ABSTRACT

Title of Document: THE ROLE OF HOST-PLANT SPECIES IN THE DIFFERENTIATION OF SYMPATRIC POPULATIONS OF HYMENOPTERAN PARASITOIDS.

Raul F. Medina, PhD, 2005

Directed By: Professor, Pedro Barbosa, Department of Entomology

The biology and ecology of insect parasitoids is strongly influenced by the host-plant species on which their herbivorous hosts occur. Hymenopteran parasitoids in particular, present a series of characteristics that made them good candidates for phenotypic and genotypic differentiation. Thus, parasitoid adaptation to plant traits may promote significant phenotypic and genotypic differences among sympatric populations of parasitoids associated with different host-plant species. The present study assessed phenotypic and genotypic differentiation in two braconid parasitoids ovipositing on the

same host species, the green cloverworm, *Plathypena scabra* Fabricius (Lepidoptera: Noctuidae) feeding on alfalfa and soybean.

Developmental time, adult weight, percent parasitism and preference for host-plant odors of the generalist parasitoid *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) and of the specialist parasitoid *Aleiodes nolophanae* Ashmead (Hymenoptera: Braconidae) were compared among individuals ovipositing in green cloverworm larvae feeding on alfalfa and soybean. In addition, amplified fragment length polymorphisms (AFLP) were used to assess if genotypic differentiation between parasitoids ovipositing on green cloverworm larvae feeding on different host-plant species (i.e., alfalfa or soybean) was present.

Phenotypic differentiation in adult mass, adult longevity and percent parasitism between parasitoids ovipositing green cloverworm larvae on alfalfa and on soybean were found. These phenotypic differences between parasitoids associated with different host-plant species were observed in both the generalist and the specialist parasitoids. No evidence of parasitoids showing preferences for the host-plant species from which their host fed was found in the generalist nor in the specialist parasitoid. Contrary to the expectations and predictions from the literature, these parasitoid species did not show evidence of reproductive isolation when associated with different host-plant species (i.e., alfalfa or soybean), as evidenced by the lack of genetic differentiation in AFLP profiles between parasitoids associated with alfalfa and soybean. In order to ensure that the number of wasps and the number of AFLP bands used were enough to provide an accurate assessment of genetic differentiation among wasps ovipositing hosts on different host-plant species, a method for determining the minimum number of individuals and

AFLP bands to include to obtain accurate genetic profiles of hypothesized populations was proposed.

THE ROLE OF HOST-PLANT SPECIES IN THE DIFFERENTIATION OF SYMPATRIC POPULATIONS OF HYMENOPTERAN PARASITOIDS.

By

Raul F. Medina.

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Advisory Committee:
Dr. Pedro Barbosa, Chair
Dr. Dale Bottrell
Dr. Galen Dively
Dr. David Hawthorne
Dr. Kerry Shaw

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1:

The Role of Host-Plants in the Differentiation of Sympatric Populations of Hymenopteran Parasitoids

Studies of herbivorous insects have played a major role in understanding how the use of different host-plant species can facilitate phenotypic and genotypic differentiation (Sword et al. 2005). In contrast, comparatively very few studies have explored what ecological factors may drive differentiation in natural enemies of herbivores. Since parasitoids of phytophagous insects forage on plants, traits of both plants and herbivore hosts influence their fitness (Souissi and Le Rü 1998). In this chapter, I will discuss the role of host-plant species in parasitoid ecology. Specifically, I will argue that parasitoid diet breath may influence phenotypic and/or genotypic differentiation and comment on the role of geographic location on parasitoid differentiation. I will discuss why parasitoids may be considered as good candidates to study host-plant based genetic differentiation, and I will argue that phenotypic and/or genotypic differentiation of herbivorous insects as well as of parasitoids can occur sympatrically among individuals associated with different host-plant species.

Importance of Host -Plant Species in Parasitoid Ecology

Natural enemies interact not only with their herbivore hosts but also with the host-plants of these herbivores. The combination of a particular herbivore host species on a particular host-plant species is referred as host-plant complex to denote this interaction.

Not only is foraging behavior of parasitoids influenced by plants but they also influence the level of parasitism of herbivores (Godfray 1994). Intraspecific phenotypic variability in hymenopteran parasitoids that use different resources (i.e., host species, and/or host-plant species) are exhibited in morphological, ecological, behavioral and physiological traits (Hopper et al. 1993, Unruh and Messing 1993). However, very few studies have explored the role that different host-plant species have in the differentiation of natural enemies when they attack the same herbivore species. There are several reports of herbivorous hosts that are attacked when occurring on one plant species but not on another (Walker 1940, Clausen 1941, Smith 1957, Zwolfer and Kraus 1957, Arthur 1962, Stary 1964, Hassell and Southwood 1978). Further, certain parasitic Hymenoptera are capable of developing on an herbivorous host only when the host is on particular host-plant species (Price et al. 1980, Vet and Dicke 1992).

Plants often provide the first cue in the chain of events that lead to host location by parasitic Hymenoptera. Parasitoids often locate their hosts by first orienting to cues emanating from the host-plant or host substrate (e.g., parasitoids responding to cues from rotten organic material fed on by drosophilid flies) (Price 1981, Vinson 1984) and are stimulated to search for hosts when on the surface that produces these cues (Carton 1977, Vinson 1981, Weseloh 1981, 1982, Mueller 1983, Vet et al.1983, Vet 1983, Dicke et al. 1984, Vet and Dicke 1992, Agelopoulos and Keller 1994, Craig 1994, Kraaijeveld et al. 1994, Baur and Yeargan 1996, Bertschy et al. 1997, Barbosa and Benrey 1998, Geervliet et al. 1998a, De Moraes et al. 1998). The ability of natural enemies to differentiate between plant species can directly influence their fitness. For example, plant species vary

in the presence and density of trichomes and these trichomes and their secretions interfere with foraging behavior (Obrycki 1986, Van Lenteren and De Ponti 1991). Indirectly, these plant traits affect parasitoids due to variation in a plant's suitability for herbivore development (Barbosa 1988). Although host-plant cues used by parasitoids to locate their herbivorous hosts can include physical and/or chemical traits (e.g., silhouette, contrast, color, leaf texture, plant architecture, tactile or volatile chemicals, etc.), chemical cues are the ones that seem to be the most important and consequently the most studied (Vet 1983).

Volatile blends produced by damaged plants and used by parasitoids to find hosts vary significantly in different plant species. For instance, tobacco, cotton and maize each produce distinct volatile blends in response to herbivore damage (De Moraes et al. 1998). Parasitoids are able to detect these differences in volatile composition and are differentially attracted towards the odors that maximize their probabilities of finding suitable hosts (Vet and Dicke 1992, De Moraes et al. 1998). Thus, differences in percentage of parasitism among conspecific parasitoids ovipositing in the same host species on different host-plant species can be the result from differences in the attraction of parasitoids to plant specific volatile compounds.

My study system involves two parasitoid species attacking the green cloverworm *Plathypena scabra* Fabricius (Lepidoptera: Noctuidae) on two of its host-plant species, alfalfa and soybean. These parasitoids are the generalist *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) and the specialist *Aleiodes nolophanae* Ashmead (Hymenoptera: Braconidae). Parasitism by *C. marginiventris* and by a species in the

same genus as *A. nolophanae* (i.e., *Aleiodes laphygmae* (Hymenoptera: Braconidae)), of *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) differs depending on whether host larvae are on corn, sorghum, bermudagrass or other turf grass species (Rajapakse et al.1991, Braman et al. 2004). Other examples of parasitoids affected by the host-plant of its host include *Apoanagyrus lopezi* De Santis (Hymenoptera: Encyrtidae) (attacking *Phenacoccus manihoti* Matile Ferrero (Hemiptera: Pseudococcidae)), *Lysiphlebus testaceipes* Cresson (Hymenoptera: Braconidae) (attacking *Aphis nerii* B de F (Homoptera: Aphididae)) and *Diadegma semiclausum* Hellen (Hymenoptera: Ichneumonidae) and *Diadromus collaris* Gravenhorst (Hymenoptera: Ichneumonidae) (attacking *Plutella xylostella* L. (Lepidoptera: Plutellidae)) (Beck and Cameron 1990, Souissi and Le Rü 1998, Helms et al. 2004).

Fidelity to the host-plant odor of the plant on which the parasitoid's host resides also may promote phenotypic differentiation among parasitoids ovipositing in hosts on different plant species (Kester and Barbosa 1991, Van Driesche and Bellows 1996). If this fidelity is strong, populations of the same species using different host-plant species may be reproductively isolated (Caillaud and Via 2000). Many parasitoid species prefer the odor (i.e., volatile profile) of the plants on which they have developed (Kester and Barbosa 1991, Turlings et al. 1993, Bogahawatte and van Emden 1996). For instance, *Aphidius smithi*, Sharma & Subba Rao (Hymenoptera: Braconidae), a parasitoid of the pea aphid, *Acyrthosiphon pisum* Harris (Homoptera: Aphididae), also attacks the green peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae) when the green peach aphid is reared on broad bean but not when reared on tobacco (Fox et al. 1967). When offered a

choice between Chinese cabbage and common cabbage, naive female *Cotesia plutellae* Kurdjumov (Hymenoptera: Braconidae) reared from hosts feeding on radish have a strong preference for *P. xylostella* larvae on Chinese cabbage (Liu and Jiang 2003).

The specificity of parasitoid responses to diverse morphological and chemical cues can occur as a response to host-plant cues, host cues, or both (Vet and Dicke 1992). Both chemical characteristics from host-plants (i.e., plant volatile compounds) or chemical cues from the host (e.g., frass, honeydew) may be critical cues in host and host-habitat finding by parasitoids (Hassell and Southwood 1978, Godfray 1994) and may influence habitat preferences after emergence (Read et al. 1970, Arthur et al. 1972, Honda and Walker 1996, Monge and Cortesero 1996). Indeed, in some parasitoids, females are attracted to the host-plant of their hosts rather than to the hosts themselves (Hassell and Southwood 1978).

Host-plant species also may mediate parasitoid's phenotypic and genotypic differentiation through their indirect effect on the parasitoid herbivorous host. Concentration of nutrients and secondary compounds in a particular host-plant species may influence the nutritional value of a parasitoid's host. Certain parasitic Hymenoptera are capable of developing on a host only when the host is feeding on particular host-plant species (Price et al. 1980, Vet and Dicke 1992). Differences in hosts nutritional quality when feeding on different host-plant species may produce phenotypic differences in parasitoid traits related to fitness, such as adult mass, adult longevity, fecundity, and developmental time (Barbosa et al. 1991, Blackburn 1991).

Therefore, host-plant specific parasitoid strains may result from a combination of selection pressures on physiological and behavioral traits in parasitoids attacking hosts on different host-plant species (Kester and Barbosa 1994, Potting et al. 1997). Preferences for host-plant species may be learned (Kester and Barbosa 1994) or inherited (Barbosa et al. 1990) and might lead to reproductive isolation of conspecific parasitoids associated with differential response to host-plant odors (Vaughn and Antolin 1998). Several braconid parasitoids, including *C. marginiventris*, and species in the genus *Aleiodes* have shown to be attracted to the odors of their most suitable host and/or host-plant species (Rajapakse et al.1991, Vet and Dicke 1992, Braman et al. 2004).

Thus, the host-plant species on which herbivores reside can mediate differentiation of parasitoids due to the central role they play in their ecology. Even though host-plants are such an important component in parasitoids' ecology, the role of host-plant species in the genetic differentiation of parasitic Hymenoptera that attack the same herbivorous species on different host-plant species has been meagerly explored.

The Influence of Parasitoid Diet Breath in Their Phenotypic and Genotypic Differentiation.

The fact that generalist and specialist parasitoids are subjected to different selective pressures may be responsible for the differences in behavior observed in specialist and generalist parasitoids when faced with the same type of host on different host-plant species (Campan and Benrey 2004). Generalist and specialist parasitoids have been found to significantly differ in phenotypic traits important to fitness. For example,

variation exists among *Zabrotes subfasciatus* Boheman (Coleoptera: Bruchidae) family lines feeding on cultivated and wild *Phaseolus vulgaris* L. (Leguminosae: Phaseolinae) seeds (Campan and Benrey 2004). *Stenocorse bruchivora* Crawford (Hymenoptera: Braconidae), a specialist parasitoid of *Z. subfasciatus*, is selective (i.e., parasitize mainly *Z. subfasciatus* individuals that feed on the seed type on which they perform best) resulting in offspring with similar sizes and development times. In contrast, the generalist parasitoid *Dinarmus basalis* Rondani (Hymenoptera: Pteromalidae) (Campan and Benrey 2004) is less selective, parasitizing almost all hosts offered and its performance (i.e., size and development time) is more variable than in the specialist parasitoid (Campan and Benrey 2004). Similarly, the behavioral response of the specialist *Leptopilina boulardi* Barbotin, Carton and Kelner-Pillault (Hymenoptera: Eucolidae) to kairomones produced by larval extracts from six different drosophilid larvae is more specific than the behavioral response of the generalist *Leptopilina heterotoma* Thompson (Hymenoptera: Eucolidae) (Vet el al. 1993). On the other hand, the generalist *C. marginiventris* is attracted to plants that released constitutive compounds (i.e. low specificity compounds), whereas *Microplitis croceipes* Cresson (Hymenoptera: Braconidae), a specialist parasitoid of *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), is unaffected by this kind of compounds (Rose et al. 1998). Instead, *M. croceipes*, is attracted to volatiles from its host's frass whereas *Campoletis sonorensis* Cameron (Hymenoptera: Ichneumonidae), a generalist parasitoid of *H. virescens*, is not (Elzen et al. 1987).

Differences in the effect that host-plants have on certain parasitoid behaviors have been found between specialist and generalist parasitoids. For instance, bean seed type

(i.e., cultivated vs wild) has a significant effect on oviposition behavior in the specialist parasitoid *S. bruchivora* but not in the generalist parasitoid *D. basalis*. Similarly, generalist parasitoids are affected by chemical defenses from host-plants to a greater degree than specialist parasitoids. For example the generalist parasitoid *Hyposoter annulipes* Cresson (Hymenoptera: Ichneumonidae) parasitizes fewer *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) larvae, is subject to reduced larval survival, prolonged development time and reduced adult size, when feeding on diets containing nicotine compared to nicotine-free diets. In contrast, adult mass differences between adults of the specialist parasitoid *Cotesia congregata* Say (Hymenoptera: Braconidae) feeding on nicotine containing and nicotine-free diets are not significant (Barbosa 1988).

Differences in the influence of learning in foraging behavior have also been found between generalist and specialist parasitoids. For example, the generalist *Cotesia glomerata* L. (Hymenoptera: Braconidae) has been shown to shift preferences when experiencing host feeding damage or oviposition on new host-plant complexes whereas the specialist *Cotesia rubecula* Marshall (Hymenoptera: Braconidae) has shown none of this behavioral variability (Geervliet et al. 1998a, 1998b). Also, the specialist parasitoid *Diadegma semiclasum* Hellén (Hymenoptera: Ichneumonidae) shows a relatively fixed behavioral pattern leading to oviposition whereas the generalist *C. plutellae* exhibits a more plastic behavior (Wang and Keller 2002).

Differences in diet breath between specialist and generalist parasitoids also may influence parasitoid foraging patterns and consequently parasitoid gene flow. Selective

specialist parasitoids may forage over larger areas than less selective generalist parasitoids. Thus, specialist parasitoids would be more prone to have greater gene flow than generalist parasitoids.

Studies comparing specialist and generalist parasitoids, suggest that differences in their foraging strategies may exist (Vet and Dicke 1992, Baur and Yeargan 1996, Campan and Benrey 2004). For example, the specialist parasitoid *C. rubecula* has a higher tendency to leave searched patches than the generalist *C. glomerata* (Vos et al. 1998). Although there are almost no studies on the correlation of diet breath with dispersal of hymenopteran parasitoids, Baur and Yeargan (1996) comparing the vagility of two parasitoids of the green cloverworm, *P. scabra*, in soybean, found that the specialist *Diolcogaster facetosa* Weed (Hymenoptera: Braconidae) was more vagile than the generalist *C. marginiventris***.** *C. marginiventris* is one of the parasitoids used in this study and given that *A. nolophanae* only attacks the green cloverworm on leguminous cultivars (Krombein et al. 1979), it is likely to be a selective specialist, unlike *C. marginiventris*. The foraging patterns of *C. marginiventris* and *A. nolophanae* are probably based on their association with their hosts prior to the establishment of modern agriculture (Baur and Yeargan 1996). The green cloverworm, *A. nolophanae* and *C. marginiventris* are native to North America (Dyar 1902, Carlson 1979, Marsh 1979). Extrapolating from their current host range to their evolutionary past suggests that *A. nolophanae* had to traverse considerable distances while foraging for few acceptable hosts on plants that were relatively uncommon and patchily distributed. In contrast, *C. marginiventris* may have been able to utilize a variety of hosts in common, widely

distributed plants, thereby eliminating the need to travel large distances (Baur and Yeargan 1996). Baur and Yeargan (1996) tested this hypothesis by comparing the vagility of the specialist parasitoid *D. facetosa* with the vagility of the generalist parasitoid *C. marginiventris* attacking green cloverworms in soybean. They found that the specialist *D. facetosa* moved significantly more than the generalist *C. marginiventris* suggesting that perhaps the specialist parasitoid *A. nolophanae* will also move more**.** However, *A. nolophanae* in our laboratory was observed to move significantly less that *C. marginiventris* when released soon after emergence in cages containing alfalfa and soybean (*A. nolophanae* stayed on the same spot in the cage for several hours whereas *C. marginiventris* was constantly moving) and the same was observed in field releases of laboratory reared *A. nolophanae* (Baur et al. 1996) and *C. marginiventris* individuals (Baur pers. comm.). If one parasitoid is more mobile than the other, differences in the level of gene flow among these two parasitoid species might exist and consequently influence the pattern of genotypic differentiation among populations of these parasitoids on different host-plant species.

The greater flexibility of generalist parasitoid foraging behavior coupled with their potential relatively reduced levels of gene flow as compared with specialist parasitoids, may lead to a greater degree of reproductive isolation and consequently a greater degree of genotypic differentiation in generalist than in specialist parasitoids.

Geographic Variation in Phenotypic and Genotypic Differentiation.

Phenotypic and genetic differentiation of distant populations of the same species have been reported for a number of taxa (Mayr 1982, Boulétreau 1986, Kraaijeveld and Van Alphen 1994, Itami et al. 1998, Thomas and Singer 1998, Althoff and Thompson 2001). Among the major causes of phenotypic and genetic differentiation among conspecific individuals from different geographic regions are climate (Crouau-Roy 1989, Karan and Parkash 1998, Holmstrup and Loeschcke 2003) relative host-plant abundance (Thompson 1994, Itami et al. 1998, Thomas and Singer 1998) host availability (Kraaijeveld and Van der Wel 1994) and presence of competitors (Kraaijeveld et al. 1994). Genetic differentiation among parasitoid populations due to geographic isolation may be exhibited in differences in physiology or behavior. For instance, the drosophilid parasitoids *L. boulardi* and *Asobara tabida* Nees (Hymenoptera: Braconidae) exhibit geographic differences in physiology, specifically, in their ability to overcome the encapsulation responses of one of their hosts, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Boulétreau 1986, Kraaijevelt and Van Alphen 1994). Similarly, strains of the parasitoid *Cotesia flavipes* Cameron (Hymenoptera: Braconidae) differ in their ability to develop in species of stem-boring Lepidoptera, primarily by overcoming the encapsulation response of their host species, to different degrees in different geographical regions (Potting et al. 1997). Althoff and Thompson (2001) working on a newly discovered *Agathis* (Hymenoptera: Braconidae) parasitoid of *Greya enchrysa* Davis and Pellmir (Lepidoptera: Prodoxidae) and *Greya piperella* Busck (Lepidoptera: Prodoxidae), found key behavioral differences in individuals of six geographically distinct

populations. Parasitoids varied in host searching time allocation and in the area of the host-plant searched. Thus, geographic variation in parasitoid traits important to parasitoid interactions with their hosts may exists across populations of a single parasitoid species at different locations.

Significant geographic variation exists in the survival probability of some parasitoids in their respective hosts, both between and within host species. Geographic differences in the abiotic and biotic environments experienced by different populations of the same parasitoid species may alter the way in which parasitoids interact with their host and host-plant species at each particular location (Thompson 1994, Kraaijeveld and Godfray 1999). This means that different populations of parasitoids may be subjected to different selection pressures in different locations (Kraaijeveld and Van Der Wel 1994).

For instance, substrate odors preferred by a parasitoid of drosophilid larvae, *Asobara rufescens* Förster (Hymenoptera: Braconidae), vary geographically. Portuguese *A. rufescens* do not have a preference for either the odor of yeast or decaying leaves, while their Dutch conspecifics prefer the odor of decaying leaves (Kraaijeveld et al. 1994). Likewise, egg load differs among *A. tabida* from different geographic origins. Swedish parasitoids have fewer eggs than western and central European parasitoids, which in turn, have fewer eggs than southern European strains (Kraaijeveld and Van Der Wel 1994). Other parasitoids exhibit geographically structuring for traits such as virulence and encapsulation defenses (Althoff and Thompson 1999). Two Swiss populations of *L. heterotoma* differ in their ability to prevent encapsulation by *D. melanogaster* (Walker 1959, 1962). In addition, the survival probability of Portuguese *A.*

rufescens in *D. melanogaster* is much higher than the survival probability of Dutch *A. rufescens* in this host species (Kraaijeveld et al.1994). Similarly, there are geographic differences in resistance to encapsulation by *D. melanogaster* between Mediterranean and northwestern and central European conspecifics of *A. tabida* (Mollema 1988, Kraaijeveld and van Alphen 1994). *A. tabida* from different locations also show variation in diapause and in egg/fat balance (Kraaijeveld and Van der Wel 1994). Evidence exists showing that host-plants from the same species growing at different sites differ in nutritional quality for herbivorous insects (Kokkini et al. 1994, Gaston et al. 2004). These differences in host-plant quality may indirectly affect parasitoids through their herbivorous hosts. Thus, the degree at which host-plant species may influence phenotypic differentiation of parasitoid species can also present a geographic component. Geographic differences in searching time and in oviposition preferences on different host-plant species have been found among *C. congregata* in sites located only 56 Km apart from each other (Kester and Barbosa 1994).

Why Might Parasitoids be Prone to Differentiate Genetically?

Parasitoids present a series of characteristics that make them good candidates for genetic differentiation. Traits such as their close association with their hosts and host-plants (Price 1980, Thompson 1982, Boulétreau 1986), the relatively short generation times of both parasitoids and their hosts (Boulétreau 1986) and their assumed low dispersal rates (Vaughn and Antolin 1998) are the type of traits that would be likely to facilitate reproductive isolation and thus contribute to genetic differentiation. Further,

many parasitoids species mate shortly after emergence (Hassell and Godfray 1992) and some at their emergence site (often the host-plant) (Godfray 1994). The latter increases the likelihood that members of one family mate among themselves (Askew 1968, Diehl and Bush 1984, Hassell and Godfray 1992). Sibling mating influences population differentiation by reducing the effective population size and by increasing the rate at which random genetic drift could lead to reproductive isolation (Unruh and Messing 1993, Conner and Hartl 2004). Effective population sizes in parasitic Hymenoptera are usually lower than in other insects (Crozier 1985). In addition, hymenopteran parasitoids' haplodiploid genetics may influence the speed of incipient genetic differentiation because the rate at which favorable mutations are fixed is assumed to be higher in haplodiploid species than in diploid species (Hartl 1972, Crozier 1985). Several of the traits that make parasitoids good candidates for the study of genetic differentiation occur in *A. nolophanae* and *C. marginiventris* (the parasitoids of my study system). They are both closely associated to their host-plants. The specialist parasitoid *A. nolophanae,* only parasitizes the green cloverworm on herbaceous legumes (Marsh 1979, Covell 1984, Johnson and Lyon 1991) and *C. marginiventris* shows a strong attraction to volatiles released by the host-plant species of its herbivorous hosts (Turlings et al. 1991). *A. nolophanae* and *C. marginiventris* mate shortly after emergence (Lentz and Pedigo 1973, Boling and Pitre 1970) and they are both haplodiploid. All these traits, suggest that intraspecific genetic differences in parasitoid populations utilizing hosts on different host-plant species might be likely. If genetic differentiation occurs not only in loci involved in performance but also in loci associated with mating and oviposition

preferences for a particular resource (i.e., host and/or host-plant species) then reproductive isolation in sympatry may promote genetic differentiation across the entire genome (Rank 1992, Feder et al. 1998, Vaughn and Antolin 1998). Alternatively, genetic differentiation may occur only at certain loci but it may be insufficient to manifest itself as an effect on the entire genome (Conner and Hartl 2004).

***Phenotypic Differentiation in Herbivorous Insects*.**

Herbivorous insect populations of the same species that are associated with different host-plant species (e.g., through feeding, sheltering or mating) exhibit significant phenotypic differences (Walsh 1864, Bush 1969, Boller and Bush 1973, Price and Willson 1976, Hsiao 1978, Sturgeon 1980, Claridge and Nixon 1981, Denno and Dingle 1981, Hsiao 1982, Lorimer 1982, Gould 1983, Mitter and Futuyma 1983, Diehl and Bush 1984, Futuyma et al. 1984, Futuyma and Peterson 1985, Carroll and Boyd 1992, Thomas and Singer 1998, Braman et al. 2004). These phenotypic differences are expressed in fitness components such as adult mass, adult longevity, fecundity and resistance to natural enemies attack as well as in differences in morphology. For example, the pea aphid, *A. pisum*, is adapted to its host-plant species. This adaptation is observed in differential survival, fecundity and resistance against parasitism when feeding on sympatric host-plant species (Via 1991a, 1991b, 1994, Henter and Via 1995). Similarly, the apple maggot, *Rhagoletis pomonella* Walsh (Diptera: Tephritidae), associated with apple (*Malus pumila*) differs significantly in adult body size, adult emergence dates and in morphological traits such as ovipositor length or the number of postorbital bristles

compared to apple maggots feeding on hawthorne (*Crataegus* spp.) (Bush 1969). Likewise, the ball-gallmaker *Eurosta solidaginis* Fitch (Diptera: Tephritidae), attacks two sympatric species of *Solidago*: *S. altissima* and *S. gigantea*. Flies prefer to mate assortatively with their own host-plant associated individuals and females oviposit on their natal host-plant species (Craig et al. 1997). Flies that oviposit on a host-plant different from the one with which they are associated have significantly lower offspring survivorship than flies that oviposit on the host-plant species with which they are associated (Craig et al. 1997). Flies associated with different *Solidago* species also differ in emergence times (Craig et al. 1993, Horner et al. 1999).

Phenotypic differences among herbivorous insects associated with different host-plant species may occur relatively quickly. For instance, it is estimated that the first *R. pomonella* switched from the native hawthorne species to introduced apples species, sometime in the mid-1800s (Feder et al. 2003a). Similarly, in the soapberry bug *Jadera haemotoloma* H.S. (Hemiptera: Rhopalidae) differences in size, development time, growth rate and beak lengths correspond to differences in the fruit sizes of different host-plant species. These changes have occurred in as little as 20 to 50 years (Carroll and Boyd 1992).

Genotypic Differentiation in Herbivorous Insects.

As with phenotypic differentiation, genetic variation within herbivorous insect species in the ability to utilize different host-plant species or varieties has been reported in nearly all cases in which it has been investigated (Hsiao 1978, Rausher 1982,

Tavormina 1982, Jaenike and Grimaldi 1983, Mitter and Futuyma 1983, Scriber 1983, Tabashnick 1983, Futuyma et al. 1984, Futuyma and Peterson 1985, Pashley 1986, Via 1989, Via 1991a, 1991b, De Barro et al. 1995, Emelianov et al. 1995, Carroll et al. 1997, Vanlerberghe-Masutti and Chavigny 1998, Rossi et al. 1999, Via 1999, Caillaud and Via 2000, Via et al. 2000, Lushai et al. 2002). Selection by a host-plant species may occur at loci or polygenic regions that are correlated with traits involved in host-plant preference so that shifts in preferences for particular host-plant species may occur rapidly (Fox and Morrow 1981). For example, QTL (quantitative trait locus) mapping in the pea aphid, *A. pisum*, has shown that QTLs for fecundity and host-plant acceptance may be correlated (Via and Hawthorne 2002). Selection may also occur for traits responsible of reproductively isolating populations associated with different host-plant species. For example, in *A. pisum* because mating occurs on the host-plant, selection on host-plant choice leads to assortative mating and is therefore responsible for reproductively isolating populations of pea aphid in alfalfa from sympatric populations of pea aphid in clover (Via 1999). Similarly, *R. pomonella*, genetic differentiation in apple and hawthorne flies (Mc Pheron et al. 1988) reflects intense natural selection for phenotypes that diverge in fruiting between the original and the introduced host-plant (Bush 1969). This divergence in fruiting reproductively isolates fly populations from apple and hawthorne trees, in time. Further, apple and hawthorne populations of *R. pomonella* display significant allele frequency differences for six allozyme loci at sympatric field sites across the eastern United States (Feder et al. 2003a). These six allozymes are all correlated with the timing of adult emergence (Feder et al. 2003a). In addition, evidence

exists that genes affecting diapause traits, involved in host race formation, reside within large complexes of rearranged genes (Feder et al. 2003a) and that substantial gametic disequilibrium exists that differentiate apple and hawthorne flies (as it has been demonstrated using allozymes and complementary DNA markers that encompass three chromosomal regions) (Feder et al. 2003b). Thus, the presence of extensive gametic disequilibrium in *R. pomonella* suggests that recognition of different host-plant species and performance on different host-plant species by herbivores may be genetically linked.

Host-Plant Driven Insect Differentiation: The Goldenrod System Case.

One of the best studied cases of phenotypic and genotypic differentiation of insects associated with different host-plant species is the one that occurs between insects associated with different species of goldenrod (*S. altissima* and *S. gigantea*). Hybridization experiments between ball-gallmakers, *E. solidaginis*, associated with *S. altissima* and *S. gigantea* suggest that differences in both plant preference and larval performance have a genetic basis (Craig et al. 1997). Allozyme and mitochondrial data indicate that ball-gallmakers from each host-plant species represent genetically distinct populations that occur in sympatry (Waring et al. 1990, Brown et al. 1996). The ball-gallmaker is not the only herbivore associated with *S. altissima* and *S. gigantea* that has differentiated by host-plant species. The goldenrod elliptical-gall moth, *Gnorimoschema gallaesolidaginis* Riley (Lepidoptera: Gelechiidae) and the beetle *Mordellistena convicta* LeConte (Coleoptera: Mordellidae), also shows genetic differentiation between individuals occurring on *S. altissima* and *S. gigantea* (Nason et al. 2002, Cronin and

Abrahamson 2001). Thus, the herbivores *E. solidaginis* and *G. gallaesolidaginis* show parallel host-associated differentiation. *M. convicta* acts like a facultative predator of gall insects, consuming them as it consumes the gall itself. Thus, parallel host-associated differentiation may be observed in two herbivore and one omnivore species co-inhabiting the goldenrod system.

Parasitoids are more highly specialized than predators and thus should show more extensive diversification (Mayr 1976, Price 1980). Phenotypic differences in behavior have been reported in the specialist parasitoid of the ball-gallmaker, *Eurytoma obtusiventris* (Hymenoptera: Eurytomidae). This parasitoid lands and spends more time on *S. altissima* than on *S. gigantea* plants and consequently parasitizes more gallmakers on the former species than on the later one (Cronin and Abrahamson 2001). Further studies exploring phenotypic and genetic differentiation of parasitoids of goldenrod herbivores are needed to better understand the role of host-plant species in the differentiation of parasitic Hymenoptera. The fact that the two herbivores, the omnivore and the parasitoid species associated with goldenrod species are in different orders and have markedly different life histories, suggests that host-associated differentiation might be more dependent on traits associated with the host-plant species than on phylogenetically constrained insect adaptations. Cronin and Abrahamson (2001) have suggested that specialization and subsequent diversification by parasitoids, as a result of specialization and subsequent diversification by their hosts, may not be the primary diversification mechanisms within parasitic Hymenoptera. Rather, parasitoids' ability to respond to specific host-plant clues may be more important.

Phenotypic Differentiation in Hymenopteran Parasitoids.

Parasitoid individuals of a particular species differ in their preference for, or ability to survive in, different herbivorous host species, herbivore host-plant species, or host-plant complexes (i.e., the combination of a particular herbivorous host species on a particular host-plant species) (Lewis et al. 1990, Guerrieri et al. 1993, Kester and Barbosa 1994, Potting et al.1997, Du et al. 1998, Souissi and Le Rü 1998, Althoff and Thompson 2001, Daza-Bustamante et al. 2002). Thus, phenotypic differentiation can occur among populations of a parasitoid species that attack different herbivorous host species. For example, *Trichogramma semblidis* Aurivillius (Hymenoptera: Trichogrammatidae) reared on different herbivorous host species exhibit marked differences in wing and antennal structure (Salt 1937). Phenotypic differentiation can also occur among conspecific parasitoids attacking different host-plant complexes. *C. flavipes* differs in reproductive success when associated with different host-plant complexes (i.e., a stemborer species attacking maize versus another stemborer species attacking sugarcane) (Potting et al. 1997). Similarly, *Aphidius ervi* Haliday (Hymenoptera: Aphidiidae) females orient more strongly towards the odor of the host-plant complex with which they have been associated as larvae (Daza-Bustamante 2002).

Phenotypic differences have also been reported among conspecific parasitoids attacking the same herbivorous host species on different host-plant species. For instance, the host selection behavior of *C. congregata* attacking *Manduca sexta* L. (Lepidoptera: Sphingidae) on tobacco differs from that of *C. congregata* attacking *M. sexta* on tomato (Kester and Barbosa 1994). Similarly, percentage of parasitism, encapsulation levels,

survival, total developmental time and size of male and female *A. lopezi* are significantly different in parasitoids reared from the same host species, *P. manihoti*, but on different host-plant species (Souissi and Le Rü 1998). Differences in the relative attractiveness of collards and beets to the parasitoid *Diaeretiella rapae* M'Intosh (Hymenoptera: Braconidae) explains different levels of parasitism of the host, *Myzus persicae* Sulzer (Homoptera: Aphididae) (Read et al. 1970). Likewise, *Itoplectis conquistor* Say (Hymenoptera: Ichneumonidae) is more attracted by the odor of Scots pine than by that of red pine and correspondingly attacks its host, *Rhyacionia buoliana* Denis and Schiffermüller (Lepidoptera: Tortricidae) to a greater extent on Scots pine than on red pine (Arthur et al. 1964).

Genotypic Differentiation in Hymenopteran Parasitoids.

Although the potential of parasitic Hymenoptera to evolve in response to selection by conspecific herbivorous hosts associated with different host-plant species has been proposed (Henter 1995), and the important role of host-plant species in parasitoid ecology has been widely recognized, no study has used genetic markers to explore the role that different host-plant species fed upon the same herbivore species, plays in the genetic differentiation of conspecific hymenopteran parasitoids. To my knowledge there is only one example of genetic differentiation in parasitoids associated with different host-plant complexes that has found genetic differentiation among parasitoids associated with different host-plant complexes, but only at a very local scale. Genetic differences in genetic marker fingerprints has been reported in sympatric *D.*

rapae attacking different aphid species on different host-plant species within populations less than 1.0 Km apart (e.g., Vaughn and Antolin 1998). However, different populations from each of the host-plant complexes tested were also genetically different from each other, indicating that differentiation did not result from separate lineages isolated on the different host-plant complexes (Baer et al. 2004). Similarly, no genetic differences in molecular markers have been found between *A. ervi* populations associated with different host-plant complexes (Daza-Bustamante et al. 2002).

Thus, despite hymenopteran parasitoids meeting several of the theoretical criteria necessary for genetic differentiation, the few studies that have searched for it have failed to find it. Although the number of studies on parasitoid genetic differentiation are too scarce to draw any generalizations yet, it could be that some parasitoid species present traits that make them unlikely candidates for genetic differentiation. For instance, it has been speculated that gene flow of some hymenopteran parasitoids could be sufficient to prevent fixation of neutral loci (Baer et al. 2004). Although sibling mating (Askew 1968, Diehl and Bush 1984, Hassell and Godfray 1992) and mating at the site of emergence (Godfray 1994) have been observed in some parasitoids, other parasitoid species may disperse relatively long distances before mating and/or ovipositing (Roland 2000, Van Nouhuys and Hanski 2002). Long distance dispersal of parasitoids attacking different host-plant complexes might diminish the probabilities that parasitoids would mate with others associated with the same host-plant species. In addition, some parasitoid species learn to associate microhabitat or host-plant cues with their hosts and change their preference for plant volatiles based on experience (Turlings et al. 1993). This behavioral

plasticity reduces fidelity to one host-plant species and might impede host-plant based genetic differentiation to be observed in certain parasitoid species.

My Study System.

The published research conducted to date and reviewed above, suggests that host-plant species can generate phenotypically and genotypically distinct parasitoid conspecifics in hymenopteran parasitoids ovipositing on the same host on different host-plant species. In summary, we know that parasitoids can be phenotypically different on different host species, on different host-plant species and on different host-plant complexes. We also know that parasitoids can be genotypically different on different host-plant complexes. However, research is lacking on the role of different host-plant species fed upon the same herbivorous host species, in the genetic differentiation of parasitoids. The present study focuses on the influence of host-plant species in the phenotypic and genotypic differentiation of populations of the generalist parasitoid *C. marginiventris* and the specialist *A. nolophanae*. Both parasitoid species are the most abundant parasitoids of the green cloverworm, *P. scabra,* on alfalfa and soybean in Maryland.

Variation in the use of resources has important implications for the evolution of ecological specialization and is relevant to host race formation and parasitoid speciation (Diehl and Bush 1984). Parasitoid insects that use different host species, different host-plant species, or different host-plant complexes can have a subdivided population structure that corresponds to use of different host species, host-plants, or host-plant

complexes. A subdivided population structure may favor local sub-population adaptations to small scale environmental differences and may promote their genetic divergence (Vaughn and Antolin 1998). Thus, over time, resource specific strains may evolve towards distinct species as suggested by Vet and Janse (1984). Parasitoid variation in the use of resources among different populations also has important implications for biological control, because the selection of appropriate strains or biotypes is a significant factor in successful biological control practices (Roush 1990, Lewis et al. 1990). Indeed there are numerous examples of differential effectiveness of biological control agents selected from different geographic regions. Thus, it is important to select a strain that has the ability or potential to search for and utilize the target host species in its micro-habitat (Lewis et al. 1990). The present study will contribute to our knowledge on phenotypic and genetic differentiation in parasitoid populations by exploring the role that host-plant species play in this process.

In this study I hypothesize that host-plant species might promote phenotypic and genotypic differentiation in hymenopteran parasitoids. In chapter two, I discuss the role of host-plant species in the phenotypic differentiation of *A. nolophanae* and *C. marginiventris*. In chapter three, I discuss the role of host-plant species on the genotypic differentiation of *A. nolophanae* and *C. marginiventris*. In chapter four, I propose a new methodology to rigorously determine the number of individuals and the number of molecular markers one needs to use in order to accurately determine genetic differentiation of field populations. This method is critical to determine if my conclusions on genetic differentiation are robust.

CHAPTER 2:

The Role of Host-Plant Species in the Phenotypic Differentiation of Sympatric Populations of *Aleiodes nolopahanae* Ashmead (Braconidae: Hymenoptera) and *Cotesia marginiventris* Cresson (Braconidae: Hymenoptera)

INTRODUCTION.

The biology of Hymenoptera that are parasitic on herbivorous insects is significantly influenced by the host herbivores they parasitized and the host-plants used by their hosts (Price et al. 1980, Benrey and Denno1997, Huffbauer and Via 1999, Vinson and Barbosa 1987, Vet and Dicke 1992, Hunter 2003). Therefore, phenotypic differentiation reflected in changes in morphology, physiology and behavior has been observed among individuals parasitizing different herbivorous host species (Salt 1937, Sato and Ohsaki 1987, Eben et al. 2000) and different herbivore species parasitized on different host-plant species (Potting et al. 1997, Eben et al 2000, Daza-Bustamante 2002). However, perhaps less expected have been observations of phenotypic differentiation of parasitoids attacking the same herbivore host species on different plant species (Kester and Barbosa 1994, Souissi and Le Rü 1998, Sznajder and Harvey 2003, Campan and Benrey 2004). These differences typically are in morphology, physiology and behavior affecting survival, ability to find and attack hosts and rate of population increase. Thus, the parasitoids emerging from each herbivore host-host plant combination (henceforth host-plant complex) in large part, is a consequence of host herbivore traits, plant traits, traits produced as a result of herbivore feeding and/or oviposition, or some combination

of these.

Studies that have looked for phenotypic differences in Hymenoptera attacking the same host species on different host-plant species have mostly compared wild and cultivated plants and have not considered the relative ratio of the plant species studied. In this study, I compared phenotypic traits of parasitic Hymenoptera attacking the same host species on two different cultivated Leguminosae species at sites that differed in their current and historic commitment of acreage devoted to the cultivation of each crop plant species. In addition, studies that have looked for phenotypic differences in Hymenoptera attacking the same host species on different host-plant species have rarely been coupled with genotypic differentiation studies. In this study, I used the same individuals from which phenotypic traits were measured to study genotypic differentiation between parasitoids associated with each crop plant species (see Chapter 3). Because generalist and specialist parasitoids have been found to differ in their foraging strategies and in the effect that plant chemicals have on their behavior and performance (Barbosa 1988, Vet and Dicke 1992), the role of host-plant species on phenotypic differentiation was explored in a generalist and in a specialist parasitoid from the same family (i.e., Braconidae).

In this chapter, I review fitness parameters in parasitoids likely to be affected by host-plant species (i.e., adult mass, adult longevity, percentage of parasitism and preference for host-plant odors) and discuss how geographic (i.e., site) differences may modulate the strength of host-plant species on parasitoid's phenotype. This overview will propose that plants may be an important agent of differentiation and that the strength of

this differentiation can be expressed differently at different locations.

Plants can influence parasitoid fitness parameters directly and/or indirectly through its effects on the development of the phytophagous host (Arthur et al. 1964, Read et al. 1970, Kester and Barbosa 1994, Fox et al. 1996, Souissi and Le Rü 1998, Turlings and Benrey 1998). An understanding of the influence of an herbivore's host-plant species on parasitoids fitness is crucial in understanding parasitoid population dynamics under field conditions and for the design of effective biological control programs (Bottrell et al. 1998, Verkerk et al. 1998, Eben et al. 2000). Parameters such as adult mass, adult longevity, percentage of parasitism and response to host-plant odors are particularly important indicators of fitness in parasitoids. Adult mass is one of the most utilized fitness parameters in studies of hymenopteran parasitoids because it is correlated to fecundity and, in some instances, to adult longevity (Charnov et al. 1981, Waage and Godfray 1985, Godfray 1994, Kraaijeveld and Van der Wel 1994). Female fitness in hymenopteran parasitoids has been shown to increase with adult size (Blackburn 1991, King 1988, Visser 1994). In the parasitoid *Apoanagyrus lopezi* De Santis (Hymenoptera: Encyrtidae) the number of oocytes in the ovarioles of females is positively correlated with body size (Van Dijken et al. 1991). Large females not only live longer than small females but also have a greater probability of successful mating (Kraaijeveld and Van Alphen 1986). Similarly, larger *Asobara tabida* Nees (Hymenoptera: Braconidae) females have more eggs in their ovarioles (Kraaijeveld and Van Der Wel 1994). In *Aphaereta minuta* Nees (Hymenoptera: Braconidae), a gregarious parasitoid of dipteran larvae, female size is positively correlated with egg load, egg size, and adult longevity

and searching efficiency (Visser 1994).

Adult longevity is widely used as a fitness indicator (Godfray 1994). Both adult longevity as well as adult mass can reflect potential reproductive output in hymenopteran parasitoids. Plants may affect parasitoids by influencing on both these fitness parameters (Bhatt and Singh 1989, Souissi and Le Rü 1998). Changes in these parameters can promote phenotypic differentiation among parasitoids ovipositing on hosts on different host-plant species (Charnov et al. 1981, Waage and Godfray 1985, Godfray 1994). For example, significant differences in adult mass have been found among *C. marginiventris* ovipositing in *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) on different host-plant species (Sznajder and Harvey 2003). Similarly, differences in development time and in adult mass have been found among *Cotesia glomerata* L. (Hymenoptera: Braconidae) ovipositing in *Pieris brassicae* L. (Lepidoptera: Pieridae) on different host-plant species (Sznajder and Harvey 2003). The host-plant on which a parasitoid's host feeds may also affect parasitoid survival. *C. marginiventris* larval parasitoid mortality is affected by the food plant species upon which its host S. *exigua* feeds. More than 50% of *C. marginiventris* wasps developing on *S. exigua* from two wild crucifer species perished during development. In contrast, parasitoid mortality on hosts reared on the domesticated *Brassica oleracea* Kale was only 9% (Sznadjer and Harvey 2003).

Parasitoid fitness also is a function of percentage of parasitism. The percentage of herbivore hosts parasitized by a parasitoid may differ when hosts are on different plant species or cultivars (Beck and Cameron 1990, Liu and Jiang 2003, Helms et al. 2004). For instance, the plant species on which *Phenacoccus manihoti* Matile Ferrero

(Hemiptera: Pseudococcidae) feeds, influences percentage of parasitism by *Apoanagyrus lopezi* De Santis (Hymenoptera: Encyrtidae) (Souissi and Le Rü 1998). Similarly, percentage of parasitism by *C. marginiventris* of *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) differs when it occurs on different host-plant species (Rajapakse et al. 1991). Likewise, percentage of parasitism of *S. frugiperda* by *Aleiodes laphygmae* Viereck (Hymenoptera: Braconidae) is significantly different when host larvae feed on different turfgrass species (Braman et al. 2004).

The ability of natural enemies to differentiate between different plant species can influence their fitness directly (e.g., differences in hairs and glands on different plant species can interfere with foraging behavior) (Obrycki 1986, Van Lenteren and De Ponti 1991) or indirectly (e.g., as a result of differences in host quality due to differences in plant species suitability for herbivore development) (Barbosa 1988). Thus, plant species have been found to influence parasitoid's host selection (Benrey et al. 1997). Further, variation in the levels of parasitism among plant species has been shown to be correlated with plant morphology and/or plant chemistry (Liu and Jiang 2003). During host selection, parasitoids use a variety of plant cues such as shape, surface structure, and internal and external (surface) chemicals to assess the quality of their hosts (Godfray 1994). The quality and the quantity of these traits vary from plant species to plant species and influence host discrimination by parasitoids (Souissi and Le Rü 1998). Plants often provide the first cue in the chain of events that leads to host location.

Out of all the potential variables that parasitoids may use to locate their hosts chemical cues are the ones that seem to be the most important and consequently have

been the most studied (Vet 1983). For example, host herbivores are attacked when they occur on one plant species but not on another plant species (Walker 1940, Clausen 1941, Smith 1957, Zwolfer and Kraus 1957, Arthur 1962, Stary 1964, Hassell and Southwood 1978). Differences in percentage of parasitism among conspecific parasitoids ovipositing in the same host species on different host-plant species may be explained by differences in the degree of attraction of parasitoids to plant specific volatile compounds (Fox et al. 1967, Liu and Jiang 2003). Further, certain parasitic Hymenoptera are capable of developing on a host only when the host has fed on particular host-plant species (Price et al. 1980, Vet and Dicke 1992).

Host-plant species may influence more than one fitness parameter. For instance, female *Diachasmimorpha longicauda* Ashmead (Hymenoptera: Braconidae) emerging from *Anastrepha ludens* Loew (Diptera: Tephritidae) reared in different host-plant species presents significant differences in size and adult longevity (Eben et al. 2000). Female *C. glomerata* are more attracted to the cultivated *B. oleracea* species than to the wild *Lunaria annua* and *C. glomerata,* parasitoids develop faster on hosts raised on cultivated *B. oleracea,* survive better and emerge as larger adults than parasitoids that developed on larvae reared on the wild plant, *L. annua* (Benrey et al. 1998). Similarly, the parasitoid *Stenocorse bruchivora* Crawford (Hymenoptera: Braconidae) prefers cultivated beans (genus *Phaseolus* L.) over wild bean subspecies and, consequently, faster developing and more parasitoids emerged from these subspecies than from the wild subspecies (Benrey et al. 1998).

Volatile blends produced by damaged plants and used by natural enemies to find hosts or prey vary significantly in different plant species. For instance, tobacco, cotton and maize each produce distinct volatile blends in response to herbivore damage (De Moraes et al. 1998). Parasitoids are able to detect these differences in volatile composition and are differentially attracted towards the odors that maximize their probabilities of finding suitable hosts (Vet and Dicke 1992, De Moraes et al. 1998). Thus, phenotypic differentiation among parasitoids ovipositing in the same host species on different host-plant species, may be reflected in differential attraction of parasitoids to the distinct volatiles of each host-plant complex. Many parasitoids prefer the odor (i.e., volatile profile) of the plants on which they have developed (Kester and Barbosa 1991, Turlings et al. 1993, Bogahawatte and van Emden 1996). This sort of fidelity to the host-plant odor of the plant on which the parasitoid's host resides may promote phenotypic differentiation among parasitoids ovipositing hosts on different plant species (Kester and Barbosa 1991).

Significant geographic variation exists in the survival probability of some parasitoids in their respective hosts. Geographic differences in the abiotic and biotic environments experienced by different populations of the same parasitoid species may alter the way in which parasitoids interact with host or their host-plants (Thompson 1994, Kraaijeveld and Godfray 1999). This means that different populations of parasitoids may represent different phenotypes as a result of geographic differences (Kraaijeveld and Van Der Wel 1994). For instance, differences in the substrate odors preferred by a parasitoid of drosophilid larvae, *Asobara rufescens* Förster (Hymenoptera: Braconidae), vary

geographically (Kraaijeveld et al. 1994). Likewise, *A. tabida* that differ in geographic origin have differences in their egg load (Kraaijeveld and Van Der Wel 1994). Geographically related differences in traits such as virulence and encapsulation defenses have been observed in other parasitoids (Walker 1959, 1962, Carton 1984, Boulétreau 1986, Mollema 1988, Carton and Nappi 1991, Kraaijeveld and van Alphen 1994, Althoff and Thompson 1999) as has geographic variation in diapause and in egg/fat balance (Kraaijeveld and Van der Wel 1994). These and other geographically related phenotypic differences may occur among individuals of populations that are relatively close to each other. For example, *Cotesia congregata* Say (Hymenoptera: Braconidae) exhibits geographic differences in searching time and in oviposition preferences for hosts on different host-plant species in sites located only 56 Km apart from each other (Kester and Barbosa 1994). Thus, the degree to which host-plant species may influence phenotypic differentiation of parasitoid species can be influenced by the geographic location of the organism.

In this chapter I determine if phenotypic differentiation due to host-plant species (alfalfa versus soybean) is observed in the specialist parasitoid *A. nolophanae* and in the generalist *C. marginiventris,* ovipositing on the same host, the green cloverworm, *Plathypena scabra* Fabricius (Lepidoptera: Noctuidae). Specifically, I address three questions relating to the role of herbivore host-plants in mediating phenotypic differentiation in parasitoids: (1) Do parasitoids ovipositing in green cloverworm larvae in alfalfa versus soybean differ in adult mass and adult longevity? (2) Do parasitoids ovipositing in green cloverworm larvae on alfalfa and soybean differ in the proportion of

larvae they parasitize? and (3) Does attraction of parasitoids to alfalfa and soybean odors differ depending on the host-plant species on which their host was reared?. Some key fitness phenotypic traits in parasitoids have been found to vary among populations from different locations (see introduction). Thus, the questions asked in the present study will be addressed for parasitoids in geographically distinct locations. This study will provide information on the role of cultivated host-plant species in the phenotypic differentiation of a specialist and a generalist parasitoid that co-occur associated with sympatrically occurring host-plant species.

METHODS.

Study Organisms.

Plathypena scabra Fabricius. (Lepidoptera: Noctuidae).

Also known as the green cloverworm, this species is a native to the US, occurring predominantly from the eastern United States westward into the Great Plains states and northward into southeastern Canada (Pedigo 1994). It is one of the most common lepidopteran species in leguminous agroecosystems (McCutcheon et al. 1997). The green cloverworm is a generalist herbivore associated with various host-plants, including alfalfa, soybean, red clover, vetch, cowpea and beans (Pedigo 1971).

In Maryland, green cloverworms start to appear in alfalfa fields approximately the first days of June and start overwintering as pupae or adults in the last days of August. They are known to overwinter at locations South of 41 $^{\circ}$ N latitude except in more southerly locations where feeding activity and reproduction occur year round. Northern

(i.e., above 41° N latitude) areas are colonized each Spring by migrating moths transported by southerly winds. Females oviposit eggs singly on the undersides of leaves. The eggs hatch in about 3 days, and the larvae undergo 6 to 7 molts in about 14 days. Eggs are 0.5 mm in diameter, translucent green and become brownish with red specks, about 48 hours before hatching. Green cloverworm larvae are about 25 mm long when fully grown and are pale green with two white stripes running horizontally along each side of the body. Larvae are distinctive from other soybean or alfalfa caterpillars by having three pairs of abdominal prolegs plus one pair of anal prolegs. After a 7 to 10 day pupal stage, moths emerge. Pupae are brown and are found inside a silk cocoon (Pedigo 1994).

There are two generations per year in the Northern United States (north of 41° N latitude) and three to four generations per year in the South (south of 41° N latitude). The green cloverworm is attacked by several species of predators and parasitoids. In Maryland *A. nolophanae* and *C. marginiventris* are the most abundant parasitoids of the green cloverworm. However, the primary factor regulating green cloverworm populations is disease caused by the fungus *Nomuraea rileyi* (Pedigo 1994).

***Aleiodes nolophanae* Ashmead (Hymenoptera: Braconidae).**

This species is native to the US. It prefers to oviposit in third instar green cloverworms (Lentz and Pedigo 1973). It oviposits one egg per host larva. Eggs of *A. nolophanae* are slightly curved and somewhat oval along the longitudinal axis (Lenz and Pedigo 1973). Once *A. nolophanae* oviposits in green cloverworms, there is no obvious

external evidence of parasitism during approximately the first eight days. After eight days a swelling appears in the abdominal region of the larva. When the larva is still green *A. nolophanae* chews an opening on the mesothoracic sternum and secretes an adhesive material that attaches the host larval skin to the substrate. Within two days, a brown mummified skin is formed in which the parasitoid completes its development. The mean developmental time from oviposition to adult emergence is 18 days for males and 18.5 days for females (Lenz and Pedigo 1973). The adult parasitoid emerges from the cocoon by chewing an exit hole in the tergum near the tip of the host's abdomen. *A. nolophanae* can be considered as a specialist parasitoid of the green cloverworm. It only has been reported to occur in green cloverworm on Leguminoceae and in *Balsa malana* Fitch (Lepidoptera: Noctuidae) on apple trees (Krombein et al. 1979).

***Cotesia marginiventris* Cresson (Hymenoptera: Braconidae).**

This species was originally described from Cuba and it is native to North America (Marsh 1979, Jalali et al. 1987). It occurs in the United States: from Delaware south to Florida, north to Wisconsin, west to Indiana, Kansas and Texas, Arizona and California. It is also present in Hawaii, Mexico and South America. A single egg is usually laid in each host. *C. marginiventris* eggs are oval, three times longer than wide, with a small projection. Parasitoid larvae eclose from eggs two days after oviposition by the adult. The larvae bear a caudal vesicle in the posterior region and are located in the host's posterior end. First instar are on average only 0.06 mm long, whereas mature (third instar) larvae are on average 5.5 mm long. Practically all caterpillar internal organs are

consumed by the parasitoid larva. The host, which feeds little throughout its life, dies approximately a day after the parasitoid has left the caterpillar. When larvae emerge from the host, about 7 to 10 days after the egg is laid, they immediately begin spinning a silky cocoon. The cocoon is white and approximately 4 mm long. The adult wasp is small (approximately 3mm in length). Females bear relatively short ovipositors and parasitize only first to second instars or even eggs (Boling and Pitre 1970, Kunnalaca and Mueller 1979). In the laboratory, *C. marginiventris* lives more than a week, but it is most active (e.g., searching and ovipositing) between two and four days of age.

C. marginiventris is a generalist parasitoid of noctuid pests. Although all *C. marginiventris* hosts are noctuids they represent species in 15 genera feeding on at least eight plant families. *C. marginiventris* is a parasitoid of *Agrotis ipsilon* Hufn. (the black cutworm) *Anagrapha falcifera* Kirby (the celery looper), *Autographa precationis* Gn., *Autoplusia egena* Guen.(the bean leaf skeletonizer), *Helicoverpa zea* Boddie (the bollworm, also called the corn earworm or tomato fruitworm), *Heliothis virescens* F. (the tobacco budworm), *Hymenia perspectalis* Hbn. (the spotted beet webworm), *Hymenia recurvalis* F., *Leucania latiuscula* H.S., *Leucania multisulca* Wlkr., *Peridroma saucia* Hbn., (the variegated cutworm), *Plathypena scabra* F. (the green cloverworm), *Pseudaletia unipuncta* Haw. (the armyworm), *Pseudoplusia includens* Wlkr.(the soybean looper), *Scotorythra caryopsis* Meyr., *Spodoptera eridania* Cram.(the southern armyworm), *Spodoptera exigua* Hbn. (the beet armyworm), *Spodoptera frugiperda* Smith (the fall armyworm), *Spodoptera ornithogalli* Guen., *Spodoptera praefica* Grote and

Trichoplusia ni Hbn. (the cabbage looper) (Krombein et al. 1979). In Maryland *C. marginiventris* attacks the green cloverworm starting in June throughout the summer and its numbers start to decline in the last weeks of August.

***Medicago sativa* L. (Fabaceae: Leguminosae).**

Commonly known as alfalfa, *Medicago sativa* L. is an herbaceous perennial legume that originated near Iran. Related varieties and species are found as wild plants throughout Central Asia and into Siberia. Alfalfa was first introduced into the eastern US by the colonists in 1736. It is the oldest cultivated forage crop in the US. Alfalfa is one of the most important forages for livestock. It has a high regrowth capacity and possesses nitrogen fixing properties (Hanson et al. 1988).

Since plant architecture may influence natural enemies foraging (Kareiva and Sahakian 1990, Legrand and Barbosa 2003) differences in plant morphology may be important in explaining differences in parasitoid fitness among different plant species. A mature alfalfa plant may have from 5 to 25 stems, which usually reach a height of 38-63 cm. Stems are branched and slender and bear pinnately trifoliolate glabrous leaves. Leaves with more than three leaflets are not uncommon. Leaves are arranged alternately on the stem. Stipules are slender and fused to the petiole. Leaflets are linear, oblong and are toothed toward their apices. Alfalfa is worldwide in its distribution and is grown in many areas of the US (Hanson et al. 1988). In Maryland, alfalfa is planted in March and April if planted in Spring and in August if planted in the Fall. Once planted, alfalfa fields typically are maintained for four years.

***Glycine soja* Sieb & Zucc. (Fabaceae: Leguminosae).**

Commonly known as soybean, it is the domesticated variety of the wild *Glycine max* L. Merr. *Glycine soja*, which is native to China but does not occur in the wild, was introduced in the US in the mid 1770's (Smith 1994). The soybean plant has a single trifoliolate leaf at each node alternatively attached to each side of the stem, except at the cotyledonary and second nodes of the main stem. Leaflets are obovate. The petiole attaches the leaf to the main stem. A pair of lance-shaped stipules are located at the base of the petiole in the petiole-stem junction. The leaves (also the stem, branches and pods) of the soybean plant are normally covered with pubescence. In Maryland, soybeans are planted in May and June and harvested in October and November.

Study Sites.

Some key fitness phenotypic traits in parasitoids have been found to vary among populations from different locations (see introduction). Thus, I sampled wasps from three counties in Maryland in order to determine if geographic location influences the phenotypic differences in alfalfa-associated parasitoids and soybean-associated parasitoids. Obviously, there may be many biotic and abiotic differences among field sites. However, I used a criterion for the selection of the three sites that was most relevant to this study, i.e., the differences in the current and historical ratio of alfalfa to soybean cultivation which should reflect the degree to which parasitoids have interacted with hosts on alfalfa versus soybean, over time. Thus, green cloverworm were sampled at the USDA Beltsville Agricultural Center in Prince George's County (PG County) in 2001, 2002 and 2003, in a private farm in Washington County in 2002 and 2003, and in a

private farm in Garrett County in 2002 and 2003. PG County has had an alfalfa: soybean ratio of 1:12.5, Washington County a 1:1 ratio and Garrett County a 20:1 ratio. According to each county's agricultural extension records, these ratios have been kept at about these levels at the three counties for at least 10 years. The closest study sites were 60 Km apart (i.e., PG and Washington county sites) and the most distant ones were 160 Km apart (i.e., PG and Garrett county sites).

In PG County there were three plots of alfalfa and three of soybean; each was 32x32 m with an average distance of about 200m between plots. These plots were created because in 2001, I did not know the scale at which phenotypic and genetic differentiation might be acting in this system. If movement among wasps was as restricted as has been demonstrated in some herbivorous insects (Mopper and Strauss 1998) genetic and phenotypic differences could have been found among plots. Preliminary analyses (2001) did not show phenotypic or genetic differentiation among field plots of the same crop. Thus, wasps collected from the PG plots were pooled in subsequent years. In Washington and Garrett Counties, a field of alfalfa and one of soybean were established, each approximately 300x300 m. I had no control over the varieties of alfalfa and soybean planted by farmers at the Maryland counties studied. The varieties used by the farmers in each of the counties were chosen by farmers according to the conditions for the particular geographic area in which their crops were planted.

Sampling.

Green cloverworm larvae were collected in each of the alfalfa and soybean fields in each of the study sites. The fields were sampled using a 38 cm diameter sweep net. In Garrett and Washington Counties collections consisted of sweeping the field while walking at an even pace along a haphazardly chosen transect within each of the alfalfa

and soybean fields. The total collection effort per transect was 30 minutes. Within each transect in each crop, sampling consisted of 5 sweeps after which green cloverworms were taken out of the net and placed in a container to reduce larval injury. Then five additional sweeps were performed, and so on, until ten minutes had elapsed. I then walked along the transect for approximately 50 meters and resumed the five sweep protocol until ten minutes had elapsed, and again walked another 50 meters, followed by one last 5 sweep protocol for 10 minutes, for a total of 30 minutes. Because there were three plots in PG County instead of one (as in Garrett and Washington Counties), sampling differed but the total sampling effort was the same. That is, a total of thirty sampling minutes was divided into three ten minute intervals; ten minutes in each of the three plots of each crop species. The same five sweep protocol was used. Thirty minutes of collection time per crop field provided the maximum number of larvae that could be reared in the laboratory on a daily basis. In 2001, only PG County was sampled, four to five days per week from the second week of June until the last week of August. Washington and Garrett Counties were not sampled in 2001. In 2002 and 2003 each crop, in each study site, was sampled once a week from the second week of June until the last week of August.

Green Cloverworm Rearing.

Green cloverworm larvae were placed individually in 473 ml clear plastic cups labeled with their site, collection date and host-plant species. Larvae were fed with the same species of host-plant from which they were collected. They were kept in the laboratory at room conditions (an average of 23.7 ˚C ± 0.14, 66.7 RH ± 0.75%). Green cloverworm larvae were reared until adult moths or until parasitoids emerged. Adult parasitoids were sexed and weighed after emergence and then frozen for subsequent

genetic analyses (see Chapter 3) in 2001 and 2002. In 2003 adult parasitoids were kept alive in individual plastic containers with honey water to measure adult longevity. Once parasitoids died they were weighed and then frozen. Alfalfa and soybean leaves used for feeding larvae were washed with running water as soon as they were brought from the field and then kept at 4 °C. When green cloverworm larvae were infected with *N. rileyi* they were immediately discarded within their closed cups to prevent the infection of other larvae in the laboratory.

Effect of Host-Plant Species in Parasitoid Adult Mass and Adult Longevity.

Mean adult mass and mean adult longevity of *A. nolophanae* and *C. marginiventris* individuals attacking green cloverworm larvae on alfalfa were compared to that of adults from the same host on soybeans. Once each adult parasitoid emerged from its pupae it was killed and then weighed (in 2001 and 2002). In 2003, adult parasitoids were weighed after their longevity was measured. Adult parasitoids were weighed in a Mettler AE 100 balance.

Every individual parasitoid obtained in the laboratory from parasitized green cloverworms was used for DNA extraction. Parasitoid DNA was used to study if there was genotypic differentiation among parasitoids associated with different host-plant species (see Chapter 3). Thus, parasitoid adult wet mass rather than dry mass was measured because the high temperatures required to obtain dry masses could damage parasitoids' DNA. A cohort of 34 *A. nolophanae* and 10 *C. marginiventris* were used to determine whether adult wet and dry mass in each of the parasitoid species were correlated. Newly emerged parasitoids were weighed and then were placed inside a laboratory oven at 60° C for 48 hours and weighed again and the correlation between wet

and dry mass was calculated. Because wet and dry mass were highly correlated (Figure 2. 1) wet mass was used. Differences in adult mass means were analyzed as a split plot design using Proc Mixed (SAS 1998). County (i.e., site), host-plant species, gender and their interactions were considered as fixed effects and collection year was considered as a random effect. The data was split in gender within host-plant species and in host-plant species within sites. Normality of the data was tested using the Shapiro-Wilkinson test and homogeneity of variances was tested by plotting residuals versus predicted values. In the *A. nolophanae* adult mass data, sites were nested within years. *C. marginiventris* adult mass data from the three years of the study were pooled in order to have enough degrees of freedom to perform the analysis.

Longevity tests were conducted in 2003. Each adult parasitoid was placed in individual 473 ml clear plastic cups with honey water. Parasitoids were kept at room conditions (an average of 23.7 ˚C ± 0.14, 66.7 RH ± 0.75%) until they died. Once parasitoids died they were weighed and stored at -80°C. Adult longevity was measured by counting the number of days either male and female adult parasitoids survived in the laboratory. The day count started immediately after parasitoid adult emergence and ended with the death of the adult parasitoids. These data were analyzed as a split plot design using Proc Mixed (SAS 1998). County, host-plant species, gender and their interactions were considered as fixed effects. The data was split in gender within host-plant species and in host-plant species within sites. Normality of the data was tested using the Shapiro-Wilkinson test and homogeneity of variances was tested by plotting residuals versus predicted values.

Effect of host-plant species on percentage of parasitism.

Differences in Percentage of Parasitism Between Alfalfa and Soybean Green Cloverworm Larvae.

Percentage of parasitism of green cloverworm larvae on alfalfa by *A. nolophanae* and by *C. marginiventris* was compared with percentage of parasitism of green cloverworm larvae on soybean at each of the study sites in 2001, 2002 and 2003. Percentage of parasitism by *A. nolophanae* and by *C. marginiventris* was calculated independently by counting the total number of parasitized and non-parasitized green cloverworm larvae collected in alfalfa and in soybean at each of the three study sites, each year. In both host-plant species and all three sites, sampling intensity was the same. Chi-square tests of association (SAS 1998) were used to analyze the data on differences in parasitism among host-plant species in each study site, each year. Differences in parasitism among host-plant species may be a reflection of differences in host density or differences in host quality among hosts feeding on different host-plant species. As described in the following sections, I evaluated whether host density and host quality explained differences in percentage of parasitism between alfalfa and soybean.

Host Density in Alfalfa and Soybean.

The comparison of percentage of parasitism as an indicator of phenotypic differentiation may be confounded by differences in host density. Thus, the relative density of green cloverworm larvae on alfalfa and soybean was assessed by counting the number of green cloverworm larvae on five alfalfa and five soybean plants selected from each of ten randomly selected $1m^2$ plots within an alfalfa field and a soybean field in PG County in the Summer of 2004. Five whole alfalfa and five whole soybean plants were

taken from each of these randomly selected alfalfa and soybean plots and each plant was immediately placed inside a sealed plastic bag. In the laboratory, each plant was searched for green cloverworm larvae. The number of green cloverworm larvae per plant was recorded and the leaf area of each plant was determined using SCION © Image for windows software to report the data as number of larva per mm^2 of leaf per plant. These data were analyzed using ANOVA (SAS 1998). Sampling date, host-plant species and date by host-plant species were included in the statistical model as main effects. ANOVA normality assumption was tested using the Shapiro-Wilkinson test and the homogeneity of variance assumption was tested by plotting residuals versus predicted values. Data were square root transformed to better fit the ANOVA model assumptions.

Because the number of alfalfa and soybean plants per unit area differed, differences in the abundance of green cloverworm larvae in alfalfa and soybean fields were determined by comparing the mean number of larvae collected in sweeps taken in 30 minutes on the same date, and county, using a pair t-test. The normality assumption of the test was assessed using the Shapiro-Wilkinson test and the homogeneity of variance assumption was tested by plotting residuals versus predicted values.

The same alfalfa and soybean fields from which the host density per plant was estimated were sampled in the summer of 2004 to calculate percentage of parasitism in alfalfa and in soybean. In the laboratory, each green cloverworm larva found was placed in an individual 473 ml clear plastic container labeled with the host-plant species from which it was collected and it was reared until an adult moth or a parasitoid emerged (as described in the “Green cloverworm rearing” section above). Differences in percentage of parasitism by *A. nolophanae* and *C. marginiventris* among green cloverworm larvae from alfalfa and soybean were calculated by counting the number of parasitized and non parasitized green cloverworms collected in alfalfa and in soybean. Chi-square tests of

association (SAS 1998) were used to analyze parasitism data from alfalfa and soybean fields.

Correlation Between Host Larval Mass and Host Adult Mass.

Data on host adult mass was taken every year of the study at each of the study sites (see above). Since the parasitoids studied oviposit in host larvae rather than in adult hosts it is important to know if adult mass correlates with larval mass. If so, adult mass can be used as a good indicator of larval mass. To determine if adult green cloverworm mass correlated to larval green cloverworm mass, the wet weight of green cloverworm larvae was obtained using a Mettler AE 100 balance right before pupation and as newly emerged adults. Larvae were collected in the field and brought to the laboratory where they were reared (as described in the "Green cloverworm rearing" section above) and checked daily. Just as green cloverworm larvae are about to pupate they assume a characteristic C-shaped position, change color, and become turgid and less mobile. At this point they are fully grown and thus were weighed and allowed to pupate and emerge as adults. Once emerged, one day-old adult moths were weighed. The correlation between larval host wet mass and adult host wet mass was calculated. A cohort of 33 host larvae were weighed right before pupation (as described above) and then were placed inside a laboratory oven at 60 ˚C for 48 hours and weighed again and the correlation between wet and dry mass was calculated. I determined that larval wet and dry mass were highly correlated (Figure 2. 2).

Host Quality in Alfalfa and Soybean.

Another factor that may explain differences in percentage of parasitism among host-plant species is the quality of the parasitoid's host. All else being equal, host mass can be considered as a good estimate of host quality for parasitoids (Cloutier et al. 1991, Godfray 1994, Jarošík et al. 2003). Thus, after checking if there was a significant adult and larval mass correlation, data collected on green cloverworm adult mass were used as an indicator of host quality.

Green cloverworms were collected as larvae at the same time (i.e., summer of 2001, 2002 and 2003) and from the same alfalfa and soybean fields from which the *A. nolophanae* and *C. marginiventris* parasitism data were collected. Green cloverworm larvae were reared in the laboratory until adult moths emerged and then they were weighed in a Mettler AE 100 balance. Differences in adult mass were analyzed as a split plot design using Proc Mixed (SAS 1998). County, host-plant species and gender were considered as fixed effects. The data was split in gender within host-plant species and in host-plant species within sites. Data from the three years of the study were pooled in order to have enough degrees of freedom to perform the analysis. Normality of the data was tested using the Shapiro-Wilkinson test and homogeneity of variances was tested by plotting residuals versus predicted values.

Host-Plant Odor Preferences.

I determined whether there was phenotypic difference in responses to plant volatiles. That is, whether *A. nolophanae* and *C. marginiventris* individuals were preferentially or exclusively attracted to volatiles from the host-plant species on which their hosts fed. *A. nolophanae* and *C. marginiventris* preferences were assessed using a

four armed olfactometer (Vet et al. 1984). The olfactometer consisted of a 33 cm diameter circular chamber in which four distinct odor fields were created and within which parasitoids could move around freely (Figure 2. 3). Choice tests were performed on newly emerged female and male wasps collected from alfalfa and soybean. *A. nolophanae* and *C. marginiventris* wasps were individually placed inside and in the center of the four armed olfactometer chamber and were given the following choices: alfalfa, soybean, alfalfa plus a 10-15 mm green cloverworm plus frass and soybean plus a 10-15 mm green cloverworm plus frass.

I gave parasitoids a choice between plant odors alone and plant odors plus herbivore odors because some parasitoids do not respond to plant odors alone, instead they respond to the odors generated by the interaction of the herbivore with the plant on which they feed (Vet and Dicke 1992). The pellet of frass was used because some parasitoids are attracted to frass odors rather than to plant or herbivore odors (Vet and Dicke 1992). Alfalfa and soybean plants used in these experiments were planted and kept in the greenhouse under the same conditions and were grown in the same kind of potting soil. An hour before the olfactometer experiments started two soybean leaflets and two alfalfa stems containing a comparable leaf area were cut from an alfalfa and soybean greenhouse plant and the cut ends were wrapped in wet cotton wool. Each cut soybean leaflet and alfalfa stem was placed in each of the four odor sample tubes of the olfactometer. After an hour, the chamber of the olfactometer was attached to the odor sample tubes and the choice experiments began. Each day the order in which the odor sample tubes were attached to the odor chamber of the olfactometer was randomly assigned to avoid position effects. At the end of the odor trials the olfactometer was cleaned with 70% alcohol and left open to dry. The laboratory windows were completely covered so daylight intensity or direction would not be a confounding factor in the

experiment. Experiments started the fourth week of June and finished the first week of September. Every testing day (i.e., five days per week) an average of about six unmated female and male *A. nolophanae* (0-17 per day) and four unmated *C. marginiventris* (0-11 per day) were tested in the olfactometer between 1100 and 1400. *A. nolophanae* and *C. marginiventris* adult wasps were taken from individual 473 ml clear plastic containers. Each container had honey water and leaves from the same host-plant species from which their respective hosts were collected. The age of the adult wasp, county of origin, host-plant species of origin (i.e., the host-plant species from which the host of the tested parasitoid was collected), room temperature, relative humidity, barometric pressure, the odor chosen and the time elapsed before a choice was made were recorded. Sixty one *A. nolophanae* parasitoids from PG County, 92 parasitoids from Washington County and 101 parasitoids from Garrett County were tested. Similarly, 52 *C. marginiventris* parasitoids from PG County, 23 parasitoids from Washington County and 54 parasitoids from Garrett County were tested. Parasitoids that did not choose within 15 minutes after their introduction in the olfactometer chamber were considered unresponsive to the odors provided. Each parasitoid was used only once. Data collected were used to determine if there was an association between the plant species on which the parasitoid's host fed and the preference of the parasitoid to go towards the odor of that particular plant species or host-plant complex. Pearson chi-square tests of equal proportions (SAS 1998) were performed to determine preference for a particular odor in wasps associated with alfalfa and soybean and Pearson chi-square tests of association (SAS 1998) were performed to test if there was an association between parasitoid host-plant species and the number of wasps that did show a response in the olfactometer. The time elapsed before a choice was made was analyzed as a split plot design using Proc Mixed ANOVA (SAS 1998) using county, host-plant species (i.e., host-plant species of origin) and gender as main effects. Host- plant species was split within counties and gender was split within host-plant

species. Data from both *A. nolophanae* and *C. marginiventris* were log transformed to fit the assumptions of the analysis. The normality assumption was tested with the Shapiro-Wilkinson test and plots of residuals versus predicted values were used to test for the homogeneity of variance assumption. Due to the lack of *C. marginiventris* wasps from alfalfa in Washington County, only data from PG and Garrett counties were considered when analyzing the time elapsed before a choice was made in *C. marginiventris.*

RESULTS.

Effect of Host-Plant Species on Parasitoid Adult Mass and Longevity.

Adult Mass.

Overall, *A. nolophanae* wasps that parasitized the green cloverworm on alfalfa had a significantly larger mass than *A. nolophanae* wasps that parasitized the green cloverworm on soybean ($F_{1,416}$=8.30; P=0.0042) (Figure 2. 4). Mean mass of adult female *A. nolophanae* was significantly larger than that of male *A. nolophanae* (1.77 mg vs 1.24 mg respectively; $F_{1,413}$=175.64; P<0.0001). No significant differences in adult mass were observed among *A. nolophanae* wasps from different counties and no significant interactions among site, host-plant species and wasp gender were observed (Table 2.1). In contrast to *A. nolophanae,* mean adult mass of female and male *C. marginiventris* did not significantly differ (Table 2. 2). Significant differences in adult mean mass were observed among *C. marginiventris* from alfalfa and soybean only in PG county ($F_{1,161}$=5.26, P=0.0231). No significant differences in adult mean mass were observed between *C. marginiventris* from alfalfa and soybean in the other two counties ($F_{1,45}$=0.22, P=0.6393 in Washington County and $F_{1,96}$=2.56, P=0.1132) (Figure 2. 5).

Adult Longevity.

Mean adult longevity of female and male *A. nolophanae* did not significantly differ (Table 2. 3). Differences in mean adult longevity of *A. nolophanae* attacking green cloverworm larvae on different host-plant species varied among sites (i.e., there was a significant site by host-plant interaction) (Table 2. 3). *A. nolophanae* associated with alfalfa in PG County lived significantly longer than those associated with soybean ($F_{1,45}$=34.55, P<0.0001) (Figure 2. 6). Parasitoids from the two host-plants in Washington and Garrett Counties, did not differ in their mean longevity ($F_{1,108}$=3.11, P=0.0805 and $F_{1,65}$=0.10, P=0.7526 respectively) (Figure 2. 6).

Female *C. marginiventris* lived longer on average than male *C. marginiventris* (Table 2. 4). As observed in *A. nolophanae*, there was a significant site by host-plant interaction for *C. marginiventris* (Table 2. 4). Female *C. marginiventris* associated with alfalfa lived longer on average than those associated with soybean in PG County ($F_{1,20}$=9.89, P=0.0051) but there were no significant differences in longevity among soybean and alfalfa female parasitoids in Washington ($F_{1,21}$=3.33, P=0.0822) or Garrett Counties ($F_{1,16}$=0.01, P=0.9171) (Figure 2. 7A). No significant differences were found in mean adult longevity between alfalfa and soybean male *C. marginiventris,* in any of the counties ($F_{1,29}$=2.43, P=0.1296 in PG County; $F_{1,9}$=1.23, P=0.2955 in Washington County, and $F_{1,43}$=0.16, P=0.6932 in Garrett County) (Figure 2. 7B).

Effect of Host-Plant Species in Percentage of Parasitism.

Percentage of Parasitism.

A. nolophanae wasps in PG County parasitized significantly more green cloverworms in alfalfa than in soybean. This greater parasitism of the green cloverworm

by *A. nolophanae* in alfalfa occurred in 2001, 2002 and 2003 (Figure 2. 8A). In Washington and Garrett Counties parasitism was more variable from year to year. In Washington County significantly more green cloverworms were parasitized in soybean than in alfalfa in 2002 but the opposite occurred in 2003 (Figure 2. 8B). In Garrett County, no significant differences in percentage of parasitism were found among host plants in 2002 but significantly more green cloverworms were parasitized in soybean than in alfalfa in 2003 (Figure 2. 8C).

For *C. marginiventris* there were no significant differences in percentage of parasitism of the green cloverworm in alfalfa or soybean in PG County in 2001, 2002, or 2003 (Figure 2. 9A). In Washington County there were no significant differences in percentage of parasitism of hosts in alfalfa and soybean in 2002 but significantly higher parasitism in soybean than in alfalfa in 2003 (Figure 2. 9B). Similarly, there were no significant differences in percentage of parasitism by *C. marginiventris* in soybean and alfalfa in Garrett County in 2002 but significantly greater parasitism in soybean in 2003 (Figure 2. 9C).

Host Density Between Alfalfa and Soybean.

Differences in mean host density were found between alfalfa and soybean individual plants in PG County (Figure 2. 10A). There were significantly more green cloverworm larvae per mm^2 of leaf per plant in soybean than in alfalfa ($F_{1,56}$=16.84; P= 0.0001). However, because there were more alfalfa than soybean plants per unit of area, no significant differences in host density were found when sampling alfalfa and soybean fields with the same collection effort (T_{19}= 0.50; P= 0.6211) (Figure 2. 10B). Consequently, percentage of parasitism of the green cloverworm larvae by *A. nolophanae* (X^2=0.0142; DF=1; P=0.9051) and *C. marginiventris* (X^2=0.0113 ; DF=1; P=0.9152) on

soybean and alfalfa did not significantly differ (Figure 2. 10C and 2. 10D). This suggests that percentage of parasitism was unlikely to be explained by differences in host abundance between alfalfa and soybean plants.

Host Quality in Alfalfa and Soybean.

Green cloverworm adult wet mass was highly correlated with green cloverworm wet larval mass ($R=0.84$, $P<0.0001$) (Figure 2. 11) and wet and dry larval mass were also highly correlated (Figure 2. 2). Thus, data on adult mass green cloverworm can be used as an indicator of host quality for the parasitoids *A. nolophanae* and *C. marginiventris*.

When all sampled individuals were considered, male green cloverworm adults weighed significantly more on average, than female green cloverworms (42.83 mg vs 33.78 mg, respectively; $F_{1, 265}=40.89$; $P<0.0001$) and mean adult mass varied from county to county (Table 2. 5). Female green cloverworms associated with alfalfa did not differ significantly in mean adult mass from female green cloverworms associated with soybean in any of the sites ($F_{1,46}=0.03$, $P=0.8726$ for PG County, $F_{1,56}=1.25$, $P=0.2677$ for Washington County and $F_{1,41}=0.18$, $P=0.6765$ for Garrett County) (Figure 2. 12A). Similarly, male green cloverworms from alfalfa and soybean did not differ significantly in adult mass in any of the sites ($F_{1,42}=2.55$, $P=0.1179$ for PG County, $F_{1,45}=0.18$, $P=0.6775$ for Washington County and $F_{1,30}=0.15$, $P=0.6974$ for Garrett County) (Figure 2. 12B).

Host-Plant Odor Preferences.

Parasitoids can differ geographically in host searching behavioral traits (Althoff and Thompson 2001). For this reason I analyzed parasitoid response to plant and host-

plant complex odors by site. In *A. nolophanae*, no evidence for a preferential attraction to any of the olfactometer odor sources was found in PG County wasps from alfalfa (X^2 =1.84; d.f.=3; P= 0.6687) or from soybean (X^2 =6.33; d.f.=3 ; P=0.0965) (Figure 2. 13A). Similarly, in Washington County no evidence for a preferential attraction to any of the olfactometer odor sources was found in wasps from alfalfa (X^2 = 6.44; d.f.=3 ; P=0.0919). However, Washington County wasps from soybean show preference towards the soybean plus green cloverworm plus frass odor in the olfactometer (X^2 =29.34; d.f.= 3 ; P< 0.0001) (Figure 2. 13B). In Garrett County, wasps from alfalfa were preferentially attracted to odors emitted by alfalfa plus green cloverworm plus frass (X^2 =8.00 ; d.f.=3 ; P=0.0460). In contrast, Garrett County wasps from soybean showed no preferential attraction to any of the odor sources (X^2 = 5.93; d.f.= 3 ; P= 0.1152) (Figure 2. 13C). Thus, preference for the odor from which their hosts were reared is site dependent. In PG County, no preferences were observed whereas in Washington County soybean wasps preferred soybean and in Garrett County alfalfa wasps preferred alfalfa.

No differences in the mean time that elapses before a choice is made were found among *A. nolophanae* from different counties or from different host-plant species (Table 2. 6). However, female *A. nolophanae* regardless of site chose an odor in the olfactometer significantly faster than male *A. nolophanae* ($F_{1,196}$= 9.09; P=0.0029) (Figure 2. 14). No significant differences in the proportion of wasps that chose an odor source and wasps that did not respond to odors in the olfactometer were found between wasps from alfalfa and soybean in any of the counties. Similarly, there were no gender- or site- based differences in the proportion of wasps that chose an odor source and wasps that did not respond to odors in the olfactometer. Overall, 82 % of the *A. nolophanae* wasps tested, chose an odor source and 18% did not respond.

Cotesia marginiventris exhibited no preference for the odor of the plant on which their hosts were reared, in any of the counties. I pooled the number of *C. marginiventris* that chose alfalfa odors alone with the number of wasps that chose the odor of alfalfa plus green cloverworm plus frass in order to have sufficient numbers to perform the X^2 tests. The same was done for the odors associated with soybean. In PG County, *C. marginiventris* did not preferentially respond to the odor of the plant species on which their hosts were reared. Whether it was alfalfa (X^2 =0.29; d.f.=1; P= 0.5930) or soybean (X^2 =0.13; d.f.=1 ; P=0.7237) (Figure 2. 15A). Similarly, in Washington County there was no evidence for a preferential attraction to soybean among *C. marginiventris* that emerged from hosts reared on soybean (X^2 = 0.25; d.f.=1 ; P=0.6171) (Figure 2. 15B). Unfortunately, the reduced number of *C. marginiventris* collected from alfalfa in Washington County in the year in which olfactometer experiments were conducted prevented me from assessing the preference of alfalfa wasps in this County. In Garrett County, *C. marginiventris* wasps from alfalfa were not preferentially attracted to alfalfa odors (X^2 =0.86 ; d.f.=1 ; P=0.3532), nor were wasps from soybean preferentially attracted to soybean odors (X^2 = 0.05; d.f.= 1 ; P= 0.8185) (Figure 2. 15C).

There were no differences in the mean time that elapsed prior to responses to a particular odor among soybean or alfalfa *C. marginiventris* from different counties (Table 2. 7). However, as observed in *A. nolophanae*, regardless of site, female *C. marginiventris* chose an odor in the olfactometer significantly faster ($F_{1,84}$ =8.70; P=0.0041) than male *C. marginiventris* (Figure 2. 16). There were no significant differences in the proportion of wasps that chose an odor field and wasps that did not show a response in the olfactometer whether wasps were from alfalfa or soybean, or regardless of the site of origin or gender. Overall, 88 % of the *C. marginiventris* tested chose an odor and 12% did not respond.

DISCUSSION.

Alfalfa produced hosts that in some way were better resources than those in soybean for both *A. nolophanae* and *C. marginiventris,* as reflected in the longer mean longevity, larger mean adult mass and greater parasitism observed in wasps associated with alfalfa rather than soybean. Thus, phenotypic differentiation occurred among wasps of both parasitoid species, ovipositing in the same host species on two different host-plant species. However, the magnitude and direction of differences were not consistent based on crop or site. Host-plant species affected the generalist and the specialist parasitoids in very similar ways. The fact that better performance in alfalfa than in soybean was observed in *A. nolophanae* and *C. marginiventris* at PG and Washington Counties but not at Garrett County may have been due to an interaction between the abiotic and biotic conditions at these study sites.

The most significant difference between alfalfa and soybean *A. nolophanae* and *C. marginiventris* was observed in adult longevity. In *A. nolophanae* the difference in adult longevity between alfalfa and soybean wasps was observed in two out of three sites. In *C. marginiventris* differences in adult longevity between alfalfa and soybean wasps were observed in PG County and a trend resembling the pattern in PG County was also observed in Washington County (Figure 2. 7). Small sample sizes in Washington County *C. marginiventris* may be responsible for the lack of significance of this trend. Adult longevity reflects reproductive output (Coyle et al. 1999, Abe and Tahara 2003) and it is an important fitness component. Long adult longevities provide parasitoids with higher probabilities to find mates (Kraaijeveld and Van Der Wel 1994) and allow them to oviposit more eggs than short lived adults (Eliopoulos and Stathas 2005).

Thus, the phenotypic differences found between alfalfa and soybean wasps in both parasitoid species varied on a spatial scale, i.e., differed from site to site, suggesting

that host plant advantage is strongly influenced by environmental factors. Abiotic differences among sites may be responsible for the observed geographic differences in the degree of phenotypic differentiation between alfalfa and soybean *A. nolophanae* and *C. marginiventris.* Environmental gradients are expected to alter the relative effects of host-plants and natural enemies on phytophagous insects (Hunter and Price 1992, Yames and Boecklen 2005). Differences in temperature and precipitation exist among the chosen study sites. The average temperatures for the months of June, July and August in the years on which the present study was conducted were higher in PG and Washington Counties (23.8 ˚C and 23.5 ˚C, respectively) than in Garrett County (19.8 ˚C). Average precipitation for the months of June, July and August in Garrett County was the highest (153 mm). Washington County had the lowest average precipitation on these months (87 mm) and PG County had intermediate levels of precipitation (119 mm). Differences in abiotic variables among sites have been found to be responsible for differences in the phenotype of other hymenopteran parasitoids. For instance, encapsulation of parasitoid larvae by some of their hosts differs among environments differing in as little as 4˚C (Salt, 1963, Salt 1970, Lynn and Vinson 1977, Van Driesche et al. 1986, Nenon et al. 1988, Blumberg and Ferkovich 1994, Fellowes et al. 1999, Blumberg and Van Driesche 2001). Differences in the efficiency of parasitoid hosts at encapsulating parasitoid larvae fed on different host-plant species may explain differences in perceived percentage of successful parasitism among sites. Further, precipitation differences may also play a role. Mollema (1988) has suggested that in dry climates *Drosophila* may be selected to have thicker puparia to prevent dessication and as a result may invest more in the biochemical pathways associated with melanin production. Melanin is involved in the encapsulation process in insects (Beresky and Hall 1977, Brewer and Vinson 1971, Nappi 1973). There is evidence for a positive geographic correlation between puparial thickness and encapsulation in braconid parasitoids of drosophilid larvae (Kraaijeveld 1994).

Moreover, Calatayud et al. (2002) have shown that water stress appears to enhance encapsulation of encyrtid parasitoid eggs and larvae by *Phenacoccus herreni* Cox & Williams (Homoptera: Pseudococcidae). Thus, differences in humidity across sites may contribute to differences in the efficiency of host to encapsulate their parasitoid larvae. Although only experimentation can resolve this issue, I hypothesize that it could be that observed geographic differences in percentage of parasitism in the present study may be due to differences in encapsulation strength resulting from the interaction of variables such as temperature and precipitation with host-plant species (i.e., alfalfa and soybean). In addition, other abiotic variables may influence other important physiological aspects of parasitoid biology besides encapsulation. For example, both *A. nolophanae* and *C. marginiventris* lived significantly less in Garrett County (the coldest and most humid site) than in the other two counties. Thus, it is possible that differences in temperature and precipitation levels could determine parasitoid adaptations to environments that affect their longevity.

Because both the braconid parasitoids I studied attack the same host species i.e., the green cloverworm, on alfalfa and on soybean, it seems that it is the host-plant species on which green cloverworms feed that is mediating the observed difference in adult longevity (and in the other fitness parameter discussed above) in both parasitoid species. Different plant species contain different types and concentrations of nutrients and secondary compounds. This difference in nutritional content may differentially affect host species feeding on different host-plant species. For example, insect hosts feeding on poor quality host-plants represent a poor quality resource for larval parasitoids and thus may affect parasitoid fitness (Stadler and Mackauer 1996, Souissi and Le Rü 1998, Van Huis and De Rooy 1998, Teder and Tammaru 2002). For many parasitoid species, host quality is assumed to be determined by host size (Bernal et al. 1998, De Jong and Van

Alphen 1989). In this study green cloverworm larvae did not differ in mass between alfalfa and soybean at any of the study sites. However, differences in host quality that were not reflected in host mass may still be present between larvae feeding on alfalfa and larvae feeding on soybean due to difference in the secondary chemistry of their hosts' host-plant (Godfray 1994), that are transferred to host tissues. The plants (and in particular the secondary chemicals in those plants) on which parasitoid hosts feed can affect parasitoid fitness (Kester and Barbosa 1994, Fox et al. 1996, Souissi and Le Rü 1998, Turlings and Benrey 1998, Eben et al. 2000). Thus, hosts of the same size may still differ in quality (Harvey et al. 1995). Differences in the presence of allelochemicals in host tissues that do not compromise the size of hosts may nevertheless impact parasitoids that attack them (Sznajder and Harvey 2003, Hunter 2003).

Chemical differences between host-plant species may not only influence parasitoid fitness components such as adult mass and adult longevity but also influence percentage of parasitism of hosts feeding on these plants. For example, the presence of nicotine may explain lower rates of parasitism of *Manduca sexta* (Lepidoptera: Sphingidae) on tobacco than on other solanaceous plants (Morgan 1910, Gilmore 1938a, 1938b) or differential rates of parasitism of *Manduca sexta* on flue and burley tobacco varieties (Thurston and Fox 1972), or on breeding lines containing high compared to low nicotine concentrations (Thorpe and Barbosa 1986). Braman et al. (2004) have hypothesized that differences in percentage of parasitism could be explained by differences in the level of attraction to herbivore-damaged plants, more favorable plant architecture for host location in susceptible plants or possible deterrence of host location in the less susceptible plants. In addition, parasitoids may experience greater mortality in hosts feeding on the more resistant plants (Campbell and Duffey 1979, Thurston and fox 1972, Barbosa et al. 1982, 1986, 1991).

Differences in parasitoid fitness on different host-plant species at different study sites did not correspond with parasitoid preference towards the plant species on which parasitoids performed best in any of the parasitoid species studied. Unmated *A. nolophanae* and *C. marginiventris* wasps did not prefer the odors from the host-plant species on which they perform best at each site. Data from the present study show that there is not an innate pre-mating behavioral tendency to respond to the odors of the plant or host-plant complex with which these two wasp species had been associated. Other studies have found similar results. For example, in choice tests the braconid parasitoid *D. longicaudata* shows no significant preference for mango or grapefruit in wind tunnel essays or in olfactometer trials. Nevertheless, host fruit significantly affect parasitoid size, longevity and reproductive success (Eben et al. 2000).

I did not find significant differences in *A. nolophanae* or *C. marginiventris* attraction to host-plant odors by unmated male and females. Mated parasitoid females might respond differently to plant odors than unmated females. Perhaps attraction to host-plant species, host species or host-plant complexes that enhance fitness is triggered after mating. In the present study, unmated individuals were used because I was interested in testing for odor preferences that could potentially influence mating patterns which, in turn, are likely to affect genotypic differentiation (see Chapter 3). There does not appear to be a preference for a particular odor by either parasitoid gender prior to mating. Thus, there are no reasons to assume that *A. nolophanae* or *C. marginiventris* might remain in close proximity to the host-plant species from which they emerged. *A. nolophanae* and *C. marginiventris* may fly away from the crop area from which they emerged to look for mates and they may mate on a host-plant species different than the one of origin. The probabilities of *A. nolophanae* and *C. marginiventris* emerging from a particular crop to mate with wasps from the same crop seem to depend on their dispersal ability and propensity more than on host-plant fidelity or their preference for staying around the

host-plant on which their host fed.

The sites picked for this study were chosen based on the relative ratio of cultivated alfalfa versus soybean in the last 10 years. Thus, I expected a higher wasp fitness on plants in areas were that plant had been more abundant, as has been observed in other insects (Fox and Morrow 1981, Thompson 1994, Itami et al. 1998, Thomas and Singer 1998). Contrary to this expectation, there was no obvious relationship between the alfalfa: soybean ratios and the phenotypic differences observed in alfalfa and soybean wasps. Indeed, alfalfa was more favorable to parasitoids when the ratio indicated a high level of soybean cultivation (e.g., in PG County) and a high historical abundance of alfalfa (in Garrett County) was not reflected in higher fitness in alfalfa wasps. Thus, factors other than the relative current and historical abundance of host-plant species fed by herbivorous hosts must explain the better performance of alfalfa wasps in PG and Washington Counties. The fact that the advantage of alfalfa over soybean was not observed at all the study sites may also be explained by the alfalfa and soybean varieties used at each site. The alfalfa and soybean varieties used in each of the three study sites were different The performance of various parasitoids may differ not only between crop species but also between crop varieties or cultivars (Verkerk and Wright 1996).

Unlike most previous studies, the study system I chose allowed me to explore phenotypic differentiation of a generalist and a specialist parasitoid ovipositing on the same host species on two different host-plant species that co-occurred geographically. This allowed me to test the role of host-plant on parasitoid fitness and the influence of both host-plant species and geographic location. My observations and experiments enable me to conclude that, host-plant species differentially affected parasitic Hymenoptera. It is clear from this study that some differences in key fitness parameters among wasps associated with these two crops exist and that the expression of these differences differ spatially. Nevertheless, one might ask if observed phenotypic differences have resulted in

genotypic differentiation in these parasitoid populations. Thus, in the next chapter I will explore the role of host-plant species in the genetic differentiation of *A. nolophanae* and *C. marginiventris*. To my knowledge no study has explored whether different host-plant species (as a consequence of their direct and indirect influences) cause phenotypic differentiation in parasitoids by determining and comparing the genetic profile and phenotypic profile of the exact same individuals.

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Table 2. 1. Results of the ANOVA for *A. nolophanae* adult mass. Adult mass data were analyzed as a split plot design. Site was split by host-plant and host-plant was split by gender. The first number in the degrees of freedom column, *df*, represents the numerator df and the second number represents the denominator df.

ANOVA			
Source of variation	*df*	*F*	*P*
Site	2, 4	0.08	0.9281
Host-plant	1, 416	8.30	0.0042
Gender	1, 413	175.64	<0.0001
Site*Host-plant	2, 415	0.59	0.5540
Site*Gender	2, 413	2.46	0.0869
Host-plant*Gender	1, 413	0.12	0.7265
Site*Host-plant*Gender	2, 413	0.97	0.3813

Table 2. 2. Results of the ANOVA for *C.* marginiventris adult mass. Adult mass data were analyzed as a split plot design. Site was split by host-plant and host-plant was split by gender. The first number in the degrees of freedom column, *df*, represents the numerator df and the second number represents the denominator df.

ANOVA			
Source of variation	*df*	*F*	*P*
Site	2, 296	2.75	0.0655
Host-plant	1, 296	0.29	0.5930
Gender	1, 296	1.09	0.2962
Site*Host-plant	2, 296	3.42	0.0339
Site*Gender	2, 296	0.53	0.5864
Host-plant*Gender	1, 296	0.18	0.6711
Site*Host-plant*Gender	2, 296	0.25	0.7782

Table 2. 3. Results of the ANOVA for *A. nolophanae* adult longevity. Adult longevity data were analyzed as a split plot design. Site was split by host-plant and host-plant was split by gender. The first number in the degrees of freedom column, *df*, represents the numerator df and the second number represents the denominator df.

ANOVA			
Source of variation	*df*	*F*	*P*
Site	2, 212	13.93	< 0.0001
Host-plant	1, 212	26.46	< 0.0001
Gender	1, 212	2.15	0.1436
Site*Host-plant	2, 212	10.92	<0.0001
Site*Gender	2, 212	2.30	0.1024
Host-plant*Gender	1, 212	1.40	0.2383
Site*Host-plant*Gender	2, 212	0.96	0.3840

Table 2. 4. Results of the ANOVA for *C. marginiventris* adult longevity. Adult longevity data were analyzed as a split plot design. Site was split by host-plant and host-plant was split by gender. The first number in the degrees of freedom column, *df*, represents the numerator df and the second number represents the denominator df.

ANOVA			
Source of variation	*df*	*F*	*P*
Site	2, 138	14.80	< 0.0001
Host-plant	1, 138	13.73	0.0003
Gender	1, 138	7.38	0.0074
Site*Host-plant	2, 138	4 .92	0.0087
Site*Gender	2, 138	0.01	0.9857
Host-plant*Gender	1, 138	1.91	0.1697
Site*Host-plant*Gender	2, 138	1.44	0.2416

Table 2. 5. Results of the ANOVA for green cloverworm adult mass. Adult mass data were analyzed as a split plot design. Site was split by host-plant and host-plant was split by gender. The first number in the degrees of freedom column, *df*, represents the numerator df and the second number represents the denominator df.

ANOVA			
Source of variation	*df*	*F*	*P*
Site	2, 266	4.81	0.0089
Host-plant	1, 266	0.00	0.9889
Gender	1, 266	40.89	<0.0001
Site*Host-plant	2, 266	0.92	0.4009
Site*Gender	2, 266	0.30	0.7385
Host-plant*Gender	1, 266	1.50	0.2214
Site*Host-plant*Gender	2, 266	0.58	0.5602

Table 2. 6. Results of the ANOVA for the time elapsed prior to *A. nolophanae* response to odors in the olfactometer. The data were analyzed as a split plot design. Site was split by host-plant and host-plant was split by gender. The first number in the degrees of freedom column, *df*, represents the numerator df and the second number represents the denominator df.

ANOVA			
Source of variation	*df*	*F*	*P*
Site	2, 196	2.03	0.1335
Host-plant	1, 196	2.30	0.1310
Gender	1, 196	9.09	0.0029
Site*Host-plant	2, 196	1.30	0.2755
Site*Gender	2, 196	1.88	0.1547
Host-plant*Gender	1, 196	3.01	0.0842
Site*Host-plant*Gender	2, 196	0.19	0.8256

Table 2. 7. Results of the ANOVA for the time elapsed prior to *C. marginiventris* response to odors in the olfactometer. The data were analyzed as a split plot design. Site was split by host-plant and host-plant was split by gender. The first number in the degrees of freedom column, *df*, represents the numerator df and the second number represents the denominator df.

ANOVA			
Source of variation	*df*	*F*	*P*
Site	1, 84	2.65	0.1070
Host-plant	1, 84	1.21	0.2749
Gender	1, 84	8.70	0.0041
Site*Host-plant	1, 84	0.53	0.4668
Site*Gender	1, 84	0.00	0.9701
Host-plant*Gender	1, 84	1.19	0.2787
Site*Host-plant*Gender	1, 84	2.80	0.0980

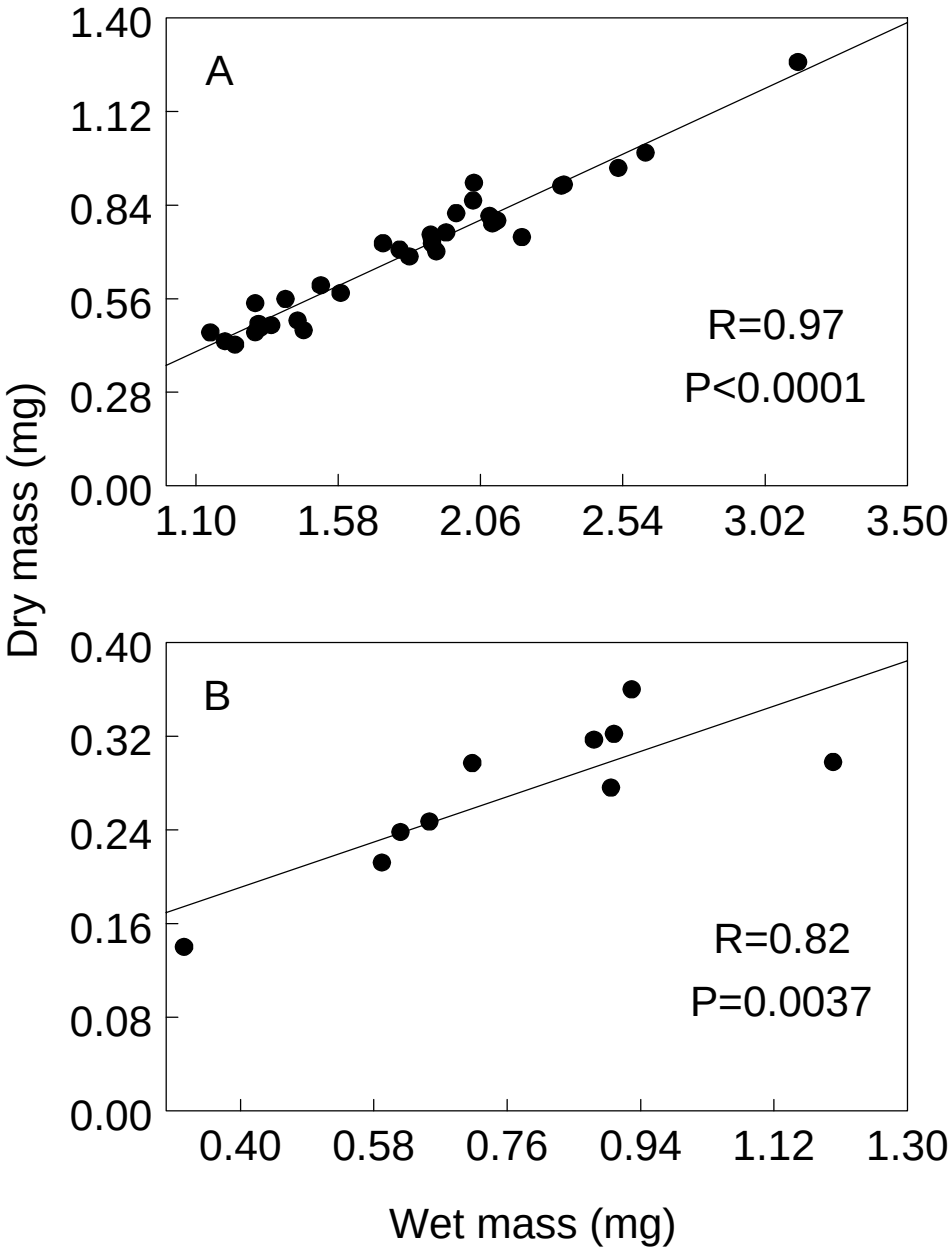

Figure 2. 1. Correlation between adult parasitoid wet and dry mass. **A.** *Aleiodes nolophanae* **B.** *Cotesia marginiventris*. Wet adult mass was a good predictor of dry adult mass in both parasitoid species.

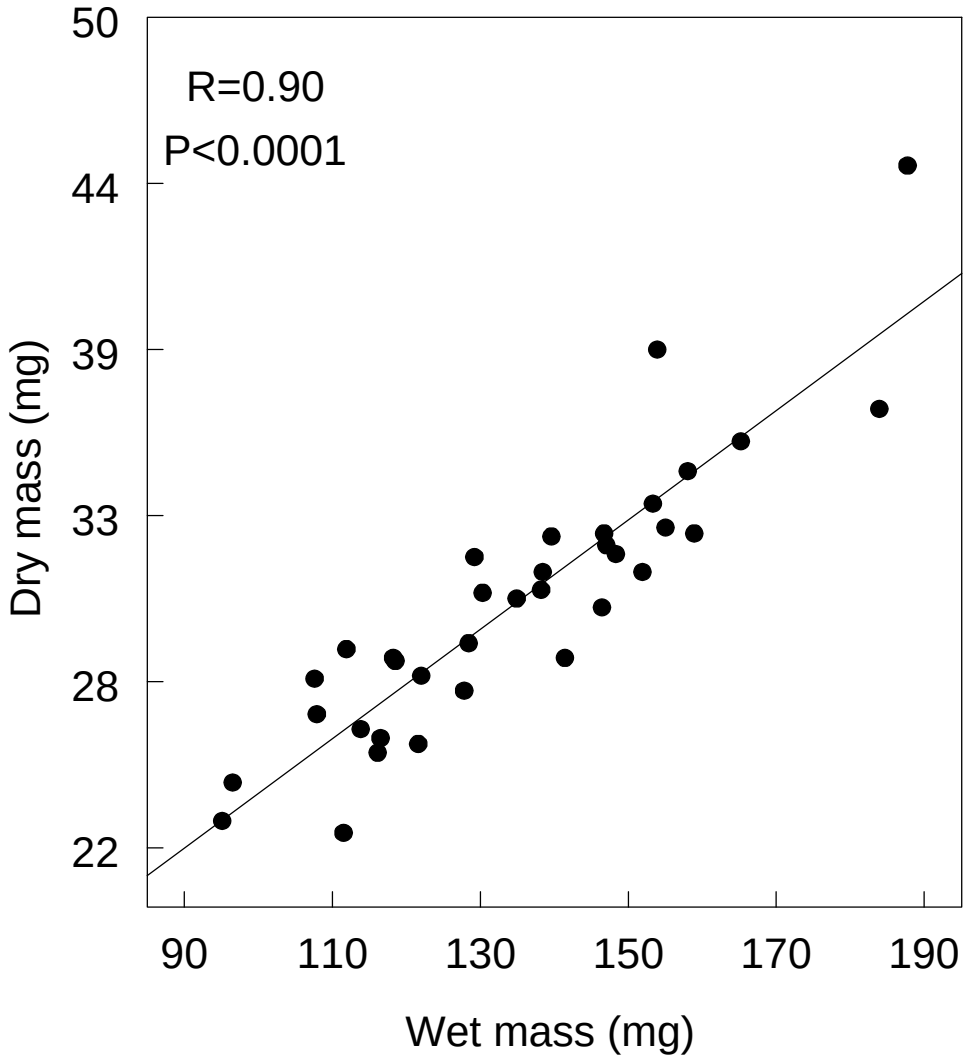

Figure 2. 2. Correlation between green cloverworm larval wet and dry mass.

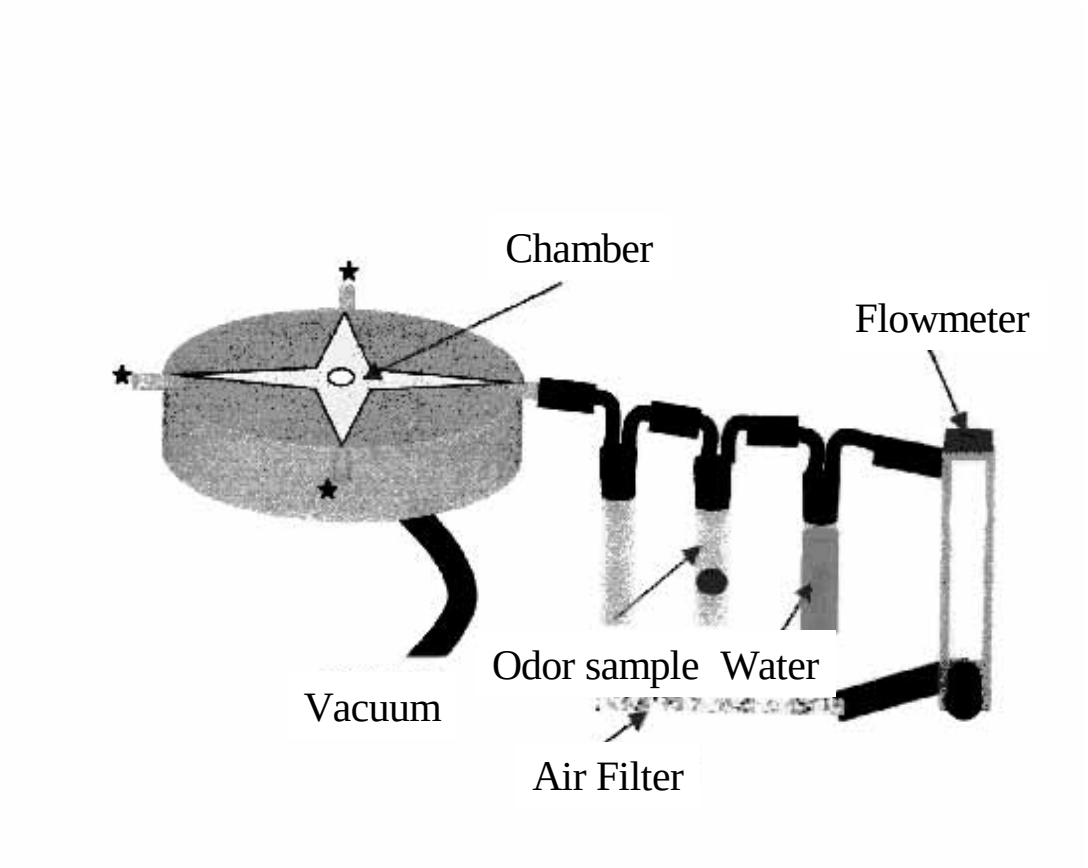

Figure 2. 3. Four armed olfactomer (Vet et al. 1983). Air is extracted by vacuuming air through a tube attached to the chamber. Air enters the system and passes through a carbon air filter and then through a flowmeter that regulates air flow. Air then goes to a tube with distilled water were the air is humidified and then it passes through the odor sample tube. An empty tube is placed in the system to keep parasitoids from getting into the odor sample tube if they walked out of the chamber. Inside the chamber four defined odor fields are generated. For clarity only one of the four arms of the olfactometer is depicted.

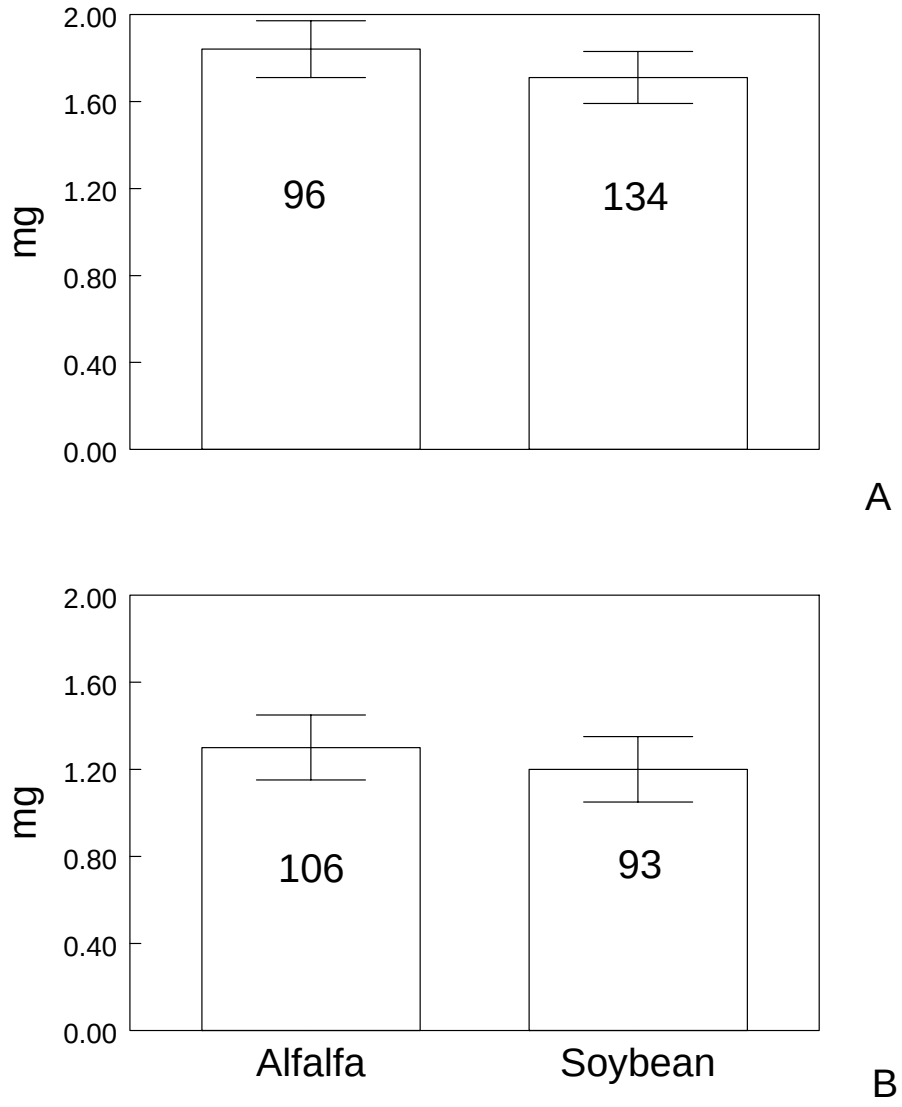

Figure 2. 4. *Aleiodes nolophanae* adult mass. Overall, alfalfa wasps had a significantly larger mean adult mass than soybean wasps ($F_{1,416}$=8.30; P=0.0042). Female wasps were significantly larger than male wasps ($F_{1,413}$=175.64; P<0.0001). **A.** Female *A. nolophanae* adult mass. **B.** Male *A. nolophanae* adult mass. Sample size within columns. Bars represent SE of the mean.

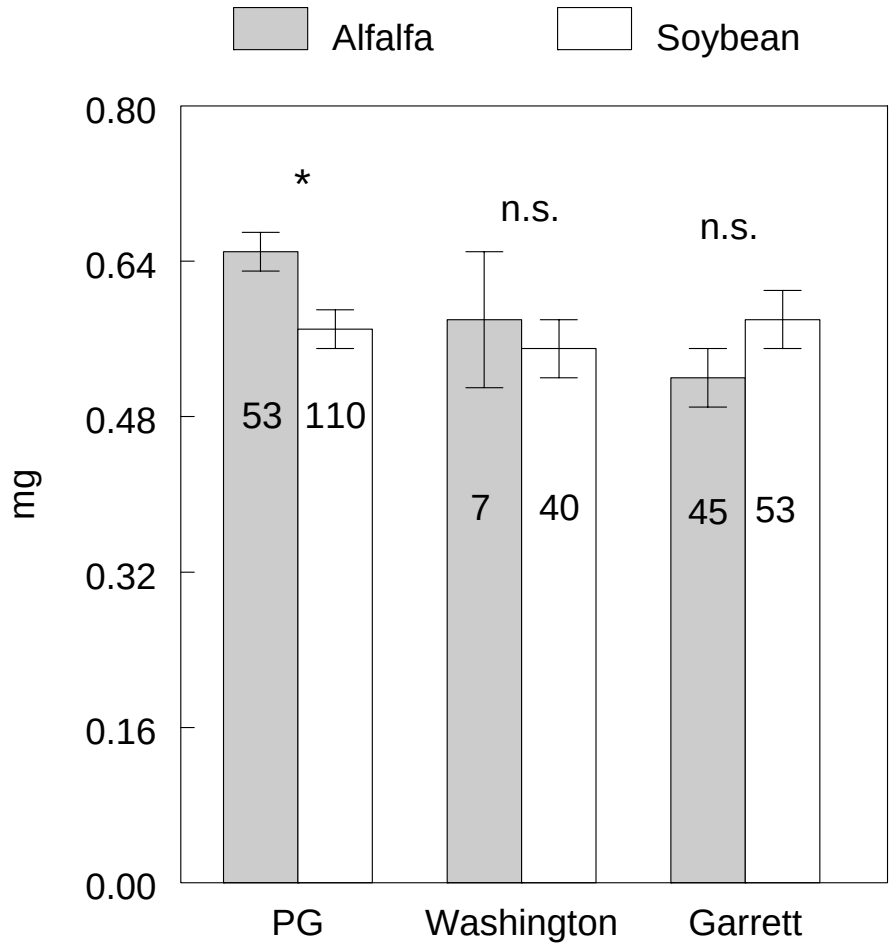

Figure 2. 5. *Cotesia marginiventris* adult mass. Significant differences in adult mass between alfalfa and soybean wasps were observed only in PG county. * = Significant difference ($P<0.05$) between adjacent columns, n.s.= non significant differences adjacent columns ($P>0.05$). Sample size within columns. Bars represent SE of the mean.

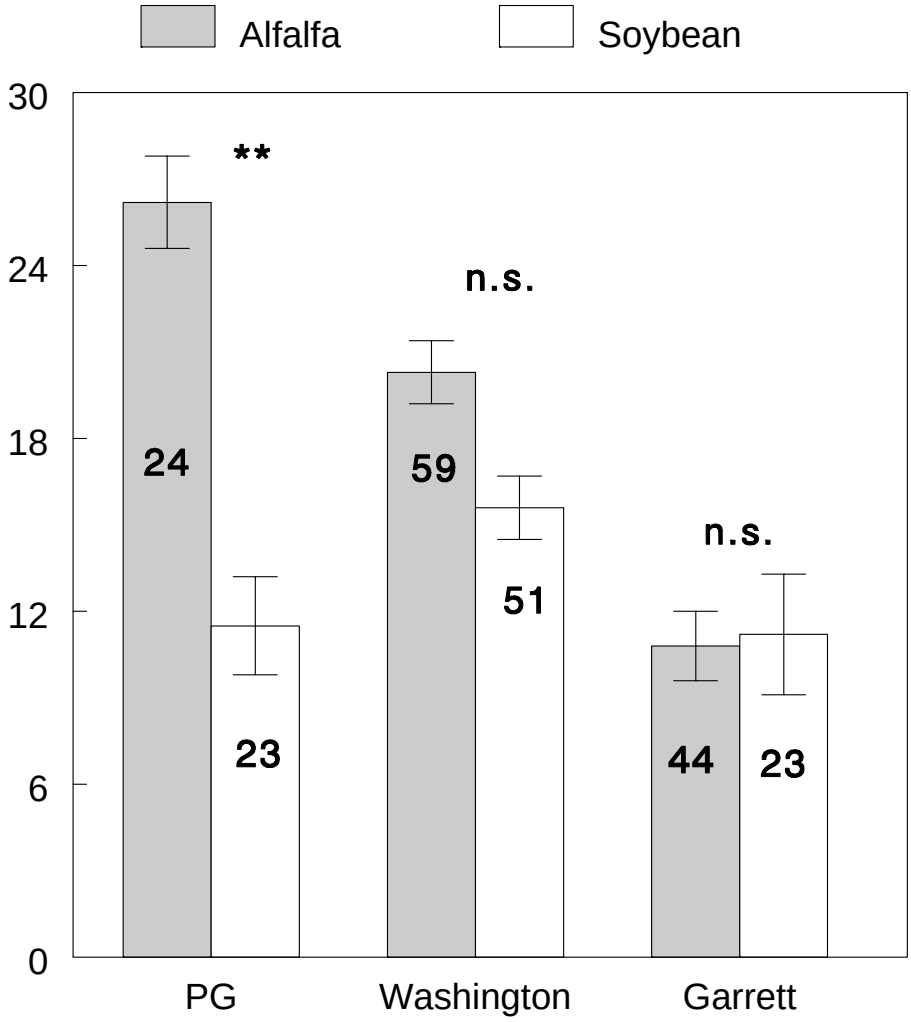

Figure 2. 6. *Aleiodes nolophanae* adult longevity. Alfalfa adult wasps in PG and Washington county lived significantly longer than soybean wasps. No significant differences in longevity between alfalfa and soybean wasps were observed in Garrett county. Different letters represent significant differences ($P<0.05$) between columns based on Tukey-Kramer test. Sample size within columns. Bars represent SE of the mean.

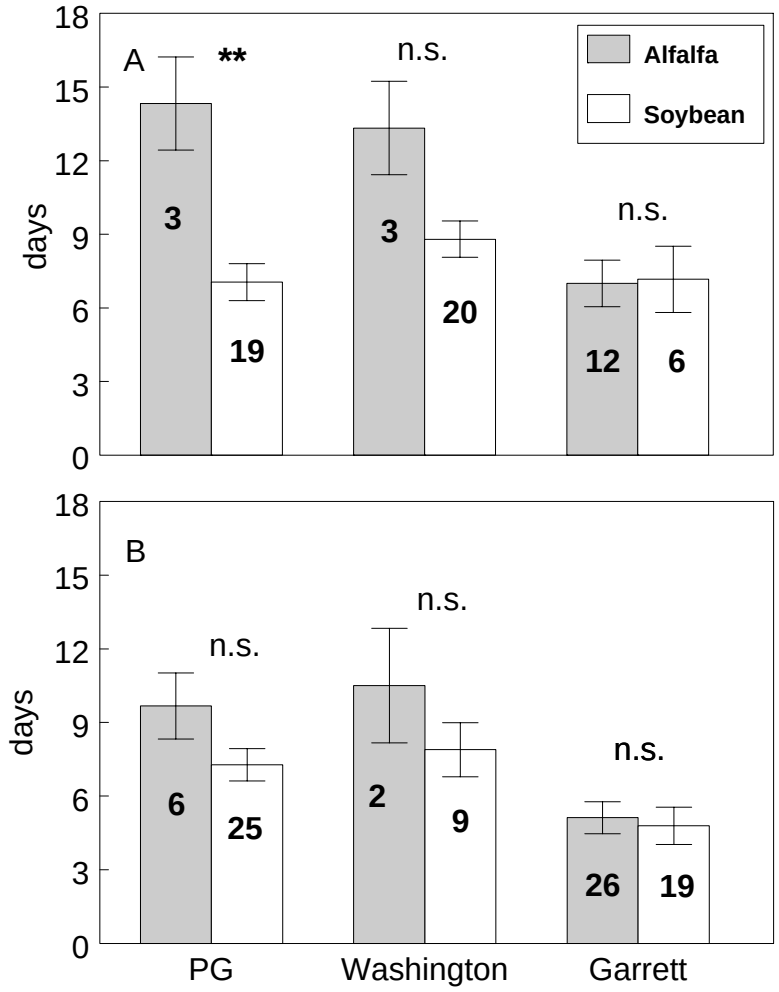

Figure 2. 7. A. Female *C. marginiventris* adult longevity. Female alfalfa wasps in PG county lived significantly longer than soybean wasps. No significant differences in longevity between alfalfa and soybean wasps were observed in Washington or Garrett county. **B.** Male *C. nolophanae* adult longevity. No significant differences in adult longevity were found between alfalfa and soybean wasps in any of the counties studied.** represents significant differences between alfalfa and soybean wasps ($P<0.01$). n.s.= no significant differences ($P>0.05$). Sample size within columns. Bars represent SE of the mean.

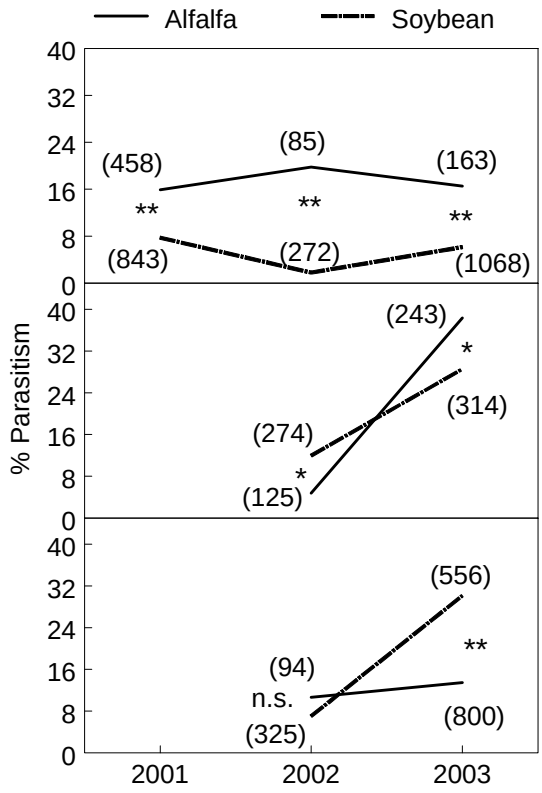

Figure 2. 8. Percent parasitism in *A. nolophanae*. **A.** In PG county significantly more parasitism occurred in alfalfa than in soybean in 2001 (X^2= 20.69; d.f.=1; P<0.0001), 2002 (X^2= 36.42; d.f.=1; P<0.0001) and 2003 (X^2=21.84; d.f.=1; P<0.0001). **B.** In Washington county significantly more parasitism occurred in soybean than in alfalfa in 2002 (X^2=5.11; d.f.=1; P=0.0238). In 2003 the opposite pattern occurred (X^2=5.04; d.f.=1; P=0.0247). **C.** In Garrett county no significant differences in parasitism in alfalfa versus soybean were observed in 2002 (X^2=1.27; d.f.=1; P=0.26). In 2003 significantly more parasitism occurred in soybean than in alfalfa (X^2=30.83; d.f=1; P<0.0001). * = P<0.05; ** = P<0.01; n.s.= non significance. Total number of green clover worm larvae (i.e. parasitized and non parasitized) in parentheses.

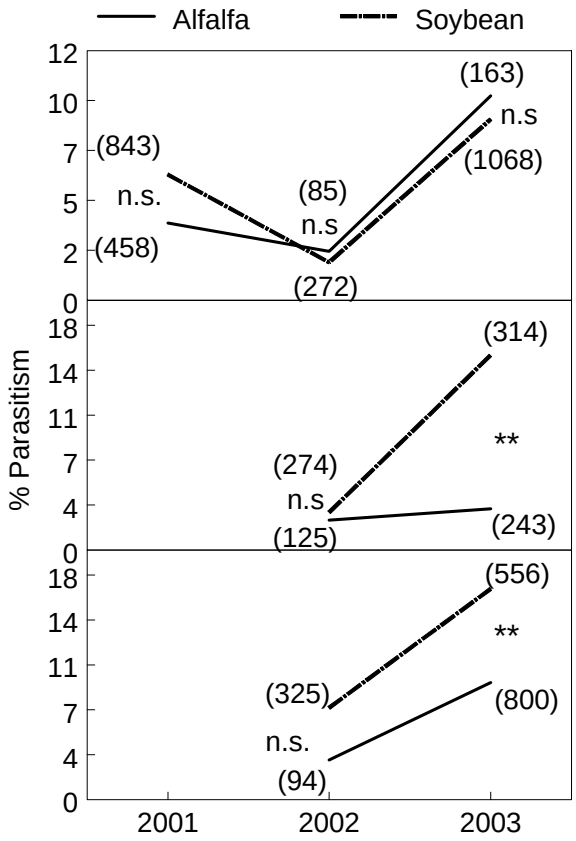

Figure 2. 9. Percent parasitism in *C. marginiventris*. **A.** In PG county no significant differences in parasitism in alfalfa versus soybean were observed in 2001 (X^2= 3.27; P=0.0703), 2002 (X^2=0.09 P=0.7651) and 2003 (X^2=0.2152; P=0.6428). **B.** In Washington county no significant differences in parasitism between alfalfa and soybean were found in 2002 (X^2=0.1227; P=0.7634). In 2003 significantly more parasitism occurred in soybean than in alfalfa (X^2=22.61; P<0.0001).**C.** In Garrett county no significant differences in parasitism were observed in 2002 (X^2=2.13; P=0.1448). In 2003 significantly more parasitism occurred in soybean than in alfalfa (X^2=17.05; P<0.0001). ** = P<0.01; n.s. = non significance. Total number of green clover worm larvae (i.e. parasitized and non parasitized) in parentheses.

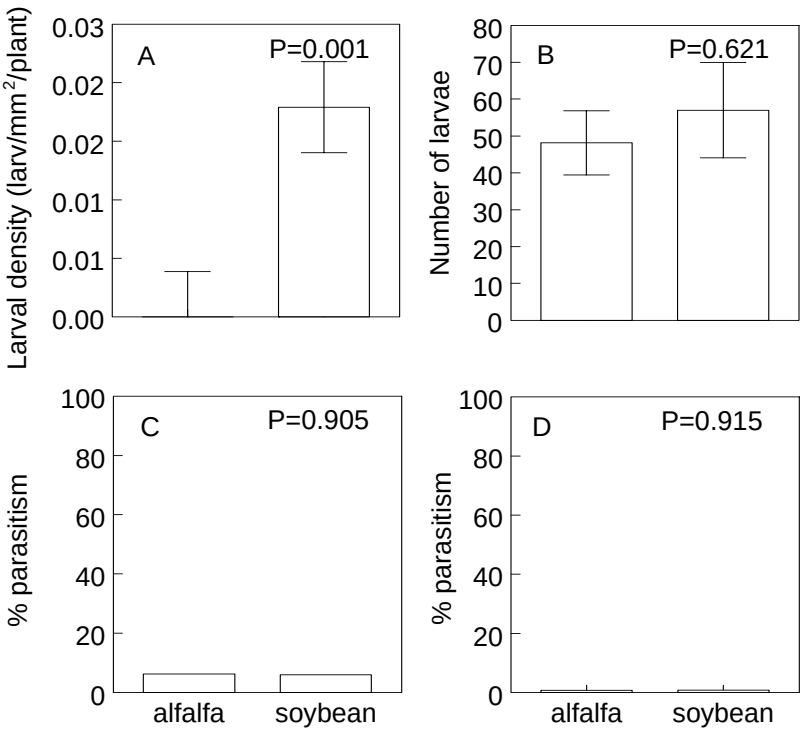

Figure 2. 10. A. Green cloverworm densitiy between alfalfa and soybean plants **B.** Green cloverworm numbers between alfalfa and soybean fields **C.** Percent parasitism by *A. nolophanae* in alfalfa and soybean **D.** Percent parasitism by *C. marginiventris* in alfalfa and soybean. Significantly more green cloverworm larvae per plant were found in soybean than in alfalfa plants (A). However, no significant differences in the number of green cloverworm found among alfalfa and soybean fields was found (B). Consequently, no significant differences in percent parasitism by *A. nolophanae* (B) and *C. marginiventris* (C) were found between alfalfa and soybean, suggesting than percent parasitism is not determined by differences in host densities between these two plants. Sample sizes within parentheses. Bars represent SE of the mean.

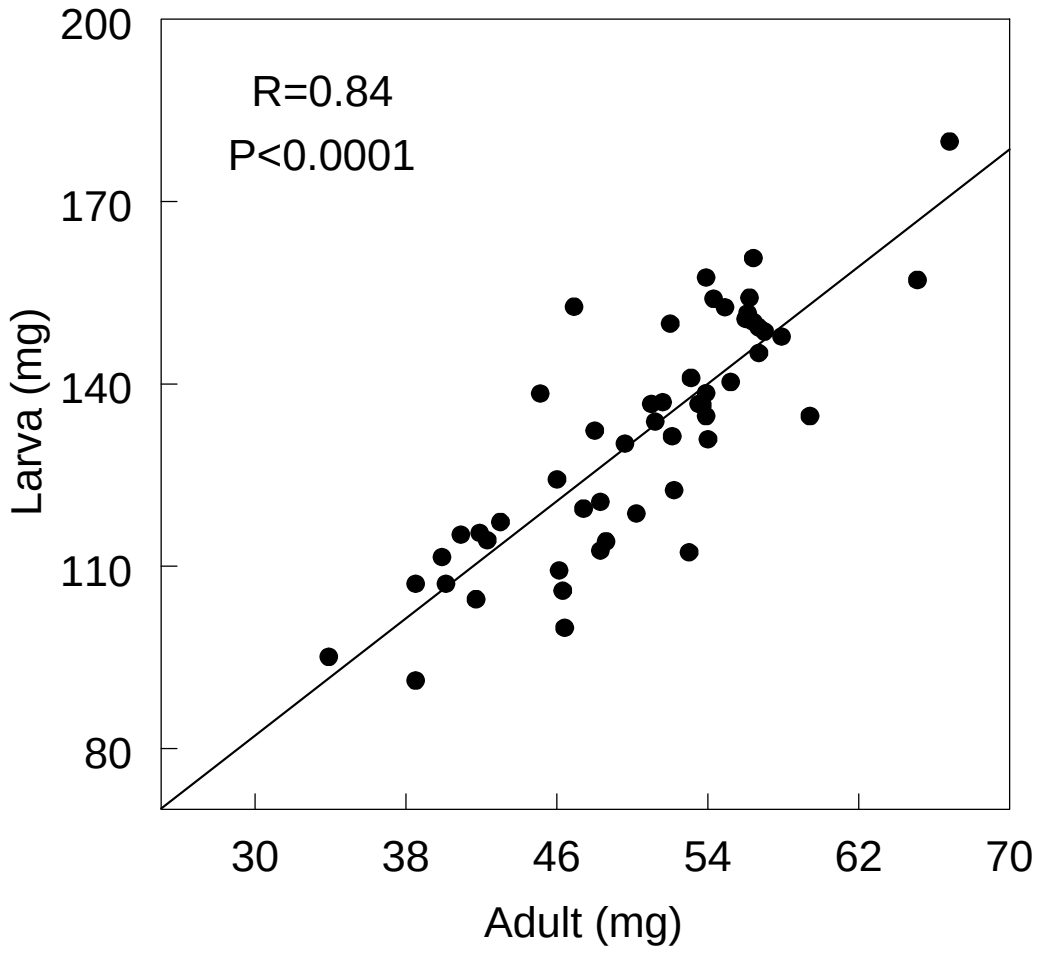

Figure 2. 11. Correlation between adult green cloverworm mass and larval green cloverworm mass. Due to the significant correlation between these two variables, adult green cloverworm mass can be used as an indicator of host quality for the parasitoids *A. nolophanae* and *C. marginiventris*.

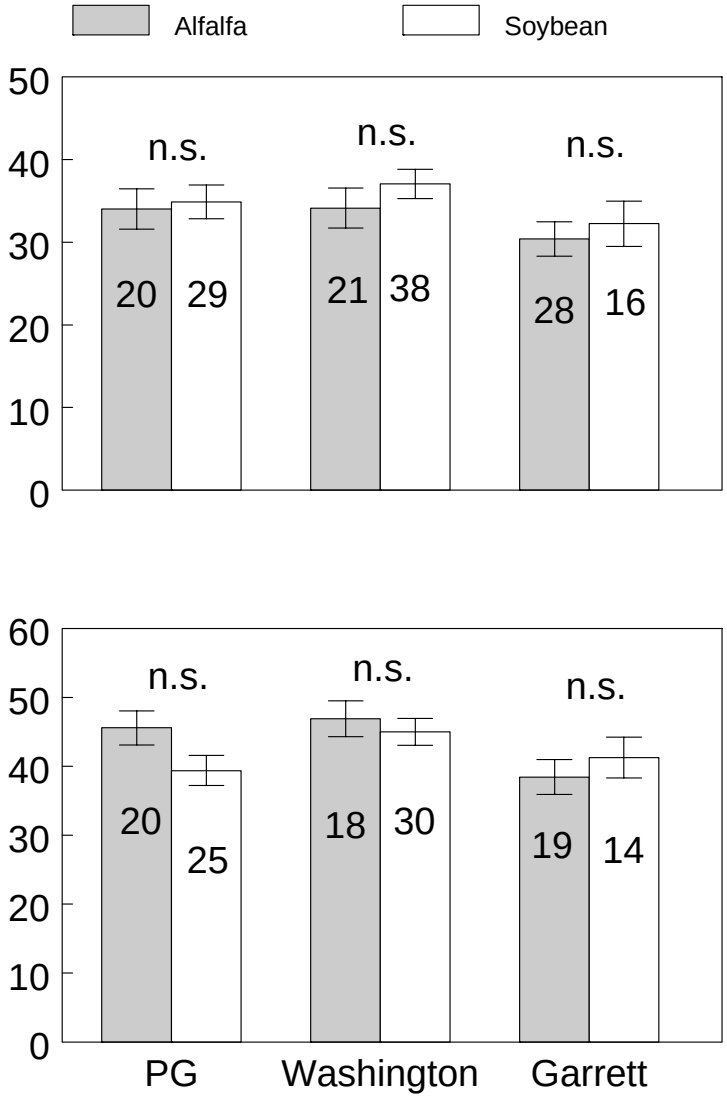

Figure 2. 12. A. Female green cloverworm adult mass. No significant differences in adult mass between alfalfa and soybean green cloverworms were observed in PG, Washington or Garrett counties. **B**. Male green cloverworm adult mass. No significant differences in adult mass between alfalfa and soybean green cloverworms were observed in PG, Washington or Garrett counties. n.s. = non significant differences between adjacent columns (P>0.05). Sample size within columns. Bars represent SE of the mean.

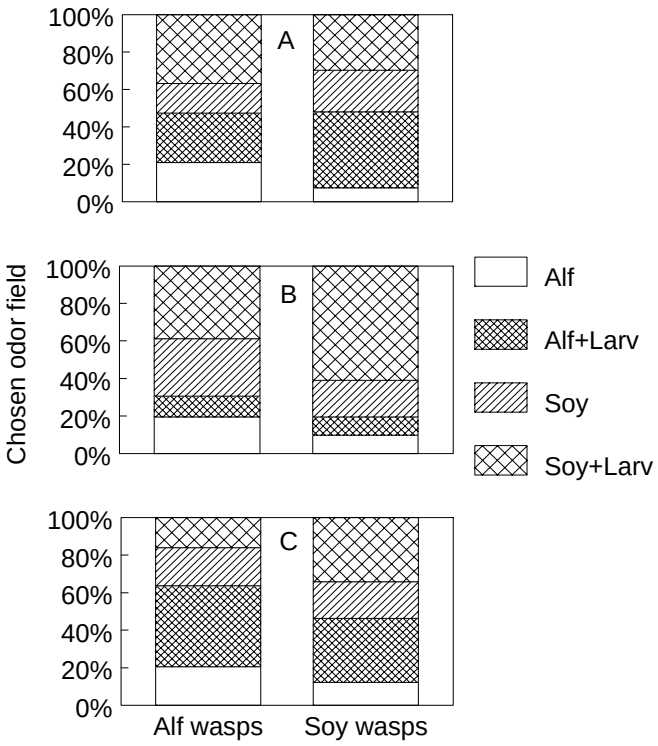

Figure 2. 13. *Aleiodes nolophanae* odor choices. Labels in the x-axis refer to the host plant from which the parasitoid was reared (i.e. the host plant from which the parasitoid's host fed) and the length of each section (odor source) represents the proportion of all wasps that chose the odor (i.e., soybean plus green cloverworm plus frass, soybean, alfalfa plus green cloverworm plus frass and alfalfa). **A.** In PG county, no preference for any particular odor was observed in alfalfa (P=0.6687; N=19) or soybean wasps (P=0.0965; N=27). **B.** In Washington county no preference was observed in alfalfa (P=0.0919; N=36) but preference for soybean plus green cloverworm plus frass odor was observed in soybean wasps (P<0.001; N=41) **C.** In Garrett county no preference was observed in soybean wasps (P=0.1152; N=41) but a preference for alfalfa plus green cloverworm plus frass was observed in alfalfa waps (P=0.0460; N=44). Alf=alfalfa, Soy=soybean, Larv= Larvae.

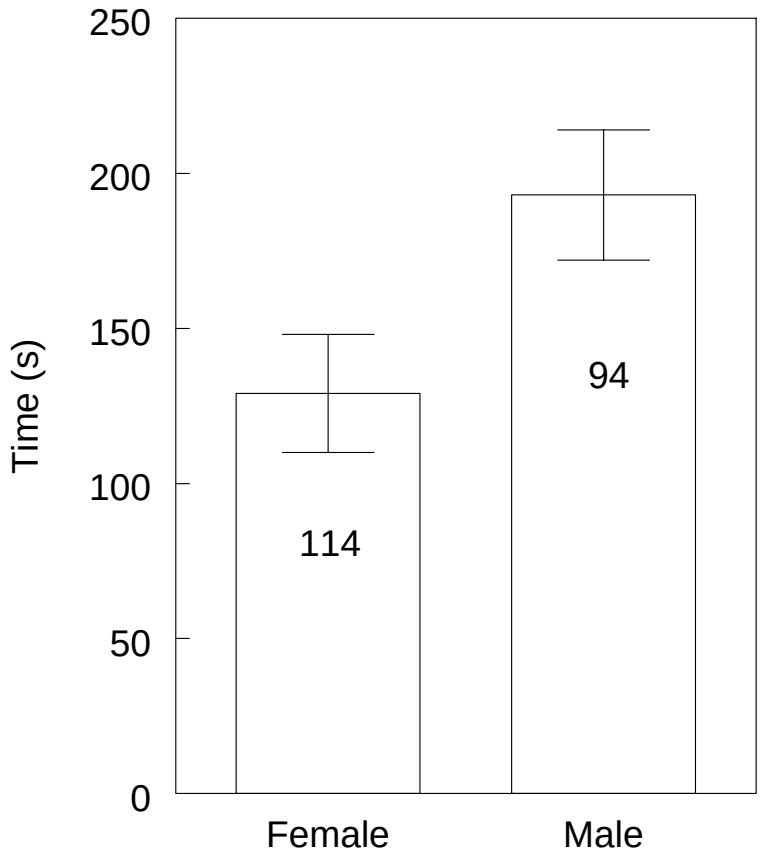

Figure 2. 14. Time elapsed before *A. nolophanae* chooses an odor in the olfactometer. Female wasps took a decision significantly faster than male wasps ($F_{1,196}$= 9.09, P=0.0029). Bars represent standard error of the mean. Sample size within columns.

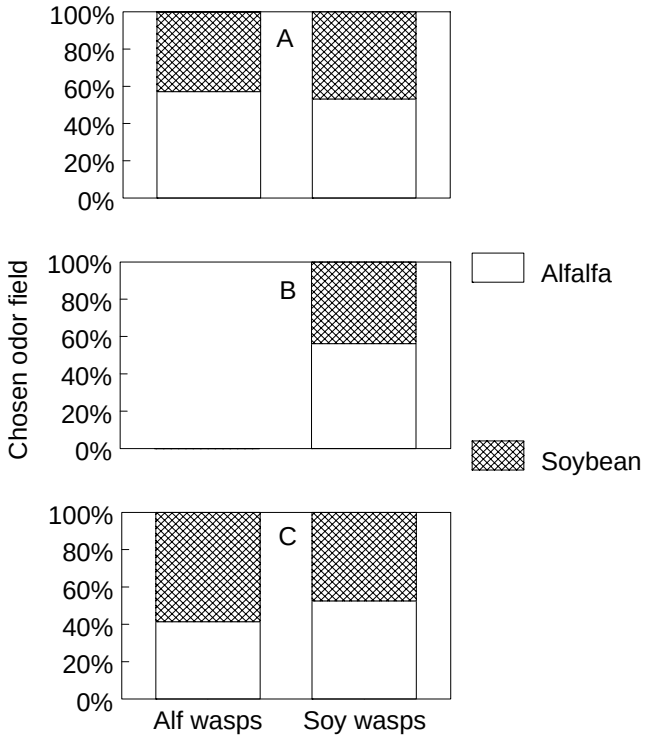

Figure 2. 15. *Cotesia marginiventris* odor choices. Labels in the x-axis refer to the host plant from which the parasitoid was reared (i.e. the host plant from which the parasitoid's host fed) and the length of each section (odor source) represents the proportion of all wasps that chose the odor (i.e., alfalfa or soybean). Due to small sample sizes the proportion of wasps that chose alfalfa and the proportion of wasps that chose alfalfa plus green cloverworm plus frass were pooled together. The same was done for soybean odors. **A.** In PG county, no preference for any particular odor was observed in alfalfa (P=0.5930; N=14) or soybean wasps (P=0.7237; N=32). **B.** In Washington county, no preference was observed in soybean wasps (P=0.6171; N=18). No alfalfa wasps were available for the olfactometer test in Washington county. **C.** In Garrett county, no preference for any particular odor was observed in alfalfa (P=0.3532; N=29) or soybean wasps (P=0.8185; N=19). Alf= Alfalfa; Soy=Soybean.

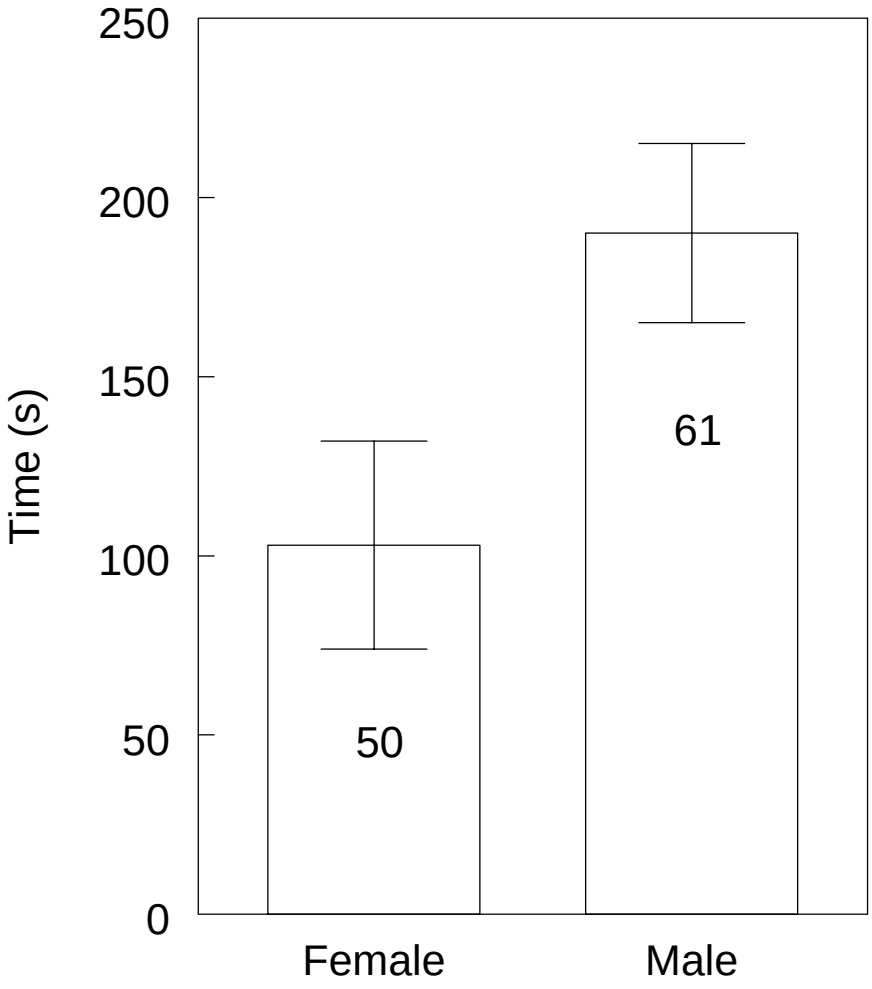

Figure 2. 16. Time elapsed before *C. marginiventris* chooses an odor field in the olfactometer. Female wasps took a decision significantly faster than male wasps ($F_{1,84}$= 8.70, P=0.0041). Bars represent standard error of the mean. Sample size within columns.

CHAPTER 3:

The Role of Host-Plant Species in the Genotypic Differentiation of Sympatric Populations of Hymenopteran Parasitoids

INTRODUCTION.

Host-plant based genetic differentiation has been demonstrated among sympatric populations of several insect herbivore species (Fox and Morrow 1981, Tavormina 1982, Gould 1983, Jaenike and Grimaldi 1983, Mitter and Futuyma 1983, Futuyma et al. 1984, Via 1984a, 1984b, Futuyma and Peterson 1985, McPheron et al. 1988, Pashley 1986, Feder et al. 1990, Via 1991a, Carroll et al. 1997, Via 1999, Hufbauer and Via 1999, Haack et al. 2000). For some insects, genetic differentiation of populations associated with different host-plant species has been established based on observed differences at several loci. Examples include the apple maggot fly *Rhagoletis pomonella* Walsh (Diptera: Tephritidae) (Feder et al. 2003a), the goldenrod gall maker *Eurosta solidaginis* Fitch (Diptera: Tephritidae) (Craig et al. 1993) and several aphid species (Via 1999, 2001, Haack et al. 2000, Via et al. 2000, Lushai et al. 2002).

The role of host-plant species in the genetic differentiation of herbivorous insects such as *R. pomonella*, *E. solidaginis* and several aphid species suggests that host-plant species may promote genetic differentiation in parasitic Hymenoptera as well. Little is known about the role of host-plant species as agents of phenotypic differentiation in hymenopteran parasitoids (But see Kester and Barbosa 1994, Fox et al. 1996, Souissi and Le Rü 1998, Sznajder and Harvey 2003, Campan and Benrey 2004). However, even less is known about the role of host-plant species as agents of genetic differentiation in parasitic Hymenoptera. Genetic differences in key fitness parameters among parasitoid populations attacking host herbivore species on different host-plant species have been

suggested (Kester and Barbosa 1994, Vaughn and Antolin 1998, Van Nouhuys and Via 1999) and there is evidence that genetic differentiation could be driven by the host substrate (and perhaps host-plant species) in some parasitoid species (Vet et al. 1984).

The important role that plants play in parasitoid ecology makes genetic differentiation in conspecific parasitoids associated with different host-plant species a plausible scenario. If among parasitoid populations, attacking herbivorous host on different host-plant species, host fidelity and/or assortative mating are associated with increased fitness then genetic differentiation may occur. Differentiation may occur not only at loci associated with traits under selection but also at neutral markers occurring across the entire genome (Johnson et al. 1996, Via 1999). Vaughn and Antolin (1998) provide an example of genetic differentiation at neutral markers among sympatric parasitoid conspecifics attacking different host-plant complexes. However, no study has explored the effect that different host-plant species, fed on by the same host herbivore species, has on the genetic differentiation of neutral genetic variation in parasitic Hymenoptera. In the present study, the exact same individuals from whom I determined phenotypic differentiation (see Chapter 2) were used to determine the occurrence of genotypic differentiation due to the host-plant in which herbivore hosts occurred.

Generalist and specialist parasitoids experience different selective pressures (Campan and Benrey 2004). Thus, the strength of the effect of host-plant species on genetic differentiation may differ between specialist and generalist parasitoids. Thus, in this study the role of host-plant species on genetic differentiation was explored in a generalist and in a specialist parasitoid from the same family (i.e., Braconidae). In the present section, I review data on the influence of host-plant species on the biology of parasitic Hymenoptera. In addition, I discuss the reasons why parasitic Hymenoptera should have a high likelihood of differentiating genetically. Following this discussion I

provide arguments justifying the study of genetic differentiation at different locations and the use of AFLP as the molecular marker for detecting genetic differentiation.

Hymenopteran parasitoids are tightly linked to the host-plant species on which their hosts occur (Godfray 1994) and thus, it is not unreasonable to assume the possibility of genetic differentiation among populations on different host-plant species. Physical factors (e.g., silhouette, contrast, color, leaf texture, plant architecture, etc.) (Price 1981) and chemical traits (Vet and Dicke 1992, Turlings et al. 1993, Rutledge 1996, Poppy et al. 1997, Powell et al. 1998) of host-plants are important host finding cues for parasitoids. Hymenopteran parasitoids often locate their hosts by first orienting to physical and/or chemical host-plant cues or host substrate cues (e.g., those used by parasitoids of drosophilid flies that orient to rotten organic material) (Vet 1983). Chemical characteristics from both host-plants (i.e., plant volatile compounds) and herbivore hosts (e.g., frass, honeydew) may be critical in host finding by parasitoids (Hassell and Southwood 1978, Godfray 1994). Thus, the specificity of parasitoid responses to diverse morphological and chemical cues can occur as a response to host-plant cues, host cues, or both (Vet and Dicke 1992, Van Driesche and Bellows 1996). Thus, in many parasitoids, odors from both the host and host-plant affect habitat preferences after emergence (Read et al. 1970, Arthur et al. 1972, Honda and Walker 1996, Monge and Cortesero 1996).

The central role played by host-plant species in parasitoid ecology may promote specialized host searching and parasitizing behaviors on different host-plant species. There are several examples of parasitoid females being attracted to the host-plant of their hosts rather than to the hosts themselves (Hassell and Southwood 1978). Further, certain parasitic Hymenoptera are capable of developing on a host only when the host is feeding on particular host-plant species in the host-plant range of the herbivore (Price et al. 1980, Vet and Dicke 1992). Preferences for host-plant species may be learned (Kester and

Barbosa 1994) or inherited (Barbosa et al. 1990) and may lead to reproductive isolation of conspecific parasitoids associated with different odors (Vaughn and Antolin 1998). Therefore, host-plant specific parasitoid strains or populations may result from a combination of selection pressures on physiological, ecological and behavioral traits of parasitoids attacking hosts on different host-plant species (Kester and Barbosa 1994, Potting et al. 1997).

Parasitic hymenoptera present a series of characteristics that make them good candidates with which to explore genetic differentiation. Traits such as their close association with their hosts and host-plants (Boulétreau 1986, Henter 1995) and their potential low dispersal rates (Vaughn and Antolin 1998) may provide the reproductive isolation that can contribute to the genetic differentiation in parasitoids attacking hosts on different host-plant species. Further, many parasitoids species mate shortly after emergence (Hassell and Godfray 1992) and do so at the emergence site (often the host-plant) (Godfray 1994), thus increasing the likelihood that members of one family mate among themselves (Askew 1968, Hassell and Godfray 1992). Sibling mating influences population differentiation by reducing the effective population size and by increasing the rate at which genetic drift could lead to reproductive isolation (Unruh and Messing 1993, Conner and Hartl 2004). Finally, hymenopteran parasitoids' haplodiploidy may influence the speed of incipient genetic differentiation because the rate at which favorable mutations are fixed is assumed to be higher in haplodiploid species than in diploid species (Hartl 1972, Crozier 1985). All these traits, suggest that intraspecific genetic differences in parasitoid populations utilizing hosts on different host-plant species is highly likely. If genetic differentiation occurs, not only in loci involved in parasitoid's performance when associated with different resources (i.e., host, host-plant, or host-plant complex) but also in loci associated with mating and oviposition preferences for a

particular host, host-plant or host-plant complex, then reproductive isolation in sympatry may promote genetic differentiation across the entire parasitoid's genome (Rank 1992, Feder et al. 1998, Vaughn and Antolin 1998).

Although plants have important direct and indirect effects on parasitoids, the spatial patterns associated with parasitoid populations (i.e., how close they are to each other) may have a significant impact on gene flow and thus on genetic differentiation. Genetic differentiation among sympatric parasitoid conspecifics associated with different host-plant species may be observed at individual loci involved in selection for traits associated with the use of hosts in these different plant species (e.g., ability to detect specific plant volatiles, ovipositor length, etc) (Hufbauer and Via 1999) or it may be the result of indirect selection on correlated loci (Conner and Hartl 2004, Via 1999). If gene flow among individual parasitoids attacking hosts on different host-plant species is low, then the effects of genetic drift acting on the entire genome will generate genetic differentiation at all the parasitoid loci (Hartl and Clark 1997, Conner and Hartl 2004). Genetic differentiation acting on genomic-wide loci may occur if conspecific parasitoids using different resources are reproductively isolated in allopatry or by distance (Slatkin 1993, Johannesen and Seitz 2003), isolated in time (as occurs in some herbivorous insects; see Bush 1969, Futuyma and Peterson 1985), or in sympatry, if reproductively isolation is due to ecological specialization (Futuyma et al. 1984, Abrahamson and Weis 1997).

Although, typically, genetic differentiation has been associated with geographically isolated or distant populations (Mayr 1982, Mc Cauley 1991, Finston and Peck 1995) genetic differentiation among conspecific hymenopteran parasitoids also can occur in sympatry if differential selection by host species and/or host-plant species, acts on loci involved in preference for a particular host-plant and/or herbivorous host species

(Smith 1966, Cavener 1979, Gelfand and McDonald 1980, Futuyma et al. 1984, Via 1990, Hawthorne and Via 2001) or if individuals within populations remain associated with the population of origin by means of low dispersal (Mopper et al. 1995, Stiling and Rossi 1998). If a preference to remain associated with a particular host-plant species exists, then reproductive isolation of conspecific parasitoids using different host-plant species may occur in sympatry (Wood 1980, Wood and Guttman 1982, Diehl and Bush 1989).

The extent to which host-plant species may influence genetic differentiation of parasitic Hymenoptera can vary geographically. Geographic variation in patterns of genetic differentiation has been observed in herbivorous insects and their host-plants and in parasitoids and their herbivorous hosts (Boulétreau 1986, Kraaijeveld and Van Alphen 1994, Itami et al. 1998, Thomas and Singer 1998, Althoff and Thompson 2001). Among the major causes modulating the pattern of genetic differentiation among conspecific individuals associated with different host or host-plant species in different geographic regions are differences in selective pressures at different sites. These selective pressures could be abiotic, such as differences in climate (Crouau-Roy 1989, Karan and Parkash 1998, Holmstrup and Loeschcke 2003) or biotic, such as differences in host-plant abundance (Thompson 1994, Itami et al. 1998, Thomas and Singer 1998) host availability (Kraaijeveld and Van der Wel 1994) and/or presence of competitors (Kraaijeveld et al. 1994).

Finding genetic differences among individuals from different populations can be done following a quantitative genetics approach, through crossing experiments or through the use of clones (Pashley 1988, Via 1989, 1991b, Henter 1995, Henter and Via 1995) or by exploring genetic variation at a few loci or at several loci (by using allozymes and molecular markers) (Futuyma et al. 1984, McPheron et al. 1988, Guttman et al. 1981,

Guttman et al. 1989, Navajas et al. 2000, Lushai et al. 2002, Salvato et al. 2002, Feder et al. 2003a, 2003b). Some parasitoid species, such as one of the two parasitoids studied here, are difficult to rear and to mate in the laboratory and thus crossing experiments are impossible to perform. The development of molecular techniques has made possible the identification of intraspecific genetic differences among individuals from different populations without the need to perform crossing experiments (Slatkin 1987). One of those molecular techniques involves the use of amplified fragment length polymorphisms or AFLP (Vos et al. 1995). This technique is ideal when nothing is known about the genome of the focus species (Busch et al. 2000, Hawthorne 2001), as it is the case in most parasitoid species. AFLP, simultaneously generates fragments from many genomic sites that are separated by gel electrophoresis and scored as dominant markers. AFLP banding patterns can then be grouped using cluster analysis techniques (Saunders et al. 2001).

In the present chapter, I will focus on the influence of host-plant species in the genetic differentiation of populations of the specialist parasitoid *Aleiodes nolophanae* Ashmead (Hymenoptera: Braconidae) and the generalist parasitoid *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae). Both parasitoid species are the most abundant parasitoids of the green cloverworm, *Plathypena scabra* Fabricius (Lepidoptera: Noctuidae), on alfalfa and soybean in Maryland. Most of the traits that make parasitoid species good candidates to study genetic differentiation are present in *A. nolophanae* and *C. marginiventris*. They are both closely associated to their host-plants. The specialist parasitoid *A. nolophanae*, only parasitizes the green cloverworm on herbaceous legumes (Marsh 1979, Covell 1984, Johnson and Lyon 1991) and although *C. marginiventris* uses dozens of host species that occur on abundant, widespread host-plant species (i.e., wild and cultivated species of grasses and herbs) (Carlson 1979, Marsh

1979) it has been reported to show a strong post-mating attraction to volatiles released by the host-plants of its hosts (Turlings et al. 1991). *A. nolophanae* and *C. marginiventris* mate shortly after emergence (Lentz and Pedigo 1973, Boling and Pitre 1970) and they are both haplodiploid.

To test if the association with different host-plant species results in parasitoid genetic differentiation, I determined if individuals of populations of *A. nolophanae* and *C. marginiventris* ovipositing green cloverworm larvae on alfalfa exhibited different AFLP fingerprints than individuals of populations of *A. nolophanae* and *C. marginiventris* ovipositing in green cloverworms on soybean. To explore if genetic differentiation among parasitoids associated with different host-plant species varied spatially and with the historical occurrence of each host-plant species, genotypic differentiation between alfalfa and soybean parasitoids was tested in three counties that differed in the historical ratio of alfalfa and soybean cultivation.

METHODS.

If reproductive isolation is present in parasitoids ovipositing on hosts on different host-plant species, differences in the genome should be present at several loci. Thus, AFLP were used to visualize potential genetic differentiation due to host-plant species at the genomic level. To determine if populations of *A. nolophanae* and *C. marginiventris* ovipositing in green cloverworms on alfalfa were genetically different from populations of *A. nolophanae* and *C. marginiventris* ovipositing *P. scabra* on soybean, AFLP fingerprints from wasp populations associated with these two plant species were compared using cluster analysis. For a description of the study organisms, the study site and the collection and rearing procedures refer to Chapter 2.

DNA Extraction and AFLP markers.

In order to obtain AFLP markers, DNA was extracted from frozen (-80 ˚C) individual wasps using Qiagen DNEasy Tissue Kit (QIAGEN Inc., Valencia, CA) following the manufacturer recommended protocol for animal tissue (Qiagen 2002). Final elution was in 100 uL of the supplied buffer AE. *A. nolophanae* and *C. marginiventris* AFLP markers (Vos et al. 1995) were obtained using components from various commercial AFLP kits. Digestion of genomic DNA by the restriction enzymes EcoRI and MseI and ligation of oligonucleotide adaptors compatible with these endonucleases were acomplished in a single reaction mixture of 11 uL. Final concentration of reagents were: 50 mM Tris-HCL (pH 7.5), 10 mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP, 25.5 ug/ml bovine serum albumin, 5 mM NaCL, 45 uL/ml MseI (900 U/mL of New England Biochemical 525CL), 45 uL/mL of EcoRI (4500 U/mL of NEB 101 CL), 30 uL/mL T4 ligase (6.1 x 105 Cohesive End Units/mL or 6700 U/reaction, of NEB 202 CL). Each 11-uL reaction aliquot contained approximately 200 ng of template DNA (at a final concentration 20 ug/L) in addition to EcoRI and MseI adaptor pairs, at concentrations recommended by the manufacturer. Prior to each use, the adaptor pairs were preheated to 95 ˚C for 5 minutes, then allowed to cool slowly, over a ten-minute period to room temperature. The mixture was incubated overnight at room temperature so that template DNA was completely digested. Then each reaction was diluted to 1:18 (11 uL + 189 uL) with 15 mM Tris (pH 8.0), 0.1 mM EDTA.

Preselective PCR amplification was performed using the Applied Biosystems AFLP kit. The 20 uL reaction contained 4 uL of the diluted restricted/ligated DNA and 16 uL of a mixture with 1 uL of EcoRI+A and MseI+C AFLP pre-selective primers with

15uL of AFLP core mix. The PCR protocol for the pre-selective amplification was: 95 ˚C for 1 minute followed by 20 repetitive cycles of 95 ˚C for 10 seconds, 56 ˚C for 30 seconds, and 72 ˚C for 1 minute 30 seconds with a final hold at 75 ˚C for five minutes. All samples were stored at 4 ˚C following amplification on a GeneAmp 9700 PCR system (Applied Biosystems). The amplified product was diluted 20-fold using 15nM Tris-HCl buffer (pH 8.0) containing 0.1 mM EDTA.

For selective PCR amplification of restriction fragments, custom primers were prepared for recognition of EcoRI and MseI adaptors. The EcoRI selective amplification primer had a sequence of 5'-GACTGCGTACCAATTCA*NN. For recognition of the MseI adaptor on the other side of the DNA fragment, primers were synthesized with a sequence of 5'- GATGAGTCCTGAGTAAC*NN. The A* and C* bases represent bases selected for primers in the initial pre-selective amplification and the N's represent user-selected bases amplified in the second selective PCR amplification. I used the subset of 64 primer combinations provided in commercial AFLP kits made by either Life Technologies or Applied Biosystems. Fragments were visualized by attaching a D4 WellRED^{TM} dye to the 5' end of each EcoRI selective amplification primer with no modification made to the MseI primer.

The second, selective, PCR amplification reaction requires a DNA template form the pre-selective PCR reaction, Taq polymerase, dNTPs, a dye-labeled primer as described above, and the standard buffers and salts optimized for the reaction. The PCR reaction mixture consists of 15 uL AFLP reaction buffer (Applied Biosystems AFLP kit), 1 uL Eco RI selective primer which is dye labeled and contains 3 user-selected nucleotides, 1 uL MseI selective primer without label that contains 3 user-selected nucleotides (at 5 uM to give final 0.25 uM final concentration), and finally 3 uL of diluted amplified product from pre-selective amplification.

The PCR protocol for the selected amplification consisted of an initial warm-up at 95 ˚C for 30 seconds , twelve cycles of 95 ˚C for 10 seconds, 65 ˚C for 40 seconds with a lowering of 0.7 ˚C per cycle, 72 ˚C for 1 minute 30 seconds, followed by 35 cycles of 95 ˚C for 11 seconds, 56 ˚C for 40 seconds, 72 ˚C for one minute 30 seconds and finally a hold of 75 ˚C for 5 minutes before storing the samples at 4 ˚C.

To prepare DNA fragments for separation by capillary electrophoresis, sample loading solution was prepared with a 400-base pair (bp) DNA size standard labeled with WellRED™ (dye D1 (approximately 100:1) Beckman Coulter 608082 and 608098). This solution was thoroughly mixed by vortexing for a minimum of 2 minutes. A 30 uL aliquot of this cocktail was added to 1.0 uL of the selective amplification product. Each well was overlaid with a drop of Sigma mineral oil (M5904) and samples were analyzed in the CEQ 8000 from Beckman Coulter.

AFLP Analysis.

The CEQ 8000 analyzes samples by electrophoresing DNA fragments through a capillary. A laser reader determines the size in base pairs (bp) of each fragment and arrange the different fragment by size and by fluorescence in an electropherogram (i.e., a graph on which DNA fragments of different sizes are visualized as peaks, see Figure 3. 1). Only fragments from 60 bp to 350 bp allow the CEQ 8000 to reliably separate DNA fragments by size on a one-bp basis. Peaks with less than 500 fluorescence units were considered as noise. The advantage of the use of the CEQ 8000 is that it allows to score AFLP bands in an objective way.

AFLP markers were arranged in presence/absence matrices (1= presence; 0= absence). Fragments with more than 500 luminescent units were considered as present.

PAUP* 4.0 (Swofford 2003) was used to calculate Nei-Li distance matrices (Nei and Li 1979) and to build neighbor joining trees (Saitou and Nei 1987) to visualized how wasps were grouped based on similarity. The Neighbor Joining method was selected because using computer simulations, this method has demonstrated to be the most efficient method to estimate a single parsimonious tree (Saitou and Nei 1987) and also because this method does not make assumptions about rates of evolution among individuals (Graur and Li 1999). Due to the haplodiploidy of the Hymenoptera studied, separate neighbor joining trees were constructed for female and male wasps.

RESULTS AND DISCUSSION.

No clear clustering by host-plant species or by study site (i.e., county) was observed in female or male *A. nolophanae* (Figures 3. 2 and 3. 3). Similarly, no clustering by host-plant or by study site was found in *C. marginiventris* female or male wasps (Figures 3. 4 and 3. 5). Thus, host-plant species (i.e., alfalfa or soybean) do no drive genetic differentiation in *A. nolophanae* and *C. marginiventris*.

The lack of correlation between genetic differences and host-plant association with alfalfa and soybean in *A. nolophanae* and *C. marginiventris,* shows that wasps attacking the green cloverworm on these two plant species are not reproductively isolated. Similarly, the lack of correlation between genetic differences and county of origin shows that *A. nolophanae* and *C. marginiventris* wasps in these distant counties behave as a panmictic population. *A. nolophanae* and *C. marginiventris* and their host, the green cloverworm, are native to the areas studied, however their host-plant species are not. Alfalfa and soybean were introduced into America through the Colony of Georgia in the mid 1700's (Russelle 2001, Hymowitz 1987). Although alfalfa and

soybean have been cultivated in Maryland in small scale since their introduction, it is only in the 1900s that alfalfa and soybean cultivation started increasing rapidly towards their current levels (Russelle 2001, Hymowitz 1987). Thus, even though alfalfa and soybean have been present in these parasitoid habitats since the mid 1700s, perhaps it is only in the 1900s that these crops have been present abundantly enough to represent an important component of these parasitoids ecology. Although some insects have differentiated genetically on different host-plant species in about 250 years, or even shorter (Carroll and Boyd 1992, Feder et al. 2003a), I did not find evidences of genetic differentiation in *A. nolophanae* and *C. marginiventris* associated with alfalfa and soybean. *A. nolophanae* and *C. marginiventris* association with alfalfa and soybean may be too recent for genetic differentiation to show at the molecular marker level.

In spite of the lack of genetic differentiation evidenced by AFLPs, *A. nolophanae* and *C. marginiventris* exhibit host-plant associated phenotypic differences (Chapter 2). These phenotypic differences may be the result of divergent selection of individual loci associated with alfalfa or soybean. Evidence is mounting from research on a variety of organisms that divergent selection can be the primary cause of rapid sympatric differentiation in organisms associated with different host-plant species (Tremblay and Penacchio 1988, Bush 1994, Tauber and Tauber 1989). However, if divergent selection of loci associated with the efficient use of different host-plant species is not associated with selection of loci that promote reproductive isolation of wasp populations associated with these different host-plant species, then genetic differentiation of neutral markers will not be evident.

In retrospect, after having collected phenotypic data (see Chapter 2), the lack of genetic differences between alfalfa and soybean wasps is not surprising. The fact that mating occurs on or within a preferred host or habitat is probably the most important

single factor promoting sympatric divergence in response to divergent ecological selection pressures in some insects (Diehl and Bush 1989, Bush and Smith 1998). Even though I found fitness differences between alfalfa and soybean *A. nolophanae* and *C. marginiventris* in the different counties studied, no obvious preference was observed for the host-plant species of hosts from which adult wasps emerged. Further, there were no host-plant associated differences in host herbivore quality and thus no likely differential effect on parasitoids due to host-plant. This suggests that reproduction may occur between wasps whose host fed on different host-plant species. Thus, gene flow between alfalfa and soybean wasps seems to be preventing genetic differentiation of neutral markers in *A. nolophanae* and *C. marginiventris*. Although selection at individual loci may be responsible for the observed phenotypic differences, preference for a particular host-plant species does not appear to be under host-plant selection in these wasp species.

On the other hand, phenotypic differences between alfalfa and soybean wasps could be the result of phenotypic plasticity acting on the physiology of parasitoids ovipositing on host herbivores feeding on different host-plant species. Although phenotypic plasticity has been invoked as the reason explaining phenotypic differences in other parasitoid species associated with different host-plant complexes (Daza-Bustamante et al. 2002, Drès and Mallet 2002), I find this unlikely for the braconid parasitoids studied here. Since no differences in host quality were found between green cloverworm larvae feeding on alfalfa and soybean (Chapter 2) host-plant species quality would not be likely to affect their parasitoids.

In order to rigorously discern between selection at individual loci and phenotypic plasticity as an explanation for the phenotypic differences observed in *A. nolophanae* and *C. marginiventris* from different host-plant species, it would be necessary to be able to rear these parasitoids. If that were possible one could perform transfer experiments to test

if the effect of host-plant species is acting at the genetic level (i.e. through selection at loci associated with host-plant utilization) or only at the physiological level (i.e. through phenotypic plasticity). Unfortunately, the green cloverworm, can not be reared continuously in the laboratory and thus neither can its parasitoids. All my attempts, and those of other researchers (Pedigo, Kemper pers. comm.), to maintain a green cloverworm colony in the laboratory have failed.

In some parasitic Hymenoptera, genetic differences occur among populations as close as 1 Km away from each other (Vaughn and Antolin 1998, Van Nouhuys and Via 1999). My study sites were at least 100 Km apart from each other. Thus, one would have expected to find some genetic differentiation among counties. I did not observed such differences. The lack of correlation between genetic differences and county of origin in *A. nolophanae* and *C. marginiventris* suggests that gene flow among wasps from the counties studied is large enough to prevent reproductive isolation of wasps from different counties. This suggests that perhaps parasitic Hymenoptera dispersal distances are not as restricted as it has been assumed (Antolin and Strong 1987, Godfray 1994).

Thus, despite the close association of *A. nolophanae* and *C. marginiventris* parasitoids and the host-plant species of their herbivore hosts and despite possessing many characteristics that would facilitate genetic differentiation, none was observed in both specialist and generalist hymenopteran parasitoid. This study has shown that the effect of the host-plant species fed upon by a parasitoid's herbivore host, although strong enough to produce fitness differences, was not strong enough to generate reproductive isolation among individuals associated with different host-plant species. This lack of reproductive isolation between alfalfa and soybean wasps could be due to the fact that the differences in fitness found in *A. nolophanae* and *C. marginiventris* were not correlated with differences in preference for a particular host-plant species as evidenced by the odor

preference experiments performed in the olfactometer (see Chapter 2). So it seems that parasitoids emerging from alfalfa could potentially mate with parasitoids from soybean and vice versa. The lack of genetic differentiation of neutral markers among wasps from different host-plant species may also be explained by the fact that parasitoid populations at the study sites are not small enough for genetic drift to show an effect in the amount of time elapsed since the introduction of these host-plant species (Van Nouhuys and Via 1999).

The results from this study failed to demonstrated genetic differentiation in neutral markers among parasitoid populations associated with different host-plant species. In contrast, many examples of genetic differentiation exist in herbivore insects. I believe that the lack of examples of genetic differentiation in parasitoids associated with different host-plant species represents lack of studies on the subject rather than lack of existence of the phenomena. Genetic differentiation of hymenopteran parasitoids associated with different host-plant species should be explored further in several hymenopteran species before sound conclusions could be drawn. Studies should be perform preferentially on species that could be maintained in laboratory colonies in order to be able to have more experimental flexibility.

A final reason why no genetic differentiation was detected could be that my sampling of parasitoid populations did not accurately reflect the genetic profile of the populations involved. Similarly, my AFLP profiling of individuals, or specifically the number of bands used to “fingerprint” parasitoid individuals could have been too small to accurately profile each individual. When dealing with cluster analyses based on molecular markers in studies of genetic differentiation of populations from the same species, the issue of how many individuals are representative of the populations studied and the issue of how many markers to use in order to accurately represent how genetic

variation is organized among the individuals studied is somewhat arbitrary. In the following chapter, I propose a method to determine the number of individuals and the number of molecular markers to use in order to accurately depict genetic variation among organism populations when using cluster analyses. Using this method I have determined that my conclusions on genetic differentiation in *A. nolophanae* and *C. marginiventris* are valid.

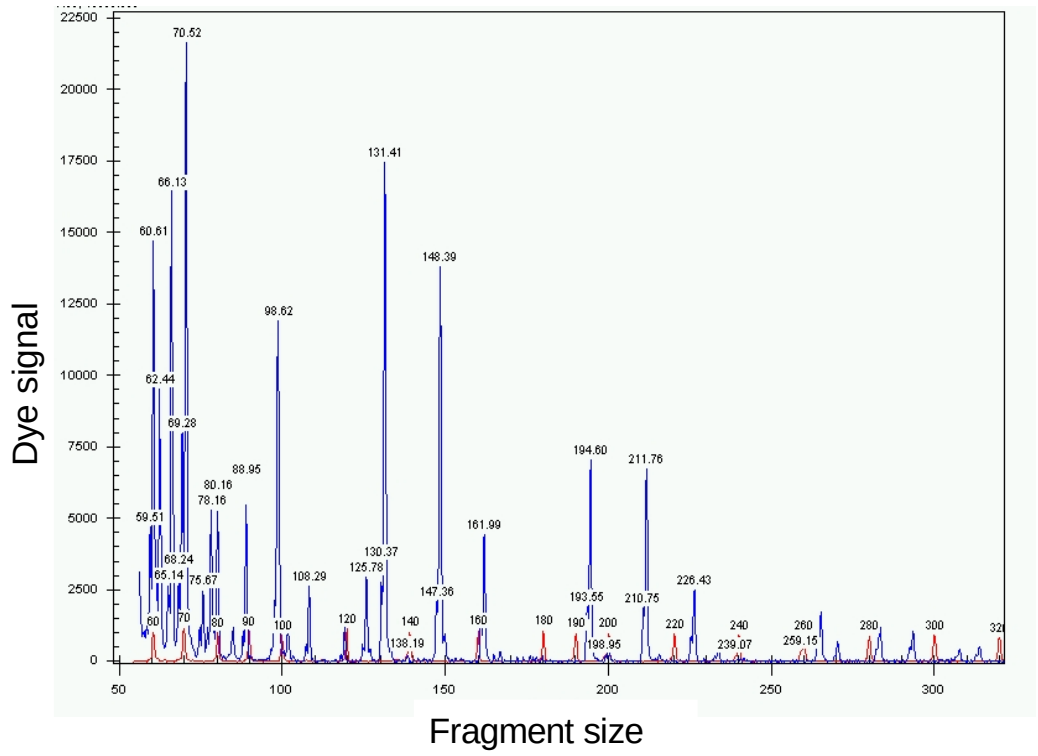

Figure 3. 1. Electropherogram showing AFLP bands as blue peaks. The x-axis shows AFLP fragment sizes in base pairs (the numbers on the peaks). The y-axis represents the strength of the signal as fluorescence units. Red peaks represent the D4WellRED^{TM} dyed standard.

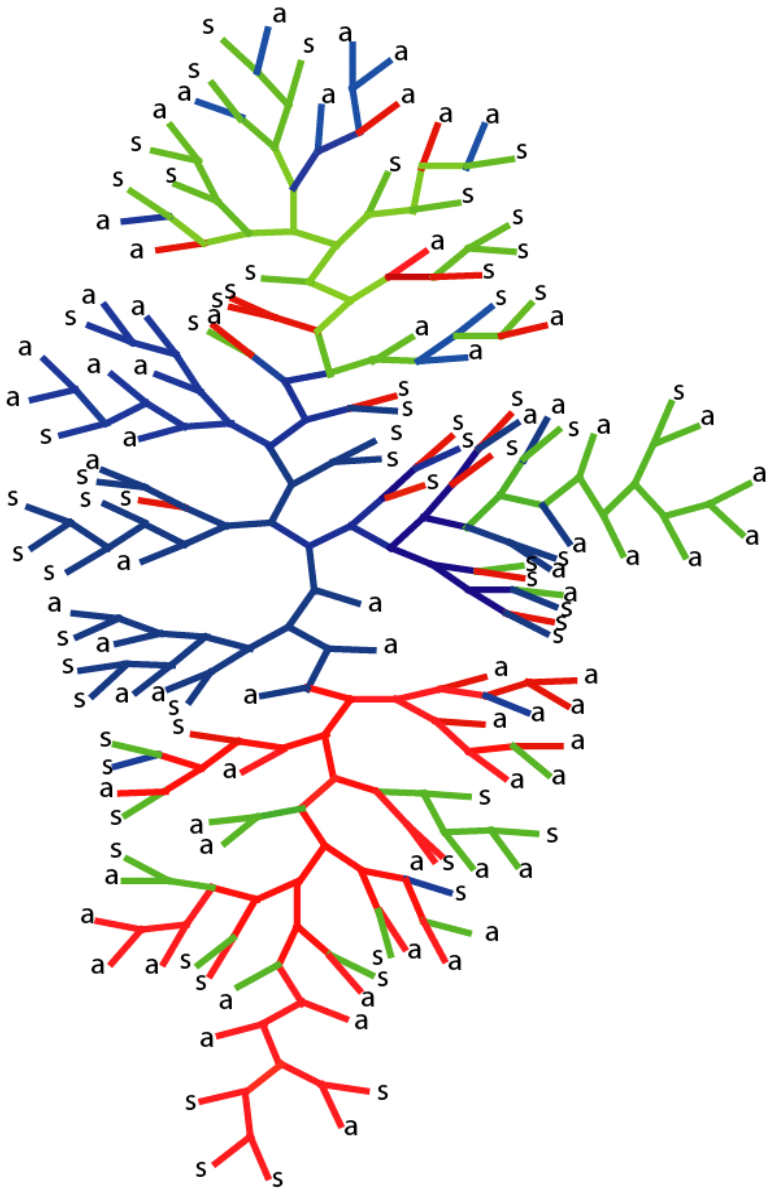

Figure 3. 2. Neighbor joining tree of female *Aleiodes nolophanae* wasps from alfalfa and soybean in three different counties in Maryland. Blue = PG county, green = Washington county and red = Garrett county. a = alfalfa and s = soybean. No clustering based on host-plant or county was observed.

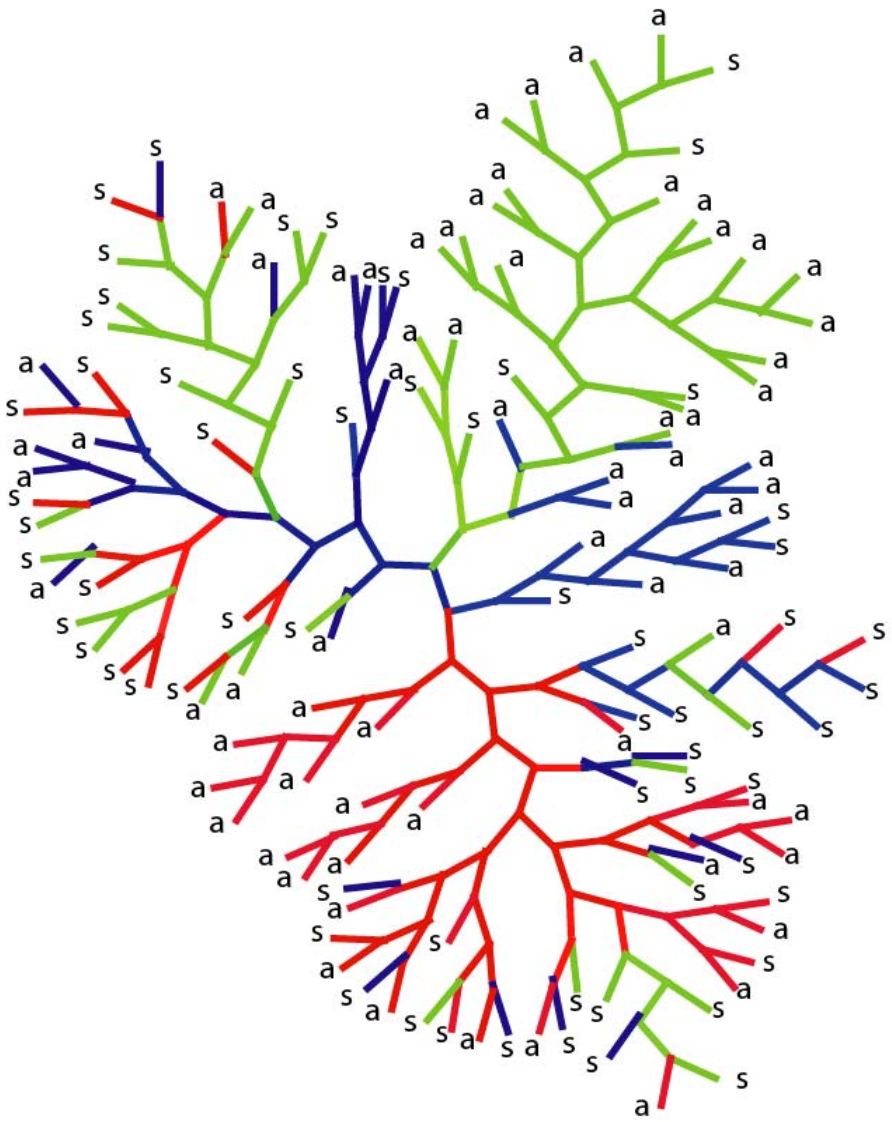

Figure 3. 3. Neighbor joining tree of male *Aleiodes nolophanae* wasps from alfalfa and soybean in three different counties in Maryland. Blue = PG county, green = Washington county and red = Garrett county. a = alfalfa and s = soybean. No clustering based on host-plant or county was observed.

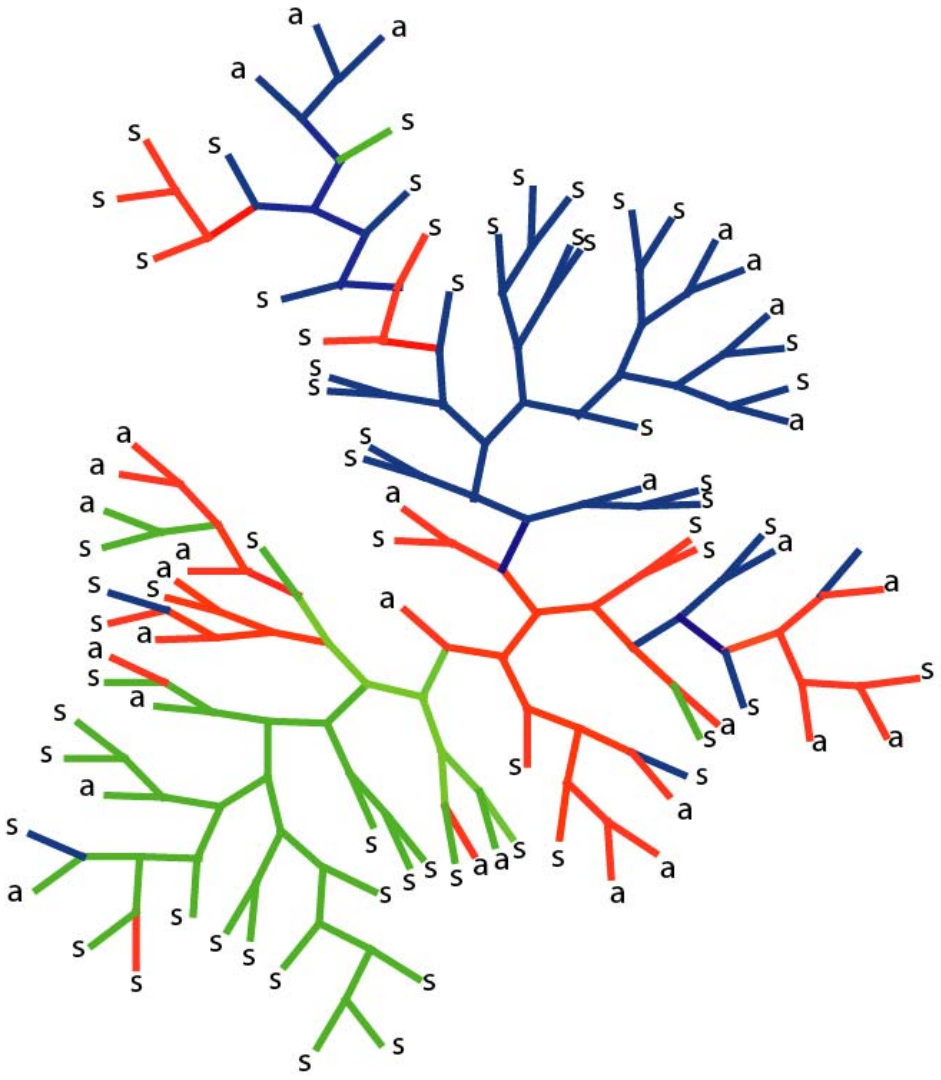

Figure 3. 4. Neighbor joining tree of female *Cotesia marginiventris* wasps from alfalfa and soybean in three different counties in Maryland. Blue = PG county, green = Washington county and red = Garrett county. a = alfalfa and s = soybean.

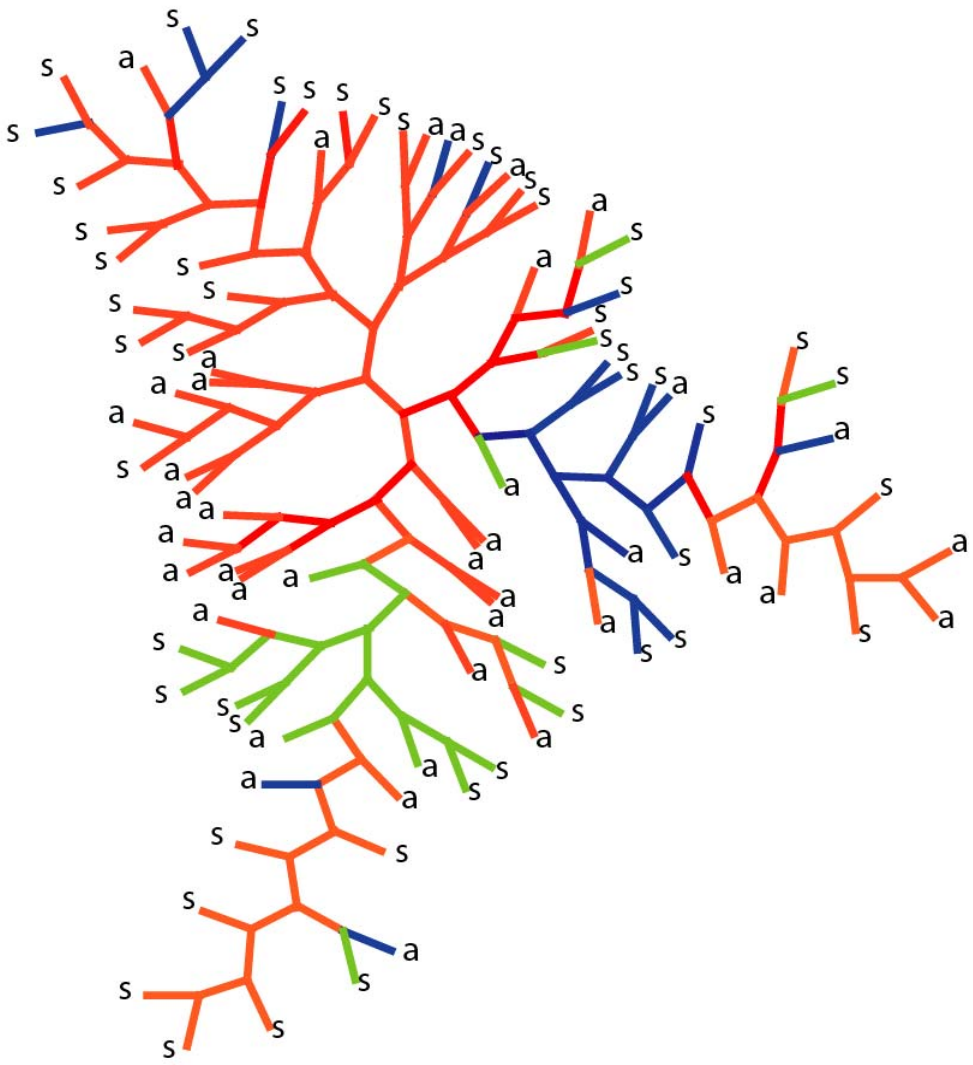

Figure 3. 5. Neighbor joining tree of male *Cotesia marginiventris* wasps from alfalfa and soybean in three different counties in Maryland. Blue = PG county, green = Washington county and red = Garrett county. a = alfalfa and s = soybean.

CHAPTER 4:

Determination of the Adequate Number of Individuals and AFLP Bands to Use When Studying Genetic Differentiation Among Intraspecific Populations

INTRODUCTION.

Molecular markers of various types have been used to answer biological and ecological questions and to study genetic variation among individuals in populations of a variety of taxa since the 1960s (Avise 1994, Loxdale and Lushai 1998, Conner and Hartl 2004). Their use continues but with a greater reliance on a variety of DNA based markers. The choice of a molecular marker depends on the question being addressed as well as on constraints associated with costs and time needed to develop markers (Parker 1998). One of the most widely used molecular marker is amplified fragment length polymorphisms (AFLP). AFLP are simultaneously generated from fragments of the total genomic DNA resulting from a digest using restriction enzymes. These restriction fragments are then amplified using polymerase chain reaction (PCR) and the amplification products are finally separated by gel electrophoresis and scored as dominant markers (Vos et al. 1995). Any individual plant or animal in a sample can be represented by its AFLP banding pattern. This banding pattern or fingerprint is defined by the presence or absence of restriction fragments or bands. Each restriction fragment is considered a locus and assumed to have two possible alleles (represented in binary form, i.e., 0 for no band present and 1 for a band present).

AFLP markers have been widely used to address key hypotheses in the study of systematics, evolution and ecology (Albertson et al. 1999, Griffiths and Orr 1999, Yan et al. 1999, Boucias et al. 2000, Gerber et al. 2000, Negi et al. 2000, Phang Loh et al. 2000, Vandermark et al. 2000, Hawthorne 2001, Jakše et al. 2001, Ren and Timko 2001,

Rogers et al. 2001, Saunders et al. 2001, Wilding et al. 2001, Young et al 2001, Orgen and Thorpe 2002, Salvato et al. 2002, Via and Hawthorne 2002). The popularity of AFLP derives from the fact that the entire genome is utilized to generate these markers and one need not know anything about the genome of the species being studied (Busch et al. 2000, Hawthorne 2001). AFLP also are one of the most efficient markers in that they yield the greatest amount of information (i.e., bands per assay) compared to other methods (Lin et al. 1996, Russell et al. 1997, Pejic et al. 1998). The reliability, reproducibility, flexibility and high degree of sensitivity of AFLP compared to other molecular markers also contribute to their widespread use (Cervera et al., 1998, Vuylsteke et al. 1999, Shim and Jorgesen 2000, Hawthorne 2001).

Differences in banding pattern among individuals can be analyzed in a multivariate fashion and then organized through cluster analysis (Rohlf 2000, Saunders et al. 2001). Using genetic differences in banding patterns provided by AFLP, individuals from populations that are presumed to be different (e.g., individuals from different geographic regions, different host-plant species, different habitats, and so on) can be grouped into distinct clusters or groups. Studies on genetic differentiation among intraspecific populations (using AFLP) typically use the Unweighted Pair Grouped Method with Arithmetic Averages (UPGMA) to cluster individuals (Sneath and Sokal 1973). UPGMA generates clusters of individuals using a similarity matrix created when making pair-wise comparisons among the individuals of interest (Sneath and Sokal 1973). By this method, individuals are always clustered in groups according to their similarity. The individuals whose genetic profiles are compared typically come from samples of arbitrary size taken from presumed populations. These arbitrary numbers of individuals from the different presumed populations are then compared (e.g., Boucias et al. 2000, Katiyar et al. 2000, Salvato et al. 2002, Abu-el Samen et al. 2003, Bennett and Mathews 2003, Ferriol et al. 2003, Gonzales et al. 2003, Kothera et al. 2003, Pester et al.

2003, Steiger et al. 2003, Tsuji et al. 2003, Ude et al. 2003, Uptmoor et al. 2003, and others). To my knowledge in all such studies the number of individuals used as well as the number of bands or markers evaluated per individual are apparently arbitrarily selected since no criteria for sample size or band number are provided. However, the number of individuals and bands is absolutely critical because the banding patterns generated directly influence the way in which individuals are organized through cluster analyses. Banding patterns from individuals are assumed to capture haplotype variation and to thus reflect the genetic profile of the population of the species under study. If the banding pattern of individuals within studied populations is not adequately sampled then cluster analyses generated with such data may produce a misleading representation.

For instance, if the similarity matrix used in UPGMA, to group individuals from what are assumed to be distinct populations, is not truly representative of the genetic variability of the individuals in these populations, the clusters generated by UPGMA might not correspond to real groups and instead may depict clusters that do not accurately reflect the genotype of field populations or species. In other words, if one uses data from an additional sample of the same size (i.e., the same number of individuals) from the same populations, it would be highly likely that a tree would be generated that differed from the original tree. Thus, it is clear that one needs to ensure that the number of individuals and the number of bands per individual used in similarity matrices are sufficient to accurately capture haplotype variation of populations.

The greater the genetic variability among individuals in a particular area, the larger the sample size needed to capture genetic variability. In the absence of knowledge of the genetic variation of the study organisms at a particular study site, reliable trees can not be obtained since the groupings they depict might be representative of an incompletely sampled marker diversity. At the same time, limited resources also make it essential that samples be comprised of only the minimum number of individuals and

bands sufficient to obtain a robust genetic profile of populations. Although we have focused on AFLP markers all other molecular markers share the same problem. Indeed, when markers such as microsatellites are used the problem is even more acute because studies based on microsatellites typically use fewer loci than studies using AFLP (Campbell et al. 2003). Thus, marker diversity might be even more unrepresented when using microsatelittes than when using AFLP.

The present paper proposes a new way to verify individual and band sample sizes in studies of genetic differentiation among individuals from populations from the same species. A limited number of attempts have been made to estimate the adequate number of loci needed to group a particular number of individuals within different populations (Pejic et al. 1998) and to assign a particular number of individuals to known candidate populations (Campbell et al. 2003). However, methods for estimating the number of loci and the number of individuals needed to generate reliable clusters of individuals belonging to different populations from the same species are lacking. In this chapter, I provide the output of a bootstrap analysis (Efron 1979) used to calculate the variability of the similarity index used to construct UPGMA trees. I used this variability estimate to provide a way of determining the optimum number of bands to include and individuals to collect in order to generate reliable trees. This method can be used with any similarity index or distance measure available to construct UPGMA or neighbor joining trees.

AFLP from *Aleiodes nolophanae* Ashmead (Hymenoptera: Braconidae) and from the processionary moth *Thaumetopoea pityocampa* Denis and Schiffermüller (Lepidoptera: Thaumetopoeidae) were used to test the proposed method. *T. pityocampa* shows clear genetic differentiation among individuals collected from different regions in Europe (Salvato et al. 2002). In contrast, AFLP data on the haplo-diploid *A. nolophanae* have found no genetic differentiation among individuals from three counties in Maryland

(Medina and Barbosa unpubl.). This distinction is important because I predict that when populations of a species are genetically distinct fewer bands per individuals should be needed to capture haplotype variation.

METHODS.

Aleiodes nolophanae Ashmead (Hymenoptera: Braconidae).

A. nolophanae is a native specialist parasitoid of the green cloverworm *Plathypena scabra* Fabricius. (Lepidoptera: Noctuidae). *A. nolophanae* was reared from host larvae collected from alfalfa and soybean at sites in three counties in the state of Maryland, in the USA. These sites where at least 100 km apart from each other. Sampling was carried out from the second week of June until the last week of August in 2001, 2002 and 2003. Adult *A. nolophanae* were preserved frozen at -80 °C until DNA extraction (for more details see Chapter 2).

Thaumetopoea pityocampa Denis and Schiffermüller (Lepidoptera: Thaumetopoeidae).

The winter pine processionary moth, *T. pityocampa* is a native pest of *Pinus* and *Cedrus* species found mainly along the Mediterranean coasts of Portugal, France, Italy and Spain (Androic 1956). Egg clusters of *T. pityocampa* were collected (by my collaborator, Dr A. Battisti) at nine different pine stands of *Pinus silvestris* L., *Pinus nigra* Arnold and *Pinus halepensis* Miller from seven northern Italian provinces, one Spanish location in the Sierra Nevada and one location in Turkey (see Salvato et al. 2002 for details). Collection sites in Europe were at least 60 km apart. To reduce the risk of sampling siblings, egg clusters were collected from different trees during the whole

emergence period of adults. The total number of egg clusters collected at each site varied from 50 to more than 200. A few neonate larvae of each egg cluster were placed in 70% ethanol immediately after hatching. One single larvae for each egg cluster was used for AFLP analysis. *T. pityocampa* AFLP data were obtained through a collaboration with Dr. Andrea Battisti (Università degli Studi di Padova).

DNA Extraction and AFLP Markers.

To obtain AFLP markers for *A. nolophanae* wasps, DNA was extracted from frozen individuals using Qiagen DNEasy Tissue Kit (QIAGEN Inc., Valencia, CA) following the manufacturer recommended protocol for animal tissue (Qiagen 2002). Final elution was in 100 uL of the supplied buffer AE. DNA from *T. pityocampa* was extracted using a salting-out protocol (Salvato et al. 2002).

A. nolophanae AFLP markers (Vos et al.1995) were obtained using components from various commercial AFLP kits (for more details see Chapter 3). AFLP markers were arranged in presence/absence matrices (1= presence; 0= absence). NTSys-pc2.1 (Rohlf 2000) was used to calculate similarity matrices and to construct UPGMA trees. Five primer combinations were used to generate bands in *A. nolophanae* and *T. pityocampa*. For details on AFLP procedures used with *T. pityocampa* see Salvato et al. (2002).

Simulations.

A. nolophanae AFLP data were bootstrapped using Matlab (Matlab 2002). The bootstrap procedure constructed 500 matrices for each of different number of individuals by number of bands designated levels. Pair-wise similarity matrices were calculated from

each of the bootstrapped matrices. For each similarity matrix the mean Jaccard index was obtained by averaging all the values within the matrix. The standard error of the mean Jaccard index (SESim) was calculated for each of the individual by band designated levels. Thus, each number of individuals by number of bands combination had associated with it a standard error value based on 500 similarity matrices. SESim can be considered a measure of the variability of the similarity index for each number of individual by number of band combination. Ideally a SESim estimate as close to zero as possible will indicate a total agreement among any of the possible Jaccard index means that could be obtained from a particular number of individuals by number of bands combination. The ideal number of individuals by bands to considered for a particular study could be estimated as the point at which SESim is at its minimum value (Figure 4. 1).

RESULTS.

The SESim curves in *A. nolophane* female simulations (Figure 4. 2A) indicate that the number of bands used had a greater impact on SESim than the number of individuals sampled. The same was observed in males. The leveling off for the SESim curves occurred at about 45 individuals for *A. nolophanae* females and at about 40 individuals for *A. nolophanae* males at any of the number of bands used. As predicted, the number of bands necessary to obtain a SESim close to zero was lower in *T. pytiocampa* than in *A. nolophanae* (204 bands versus 765 bands, respectively, to obtain a SESim=0.03). In addition, variability among SESim curves due to the numbers of bands considered per individual was lower in *T. pityocampa* than in *A. nolophanae* (Figure 4. 2). In other words, less AFLP bands per individuals were necessary to capture haplotype variation in *T. pityocampa* than to capture haplotype variation in *A. nolophanae*. The SESim curves leveled off at about 60 individuals for *T. pityocampa*.

UPGMA trees constructed with different number of individuals and different number of bands started to produced the same general pattern when the SESim curves reached a value lower than 0.045 (Figure 4. 3). This occurred at around 600 bands per 40 individuals in *A nolophanae*, and at about 100 bands per 50 individuals in *T. pityocampa*. Indicating these as adequate numbers of individuals and numbers of bands per individual to sample in the respective species. However, note that SESim<0.045 can be achieved using different combinations of number of bands by individuals than the ones just mentioned.

DISCUSSION.

In panmictic populations, gene flow among individuals prevents genetic differentiation. In contrast, when reproductive isolation occurs genetic differentiation can be observed in neutral markers due to the effect of genetic drift. Genetic drift produces a random fixation of different alleles in different populations. Because genetic drift acts throughout the genome of individuals within populations (Conner and Hartl 2004), neutral markers from individuals that belong to the same population will be more similar among them than they are to markers from individuals from different populations. Thus, as one might expect, fewer markers are necessary to capture haplotype diversity among individuals from differentiated populations than from individuals belonging to panmictic populations. Similarly, individuals from undifferentiated populations will required more markers than individuals from differentiated populations in order produce a reliable picture of the way they are organized within populations (compare *A. nolophanae* and *T. pityocampa* curves in Figure 4. 2). Individuals from panmictic populations will require a comparatively higher number of loci before reaching SESim curves close to zero. Thus, in my example, the effect of increasing the number of bands on the undifferentiated *A.*

nolopahanae populations produces a more pronounced reduction in SESim than the one produced in the differentiated populations of *T. pityocampa* due to the proportionally higher agreement among markers in *T. pityocampa* than among markers in *A. nolophanae*, indicating that SESim variation is larger in undifferentiated populations than in differentiated populations. This method allows researchers to know, specifically, the number of individuals and bands required for a robust representation of the genetic profile of a species. Further, this same methodology also can be used for data based on distance matrices, such as the data used to generate neighbor joining trees.

The number of individuals required to capture haplotype variation at a particular study location depends on the amount of variation characteristic of a particular species and on the number of candidate populations present (Campbell et al 2003) and thus it is idiosyncratic for each population studied at a particular location. My method provides a way to evaluate the variation in banding pattern in specific populations and consequently provides a statistical baseline to which specific samples can be compared. For instance, if the SESim curves do not level off close enough to zero at the number of individuals sampled one needs to sample more individuals or increase the number of molecular markers used. One can decide to increase the number of bands, the number of individuals collected, or both, after examining the SESim curves generated from the data. Once a value of SESim close enough to zero is achieved (e.g., SESim< 0.045) then one can be relatively confident that trees generated by a dataset reflect real genetic differences in populations or species. The method presented here provides a practical way to evaluate the reliability of trees based on similarity matrices and considers and captures the idiosyncratic genetic variation found in nature.

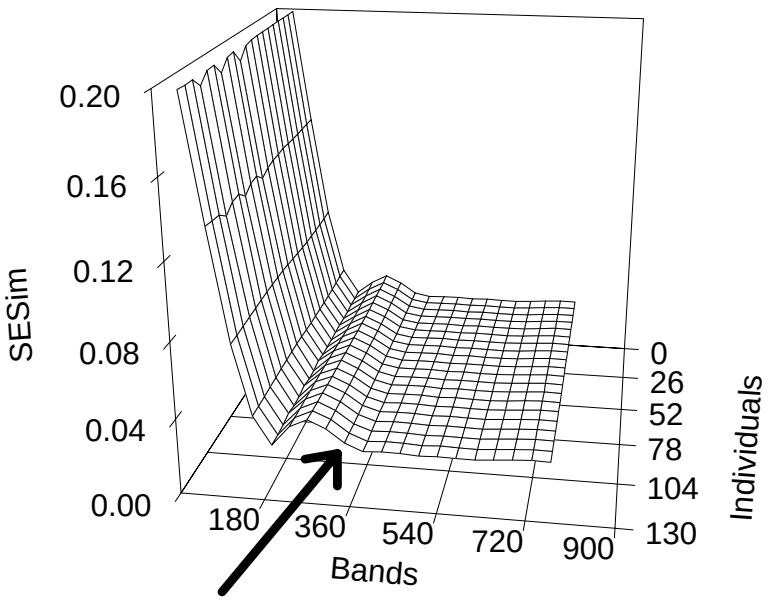

Figure 4. 1. Theoretical relationship among the number of individuals in a similarity matrix, the number of AFLP bands and the standard error of the mean similarity index (SESim). The arrow points at the number of individual by band combination at which SESim is the lowest.

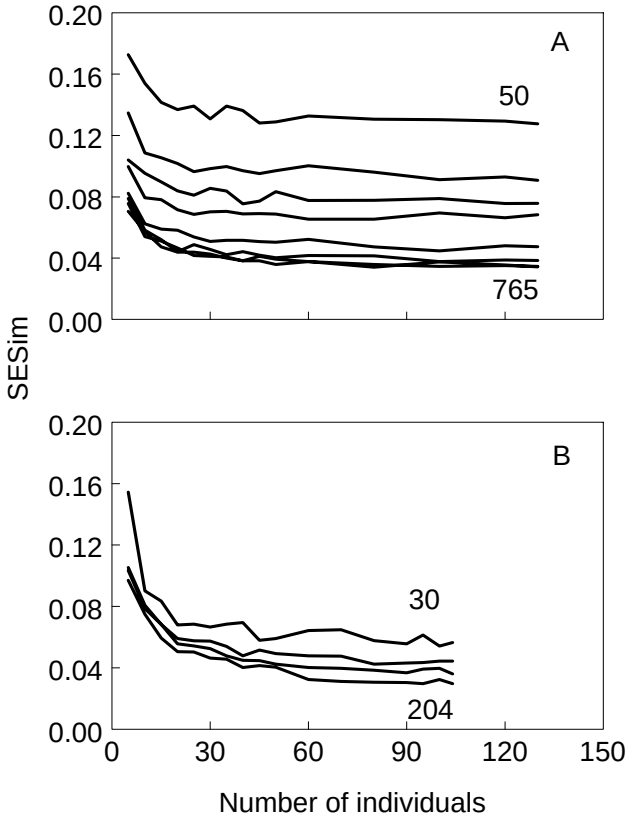

Figure 4. 2. Standard error of the mean Jaccard index (SESim) curves for different number of bands and number of individuals. Each line in the graph represents different number of AFLP bands used. A. SESim curves based on *A. nolophanae* AFLP. Data from female wasps. Males depicted the same pattern. Only data from females is shown. Lines represent 50, 100, 150, 200, 400, 600 and 700 and 765 bands. B. SESim curves based on *T. pityocampa* AFLP. Lines represent 30, 60, 100 and 204 bands. For both species as the number of individuals increases SESim decreases down to a point after which it does not further decrease (around 40 individuals in *A. nolophanae* and around 60 individuals in *T. pityocampa*). The increase in the number of bands has a greater impact in *A. nolophanae* than in *T. pytiocampa*.

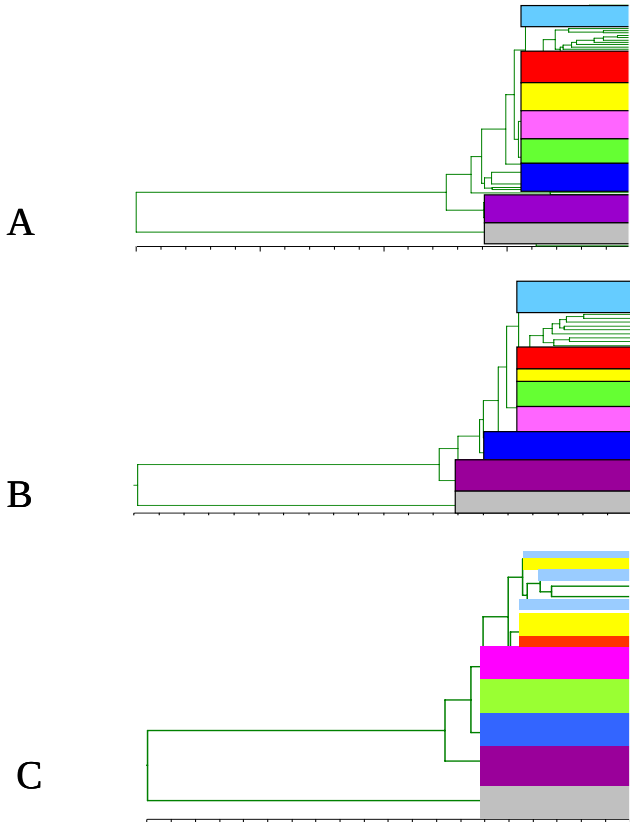

Figure 4. 3. UPGMA trees for *Thaumatopea pityocampa*. Each colored square represent a cluster from a particular sampling region. Starting from the bottom the gray area represent the Turkish samples, the purple area the Spanish samples, the colors above represent different provinces within Italy. A. UPGMA tree generated at a SESim=0.03 with 204 bands and 104 individuals. There are nine defined clusters corresponding to the nine sampled regions. B. UPGMA tree generated at a SESim=0.03 but this time with 204 bands and 60 individuals. There are still nine clearly defined clusters corresponding to the nine sampled regions. C. UPGMA tree generated at a SESim=0.05 with 204 bands and 25 individuals. Although the Turkish and Spanish clusters are still obvious some of the clusters within Italy start to decompose at SESim=0.05.

EPILOGUE

The present study found phenotypic differentiation in important fitness traits between wasp populations associated with the green cloverworm, *Plathypena scabra* Fabricius (Lepidoptera: Noctuidae) in alfalfa and in soybean, in both the generalist parasitoid *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) and the specialist parasitoid *Aleiodes nolophanae* Ashmead (Hymenoptera: Braconidae). Contrary to the expectations and predictions from the literature, these parasitoids did not show evidence of reproductive isolation when associated with these different host-plant species, as evidenced by the lack of genetic differentiation between parasitoids associated with alfalfa and soybean. By no means do I think that this should be taken as an argument against the possibility that genetic differentiation of parasitoid populations associated with different host-plant species actually occurs. The evidence of this phenomena in herbivorous insects is already vast and it keeps growing. It seems unlikely than in organisms so highly tuned to their host-plants as parasitoids, genetic differentiation of populations associated with different host-plant species should not occur. I think that the lack of examples of genetic differentiation of parasitoids associated with different host-plant species, is due more to the lack of studies on these organisms than to the lack of genetic differentiation among these parasitoid populations. Studies on other parasitoid species are needed before the view that host-plant association can drive parasitoid diversity is accepted. However, all we know about genetic differentiation in insects support the view that parasitoids should be prone to genetically differentiate when associated with different host-plant species.

One reason that might explain the failure to find genetic differentiation in the parasitoids of the green cloverworm feeding on alfalfa and soybean might have to do with the duration of the interaction. Although the green cloverworm, *A. nolophanae* and *C. marginiventris* are native to North America, the host-plant species with which they are associated are not. Alfalfa was introduced from Iran in 1736 and soybean was introduced from China in the mid 1770's. Although genetic differentiation in some herbivorous insects has occurred relatively fast (e.g., in as fast as 20 years in some instances) the time required for such changes in this system may be much larger. Studies of phenotypic and genotypic differentiation of native parasitoid species on native herbivorous host and host-plant species may provide interesting insights into the potential role of time in the differentiation of parasitoids associated with different resources. Parasitoids of the ball-gallmaker *Eurosta solidaginis* Fitch (Diptera: Tephritidae) appear to be especially suitable for this purpose as all the players involved in this system are native of North America. A future collaboration with Dr. Warren Abrahamson will explore this possibility in the future.

Studies performed on parasitoids that could be maintained in colonies on their natural herbivore hosts would be suitable candidates to study host-plant based genetic differentiation. Although several parasitoid species and their herbivorous hosts are difficult to rear in the laboratory, laboratory colonies of parasitoids and their herbivorous hosts provide the advantage of allowing the use of quantitative genetics in understanding the inheritance of important phenotypic traits and the role of selection versus phenotypic plasticity in generating phenotypic differentiation.

Another interesting venue of exploration has to do with the role of the herbivore host differentiation on its parasitoids differentiation. It could be that the selective forces that promote differentiation in parasitoids act on parasitoid larvae rather than on adults.

Thus, only herbivorous hosts that are differentiated by host-plant species will drive parasitoid genetic differentiation. My future research will determine if the green cloverworm is differentiated in alfalfa and soybean in the study sites on which I performed my studies. Also, I will study parasitoids of herbivores that show genetic differentiation on different host-plant species. The processionary moth *Thaumetopoea pityocampa* Denis and Schiffermüller (Lepidoptera: Thaumetopoeidae) shows genetic differentiation at a very local scale and it is suspected to present genetic differences also depending on the pine species it attacks. In collaboration with Dr. Andrea Battisti I will determine if genetic differentiation of *T. pityocampa* parasitoids follows the genetic differentiation found in the moth.

Understanding the role of host-plant species in the differentiation of parasitoid populations has theoretical implications as it might throw light into the process of sympatric speciation and parasitoid diversification. Further, understanding the role of host-plant species in the differentiation of parasitoids has also practical implications since parasitoids are important natural enemies of crop pests. If parasitoids attacking the same pest species on different crop species perform differently or are genetically differentiated or reproductively isolated, then parasitoid introductions to control generalist pests should consider this potential differentiation. Similarly, the role of alternative host-plant species as parasitoid's refuges need to be assessed in the light of the possibility of parasitoids not moving and reproducing as freely among crop species as previously thought.

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