chrysochus auratus*, CHCO = *C. cobaltinus*.

Table 4: The contribution of each CHC to principal components 1-6. Pearson correlation coefficients between relative peak area and principal components are shown, with significant contributors (see Methods) indicated by an asterisk.

Compound	PC1	PC2	PC3	PC4	PC5	PC6
2-methyldocosane	0.222	-0.230	0.296	0.826*	0.044	-0.202
9-tricosene	-0.050	0.044	0.463	0.812*	-0.006	-0.108
n-tricosane	0.087	0.278	-0.121	0.856*	-0.115	0.139
n-tetracosane	0.057	0.831*	0.149	0.234	0.337	0.123
2-methyltetracosane	-0.032	0.980*	0.043	0.024	-0.034	-0.056
9-pentacosene	-0.112	0.863*	0.272	-0.036	-0.077	-0.185
n-pentacosane	-0.178	0.944*	-0.125	-0.065	-0.034	-0.054
n-hexacosane	0.220	0.598	0.132	0.143	0.698*	-0.020
2-methylhexacosane	0.424	0.731*	0.306	0.093	0.370	0.046
n-heptacosane	-0.114	0.722*	-0.560	-0.049	0.190	0.139
2-methylheptacosane	0.398	0.093	0.501	0.295	0.676*	0.064
n-octacosane	-0.018	-0.073	-0.237	-0.280	0.883*	0.055
2-methyloctacosane	0.137	-0.044	0.101	-0.081	0.052	0.959*
9-nonacosene	-0.926*	0.082	0.122	0.040	-0.068	-0.060
n-nonacosane	-0.237	0.000	-0.856*	-0.356	0.006	0.013
2-methylnonacosane	-0.857*	-0.382	0.192	-0.063	0.074	-0.178
n-triacontane	0.730*	-0.220	-0.153	-0.101	0.386	-0.098
2-methyltriacontane	0.649	-0.669	0.017	0.049	-0.051	0.063
9-hentriacontene	0.891*	-0.232	0.193	0.018	-0.072	-0.039
n-hentriacontane	0.339	-0.105	-0.849*	-0.055	0.009	-0.194
unknown 12	0.167	-0.862*	0.196	-0.074	0.156	-0.124
unknown 33 (2-methyldotriacontane)[†]	0.932*	0.105	0.042	0.156	0.076	0.015
unknown 29	0.934*	-0.027	0.068	0.226	-0.038	-0.015
n-tritriacontane	0.947*	-0.131	0.039	0.063	0.162	0.039
unknown 14 (15,19-dimethyltritriacontane)[†]	0.953*	-0.102	0.095	0.001	0.142	0.093

[†] Tentatively identified based on MS spectra and information from Peterson et al. 2007

Genetic Analysis

Allozyme loci were scored for 781 *C. cobaltinus* individuals, and allele frequencies are listed in the appendix (Table A3). Comparing F_{ST} values to the geographic distance between populations revealed a pattern of genetic isolation by distance (Figure 8). Specifically, there was a significant positive relationship between population genetic structure (log F_{ST}) and the geographic distance between populations (log geographic distance) ($r = 0.7347$, $p < 0.001$). This relationship can be described by the equation log F_{ST} = 1.264(log (geographic distance)) – 3.919, when jackknifed over all populations (95% confidence interval = -4.798 to -3.041 for intercept and 0.872 to 1.656 for slope).

Global AMOVA revealed that most genetic variation (86.23%) was due to differences among individuals within a population rather than among geographic groups or populations within geographic groups ($p< 0.00001$) (locus-by-locus results were qualitatively identical). However, there were still significant differences between geographic groups, accounting for 6.09% of the total variation ($p < 0.001$). An additional 7.67% of the variation was among populations within these groups ($p < 0.00001$) (Table 5). Fixation indices revealed significant population genetic structuring at multiple levels. Genetic structure was most pronounced among all populations ($F_{ST} = 0.13765$, $p < 0.00001$), but was also seen among geographic groups ($F_{CT} = 0.06093$, $p < 0.001$) and among populations within groups ($F_{SC} = 0.08170$, $p < 0.00001$).

I compared genetic distance to CHC profile similarity to test the hypothesis that variation in CHC profiles could be explained by population genetic structure. I found that the genetic distance between populations did not correlate significantly with CHC profile similarity ($r = 0.176$, $p = 0.125$) (Appendix Figure A2). This result indicates that overall

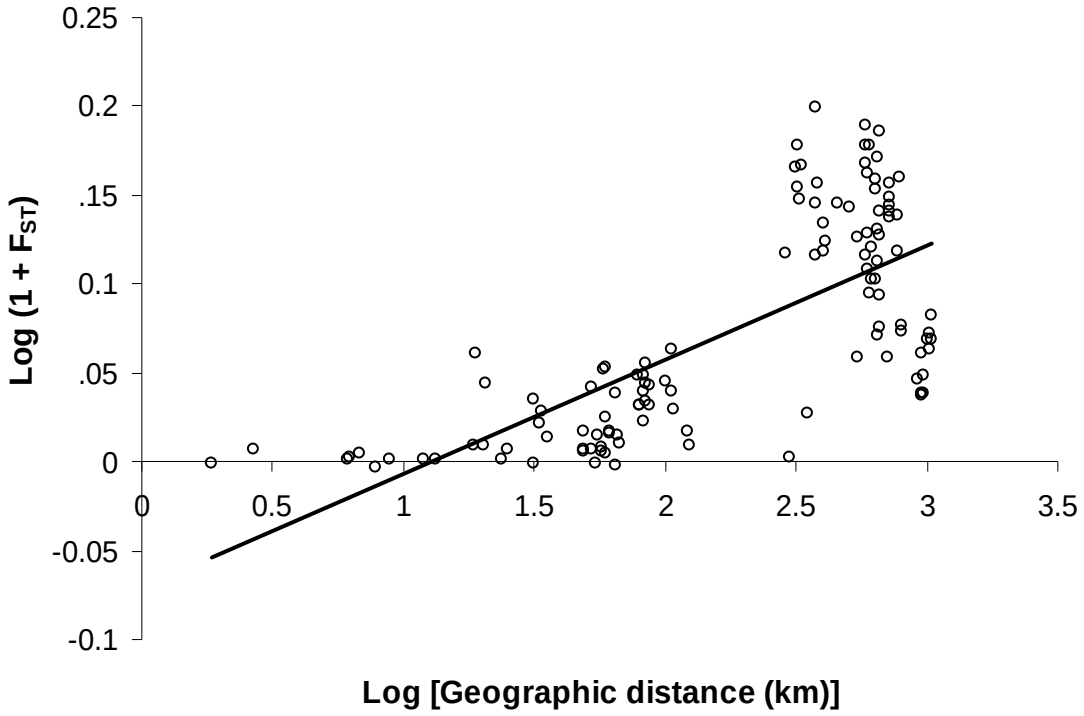


Figure 8: Isolation by distance among *C. cobaltinus* populations. Each point represents the genetic distance (F_{ST}) and geographic distance between two populations. Statistics (see Results text) are reported for the relationship between log (F_{ST}) and log (geographic distance), but log (1 + F_{ST}) is used here for graphical purposes to avoid negative values and simplify interpretation.

Table 5: AMOVA design and results. Fixation index values can range from 0, when allele frequencies are identical, to 1, when populations or groups are fixed for different alleles.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage variation
Among groups	2	33.672	0.02562*	6.09
Among populations within groups	13	45.584	0.03225**	7.67
Within populations	1546	560.496	0.36255**	86.23
Total	1561	639.752	0.42042	
Fixation indices				
FSC (among populations within group)		0.08170**		
FST (among all populations)		0.13765**		
FCT (among groups)		0.06093*		

* $p < 0.001$
** $p < 0.00001$

population genetic subdivision is not a likely explanation for *C. cobaltinus* CHC divergence. However, the CHC profiles of allopatric populations near the hybrid zone apparently have diverged more from hybrid zone populations than have allopatric populations far from the hybrid zone, after controlling for genetic distance. Specifically, for pairwise comparisons among populations with $F_{ST} \approx 0.10$, the pairs with allopatric populations near the hybrid zone had greater Bray-Curtis dissimilarity to sympatric populations than did the pairs with allopatric populations far from the hybrid zone (Figure 9). This pattern was obscured in the complete pairwise comparison in which all allopatric populations were lumped together (Appendix I, Figure A2).

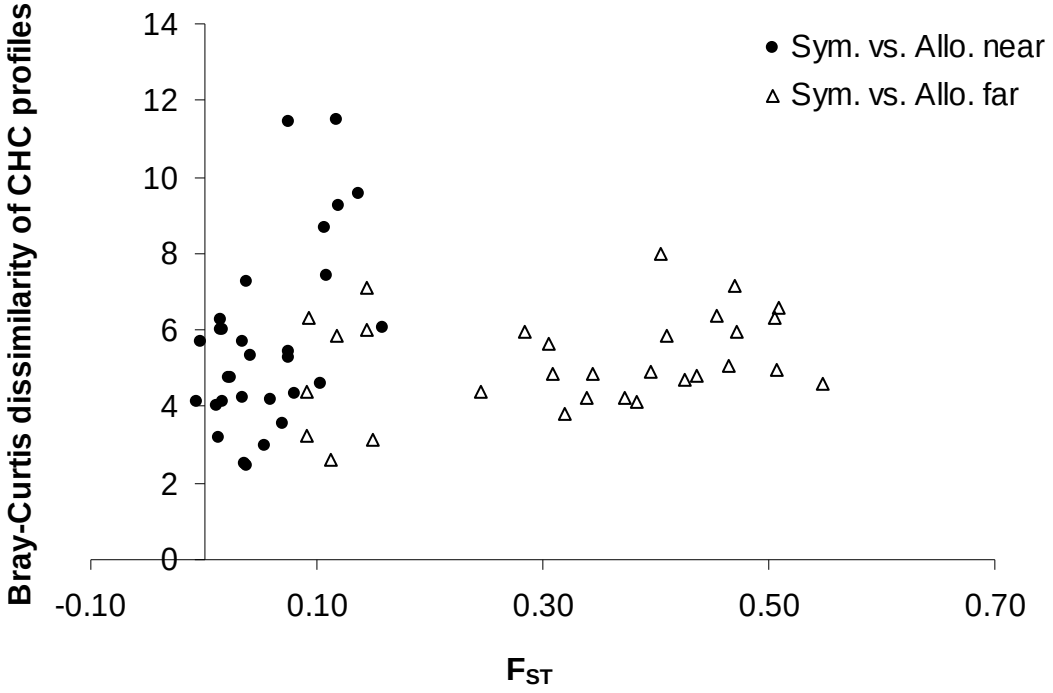


Figure 9: Relationship between genetic distance and CHC profile differences for *C. cobaltinus*. Pairs consist of sympatric populations compared to allopatric populations near the hybrid zone (●) and sympatric populations compared to allopatric populations far from the hybrid zone (Δ).

Discussion

Overview

The overall goal of this study was to determine if geographic patterns of CHC variation are consistent with a pattern of reproductive character displacement that was previously observed in the *Chrysochus* hybrid zone (Peterson et al. 2005a). CHC variation was implicated in that case of reproductive character displacement, because *Chrysochus* male mate choice is governed by species-specific CHC differences (Peterson et al. 2007). Furthermore, females of the two species were apparently more distinguishable if they were from the hybrid zone than if they were from allopatric populations (Peterson et al. 2005).

The results of my study confirmed that CHC profiles of *C. cobaltinus* and *C. auratus* are significantly different. I did not find increased CHC profile divergence in hybrid zone populations, but that could be explained by the presence of other selective forces or uncertainty about which compounds drive mate choice. An analysis of intraspecific variation in CHC profiles found increased homogeneity of sympatric *C. cobaltinus*, which caused these populations to be significantly different from allopatric *C. cobaltinus* near the hybrid zone. This pattern was not explained by allozyme-based measures of genetic relatedness, and could be caused by reinforcing selection in the hybrid zone. Although there are other possible explanations, the results of this study are consistent with the hypothesis that the pattern of reproductive character displacement observed in the *Chrysochus* hybrid zone is due to changes in CHC profiles.

Cuticular Hydrocarbon Divergence Between Species

Studies in other insect systems have indicated that divergence of cuticular hydrocarbons can play a key role in reproductive character displacement. One well studied example is the previously mentioned case of *Drosophila serrata* (Higgie et al. 2000), which co-occurs with *D. birchii* in eastern Australia. Higgie and colleagues found that when allopatric populations of *D. serrata* were forced into sympatry with *D. birchii* in the lab, the CHC profiles of *D. serrata* evolved to mirror the pattern of reproductive character displacement observed in the field. In a similar example, the CHCs of mosquitoes were studied by Milligan et al. (1993), who used gas chromatography to distinguish between populations of *Anopheles arabiensis* and *A. gambiae sensu stricto*. They found that sympatric populations could be more clearly distinguished from each other than allopatric populations. Studies of the nun moth, *Lymantria monacha* (Gries et al. 2001), have also gathered evidence that selection pressures in sympatry can lead to the divergence of CHCs. Gries and colleagues observed CHC divergence between central European and Japanese populations of *L. monacha*. They hypothesized that this divergence was caused by the presence in Japan of a cogener, *L. fumida*, that uses pheromone signals similar to *L. monacha*. However, further research documenting the divergence of sympatric *L. fumida* and *L. monacha* CHC profiles is needed to demonstrate reproductive character displacement in this system.

In the *Chrysochus* system I looked for a similar link between CHC variation and reproductive character displacement by testing the hypothesis that sympatric populations of *C. cobaltinus* and *C. auratus* would have more divergent CHC profiles than allopatric populations of *C. cobaltinus* and *C. auratus*. While I confirmed that *C. cobaltinus* and *C.*

auratus do have significantly different CHC profiles, the overall CHC divergence between species was not higher among hybrid zone populations. The lack of greater divergence in CHC profiles in the hybrid zone could have several explanations, including 1) the presence of other evolutionary forces shaping CHC profiles, 2) uncertainty regarding which specific compounds are most important for mate choice, and 3) the possibility that reproductive character displacement is caused mainly by selection on other character traits, such as male preference genes.

Cuticular hydrocarbons are subject to a variety of natural and sexual selection pressures, and the result of such selection may mask or overpower reinforcing selection. Natural selection may cause or prevent changes in CHCs due to their important role in desiccation prevention. In their study of *Drosophila melanogaster* and *D. simulans,* Rouault et al. (2004) found a significant correlation between latitude, temperature, and certain CHCs. They demonstrated that those populations with an excess of C25 compounds had a higher resistance to desiccation, and originated from areas with historically higher temperatures. Moreover, increasing the breeding temperature from 18 ºC to 29 ºC led to an increase in C25 compounds and a decrease in C23 compounds over multiple generations. This type of natural selection on CHCs, which could vary among populations in the Pacific Northwest, could confound the search for signs of reinforcing selection in the *Chrysochus* hybrid zone.

It is also possible that CHC profiles may reflect a balance between reinforcing selection and other types of selection, which may run counter to reinforcing selection. For example, field studies and manipulative experiments have shown that *Drosophila serrata* undergoes reinforcing selection that causes it to evolve a distinct sympatric CHC profile whenever it co-occurs with *D. birchii* (Higgie et al. 2000). Higgie and Blows (2008) did a subsequent series

of selection experiments using sympatric-allopatric hybrids of *D. serrata* with an intermediate CHC profile. They found that when sexual selection was allowed (and *D. birchii* were excluded), male CHCs evolved to resemble the CHCs of allopatric populations. They concluded that, in this system, sexual selection operates in conflict with reproductive character displacement. It is possible that a similar conflict between sexual selection and reinforcing selection is occurring in the *Chrysochus* hybrid zone. Under this scenario, the CHC profiles of *C. cobaltinus* and *C. auratus* may not diverge (despite the cost of producing unfit hybrids), because stronger sexual selection forces favor the current pheromone blend.

Another possible explanation for the lack of greater CHC divergence between species in the hybrid zone is that my analysis focused on general CHC profiles, rather than on specific compounds that are known to be important for mate recognition. Previous research by Peterson et al. (2007) found that *Chrysochus* CHC profiles are species-specific and known to influence mate choice. However, that study did not determine whether it was the presence or absence of a particular compound, the relative abundance of multiple compounds, or enantiomers of single compounds that most influenced mate choice. This type of uncertainty has been encountered in similar studies, such as those dealing with the walking stick insect *Timena cristinae*. Nosil et al. (2003) found strong reproductive character displacement in *T. cristinae*, and documented evidence that this pattern was likely due to reinforcing selection. Behavioral experiments demonstrated that mate discrimination is at least partly based on pheromones, but GC/MS analysis did not find significant differences in pheromone profiles among test populations (Nosil et al. 2007). Based on candidate pheromones, Nosil and colleagues hypothesized that the differences observed in the behavioral experiments could be due to a qualitative aspect of pheromones, such as enantiomer composition. If a similar

situation occurred in *Chrysochus*, I would have been unable to detect the divergence with my methods.

Interestingly, the compound which contributes most to dissimilarity between the two species, unknown 14, has been tentatively identified as 15,19-dimethyltritriacontane. 15,19-dimethyltritriacontane would be the only chiral compound in the *Chrysochus* CHC profile, and it can exist as three different stereoisomers. The chemical communication systems of insects are known for their specificity, and can frequently distinguish between stereoisomers or the enantiomers of a chiral compound (e.g. Millar et al. 1990, Gries et al. 1999, Zhang et al. 2006). For example, studies of the bark beetle, *Ips pini*, have found enantiomer-specific responses which resulted in assortative mating (Teale et al. 1994) and partial barriers to gene flow between groups (Cognato et al. 1999). In the *Chrysochus* system, a more detailed analysis of isomer blends of 15,19-dimethyltritriacontane would be a fruitful area for future research. Another area for future research is to determine whether 15,19-dimethyltritriacontane is one of the compounds most important for species identification and mate choice. 15,19-dimethyltritriacontane is known to influence mate choice in insects such as the stable fly, *Stomoxys calcitrans* (Sonnet et al. 1977) and the tsetse fly, *Glossina austeni* (Huyton et al. 1980), but its exact role in *Chrysochus* remains unknown. Upon resolving which specific compound(s) drive mate choice, the data collected for the present study could be reanalyzed in a compound-specific analysis.

An alternative approach to analyzing the data in a compound-specific manner could be based upon the results of the principle component analysis. PC 2 was associated with geographic patterns within each species, and future research could involve a more detailed analysis of those compounds which were significant contributors to this principle component.

Although overall CHC profiles were not more divergent in sympatry, the two species may have diverged only in those compounds strongly associated with PC 2. A visual analysis of Figure 7 indicates that any divergence would have been slight, and could have been overlooked in an analysis focusing on the complete CHC profile.

Finally, it is possible that the pattern of reproductive character displacement previously observed in *Chrysochus* is due mainly to selection on character traits not tested in this study. Reproductive character displacement can be influenced by a variety of character traits involved in mate choice, and has been linked in previous studies to changes in plumage color in birds (Saetre et al. 1997), acoustic communication in frogs (Höbel and Gerhardt 2003), and gamete recognition proteins in sea urchins (Geyer and Palumbi 2003). In the *Chrysochus* system, reproductive character displacement could also be caused by changes in male preference genes. Peterson and colleagues (2005a) argue that reproductive character displacement in *Chrysochus* is due to both increased choosiness of sympatric *C. cobaltinus* males, as well as more distinguishable hybrid zone females. However, the relative importance of these two contributors to reproductive character displacement was not assessed. Thus, if hybrid zone females are only slightly more distinguishable than allopatric females, it might be difficult to find statistical evidence of greater CHC divergence in sympatry. In this case, reproductive character displacement could be caused mostly by selection on male preference genes. Previous studies have found evidence for reinforcing selection on preference genes (McPeek and Gavrilets 2006, Kronforst et al. 2007), and this type of scenario could explain the lack of increased divergence in sympatric *C. cobaltinus* and *C. auratus* CHC profiles.

Intraspecific Variation in CHC Profiles

Although this study did not find evidence of greater divergence between *C. cobaltinus* and *C. auratus* in sympatry, I did find evidence that CHC profiles of hybrid zone beetles differ from those of conspecific allopatric populations. Such a pattern could reflect underlying reinforcing selection. Specifically, I found that sympatric *C. cobaltinus* populations had CHC profiles that were significantly different from allopatric *C. cobaltinus* near the hybrid zone. Interestingly, this difference was not due to divergence of the two groups, but instead resulted from *C. cobaltinus* populations forming a more homogeneous profile within the hybrid zone (Figures 5 and 7; Table 3). A potential explanation for this phenomenon is provided by Noor (1999), who suggests that reinforcement can act by reducing the range of acceptable phenotypes instead of shifting the mean phenotype. If CHC variation in hybrid zone *C. cobaltinus* females has indeed been canalized by reinforcing selection in this manner, it would make it a challenge to document greater overall *divergence* between the two species in sympatry.

Unlike the results for *C. cobaltinus,* the CHC profiles of *C. auratus* did not differ between sympatric populations and allopatric populations near the hybrid zone. Instead, *C. auratus* CHC profiles followed a geographic gradient in that the profiles of sympatric populations and allopatric populations far from the hybrid zone were most divergent from one another, with those of allopatric populations near the hybrid zone showing intermediate CHC profiles (Figures 5 and 7). Such differing patterns for the two species could be due to several factors, including: 1) species-specific patterns of reinforcing selection acting on females, 2) a lack of genetic variation for selection to act on in *C. auratus*, 3) a greater geographic spread amongst *C. auratus* populations, 4) an underlying gradient in natural

selection imposed by an environmental gradient, or 5) an underlying gradient in sexual selection.

The strength of reinforcing selection acting on the two *Chrysochus* species might differ due to their relative abundance. Indeed, it has been argued that if one species is relatively rare, that species should be under stronger selection to avoid hybridization, because it is more likely to encounter heterospecific mates in the field (Howard 1993). Similar studies have found an asymmetrical pattern of reproductive character displacement which is consistent with this relative abundance hypothesis (Waage 1979, Noor 1995). Previous work in the *Chrysochus* hybrid zone found reproductive character displacement in *C. cobaltinus* but not *C. auratus* (Peterson et al. 2005a). Uneven abundance was viewed as a possible explanation in this situation, because all sympatric beetles were taken from a site where *C. auratus* was numerically dominant. My data were complicated by the fact that beetles were collected from seven different sites within the hybrid zone. *Chrysochus auratus* is known to be the dominant species at two of those sites (site codes AR and S, Appendix Table A1), *C. cobaltinus* is dominant at one site (AQ), and relative abundance at four others has not been quantified (Peterson et al. 2005a). Interestingly, at the site where *C. cobaltinus* was dominant, CHC profiles of *C. auratus* were most divergent from allopatric *C. auratus*. At the sites where *C. auratus* was dominant, CHC profiles of *C. auratus* were least divergent from allopatric *C. auratus*. Although the sympatric and allopatric near groups were not significantly different, these data provide tentative support for the hypothesis that relative abundance has influenced the species-specificity of reinforcing selection in this system. A more detailed analysis of site specific differences, in conjunction with relative abundance data, could be a fruitful area for further research.

Another factor that could lead to asymmetries in reinforcing selection is conspecific sperm precedence. Conspecific sperm precedence refers to the favored utilization of sperm from conspecific males, when a female has been inseminated by both conspecific and heterospecific individuals (Howard 1999). The result is fewer hybrid progeny than expected, based on the frequency of heterospecific matings. Several studies have found conspecific sperm precedence to play an important role in isolating closely related taxa (Howard 1999 and references therein). Marshall et al. (2002) argued that, by buffering females from the cost of mating 'mistakes', conspecific sperm precedence might reduce the strength of reinforcing selection in systems where hybrids have low fitness. This idea has been supported by subsequent theoretical analyses (Lorch and Servedio 2007). Previous work in the Peterson lab (unpublished data) found that, in a controlled study featuring a wide range of heterospecific mating frequencies, *C. auratus* females were much less likely to produce hybrid offspring than *C. cobaltinus* females, and that the fitness of *C. auratus* females was less impacted by heterospecific matings. Thus, *C. cobaltinus* females may be experiencing greater reinforcing selection than *C. auratus* females due to asymmetries in conspecific sperm precedence.

Differences in CHC divergence patterns could also be explained by species-specific differences in levels of genetic variation within populations. Previous work by Dobler and Farrell (1999) found relatively little genetic variation in *C. auratus* compared to *C. cobaltinus*. This low genetic variation could be due to bottlenecks associated with range expansion, or lower species mobility. Regardless of the cause, selection against hybridization may not lead to reproductive character displacement in *C. auratus*, if the

species lacks the genetic variation needed to respond to such selection, as argued by Peterson et al. (2005a).

Sympatric beetles of the two species also differed in their degree of divergence from allopatric populations far from the hybrid zone. CHC profiles of sympatric *C. auratus* were significantly different from CHC profiles of *C. auratus* far from the hybrid zone, a pattern not observed in *C. cobaltinus*. This pattern may or may not reflect the influence of reinforcing selection. It is possible that reinforcing selection is influencing *C. auratus* CHC profiles in the hybrid zone, and gene flow from sympatric populations to adjacent allopatric populations is leading to a similar "central Washington" phenotype, which is distinct from *C. auratus* populations far from the hybrid zone. Alternatively, the difference in CHC profiles may simply reflect a greater geographic distance between the hybrid zone and some *C. auratus* populations, and be unrelated to reinforcing selection. The mean distance between the hybrid zone and those *C. auratus* classified as "allopatric far" was close to three times greater than the mean distance between the hybrid zone and *C. cobaltinus* classified as "allopatric far" (1522 km vs. 562 km). The significant CHC divergence could reflect genetic differences resulting from the large geographic distance between some *C. auratus* populations.

Finally, the unique patterns of CHC divergence observed in each *Chrysochus* species could be due to underlying gradients in natural selection or sexual selection that are unique to each species. CHC profiles have been shown to vary in response to precipitation and temperature gradients (Buckley et al. 2003, Rouault et al. 2004), due to the resulting gradient of natural selection along population transects. CHC profiles can be additionally influenced by sexual selection (Ferveur 2005, Higgie and Blows 2008). It is possible that the

asymmetrical pattern of CHC variation in *C. auratus* and *C. cobaltinus* is due to unique clines of natural and/or sexual selection for each species. The existence of such clines could likely obscure the effects of reinforcing selection.

Causes of CHC Profile Variability: Genes or Geography?

To examine more thoroughly the factors underlying CHC variation in this system, I combined CHC data, allozyme data, and geographic information for *C. cobaltinus* to test the contrasting hypotheses that: (1) geographic variability in CHC profiles is best explained by population genetic structure, or (2) geographic variability of CHC profiles is best explained by proximity to the hybrid zone. If variability of CHC profiles is best explained by proximity to the hybrid zone, this would provide support for reinforcing selection in regions of sympatry.

The allozyme data indicate that population genetic structure does exist in *C. cobaltinus*, and a pattern of genetic isolation by distance was found, which is consistent with previous studies (Dobler and Farrell 1999). However, this population genetic structure is not a likely explanation for CHC divergence between conspecific populations, because there was no correlation between genetic distance and CHC profile divergence. Thus the patterns of geographic CHC variation I observed in *C. cobaltinus* cannot be explained solely by the relatedness of populations.

After rejecting the hypothesis that variability in CHC profiles is best explained by population genetic structure, I tested the contrasting hypothesis that geographic variability of CHC profiles is best explained by proximity to the hybrid zone. This hypothesis was supported by the comparison of sympatric vs. allopatric *C. cobaltinus* populations. When

controlling for genetic distance, the CHC profile divergence between sympatric and allopatric populations near the hybrid zone was *greater* than the CHC profile divergence between sympatric and allopatric populations far from the hybrid zone.

Analyses of population genetic structure and reproductive character displacement have been combined in a limited number of studies (Geyer and Palumbi 2003, Gabor et al. 2005). But to my knowledge, no studies have explicitly tested the hypothesis that genetic relatedness can explain variation of reproductive traits associated with reproductive character displacement. Research that incorporates population genetic structure and CHC variation tend to fall into one of two categories. Such studies either: (1) analyze whether population genetic structure can explain widespread CHC variation in several insect populations, in the absence of a hybrid zone context (e.g. Dalecky et al. 2007), or (2) integrate population genetic structure with CHC analysis for taxonomic or historical purposes (e.g. Ugelvig et al. 2008). However, using population genetic structure to explain reproductive character displacement of CHCs is a novel approach to studying reinforcement. Thus, while other studies have documented increased CHC divergence in hybrid zone regions (e.g. Milligan et al. 1993), the *Chrysochus* system differs in that I can provisionally eliminate genetic relatedness as a cause of such divergence. Because genetic relatedness apparently does not explain this divergence, these data support the idea that selection pressures in the hybrid zone region could be influencing intraspecific variation in CHC profiles.

Summary

This thesis has built on a previous study which found that *C. cobaltinus* males could more easily distinguish between *C. cobaltinus* and *C. auratus* females from sympatric, as

opposed to allopatric, populations (Peterson et al. 2005a). Peterson and colleagues argued that this pattern of reproductive character displacement was due to selection against unfit *Chrysochus* hybrids (Peterson et al. 2005b) in regions of sympatry. Given these results, along with the importance of CHCs in sexual isolation in this system (Peterson et al. 2007), it appeared likely that reinforcing selection had driven the divergence of Chrysochus CHC profiles in sympatry.

Although I did not find a pattern of increased CHC divergence between sympatric populations of *C. cobaltinus* and *C. auratus*, I did find that the CHC profiles of sympatric *C. cobaltinus* are significantly different from allopatric *C. cobaltinus* near the hybrid zone. The increased homogeneity of sympatric *C. cobaltinus* CHC profiles could be responsible for the fact that *C. cobaltinus* males found them easier to identify in mating trials (Peterson et al. 2005a). After taking into account genetic distance, this study also found that allopatric populations near the hybrid zone were more divergent from sympatric populations than were allopatric populations far from the hybrid zone. Furthermore, species-specific differences in geographic patterns of CHC variation were consistent with recent evidence that the strength of reinforcing selection on females is species-specific. Although there are other possible explanations for these patterns, the results of this study are consistent with the hypothesis that the pattern of reproductive character displacement observed in the hybrid zone is due to changes in CHC profiles. Once the specific compounds governing mate choice are determined, it will be possible to test this hypothesis more rigorously. Further research could test the hypothesis that reproductive character displacement in *Chrysochus* is also due to selection on male preference genes. In addition, the population-genetic perspective that aided

in the interpretation of the results in this study could prove fruitful in other studies of reproductive character displacement.

Table A1: Details of *Chrysochus* samples collected from each population for *C. cobaltinus* (a) and *C. auratus* (b). Host plant abbreviations are APCA = *Apocynum cannabinum*, APAN = *Apocynum androsaemifolium*, and ASSP = *Asclepias speciosa*. Individuals collected prior to 2005 or from ASSP were not used for GCMS analysis. GC/MS samples were all females, while allozyme samples were mixed sexes.

(a)

	Category	Site code	County, State	Latitude	Longitude	Year(s) collected	Host plant	GCMS samples	Allozyme samples
Chrysochus cobaltinus	Sympatric	AA	Yakima County, WA	46º15'04.57"N	120º01'52.25"W	2005, 2006	APCA	10	50
		AQ	Yakima County, WA	46º13'27.47"N	120º07'33.53"W	2005	APCA	10	48
		AR	Yakima County, WA	46º13'06.35"N	120º26'12.34"W	2005	APCA	10	50
		CF	Yakima County, WA	46º16'12.62"N	120º11'03.11"W	2005, 2006	APCA, ASSP	8	47
		S	Yakima County, WA	46º14'47.56"N	120º06'44.12"W	2005	APCA	10	53
		CM	Benton County, WA	46º11'31.81"N	119º21'21.67"W	2006	APCA	10	38
	Allopatric Near	A	Kittitas County, WA	46º57'18.69"N	120º31'12.46"W	1998, 2005	APCA	10	50
		B	Kittitas County, WA	46º56'19.31"N	120º30'59.91"W	1998, 2005, 2006	APCA	10	51
		E	Kittitas County, WA	46º56'36.10"N	120º31'11.04"W	2005	APCA	11	
		L	Yakima County, WA	46º39'29.54"N	120º29'05.91"W	1999, 2005	APCA	10	51
		M	Yakima County, WA	46º38'31.17"N	120º35'52.93"W	1999, 2003, 2004, 2005	APCA	10	51
		P	Kittitas County, WA	46º46'30.80"N	120º27'10.47"W	1999, 2005	APCA	10	51
	Allopatric Far	CP	Blaine County, ID	43º22'00.56"N	113º45'50.46"W	2006	APCA	10	50
		CQ	Gooding County, ID	42º39'45.83"N	114º39'47.95"W	2006	APCA	10	
		CR	Gooding County, ID	42º39'36.22"N	114º38'47.96"W	2006	APCA	10	50
		CS	Utah County, UT	39º57'45.10"N	111º55'02.11"W	2006	APCA	10	41
		CU	Washoe County, NV	39º47'31.80"N	119º20'46.20"W	2006	APCA	10	50
		CV	Malheur County, OR	43º45'11.93"N	118º05'00.74"W	2006	APCA	10	
		CW	Malheur County, OR	43º46'04.37"N	118º02'48.68"W	2006	APCA	10	50
		CX	Ada County, ID	43º34'52.80"N	116º06'22.20"W	2006	APCA	10	

Table A1: Details of *Chrysochus* samples collected from each population for *C. cobaltinus* (a) and *C. auratus* (b). Host plant abbreviations are APCA = *Apocynum cannabinum* and APAN = *Apocynum androsaemifolium*. * indicates regional district and province in Canada.

(b)

	Category	Site code	County, State	Latitude	Longitude	Year(s) collected	Host plant	GCMS samples	Allozyme samples
Chrysochus auratus	Sympatric	AQ	Yakima County, WA	46º13'27.47"N	120º07'33.53"W	2005	APCA	9	-
		AR	Yakima County, WA	46º13'06.35"N	120º26'12.34"W	2005	APCA	10	-
		CF	Yakima County, WA	46º16'12.62"N	120º11'03.11"W	2005, 2006	APCA	10	-
		CG	Benton County, WA	46º10'49.16"N	119º20'45.24"W	2005, 2006	APCA	10	-
		S	Yakima County, WA	46º14'47.56"N	120º06'44.12"W	2004, 2005	APCA	10	-
		CM	Benton County, WA	46º11'31.81"N	119º21'21.67"W	2006	APCA	8	-
	Allopatric Near	AU	Adams County, WA	46º44'33.23"N	119º05'45.16"W	2005	APCA	10	-
		AW	Grant County, WA	46º54'50.23"N	119º40'49.48"W	2005	APCA	10	-
		BK	Grant County, WA	46º56'25.23"N	119º36'02.55"W	2005, 2006	APCA	10	-
		Y	Adams County, WA	46º48'35.90"N	119º07'06.94"W	2005	APCA	10	-
		DD	Sanders County, MT	47º22'06.78"N	114º34'41.70"W	2006	APCA	10	-
	Allopatric Far	CY	Lancaster County, NB	40º47'04.67"N	96º44'57.73"W	2006	APCA	10	-
		CZ	Knox County, TN	-	-	2006	APCA	10	-
		AB	Fraser Fort George RD, BC*	52º47'18.84"N	119º15'20.10"W	2006	APAN	10	-
		AV	Fraser Fort George RD, BC*	52º58'17.36"N	119º25'29.96"W	2006	APAN	10	-
		DA	Poweshiek County, IA	-	-	2006	APCA	10	-
		DF	Boulder County, CO	-	-	2007	APCA	10	-

Table A2 (a-c): Compounds contributing to dissimilarity between *C. cobaltinus* (CHCO) and *C. auratus* (CHAU) in sympatry (a), allopatric areas near the hybrid zone (b), and allopatric areas far from the hybrid zone (c). * indicates tentatively identified compounds.

(a)

Comparison: CHCO sympatric & CHAU sympatric

Average dissimilarity = 18.47

Compound	CHCO average abundance	CHAU average abundance	Average dissimilarity (±SD)	% Contribution	Cumulative % contribution
n-tritriacontane	0.03	0.00	2.20 (0.64)	11.91	11.91
unknown 14 (15,19-dimethyltritriacontane*)	0.03	0.00	2.17 (0.60)	11.73	23.64
2-methylnonacosane	0.01	0.07	1.16 (0.78)	6.30	29.94
n-triacontane	0.01	0.00	1.11 (0.80)	6.02	35.96
unknown 29	0.00	0.00	0.96 (0.72)	5.22	41.18
2-methyltetracosane	0.03	0.06	0.92 (0.63)	4.99	46.17
unknown 33 (2-methyldotriacontane*)	0.00	0.00	0.83 (0.70)	4.47	50.64
9-hentriacontene	0.14	0.05	0.82 (0.35)	4.45	55.09
n-pentacosane	0.03	0.06	0.80 (0.50)	4.33	59.42
9-pentacosene	0.01	0.03	0.78 (0.64)	4.21	63.64
2-methylheptacosane	0.00	0.00	0.77 (0.67)	4.20	67.83
9-nonacosene	0.07	0.16	0.75 (0.36)	4.04	71.87
n-hexacosane	0.00	0.00	0.67 (0.66)	3.63	75.50
n-tetracosane	0.00	0.01	0.62 (0.61)	3.36	78.86
2-methyldocosane	0.01	0.01	0.51 (0.61)	2.78	81.64
2-methyltriacontane	0.16	0.10	0.47 (0.27)	2.56	84.20
n-octacosane	0.01	0.01	0.47 (0.66)	2.56	86.76
9-tricosene	0.01	0.02	0.46 (0.52)	2.47	89.23
unknown 12	0.07	0.06	0.42 (0.30)	2.28	91.51

Table A2 (b)

Comparison: CHCO allopatric near & CHAU allopatric near Average dissimilarity = 19.21					
Compound	**CHCO average abundance**	**CHAU average abundance**	**Average dissimilarity (±SD)**	**% Contribution**	**Cumulative % contribution**
unknown 14 (15,19-dimethyltritriacontane*)	0.02	0.00	2.19 (0.68)	11.38	11.38
n-tritriacontane	0.03	0.00	1.92 (0.83)	9.98	21.36
2-methylnonacosane	0.01	0.09	1.82 (1.07)	9.48	30.84
9-pentacosene	0.01	0.02	1.28 (0.87)	6.66	37.50
n-triacontane	0.01	0.00	1.10 (0.82)	5.73	43.23
9-nonacosene	0.05	0.16	1.08 (0.35)	5.60	48.83
2-methyltetracosane	0.03	0.03	0.91 (0.77)	4.72	53.55
n-pentacosane	0.03	0.03	0.73 (0.60)	3.83	57.38
n-tetracosane	0.00	0.00	0.73 (0.64)	3.82	61.20
unknown 33 (2-methyldotriacontane*)	0.00	0.00	0.68 (0.69)	3.55	64.75
2-methyldocosane	0.01	0.00	0.65 (0.66)	3.40	68.14
unknown 29	0.00	0.00	0.65 (0.75)	3.37	71.52
9-tricosene	0.01	0.01	0.63 (0.72)	3.29	74.80
2-methylheptacosane	0.00	0.00	0.60 (0.65)	3.15	77.95
n-hexacosane	0.00	0.00	0.60 (0.63)	3.12	81.07
9-hentriacontene	0.11	0.06	0.54 (0.35)	2.79	83.86
2-methylhexacosane	0.01	0.01	0.50 (0.51)	2.62	86.48
2-methyltriacontane	0.17	0.12	0.49 (0.35)	2.55	89.03
n-hentriacontane	0.03	0.02	0.41 (0.38)	2.11	91.15

Table A2 (c)

Comparison: CHCO allopatric far & CHAU allopatric far Average dissimilarity = 22.22					
Compound	**CHCO average abundance**	**CHAU average abundance**	**Average dissimilarity (±SD)**	**% Contribution**	**Cumulative % contribution**
unknown 14 (15,19-dimethyltritriacontane*)	0.02	0.00	2.17 (0.62)	9.77	9.77
n-tritriacontane	0.02	0.00	2.06 (0.73)	9.28	19.05
2-methylnonacosane	0.02	0.10	1.54 (1.02)	6.92	25.97
2-methylhexacosane	0.02	0.00	1.42 (0.93)	6.40	32.37
2-methyltetracosane	0.03	0.01	1.29 (1.02)	5.83	38.20
9-pentacosene	0.02	0.01	1.24 (0.98)	5.56	43.76
unknown 29	0.00	0.00	1.03 (0.81)	4.63	48.39
n-tetracosane	0.00	0.00	0.89 (0.71)	4.02	52.41
9-nonacosene	0.05	0.15	0.88 (0.42)	3.95	56.36
9-tricosene	0.01	0.01	0.86 (0.86)	3.86	60.22
2-methyldocosane	0.01	0.00	0.84 (0.82)	3.80	64.02
unknown 33 (2-methyldotriacontane*)	0.00	0.00	0.83 (0.77)	3.73	67.75
n-hexacosane	0.00	0.00	0.82 (0.69)	3.70	71.45
2-methylheptacosane	0.00	0.00	0.82 (0.73)	3.68	75.13
n-triacontane	0.01	0.00	0.80 (0.86)	3.60	78.74
n-pentacosane	0.03	0.02	0.75 (0.67)	3.38	82.12
9-hentriacontene	0.14	0.07	0.61 (0.36)	2.76	84.88
n-tricosane	0.01	0.01	0.57 (0.71)	2.54	87.42
n-octacosane	0.01	0.01	0.50 (0.68)	2.27	89.69
2-methyltriacontane	0.17	0.13	0.44 (0.31)	1.97	91.66

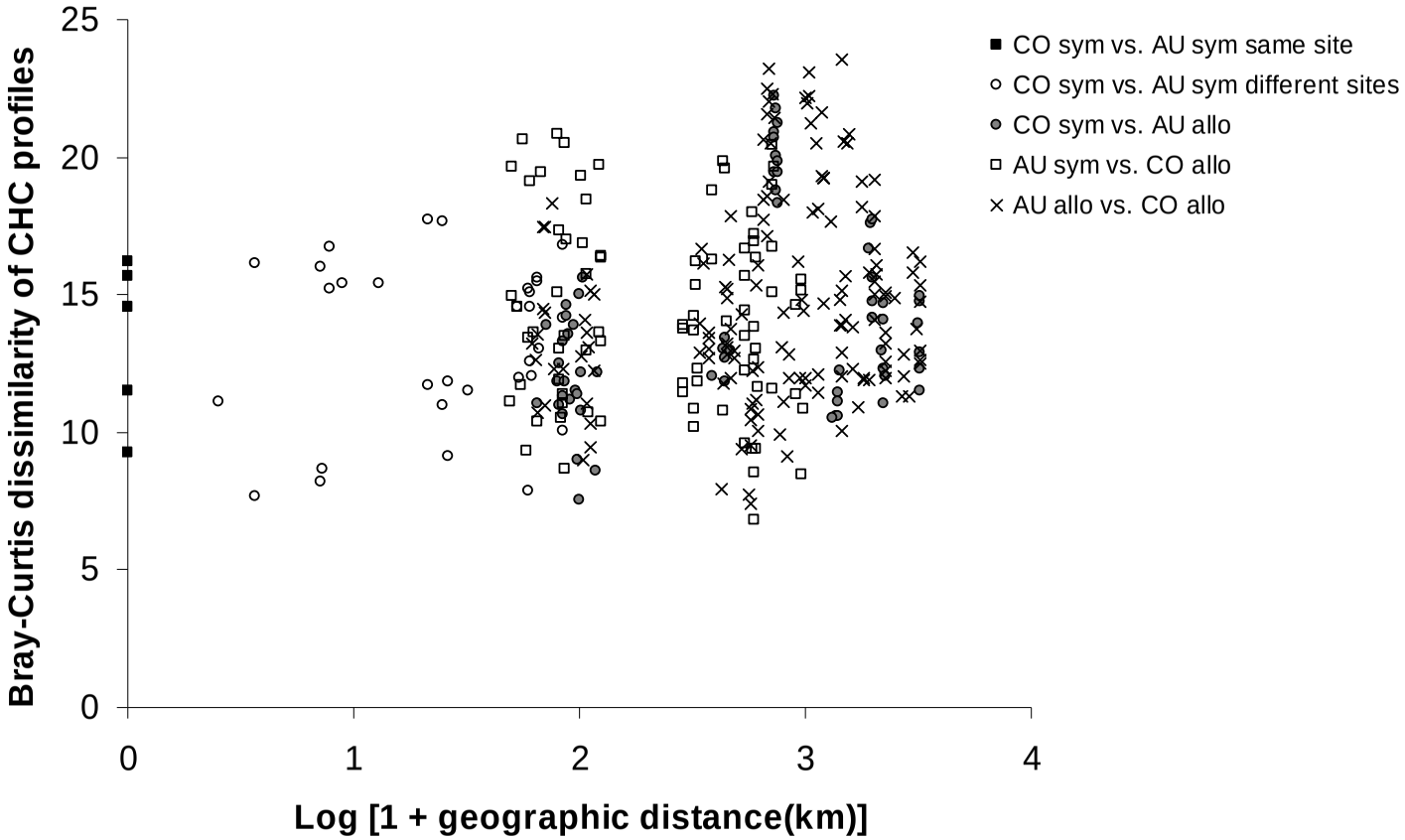


Figure A1: Pairwise comparisons between *C. cobaltinus* and *C. auratus* populations broken down by geographic group.

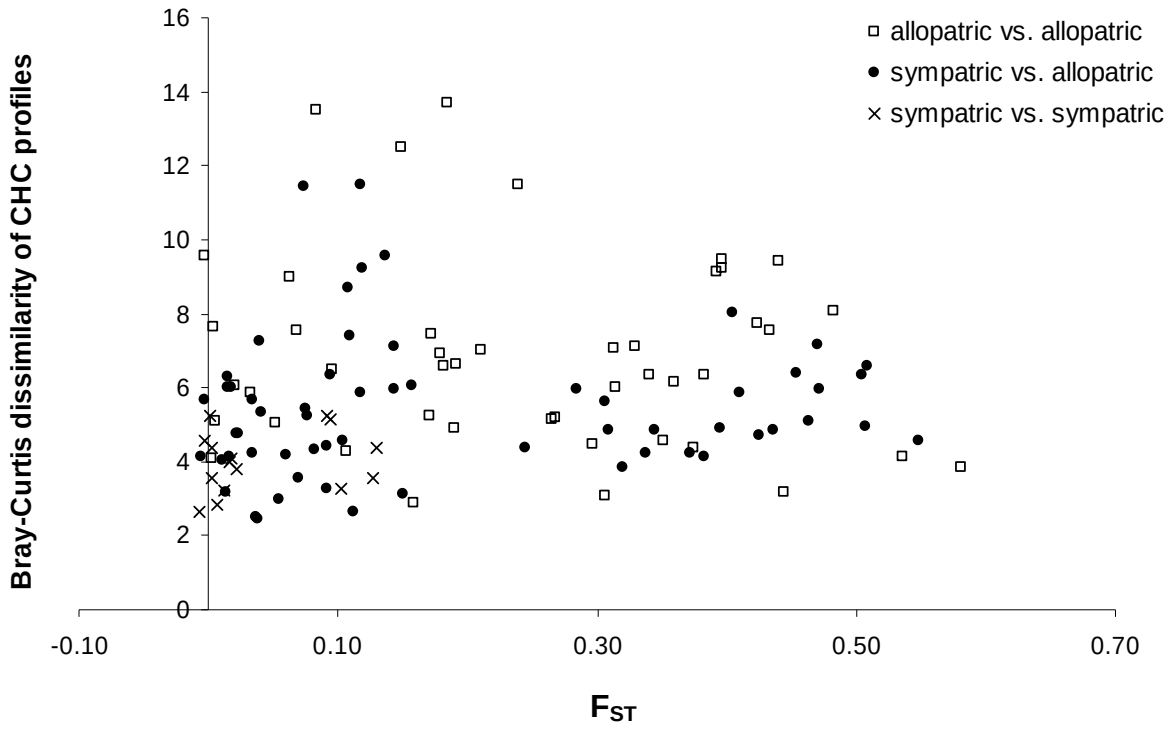


Figure A2: Relationship between genetic distance and CHC profile differences for *C. cobaltinus*. Symbols indicate whether comparison was between two sympatric, two allopatric, or a sympatric and allopatric population.

Table A3: Allele frequencies for the 3 allozyme loci scored for *C. cobaltinus*. Also listed are the sample sizes for each locus at each site. Site codes across the top of the table correspond with site codes in table A1 and Figure 3.

		Sympatric						Allopatric near hybrid zone					Allopatric far from hybrid zone				
		AA	AQ	AR	CF	S	CM	A	B	L	M	P	CP	CR	CS	CU	CW
PepGL	Allele 1	0.86	0.91	0.88	0.76	0.76	0.67	0.67	0.71	0.73	0.62	0.74	0.28	0.05	0.90	0.56	0.05
	Allele 2	0.14	0.09	0.12	0.24	0.24	0.33	0.33	0.29	0.27	0.38	0.26	0.72	0.95	0.10	0.44	0.95
	% heterozygosity	20%	14%	16%	45%	36%	45%	35%	39%	47%	49%	37%	40%	10%	20%	56%	10%
	N	50	50	50	47	53	38	51	51	51	51	51	50	50	41	50	50
PGI	Allele 1	0.32	0.31	0.36	0.29	0.41	0.13	0.33	0.37	0.45	0.37	0.43	0	0.01	0	0	0.03
	Allele 2	0.68	0.69	0.62	0.71	0.59	0.87	0.66	0.63	0.55	0.63	0.57	1	0.99	1	1	0.95
	Allele 4	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	0.02
	Allele 5	-	-	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-
	% heterozygosity	44%	38%	38%	45%	58%	21%	57%	45%	59%	39%	63%	0%	2%	0%	0%	10%
	N	50	50	50	47	53	38	51	51	51	51	51	50	50	41	50	50
PGM	Allele 1	0.60	0.63	0.52	0.65	0.66	0.33	0.32	0.24	0.59	0.56	0.72	0.43	0.26	0.56	0	0.35
	Allele 2	0.40	0.38	0.48	0.35	0.34	0.67	0.68	0.76	0.41	0.44	0.28	0.57	0.74	0.44	1	0.65
	% heterozygosity	52%	50%	48%	53%	38%	34%	44%	31%	39%	49%	37%	42%	32%	44%	0%	38%
	N	50	48	50	47	53	38	50	51	51	51	51	50	50	41	50	50

Literature Cited

Barluenga, M., K. N. Stolting, W. Salzburger, M. Muschick, and A. Meyer. 2006. Sympatric speciation in Nicaraguan crater lake cichlid fish. Nature **439**:719-723.

Barton, N. H., and G. M. Hewitt. 1985. Analysis of hybrid zones. Annual Review of Ecology & Systematics **16**:113-148.

Bolnick, D. I., and B. M. Fitzpatrick. 2007. Sympatric speciation: Models and empirical evidence. Annual Review of Ecology Evolution and Systematics **38**:459-487.

Buckley, S. H., T. O. M. Tregenza, and R. K. Butlin. 2003. Transitions in cuticular composition across a hybrid zone: historical accident or environmental adaptation? Biological Journal of the Linnean Society **78**:193-201.

Clarke, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology **18**:117-143.

Clarke, K. R., and R. M. Warwick. 2001. Change in marine communities: an approach to statistical analysis and interpretation, 2nd edition. PRIMER-E Ltd., Plymouth, United Kingdom.

Cognato, A. I., S. J. Seybold, and F. A. H. Sperling. 1999. Incomplete barriers to mitochondrial gene flow between pheromone races of the North American pine engraver, *Ips pini* (Say) (Coleoptera : Scolytidae). Proceedings of the Royal Society of London Series B-Biological Sciences **266**:1843-1850.

Coyne, J. A. 1992. Genetics and speciation. Nature **355**:511-515.

Coyne, J. A., A. P. Crittenden, and K. Mah. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. Science **265**:1461-1464.

Coyne, J. A., and H. A. Orr. 1997. "Patterns of speciation in *Drosophila*" revisited. Evolution **51**:295-303.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associate, Inc., Sunderland, MA.

Dalecky, A., M. Renucci, A. Tirard, G. Debout, M. Roux, F. Kjellberg, and E. Provost. 2007. Changes in composition of cuticular biochemicals of the facultatively polygynous ant *Petalomyrmex phylax* during range expansion in Cameroon with respect to social, spatial and genetic variation. Molecular Ecology **16**:3778-3791.

de Queiroz, K., and M. J. Donoghue. 1988. Phylogenetic systematics and the species problem. Cladistics **4**:317-338.

Dickinson, J. L. 1995. Trade-offs between postcopulatory riding and mate location in the Blue Milkweed Beetle. Behavioral Ecology **6**:280-286.

Dobler, S., and B. D. Farrell. 1999. Host use evolution in *Chrysochus* milkweed beetles: evidence from behaviour, population genetics and phylogeny. Molecular Ecology **8**:1297-1307.

Dobzhansky, T. 1940. Speciation as a stage in evolutionary divergence. The American Naturalist **74**:312-321.

Elmes, G. W., T. Akino, J. A. Thomas, R. T. Clarke, and J. J. Knapp. 2002. Interspecific differences in cuticular hydrocarbon profiles of *Myrmica* ants are sufficiently consistent to explain host specificity by *Maculinea* (large blue) butterflies. Oecologia **130**:525-535.

Endler, J. A. 1977. Geographic variation, speciation, and clines. Princeton University Press, Princeton, NJ.

Estoup, A., F. Rousset, Y. Michalakis, J. M. Cornuet, M. Adriamanga, and R. Guyomard. 1998. Comparative analysis of microsatellite and allozyme markers: a case study investigating microgeographic differentiation in brown trout (*Salmo trutta*). Molecular Ecology **7**:339-353.

Feder, J. L. 1998. The apple maggot fly *Rhagoletis pomonella*: Flies in the face of conventional wisdom about speciation? *in* D. J. Howard and S. H. Berlocher, editors. Endless forms: Species and speciation. Oxford University Press, New York.

Ferveur, J. F. 2005. Cuticular hydrocarbons: Their evolution and roles in *Drosophila* pheromonal communication. Behavior Genetics **35**:279-295.

Funk, D. J. 1998. Isolating a role for natural selection in speciation: Host adaptation and sexual isolation in *Neochlamisus bebbianae* Leaf Beetles. Evolution **52**:1744-1759.

Gabor, C. R., M. J. Ryan, and D. C. Morizot. 2005. Character displacement in sailfin mollies, *Poecilia latipinna*: allozymes and behavior. Environmental Biology of Fishes **73**:75-88.

Gavrilets, S., H. Li, and M. D. Vose. 2000. Patterns of parapatric speciation. Evolution **54**:1126-1134.

Geyer, L. B., and S. R. Palumbi. 2003. Reproductive character displacement and the genetics of gamete recognition in tropical sea urchins. Evolution **57**:1049-1060.

Goldberg, E. E., and R. Lande. 2006. Ecological and reproductive character displacement on an environmental gradient. Evolution **60**:1344-1357.

Gries, G., R. Gries, P. W. Schaefer, T. Gotoh, and Y. Higashiura. 1999. Sex pheromone components of pink gypsy moth, *Lymantria mathura*. Naturwissenschaften **86**:235-238.

Gries, G., P. W. Schaefer, R. Gries, J. Liska, and T. Gotoh. 2001. Reproductive character displacement in *Lymantria monacha* from northern Japan? Journal of Chemical Ecology **27**:1163-1176.

Harrison, R. G., editor. 1993. Hybrid zones and the evolutionary process. Oxford University Press, Inc., New York.

Hatch, M. H. 1971. The beetles of the Pacific Northwest: Part V. University of Washington Press, Seattle, WA.

Hebert, P. D. N., and M. J. Beaton. 1993. Methodologies for allozyme analysis using cellulose acetate electrophoresis. Helena Laboratories, Beaumont, TX.

Higgie, M., and M. W. Blows. 2008. The evolution of reproductive character displacement conflicts with how sexual selection operates within a species. Evolution **62**:1192-1203.

Higgie, M., S. Chenoweth, and M. W. Blows. 2000. Natural selection and the reinforcement of mate recognition. Science **290**:519-521.

Höbel, G., and H. C. Gerhardt. 2003. Reproductive character displacement in the acoustic communication system of green tree frogs (*Hyla cinerea*). Evolution **57**:894-904.

Howard, D. J. 1993. Reinforcement: Origin, dynamics, and fate of an evolutionary hypothesis. *in* R. G. Harrison, editor. Hybrid zones and the evolutionary process. Oxford University Press, New York.

Howard, D. J. 1999. Conspecific sperm and pollen precedence and speciation. Annual Review of Ecology and Systematics **30**:109-132.

Howard, R. W., and G. J. Blomquist. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annual Review of Entomology **50**:371-393.

Huyton, P. M., P. A. Langley, D. A. Carlson, and M. Schwarz. 1980. Specificity of contact sex pheromones in tsetse flies, *Glossina* spp. Physiological Entomology **5**:253-264.

Jensen, J. L., A. J. Bohonak, and S. T. Kelley. 2005. Isolation by distance, web service. BMC Genetics: 6:13 v. 3.15 http://ibdws.sdsu.edu/.

Kronforst, M. R., L. G. Young, and L. E. Gilbert. 2007. Reinforcement of mate preference among hybridizing *Heliconius* butterflies. Journal of Evolutionary Biology **20**:278-285.

Le Gac, M., and T. Giraud. 2008. Existence of a pattern of reproductive character displacement in *Homobasidiomycota* but not in *Ascomycota*. Journal of Evolutionary Biology **21**:761-772.

Lemmon, A. R., C. Smadja, and M. Kirkpatrick. 2004. Reproductive character displacement is not the only possible outcome of reinforcement. Journal of Evolutionary Biology **17**:177-183.

Lessios, H. A. 1998. The first stage of speciation as seen in organisms separated by the Isthumus of Panama. Pages 186-201 *in* D. J. Howard and S. H. Berlocher, editors. Endless forms: species and speciation. Oxford University Press, New York.

Lorch, P. D., and M. R. Servedio. 2007. The evolution of conspecific gamete precedence and its effect on reinforcement. Journal of Evolutionary Biology **20**:937-949.

Losos, J. B. 2000. Ecological character displacement and the study of adaptation. Proceedings of the National Academy of Sciences of the United States of America **97**:5693-5695.

MacNair, M. R., and P. Christie. 1983. Reproductive isolation as a pleiotropic effect of copper tolerance in *Mimulus guttatus*? Heredity **50**:295-302.

Manly, B. F. J. 1994. Multivariate statistical methods: a primer, 2nd edition. Champman and Hall / CRC Press, Boca Raton, FL.

Mardia, K., J. Kent, and J. Bibby. 1979. Multivariate analysis. Academic Press, London.

Marshall, J. L., M. L. Arnold, and D. J. Howard. 2002. Reinforcement: the road not taken. Trends in Ecology & Evolution **17**:558-563.

Mayr, E. 1963. Animal species and evolution. Harvard University Press, Camridge, MA.

Mayr, E. 1982. Processes of speciation in animals. Pages 1-19 *in* C. Barigozzi, editor. Mechanisms of speciation. Alan R. Liss, Inc., New York.

McNeilly, T., and J. Antonovics. 1968. Evolution in closely adjacent plant populations IV. Barriers to gene flow. Heredity **23**:205-218.

McPeek, M. A., and S. Gavrilets. 2006. The evolution of female mating preferences: Differentiation from species with promiscuous males can promote speciation. Evolution **60**:1967-1980.

McPhail, J. D. 1992. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*): evidence for a species-pair in Paxton Lake, Texada Island, British Columbia. Canadian Journal of Zoology-Revue Canadienne De Zoologie **70**:361-369.

Millar, J. G., M. Giblin, D. Barton, A. Morrison, and E. W. Underhill. 1990. Synthesis and field testing of enantiomers of 6z,9z-cis-3,4-epoxydienes as sex attractants for geometrid moths: Interactions of enantiomers and regioisomers. Journal of Chemical Ecology **16**:2317-2339.

Milligan, P. J. M., A. Phillips, G. Broomfield, D. H. Molyneux, Y. Toure, and M. Coluzzi. 1993. A study of the use of gas chromatography of cuticular hydrocarbons for identifying members of the *Anopheles gambiae* (Diptera: Culicidae) complex. Bulletin of Entomological Research **83**:613-624.

Monsen, K. J., B. M. Honchak, S. E. Locke, and M. A. Peterson. 2007. Cytonuclear disequilibrium in *Chrysochus* hybrids is not due to patterns of mate choice. Journal of Heredity **98**:325-330.

Mullen, S. P., T. C. Mendelson, C. Schal, and K. L. Shaw. 2007. Rapid evolution of cuticular hydrocarbons in a species radiation of acoustically diverse Hawaiian crickets (Gryllidae: Trigonidiinae: Laupala). Evolution **61**:223-231.

Mullen, S. P., J. G. Millar, C. Schal, and K. L. Shaw. 2008. Identification and characterization of cuticular hydrocarbons from a rapid species radiation of Hawaiian Swordtailed Crickets (Gryllidae: Trigonidiinae: *Laupala*) Journal of Chemical Ecology **34**:198-204.

Noor, M. A. 1995. Speciation driven by natural selection in *Drosophila*. Nature **375**:674-675.

Noor, M. A. F. 1999. Reinforcement and other consequences of sympatry. Heredity **83**:503-508.

Nosil, P., B. J. Crespi, R. Gries, and G. Gries. 2007. Natural selection and divergence in mate preference during speciation. Genetica **129**:309-327.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proceedings of the Royal Society of London Series B-Biological Sciences **270**:1911-1918.

Peterson, M. A., S. Dobler, J. Holland, L. Tantalo, and S. Locke. 2001. Behavioral, molecular, and morphological evidence for a hybrid zone between *Chrysochus auratus* and *C. cobaltinus* (Coleoptera : Chrysomelidae). Annals of the Entomological Society of America **94**:1-9.

Peterson, M. A., S. Dobler, E. L. Larson, D. Juárez, T. Schlarbaum, K. J. Monsen, and W. Francke. 2007. Profiles of cuticular hydrocarbons mediate male mate choice and sexual isolation between hybridising *Chrysochus* (Coleoptera: Chrysomelidae). Chemoecology **17**:87-96.

Peterson, M. A., B. M. Honchak, S. E. Locke, T. E. Beeman, J. Mendoza, J. Green, K. J. Buckingham, M. A. White, and K. J. Monsen. 2005a. Relative abundance and the

species-specific reinforcement of male mating preference in the *Chrysochus* (Coleoptera : Chrysomelidae) hybrid zone. Evolution **59**:2639-2655.

Peterson, M. A., K. J. Monsen, H. Pedersen, T. McFarland, and J. Bearden. 2005b. Direct and indirect analysis of the fitness of *Chrysochus* (Coleoptera: Chrysomelidae) hybrids. Biological Journal of the Linnean Society **84**:273-286.

Rouault, J. D., C. Marican, C. Wicker-Thomas, and J. M. Jallon. 2004. Relations between cuticular hydrocarbon (HC) polymorphism, resistance against desiccation and breeding temperature; a model for HC evolution in *Drosophila melanogaster* and *D. simulans*. Genetica **120**:195-212.

Rundle, H. D., and D. Schluter. 1998. Reinforcement of stickleback mate preferences: Sympatry breeds contempt. Evolution **52**:200-208.

Sady, M. B. 1994. Survey of the Blue Milkweed Beetle, *Chrysochus colbaltinus* LeConte. Coleopterists Bulletin **48**:299-299.

Saetre, G. P., T. Moum, S. Bures, M. Kral, M. Adamjan, and J. Moreno. 1997. A sexually selected character displacement in flycatchers reinforces premating isolation. Nature **387**:589-592.

Schlick-Steiner, B. C., F. M. Steiner, H. Hottinger, A. Nikiforov, R. Mistrik, C. Schafellner, P. Baier, and E. Christian. 2004. A butterfly's chemical key to various ant forts: intersection-odour or aggregate-odour multi-host mimicry? Naturwissenschaften **91**:209-214.

Schluter, D. 1995. Adaptive radiation in sticklebacks: Trade-offs in feeding performance and growth. Ecology **76**:82-90.

Schonrogge, K., J. C. Wardlaw, A. J. Peters, S. Everett, J. A. Thomas, and G. W. Elmes. 2004. Changes in chemical signature and host specificity from larval retrieval to full social integration in the myrmecophilous butterfly *Maculinea rebeli*. Journal of Chemical Ecology **30**:91-107.

Servedio, M. R., and M. A. F. Noor. 2003. The role of reinforcement in speciation: Theory and data. Annual Review of Ecology Evolution and Systematics **34**:339-364.

Singer, T. L. 1998. Roles of hydrocarbons in the recognition systems of insects. American Zoologist **38**:394-405.

Smadja, C., and R. K. Butlin. 2008. On the scent of speciation: The chemosensory system and its role in premating isolation. Heredity.

Sonnet, P. E., E. C. Uebel, R. L. Harris, and R. W. Miller. 1977. Sex pheromone of the stable fly: Evaluation of methyl- and 1,5-dimethylalkanes as mating stimulants Journal of Chemical Ecology **3**:245-249.

Stennett, M. D., and W. J. Etges. 1997. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. III. Epicuticular hydrocarbon variation is determined by use of different host plants in *Drosophila mojavensis* and *Drosophila arizonae*. Journal of Chemical Ecology **23**:2803-2824.

Teale, S. A., B. J. Hager, and F. X. Webster. 1994. Pheromone-based assortative mating in a bark beetle. Animal Behaviour **48**:569-578.

Ugelvig, L. V., F. P. Drijfhout, D. J. C. Kronauer, J. J. Boomsma, J. S. Pedersen, and S. Cremer. 2008. The introduction history of invasive garden ants in Europe: Integrating genetic, chemical and behavioural approaches. Bmc Biology **6**.

Urbanelli, S., and D. Porretta. 2008. Evidence of reinforcement of premating isolation between two species of the genus *Ochthebius* (Coleoptera : Hydraenidae). Evolution **62**:1520-1527.

Waage, J. K. 1979. Reproductive Character Displacement in *Calopteryx* (Odonata: Calopterygidae). Evolution **33**:104-116.

Weiss, H., and E. West. 1921. Notes on insects of the spreading dogbane, *Apocynum androsaemifolium* L., with a description of a new dogbane midge by Dr. E.P. Felt. Canadian Entomologist **53**:146-152.

Wiley, E. O. 1978. The evolutionary species concept reconsidered. Systematic Zoology **27**:17-26.

Wright, S. 1951. The genetical structure of populations. Annals of Eugenics **15**:323-354.

Youngsteadt, E., and P. J. DeVries. 2005. The effects of ants on the entomophagous butterfly caterpillar *Feniseca tarquinius*, and the putative role of chemical camouflage in the *Feniseca*–ant interaction. Journal of Chemical Ecology **31**:2091 - 2109.

Zhang, A. J., S. F. Wang, J. Vitullo, A. Roda, C. Mannion, and J. C. Bergh. 2006. Olfactory discrimination among sex pheromone stereoisomers: Chirality recognition by pink hibiscus mealybug males. Chemical Senses **31**:621-626.