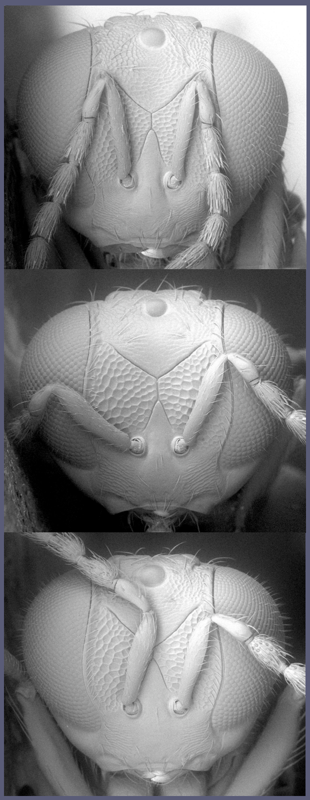
THE INFLUENCE OF HOST PLANTS ON THE ECOLOGICAL GENETICS OF THE THIRD TROPHIC LEVEL: THE CASE OF BEANS, BRUCHIDS AND THEIR PARASITOIDS



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IMPRIMATUR POUR LA THESE

The influence of host plants on the ecological genetics of the third trophic level: the case of beans, bruchids and their parasitoids

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La doyenne:

Martine Rahier

Ce travail n’aurait jamais vu le jour sans l’aide de...

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“It is interesting to contemplate an entangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent on each other in so complex manner, have all been produced by laws acting around us.” (Darwin, 1859)

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“Horismenus Species (Hymenoptera: Eulophidae) in a Bruchid Beetle Parasitoid Guild, Including the Description of a New Species”. *Zootaxa* 548: 1-16

This chapter describes a new parasitoids complex of the genus *Horismenus* attacking bruchid beetles on *Phaseolus* beans in Mexico with the description of a new species and a taxonomic revision of the parasitoids used in this study.

“Microsatellite Markers in a Complex of *Horismenus sp*. (Hymenoptera: Eulophidae), Parasitoids of Bruchid Beetles.” *Molecular Ecology Notes*. 4: 707-709

This chapter describes the isolation and characterization of new microsatellite markers for parasitoids of the genus *Horismenus*.

"Molecular Differentiation and Phylogenetic Relationships among *Horismenus spp.* (Hymenoptera: Eulophidae) Attacking Bruchid Beetles in Mexico."

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This chapter describes the results from a multidisciplinary approach aiming at understanding the role of host-plants on the ecological genetics of parasitoids of the genus *Horismenus*.

"Bruchids and Parasitoids from Wild and Cultivated Beans: An Ecological and Genetic Approach."

This chapter reviews the interactions among beans, bruchids and parasitoids in Mexico, describes the ecological differentiation of sibling *Zabrotes* species and analyses the phylogenetic relation of a potential biological control agent reared from various host-plants.

"Isolation and Characterization of Polymorphic Microsatellite Markers in *Zabrotes subfasciatus* Boheman (Coleoptera: Bruchidae)." *Molecular Ecology Notes*, 4: 752-754

In this annex I included a description of the isolation and characterization of new microsatellite markers for bruchid beetles of the genus *Zabrotes.* These results were obtained during a one-year diploma project (T. Shani) and are now being used in an ongoing population genetics project on bruchid beetles of the genus *Zabrotes*.

Foreword

The work presented here is the result of a four year PhD project conducted at the University of Neuchâtel, under the supervision of Dr Betty Benrey and Professor Martine Rahier, on the ecological genetics of a complex of *Horismenus* parasitoids specialized on beans of the genus *Phaseolus*.

This manuscript presents all the results gathered during these four years. It is divided in six chapters that I hope follow a logical line. Chapter one is an outline of the thesis that places this work in the context of tritrophic interactions. It comprises a description of the organisms studied, followed by the different techniques used, and an overview of the results. It finishes on a general conclusion of this work and lists future perspectives and open questions emerging from this work. The following chapters are constituted of the publications that are already published or ready for submission, in which material and methods and results are detailed and where the results are discussed in the light of the current literature.

Abstract

Many species are distributed over populations that live in a variety of habitats in which they may experience different environmental conditions. It can be expected that natural selection operates differently in these different habitats and if gene flow among these populations is limited, this can lead to genetic divergence and local adaptation. For parasitoids of herbivores, the host species that they attack and the plant species that the host feeds upon are likely to be the two most important external factors that will influence their fitness. Variation in these two trophic levels due to habitat characteristics may largely determine the type and evolution of the interaction with the hosts. Evidence is accumulating that plant attributes can have major impacts on parasitoid behavior and performance, but it is still unclear how these may affect parasitoid population structure. I examined the influence of plants on the ecological genetics of the third trophic level using a model system that comprises wild beans of the genus *Phaseolus* (*P. vulgaris*, *P. coccineus* and *P. lunatus*), several bruchid species in the genera *Acanthoscelides* and *Zabrotes* and a complex of hymenopteran parasitoids that attack these bruchids. Combining ecological, behavioral and genetical approaches, I investigated the influence of biotic and abiotic factors on the genetic structure of parasitoid populations and determined the geographic scale at which genetic differentiation occurs. In Mexico, bruchids in these two genera are attacked by three species of the genus *Horismenus* (Hymenoptera: Eulophidae). A taxonomic key based on morphological characters was developed and used to determine which are the *Horismenus* species associated with *Acanthoscelides spp.* and *Zabrotes spp.* on *Phaseolus* beans. Subsequently, molecular markers (microsatellites and mitochondrial DNA) were used to determine the phylogenetic relationships and degree of genetic similarity between *H. depressus* Gahan, *H. missourensis* Ashmead and *H. butcheri* Hansson and Aebi, and to confirm the status of the third as a new and distinct species. These molecular markers were also used to determine the genetic population structure of parasitoids and to estimate the degree of gene flow among and within parasitoid populations. Despite their close taxonomic position, the three *Horismenus* species have specific ecological requirements allowing them to take advantage of different ecological niches scattered along the altitudinal gradient. *H. depressus* is the most abundant parasitoid below 1700 m and the only one found on *P. lunatus*, a bean common at low altitudes. *H. missouriensis* is the most abundant above 1700 m and the only parasitoid reared on *P. coccineus*, a bean growing at high altitudes. Finally, *H. butcheri* is the only species displaying strict host plant specialization as it was only collected on *P. vulgaris* seeds, with the notable exception of samples collected on *P. coccineus* in sympatry with *P. vulgaris*. The strength of the parasitoid-host plant association is also very different among *Horismenus* species. Although all species displayed preference for their host plant of origin, host plants only had an effect on the population genetic structure of one species of parasitoid. Two very distinct genetic groups were detected for *H. butcheri* individuals that emerged from different plant species in sympatry, strongly suggesting that plants can mediate the formation of host races at the third trophic level in this system.

This work not only has an importance for a fundamental understanding of parasitoid ecology and evolution, but also has an applied significance. The understanding of how herbivore and parasitoid populations are spatially and temporally structured has broad implications for the theory and application of biological control. A natural enemy's long-term success in controlling a pest species is contingent on its ability to adapt to novel conditions.

Résumé

La plupart des espèces sont distribuées dans des populations rencontrant des conditions environnementales très variées. De ce fait, la sélection naturelle agit différemment sur les populations et, en l'absence de flux de gènes entre ces dernières, peut induire une différentiation génétique et/ou une adaptation locale. Chez les parasitoïdes s'attaquant à des insectes herbivores, l'hôte ainsi que la plante sur laquelle se nourrit celui-ci, sont les deux facteurs majeurs qui régissent leur fitness. Des variations au niveau de l'un de ces deux maillons de la chaîne alimentaire peuvent largement dicter la nature et l'évolution des interactions entre un parasitoïde et son hôte. De nombreux exemples ont montré l'impact des caractéristiques de la plante sur les performances et le comportement de parasitoïdes. Néanmoins, l'influence des plantes sur la structuration de populations de parasitoïdes n'est pas connue à ce jour. Durant ce travail réalisé entre Neuchâtel et le Mexique, j'ai étudié l'influence du premier niveau trophique sur l'écologie et la génétique des parasitoïdes, en utilisant un système biologique comprenant des haricots sauvages du genre *Phaseolus* (*P. vulgaris*, *P. coccineus* et *P. lunatus*), sur lesquelles se nourrissent plusieurs espèces de bruchides des genres *Acanthoscelides* et *Zabrotes,* elles-mêmes attaquées par un complexe de parasitoïdes hyménoptères du genre *Horismenus* (Eulophidae). Par une approche écologique, comportementale et génétique, j'ai analysé l'influence de facteurs environnementaux biotiques et abiotiques sur la structuration génétique des populations de parasitoïdes et déterminé l'échelle géographique à laquelle une différentiation génétique pouvait intervenir. Une clé de détermination basée sur les caractéristiques morphologiques a été spécialement développée et utilisée pour identifier trois espèces d'*Horismenus* intervenant dans le système, *H. depressus* Gahan, *H. missourensis* Ashmead et *H. butcheri* Hansson et Aebi. Des marqueurs moléculaires (microsatellites et ADN mitochondrial) ayant confirmé la découverte d'une nouvelle espèce d'*Horismenus*, ont été utilisés afin d'analyser la structuration des

populations de parasitoïdes et d'estimer les flux de gènes entre et au sein de ces populations. Malgré leur proche parenté, les trois espèce d'*Horismenus* ont présenté des besoins écologiques spécifiques leur permettant de coloniser des niches très différentes le long d'un gradient altitudinal. En effet, *H. depressus* est le parasitoïde le plus abondant en dessous de 1700 m et le seul capable de se développer sur *P. lunatus*, une espèce de haricot vivant en basse altitude. *Horismenus missouriensis* est le parasitoïde le plus abondant en dessus de 1700 m et le seul capable de se développer sur *P. coccineus*, une espèce de haricot limitée à de hautes altitudes. Enfin, *H. butcheri* est la seule espèce montrant une stricte association avec sa plante hôte. En effet, cette espèce a été trouvée sur *P. vulgaris* uniquement, à l'exception notable d'échantillons collectés sur des plantes hybrides provenant de croisements entre *P. vulgaris* et *P. coccineus*. La nature de l'association entre les parasitoïdes du genre *Horismenus* et leur plante hôte est très variable. Malgré le fait que chacune des espèces montre une préférence pour sa plante hôte d'origine, celle-çi a un impact sur la structuration génétique des populations uniquement sur une espèce de parasitoïde. En effet, deux groupe génétiques très distincts ont été détectés pour *H. butcheri* selon la plante sur laquelle ils se sont développés, et ceci en sympatrie, ce qui suggère fortement que, dans ce système, les plantes ont le pouvoir d'induire la formation de races d'hôtes au troisième niveau trophique.

Ce travail ne se limite pas à étudier des questions fondamentales sur l'écologie d'espèce de parasitoïdes, mais comporte également une application pratique. La compréhension de la structuration spatiale et temporelle de populations d'herbivores et de leurs parasitoïdes est importante tant pour la théorie que pour l'application de programmes de lutte biologique. En effet, le succès d'un ennemi naturel à contrôler une peste est intimement lié à sa capacité d'adaptation à de nouvelles conditions écologiques.

CHAPTER one

Thesis outline

Thesis Outline

Introduction

Tritrophic interactions among plants, insect herbivores and their natural enemies represent the vast majority of interactions among living organisms on earth, as plants and insects represent 79% of the world's biota (Strong et al. 1984). Of the wide diversity found within the Insecta, herbivores (46%; (Strong et al. 1984) and parasitoids (20-25%;(Godfray 1994) comprise the majority of species.

Among phytophagous insects, host-plant specialization is a common phenomenon (Bernays and Graham 1988). More than 90% of herbivorous insects are specialized on a very restricted number of plant families and plant secondary chemistry has been argued to be one of the major forces shaping the tight interaction between herbivores and their host plants (Ehrlich and Raven 1964; Farrell and Mitter 1994; Thompson 1988). While herbivores and plants have an intimate and unambiguous trophic relationship, the reproductive success of the herbivores' natural enemies such as parasitoids depends highly on plant traits. Indeed, parasitoids of herbivores are known to display high plant specialization (Godfray 1994). To find their often specialized and concealed host, female parasitoids often rely on a combination of sensory modalities (Wäckers and Lewis 1994). Mechano-reception allows them to detect vibrations produced by their host while feeding on its substrate (Meyhöfer et al. 1994) and vision helps them to recognize forms, shapes and colours of their host or its direct environment (Wäckers and Lewis 1994). Nevertheless, chemoreception (olfaction and taste) appears to be the most important sensory modality used by hymenopteran parasitoids to localize and identify their hosts (Turlings and Benrey 1998; Turlings and Wäckers 2004; Turlings et al. 1993). Parasitoids are short-lived organisms and therefore their time-allocation for host-location can be greatly optimized by relying on cues available in great quantities and reliably associated with the presence of their hosts (Vet 1992; Vet et al. 1991).

Several behavioural studies have shown that parasitoids often rely on cues emanating from the plant on which their host feeds rather than on cues emanating from their host itself (De Moraes and Mescher 1999; McCall et al. 1993; Turlings et al. 1991). Parasitoids of bruchid beetles such as, *Uscana lariophaga* (Trichogrammatidae) (van Huis et al. 1994) *Eupelmus vuilleti* (Eupelmidae) (Cortesero et al. 1993) and *Dinarmus basalis* (Pteromalidae) (Aebi 1999) were all more attracted to plant volatiles than to volatiles emanating from their hosts. Moreover, studies conducted with lima beans, corn, cabbage and cotton have demonstrated that plants are actively involved in the production and release of the chemicals used by parasitoids to localize their host (Agelopoulos and Keller 1994; Dicke 1995; Dicke and Sabelis 1988; Dicke and Sabelis 1989; Mattiacci et al. 1994; Takabayashi et al. 1991; Turlings et al. 1995; Turlings et al. 1991; Turlings et al. 1990). Studies on the mechanisms of volatile emission revealed that chemical compounds of the herbivore's saliva were responsible for the induction of volatiles emitted by the host plant (Alborn et al. 1997; Mattiacci et al. 1995). Apart from being emitted in large quantities through a systemic response (Cortesero et al. 1997a; Dicke et al. 1993; Turlings and Tumlinson 1992), the released odour cues are very informative for parasitoids. Indeed, the blend of herbivore-induced volatiles is specific to the plant species (Turlings et al. 1993), to the herbivore species (De Moraes et al. 1998; Dicke 1995) or to the different instars of the same herbivore (Takabayashi et al. 1995). The role of plants carries on after host location as plants have evolved a wide array of chemical defences against herbivores that directly or indirectly affect the parasitoid. The modes of action of plant secondary compounds are very diverse. Some compounds are known to repel insects or inhibit feeding while others aim to poison the insect or reduce its ability to digest food (Futuyma 1983; Giamoustaris and Mithen 1995; van Dam et al. 2000). In turn, herbivores have evolved various strategies to defeat these defence mechanisms. While many toxic compounds are detoxified by physiological means, the negative effect of other compounds can be avoided by means of behavioural adaptations (Evans et al. 2000; Futuyma 1983). Some insects have evolved a more subtle strategy consisting on the sequestration of toxic compounds produced by the plant for their own defence against natural enemies (Ehmke et al. 1999; Futuyma 1983; Rowell-Rahier et al. 1991). Because of the intimate trophic interactions between immature parasitoid and their hosts, plant chemistry indirectly affects the fitness of parasitoids (Bottrell et al. 1998; Turlings and Benrey 1998). The survival, development, size, fecundity, longevity and the sex ratio of parasitoids of herbivores is affected by plant chemistry (Godfray 1994; Vinson and Iwantsch 1980). The effects of sequestered plant secondary compounds on the parasitoid have been studied in the case of cultivated tomato and tobacco and their associated herbivores and parasitoids (Barbosa and Saunders 1985; Barbosa et al. 1986; Campbell and Duffey 1979; Campbell and Duffey 1981; Duffey et al. 1986; Kester and Barbosa 1991; Thurston and Fox 1972). The development time, survival and adult size of the ichneumonid *Hyposoter exiguae* is altered by the concentration of tomatin, an allelochemical present in tomato plants and sequestered by the noctuid *Heliothis zea* (Campbell and Duffey 1979; Campbell and Duffey 1981). Similarly, high levels of nicotine in the diet of the tobacco hornworm, *Manduca sexta*, decrease the survivorship of the parasitoid *Cotesia congregata* (Thurston and Fox 1972).

The quality of an herbivore as a host for a parasitoid depends heavily on the plants or plant parts that they feed on. Variation in plant nutritional quality due to genetic or environmental factors may also affect parasitoids indirectly by affecting the host's suitability and vulnerability (Benrey and Denno 1997; Benrey et al. 1998). There is evidence that variation in plant nutritional and chemical quality do not affect the performance of interacting organisms across several trophic levels (Harvey et al. 2003). For example, a study of a four-trophic-level system comprising the solitary secondary hyperparasitoid *Lysibia nana* (fourth trophic level), its primary endoparasitoid host, *Cotesia glomerata* (third trophic level) and the herbivore host *Pieris brassicae* (second trophic level) feeding on two cruciferous plants, *Brassica oleracea* and *B. nigra* (first trophic level) which vary in glucosinolates concentrations revealed that plant variability had no effect on the second and third trophic levels but dramatically affected the performance of the fourth trophic level (Harvey et al. 2003). The poor correspondence between the performance of the second, third and fourth trophic levels can be explained by their host specialization. Indeed, *L. nana* has a broad host range (Harvey et al. 2003) while *C. glomerata* only attacks pierid butterflies feeding on plants containing glucosinolates (van Loon and Schoonhoven 1999) and *P. brassica* on its turn, is highly specialized on crucifers (Schoonhoven 1967). Consequently *L. nana* is likely to be less adapted to the highly chemically defended *B. nigra* than are the specialized *P. brassicae* and *C. glomerata*. Another explanation for the poor correspondence reported between the performance of parasitoids and their host on certain plant species (Englishloeb et al. 1993) could be that variation in plant quality can interfere with the herbivore's immune response thereby affecting the host's ability to encapsulate parasitoid eggs (Benrey and Denno 1997; Cheng 1970; Rhoades 1983). Indeed, the effectiveness of the encapsulation reaction depends on the physiological condition of the host, which can be weakened by poor nutritive quality or the presence of toxins (Muldrew 1953; Salt 1956, 1964; van den Bosch 1964; Vinson and Barbosa 1987).

Herbivore's growth rate and development depend on the nutritional quality of the plant they feed on. Since prolonged development may result in greater mortality caused by natural enemies (Clancy and Price 1987) plant nutritional quality can indirectly affect parasitoids as herbivores feeding on different plants that vary in nutritional quality may grow at different rates.

As exemplified above, food webs are not restricted to three trophic levels. Parasitoids are often attacked by hyperparasitoids and may therefore benefit from sequestered secondary plant compounds for their own defence. Sequestration of plant secondary compounds by parasitoids via their herbivore host was first proposed by (Meiners et al. 1997) and evidence for such process is reported for the first time by Harvey and colleagues (2003).

The evidence presented in the above sections leaves little doubt that plant traits can affect parasitoid performance and behaviour. Therefore, it could be expected that plants may also affect the genetic population structure of parasitoids. The importance of population structure in the evolution and ecology of species has been long recognized (reviewed in Barton and Clark 1990, and in Hastings and Harrison 1994). Numerous studies have examined the geographic structure of single species, but only recently has the importance of the interactions among species been incorporated into research on population structure (Althoff and Thompson 1999; Thompson 1994).

Population structure arises from the genetic differentiation of populations due to processes such as natural selection, genetic drift, inbreeding, assortative mating and gene flow (Roderick 1996). Natural selection is the driving force of evolution. Adaptation to one's environment is attained by natural selection of a specific gene combination that will provide a fitness advantage to its carrier and therefore allow it to transmit more genes to the next generation. Population genetic structure can be greatly affected by the type of environment and the ability of its inhabitant to adapt to it. A species may be subject to varying environments within its distribution range. These environmental variations may result in locally very different selective forces. The different output of natural selection in local conditions will lead to genetic divergence through disruptive selection. In contrast, in the case of a stable environment, natural selection will prevent genetic population structure by normalizing selection.

Divergent selective forces may lead to local adaptation as adaptation to a given environment often has a cost and prevents simultaneous adaptation to an alternative environment (Thompson 1996). Studies of local adaptation in phytophagous insects have shown that local adaptation can occur at a very fine scale (Edmunds and Alstad 1978, Karban 1989, Hanks and Denno 1994, Alstad and Corbin 1990), and therefore induce genetic population structure. Although numerous studies have sought genetic evidence for host plant specialization by herbivorous arthropods (Jaenike 1990, Via 1990, Mopper 1996), providing mixed results, very few studies have examined the parallel situation with their natural enemies.

Adaptive differentiation to ecological conditions may lead to the occurrence of biotypes or ecological races, which are populations that have some genetic of phenotypic differentiation from another population within a species (Ford et al. 1998; Gallun et al. 1975; Gould 1991; Johnsson and Johnsson 2001; Kondrashov and Mina 1986; Lu and Bernatchez 1999; Macnair 1989; Quicke et al. 1983; Schluter and Nagel 1995; Taylor and McPhail 1999; Wang et al. 1999). The close interactions between parasites and their hosts triggered the occurrence of host-associated biotypes referred to as host races. Bush, in 1969 proposed the first definition of host race. Since then many definitions were proposed (Berlocher 1999; Bush 1969; Bush 1992; Diehl and Bush

1984; Jaenike 1981) and a recent review from Drès and Mallet (2002) to the following definition: "Host races are genetically differentiated, sympatric populations of parasites that use different hosts, and between which there is appreciable gene flow" (Drès and Mallet 2002).

Genetic drift is the random variation in allele frequencies observed from one generation to the next. Genetic drift can have a profound effect on the genetic population structure of small populations. Smaller populations may suffer larger gene frequencies fluctuations than larger populations and result in a reduction of genetic diversity by loss of certain alleles or by fixation of others alleles. Wright F-statistics that examine heterozygote deficiencies within and among populations constitute a powerful indirect tool to detect population subdivision and thereby random genetic drift. The inbreeding coefficient Fis examines heterozygote deficiencies within populations, Fst measures heterozygote deficiencies among populations and Fit evaluates the overall heterozygote deficit (Wright 1921, Wright 1969).

Colonization of suitable environments by specialist parasitoids and thereby population size heavily depends on the spatial distribution of their hosts and of their host-plants. Fluctuating host plant and/or host populations (Pimm et al. 1988; Schoener and Spiller 1992), distance between suitable patches (Hanski 1999), and weather conditions (Hanski 1994, Van Nouhuys and Hanski 1999) may limit colonization of suitable environments by parasitoids and therefore affect their population size and result in genetic drift.

One of Hardy-Weinberg main assumption of random mating is often not fulfilled in nature, as real panmictic species are rare. Inbreeding is caused by mating between relatives in a population and leads to a loss of heterozygosity. While inbreeding depression is caused by the segregation of deleterious recessive alleles, the overall viability of an inbred population tend to decline. Nevertheless, the effect of inbreeding depression on the fitness of a population varies. While it can be detrimental to certain species, others commonly undergo inbreeding without noticeable changes in the population viability.

Like inbreeding, assortative mating causes a non-random association of alleles. In contrast with inbreeding it implies individuals sharing similar phenotypes, which are not necessarily relatives.

There are two types of assortative mating with very different outputs. While positive assortative mating between individuals sharing phenotypes will increase homozygote frequency and hence reduce genetic variability, a negative assortative mating between genetically different individuals will increase heterozygote frequency.

Mating frequency depends on the spatial and temporal distribution of males and females in the environment. Parasitoids are known to rely on several environmental or species- specific cues to increase their mate encounter rate. Mating at the emergence site is very common in insects (Landolt and Phillips 1997), and specifically among parasitic Hymenoptera (Askew 1968; Godfray 1994). Assortative mating and inbreeding are favoured by the fact that female parasitoids often parasitize a series of hosts in close proximity and by the behaviour of males which emerge earlier than females and wait for mates at the emergence sites (Askew 1968).

While natural selection, genetic drift, inbreeding and assortative mating tend to induce genetic differentiation in a population, gene flow has a homogenizing effect. Gene flow is the process by which new alleles are injected in a population through migration of some individuals between populations. The homogenizing effect of gene flow is rapid as one successful migrant every two generations is sufficient to erase the genetic differentiation caused by genetic drift.

There are four models of gene flow. The continent-island model describes one-way allele movement from a large population to a small population and predicts convergence of allele distribution similar to the one of the large population. The island model describes random allele movement among a set of populations and predicts convergence of allele distribution to the average of the set of populations. The stepping-stone model is a particular case of the island model, as migration occurs along a linear set of populations. In this case each population only receives genes from neighbouring populations. Finally, the isolation by distance model describes gene flow among a continuous set of populations. The output of both stepping-stone and isolation by distance models predicts a continuous variation of allele frequencies among populations.

Chalcidoid parasitoids are known to be very efficient dispersers (Askew 1968). Adult dispersal is an adaptation to localize small isolated host populations and is achieved by their small size enabling them to be carried by air current or by accidental human transportation. As a consequence of high dispersal ability chalcidoid populations are less susceptible to be found in prolonged geographical isolation.

Numerous studies have examined the geographic structure of single species, but only recently has the importance of the interaction among species has been incorporated into research on population structure (Thompson 1994). Studies conducted on parasitoids have approached this either by comparing the population structure of parasitoids with the population structure of their hosts (Althoff and Thompson 1999; Hufbauer and Via 1999), or by examining the spatial distribution and genetic basis of traits influencing the interaction between parasitoids and their hosts (Carton and Nappi 1991; Kester and Barbosa 1991; Kraaijeveld and Vanalphen 1995).

Using a model system comprising beans of the genus *Phaseolus*, bruchid beetles and their associated parasitoids, we aimed to assess the relative role of biotic factors such as, host species, and host-plant species and abiotic factors such as, geographical distance, altitude and climatic traits on the genetic population structure of a complex of hymenopteran parasitoids.

Biological System

Host plants

The Mexican mountains are the centre of origin of several bean species of the genus *Phaseolus* (Fabales: Fabaceae) (Fig. 1) and therefore harbour a large number of bean species and varieties (Freytag and Debouck 2002). Although the bean species collected for this study can be found in sympatry, they differ greatly in their normal habitat characteristics. The wild relative of the Lima bean, *P. lunatus* grows between sea level and 1600 m in deciduous tropical forests, in moist areas. The ancestor of the common black bean, *P. vulgaris* has a wider altitudinal range and can be encountered between 50 and 3000 meters in dry deciduous forests, but especially between 1500 and 1900 m (Delgado-Salinas et al 1988). Finally the ancestor of the ayocote, *P. coccineus*, is restricted between 1400 and 2800 m in pine-oak forests (Freytag and Debouck 2002).

Figure 1. Wild *P. coccineus, P. vulgaris* and *P. lunatus* (from top to bottom). Habitus. Photo: Aebi & Heil.

Herbivores

Bruchid beetles (Coleoptera: Bruchidae) are common pests of beans in the field and in storage conditions and cause enormous economic losses to cultivated beans (35% in Mexico and Central America, 7.4% in Colombia and 13% in Brazil, (Van Schoonhoven and Cardona 1986)). Female bruchids deposit their eggs in the bean pod or on the surface of the seeds. When the egg hatches, the larvae bore the seed coat, penetrate inside the seed where they will develop and from which they will emerge as adults. The Mexican mountains harbour very diverse environmental conditions varying from hot/humid to cold/dry regions distributed along a wide altitudinal gradient. Beans of the genus *Phaseolus* are attacked by several bruchid species in two main genera: *Acanthoscelides* and *Zabrotes* (Fig. 2). Among the species of *Acanthoscelides*, *A. argillaceus* Sharp, has a distinct bright orange coloration of the elytra and is specialized on *P. lunatus* (Leroi et al. 1990) While *A. obtectus* Say and *A. obvelatus* Bridwell, which feed on *P. vulgaris* and *P. coccineus*, are only distinguishable by very few morphological characters and share identical environments as both species can be found in sympatry on the same host-plant, and even in the same pod (Alvarez et al. 2004a). The maintenance of both species through reproductive isolation (Alvarez et al. 2004a) in the same environment is thought to be due to their differentiated ecological distribution and niche segregation (Alvarez et al. 2004b). *A. obtectus* is more common at low altitudes, on cultivated bean populations whereas *A. obvelatus* is more common at high altitudes, on wild bean populations (Alvarez et al. 2004b). Furthermore their differences in reproductive biology explain partially their distribution (Alvarez et al. 2004b). *A. obtectus* is multivoltine and the ability to produce several generations per year gives it an advantage in low-altitude granaries. In contrast, *A. obvelatus* is univoltine and its capacity to enter into reproductive diapause associated with a prolonged lifetime enables it to survive between two time restricted fruiting seasons and thereby to maintain itself on wild bean populations (Alvarez et al. 2004b).

For the genus *Zabrotes*, the existence of two sibling species *Z. subfasciatus* and *Z. sylvestris,* attacking beans in Mexico was discovered (Romero and Johnson 1999) and their sibling species status was confirmed by the absence of gene flow between them (Gonzalez-Rodriguez et al. 2002). Like their host plants, both *Zabrotes* bruchid beetles originated in Mexico and Central America (refs) and may therefore have a common coevolutionary history.

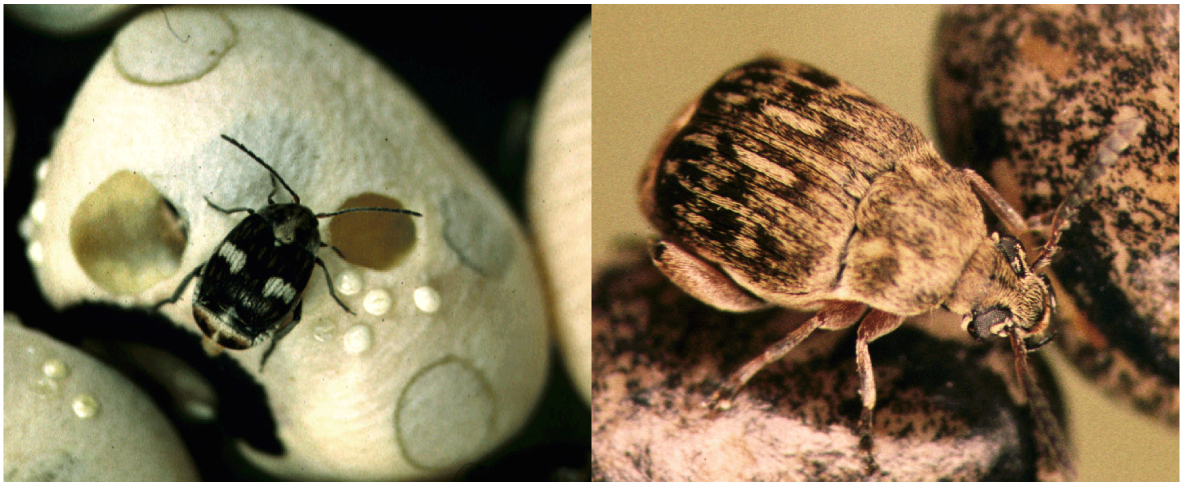

Figure 2. Bruchid beetles of the genus *Zabrotes* (left) and *Acanthoscelides* (right). Habitus. Photo: Borcard.

Parasitoids

The parasitoid guild that attacks this bruchid complex on wild and cultivated beans comprises 6 families of parasitic wasps (Campan and Benrey 2004; Delgado-Salinas et al. 1988; Rios, 1998). Among the various families, the genus *Horismenus* represents the most abundant parasitoid in that system (Fig. 4). A taxonomic survey revealed that the genus *Horismenus* was constituted of three species: *H. depressus* Gahan, *H. missouriensis* Ashmead and *H. butcheri* Hansson & Aebi (Hansson et al. 2004) (CHAPTER one).

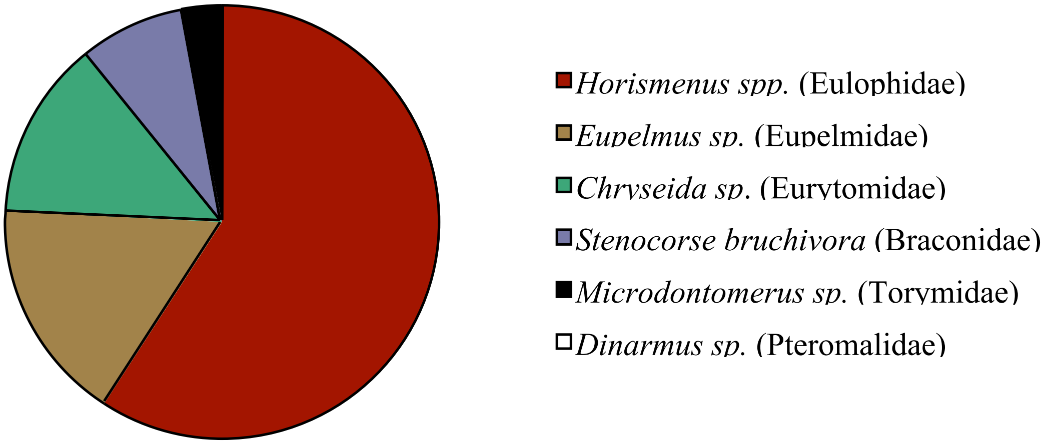

Figure 3. Parasitoid guild attacking *Acanthoscelides sp.* and *Zabrotes sp.* on beans in Mexico.

Parasitoids, as the natural enemies of bruchid beetles, have the potential to provide biological control under stored conditions. Although the confined space in seed granaries presents harsh conditions (reduced air flow, humidity and light) for parasitoids, one study showed their ability to reduce bruchid infestation in such environments. *Eupelmus vuilleti* Crawford (Hymenoptera: Eupelmidae) displayed high efficiency in finding their patchily distributed hosts, thereby demonstrating its ability to establish populations in granaries (Cortesero et al. 1997b).

Various studies have characterized the use of endemic parasitoids in augmentative and conservation biological control of bruchid populations in Africa (Dugravot et al. 2002; Ketoh et al. 2002; Leveque et al. 1993; Sanon et al. 1998; Sanon et al. 1999; van Huis et al. 2002), but little attention has been paid to hymenopteran parasitoids as control agents in Mexico, Central and South America. Female parasitoids drill a hole in the seed coat with their ovipositor and usually deposit their eggs on the surface or inside their host. At emergence, the parasitoid larva will consume its host, pupate and the imago will emerge from the seed leaving the remains of its host behind.

Horismenus missouriensis (Fig. 4.) was the only parasitoid species found in storage conditions, on cultivated beans and are therefore a potential candidate for a biological control program (see CHAPTER four).

Figure. 4. *Horismenus missouriensis*. Habitus. Photo: Borcard.

Experimental procedure

Collection

Parasitoids and their hosts were reared from wild bean samples of *P. lunatus, P. vulgaris* and *P. coccineus* collected in 66 populations from various states of Mexico (Federal District, Mexico, Morelos, Puebla and Michoacan) following a hierarchical sampling scheme (Fig, 5) (see CHAPTER five). Populations were characterized according to biotic (host-plant species, host abundance, parasitoid species composition) and abiotic (location, altitude, climatic traits) variables.

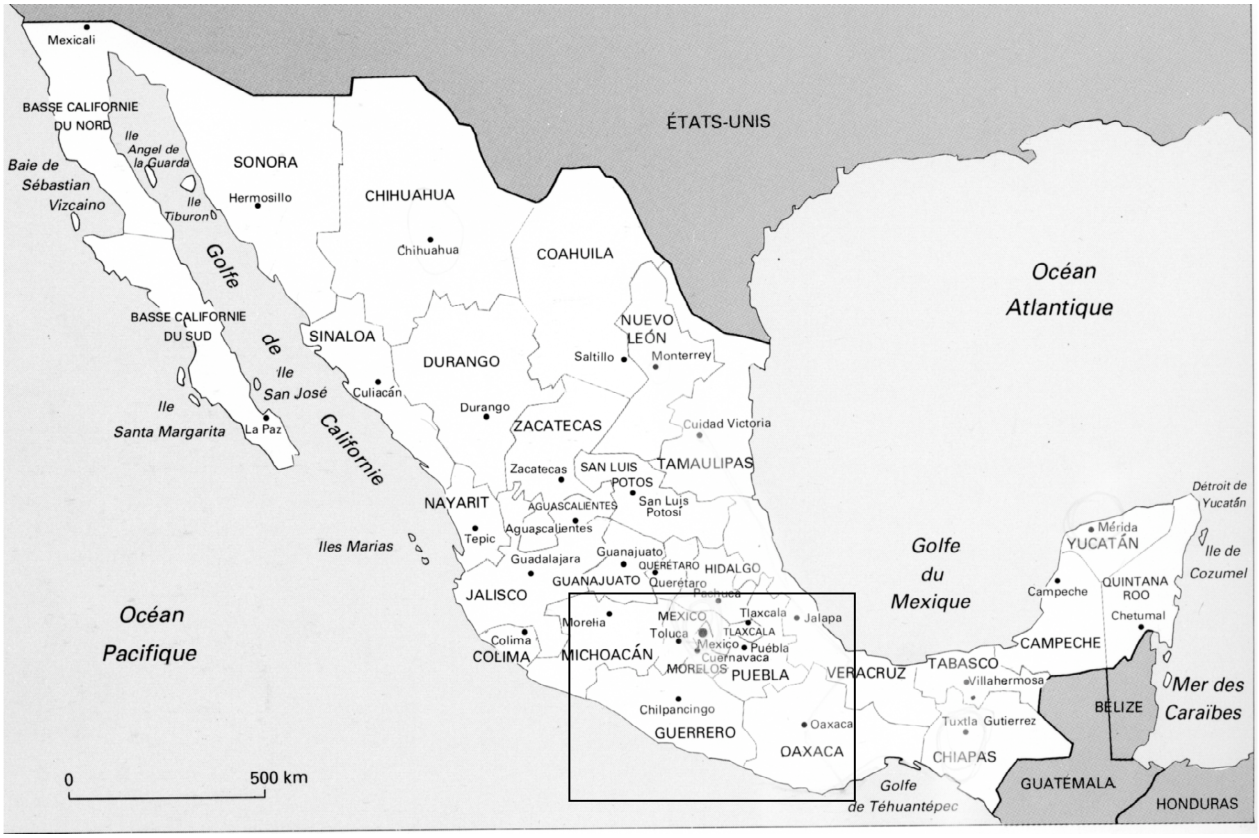

Figure 5. Study zone in Mexico.

Taxonomic identification

The genus *Horismenus* is predominantly a New World group, with its main distribution in the Neotropical region. Currently there are 53 species from the Americas (ten from the Nearctic, 39 from the Neotropics, and four from both regions), and one species from Europe. The species are parasitoids or hyperparasitoids on a variety of hosts, most commonly on larvae of Coleoptera, Diptera and Lepidoptera (LaSalle and Schauff 1995). Even though *Horismenus* is one of the most frequently encountered groups of Eulophidae in the Neotropical region very little is known of the genus from this biogeographical region. The majority of the species remain undescribed and the identities of many of the about 50 already described species are unclear due to poor original descriptions, missing type specimens, lack of revisions, etc.

A taxonomic key based on morphological characters (shape and ornamentation of the scutelum, shape and length of the first funicular segment, shape of the gaster and coloration of the frons) was developed and used to determine the *Horismenus* species associated with *Acanthoscelides spp*. on *Phaseolus spp*. in Mexico (see CHAPTER two).

Microsatellite molecular markers

Microsatellite markers were developed, characterized and used to determine the genetic population structure of the parasitoids. Microsatellites (also referred to as "SSRs" simple sequence repeats, "STR" short tandem repeats or simple repeats) are tandem repeats of short (1-6 bp) motifs (e.g. (CA)n, where n = number of repeats). The type of repeated motif is designated by terms as mono- (e.g. (A)n), di- (e.g. (CA)n), trinucleotide (e.g (AAT)n) etc. Microsatellite DNA sequences mutate at a rate several magnitudes higher than "normal" DNA which ensures a great polymorphism, crucial for population genetic analysis (Ellegren 2000). Microsatellites are genotyped by PCR using primers developed in the flanking region of the studied locus, which is usually conserved across closely related taxa. Electrophoresis through an acrylamide gel allows resolution of alleles that may differ in size by as few as two base pairs. Microsatellites have been successfully used to investigate divergence over microgeographic scales, and to detect high levels of genetic variation when other methods (e. g. allozyme electrophoresis) fail to do so (Hughes and Queller 1993; Roderick 1996). In addition, their Mendelian, co-dominant and selectively neutral characteristics define them as powerful tools for the study of population structure (Strassmann et al. 1996). (see CHAPTER three)

Molecular Differentiation and Phylogenetic Relationships among Horismenus spp.

Horismenus depressus, H. missouriensis and *H. butcheri* are morphologically very similar and differ only in a few morphological characters (see chapter two). Additionally, they have very diverse ecological distributions that confirms their species status. We used microsatellite and mt-DNA data to determine the degree of genetic similarity between *H. missourensis*, *H. depressus* and *H. butcheri*, to confirm the status of the third as a new and distinct species and to provide additional characters that can be used to differentiate them. Both molecular markers, the nuclear microsatellites and the mt-DNA haplotype sequences, further support the existence of *Horismenus depressus*, *H. missouriensis* and *H. butcheri* as differentiated species although potential hybridization may occur among them. The genetic analysis showed that *H. butcheri* form a well differentiated clade while *H. depressus* and *H. missouriensis* did not cluster in well separated clades, probably due to ancestral polymorphism retained by these very closely related species. (see CHAPTER four)

Ecological description

Multivariate analysis is aimed to extract comprehensive patterns from large, complex data sets. Therefore, such techniques have been widely used in the analysis of ecological data. The most common use of multivariate analysis is to try to match environmental data with species abundance data in order to explain the environmental requirements of a species. A redundancy analysis (RDA) was performed using counts of *H. depressus, H. missouriensis* and *H. butcheri* as species data and altitude, annual mean temperature (obtained from INEGI, Instituto Nacional de Estadistica Geografia e Informatica, Medico), plant of origin and infestation rate (where B= number of bruchids and P= number of pods) as environmental variables (see CHAPTER five).

Behavioural analysis

The degree of host-plant fidelity was tested for each *Horismenus* species separately in a four-chamber olfactometer. Attraction of males and females to volatile cues emanating from their plant of origin and an alternative plant was tested. Walking and resting time on the sectors containing the different chemical stimulus were analysed using the software THE OBSERVER (Noldus 1988) (see CHAPTER five).

An olfactometer is an apparatus used by behavioural ecologists to measure the behaviour of organisms when confronted to an olfactory stimulus. Two classes of olfactometer exist. The first one utilizes an air stream and the volatiles tested are carried downstream in a regular odour plume. The behavioural response of the studied organisms is then recorded as they enter or leave the plume. The second class of olfactometer is much simpler as no air stream is generated. Odour sources are placed in a dispenser and evaporate from it creating a gradient of concentration in which the behaviour of the organisms is measured. Depending on the displacement mode of the organisms, behavioural ecologists developed olfactometers that measure changes in direction while walking and wind tunnels that are used with flying organisms. It is crucial that the apparatus used is suitable for the organism tested. Ecologists have been very inventive, as many forms of olfactometer have been designed to perform choice tests. Olfactometers enabling testing one odour (one-way olfactometer) to more complex ones enabling testing six odour sources simultaneously (six-way olfactometer) have been developed (Cortesero et al. 1993; Steinberg et al. 1992; Turlings et al. 2004). Increasing the number of odour source increases their resolving power as many trials may be necessary to demonstrate non-random choice when testing two odour sources in a two-way olfactometer (Hare 1998). Nevertheless, olfactometers have been widely used in entomology and provide a wonderful tool to test hypotheses that cannot be verified in the field under natural conditions. Behavioural ecologists have described a wide variety of behaviour clearly associated with searching. While movement and turning rate can easily be measured, complex behaviours such as probing (insertion of the ovipositor into a host to assess its quality), oviposition, antennating (drumming of the substrate with the antennae to detect semiochemicals), grooming have been described for parasitoids (Vinson 1976) (see CHAPTER five).

Determinants of population genetic structure

A hierarchical sampling of infested bean samples on various host-plant patches within populations in various regions located in various states of Mexico enabled us to analyze the population structure of parasitoids of the genus *Horismenus* in relation to host-plant species and to isolation by distance. Descriptive statistics were obtained using the software FSTAT (Goudet 2001) and GENEPOP (Raymond and Rousset 1995) and population structure was evaluated with STRUCTURE (Pritchard et al. 2000) (see CHAPTER five).

Ecological differentiation and niche segregation of Z. subfasciatus.

Correct identification of both the pest and the natural enemies is crucial for the establishment of a successful biological control program (Rosen 1986). The ecological distribution of species in the genus *Acanthoscelides* has been recently documented (Aebi et al. 2004; Alvarez et al. 2004b). Therefore, this study was undertaken to describe the ecology of *Zabrores spp.*, the second main genus in this complex tri-trophic system. *Phaseolus lunatus*, the ancestor of lima bean is a new-world legume that was domesticated in this region of the world (Kaplan 1971). The fact that *Z. subfasciatus* attacks wild and cultivated *P. lunatus* suggests that through their long evolutionary history, this bruchid species has evolved resistance to the chemical defence of the plant. A multivariate analysis approach was used to determine the role of altitude and host-plant species on the ecological distribution of individuals of *Z. subfasciatus* and *Z. sylvestris* collected on the different host-plant species along an altitudinal gradient. Specimens were determined using a taxonomic key based on genitalia morphology (Romero and Johnson 1999) (see CHAPTER six).

Genetic similarity between H. missouriensis from wild and cultivated beans.

To determine the origin and genetic similarity of individuals of *H. missouriensis* attacking bruchids in storage conditions and in the wild, we used mitochondrial DNA sequences. Mitochondrial DNA (Mt-DNA) is constituted of a small, circular molecule of 15-17 kb inherited through non-Mendelian, cytoplasmic and often uniparental transmission. The rate of mutation of Mt-DNA is greater than in coding regions of nuclear DNA and therefore displays considerable variation within and among populations (Parker et al. 1998). Mt-DNA sequences have been widely used to infer genetic population structure or to construct phylogenies.

We used cytochrome oxydase I gene sequences to construct a phylogenetic tree based on distance, to evaluate the genetic distance between *H. missouriensis* individuals collected on various host plants (wild and cultivated *P. vulgaris,* wild *P. coccineus* and *Leucaena sp.* an alternative host plant of the family Mimosacea) (see CHAPTER six).

Summary of the Results

Ecological description

Despite their close taxonomic position, the three *Horismenus* species have specific ecological requirements allowing them to take advantage of different ecological niches scattered along the altitudinal gradient. *H. depressus* is the most abundant parasitoid below 1700 m and the only one found on *P. lunatus*, a bean common at low altitudes. *H. missouriensis* the most abundant above 1700 m and the only parasitoid reared on *P. coccineus*, a bean growing at high altitudes. Finally, *H. butcheri* is the only species displaying strict host plant specialization as it was only collected on *P. vulgaris* seeds, with the notable exception of samples collected on *P. coccineus* in sympatry with *P. vulgaris* (see CHAPTER five).

Behavioural analysis

The olfactometer assays revealed that all three *Horismenus* species are more attracted by odours from their host plant of origin than by the odours of alternative plant species. Indeed, males and females of each species spent significantly more time walking and resting on the sector containing pods of their host plant of origin (see CHAPTER five).

Determinants of population genetic structure

Results from the analysis on population structure gave contrasting results for the three *Horismenus* species. No isolation by distance was found for *H. depressus*. Genetic differentiation was only detected between very distant populations or between regions separated by a physical barrier (mountain peaking at 4578 m). No signature of host-beetle mediated genetic differentiation was detected. Host-plant mediated genetic differentiation could not be tested for this parasitoid because of low sample sizes. Nevertheless, the strong association of this species with *P. lunatus* and its ability to use *P. vulgaris* as an alternative plant in sympatry warrants further genetic analyses on this system.

No isolation by distance was detected for *H. missouriensis*. As the only species found on cultivated beans, its high dispersal ability is probably due to bean trade among farmers. The result from STRUCTURE revealed the existence of two genetic pools among all the genotyped populations independently of the host plant, suggesting a host-beetle mediated genetic differentiation. No evidence for host plant mediated genetic differentiation was found.

Finally, *H. butcheri* was the only parasitoid showing host plant mediated genetic differentiation, as the level of genetic differentiation was similar between populations and between host plants within populations. The results from STRUCTURE support the role of the host plant in having determined genetic differentiation. These results strongly suggest a shift of part of the population growing on *P. vulgaris* to *P. coccineus* and a subsequent genetic differentiation triggered by assortative mating (see CHAPTER five).

Ecological differentiation and niche segregation in Z. subfasciatus.

Counts of *Zabrotes* on the different bean species sampled revealed that *P. lunatus* is the bean species harbouring the major number of *Zabrotes* bruchid beetles while only a few individuals were reared from *P. vulgaris* seeds and no *Zabrotes* were collected on *P. coccineus*.

The identification of the *Zabrotes* species collected on *P. lunatus* and *P. vulgaris* along an altitudinal gradient revealed a clear altitude and host-plant segregation pattern. Most *Z. subfasciatus* were collected from sea level to 1000 m, on *P. lunatus* with some individuals collected on *P. vulgaris* while all *Z. sylvestris* were collected from 1200 m to 2000 m on *P. vulgaris*. These results show that as in the case of *Acanthoscelides obtectus* and *A. obvelatus*, *Zabrotes subfasciatus* and *Z. sylvestris* have well differentiated ecological distribution and niche segregation (see CHAPTER six)

Genetic similarity between H. missouriensis from wild and cultivated beans

The phylogenetic analysis of *H. missouriensis* individuals collected on wild and cultivated *P. vulgaris*, wild *P. coccineus* and an alternative host plant revealed no host-plant specialization. In fact, the well-supported branches of the tree harboured individuals from various origins. Furthermore, close genetic similarity was observed between individuals collected from wild and cultivated bean samples showing that *H. missouriensis* individuals attacking bruchids in granaries come from wild bean populations. These results suggest that *H. missouriensis* is a generalist parasitoid attacking bruchid beetles on a wide variety of plants (see CHAPTER six).

Conclusion

Plants at the base of tritrophic interactions can have a profound effect on the ecological genetics of parasitoids. Moreover, closely related organisms can have very different ecological requirements. The interactions between parasitoids of the genus *Horismenus* and their host plants are very diverse. Two of the parasitoid species studied here appear to have benefited from their wide altitudinal range and have evolved the unique capacity to colonize chemically defended plants. While the third species, *H. butcheri* appears to be much more specialized, parasitizing bruchids on only one bean species (*P. vulgaris*). The strength of the parasitoid-host plant association is also very broad among *Horismenus* species. Although all species displayed preference for their host plant of origin, host plants only had an effect on the population genetic structure of one species of parasitoid. The two very distinct genetic groups found for *H. butcheri* when emerging from different plant species within the same population, strongly suggest that plants could mediate the formation of host races at the third trophic level in this system.

Sympatric speciation has only recently been accepted as an important evolutionary mechanism (Via, 2001). Since then, many examples of sympatric speciation via host race formation have been described for phytophagous insects (Abrahamson et al. 2001; Carroll and Boyd 1992; Feder et al. 1988; Mc-Pheron et al. 1988; Via 1999; Via 2000; Wood and Guttmann 1982). However, we believe that with this study, we present the first evidence of host plant mediated host race formation at the third trophic level.

This work not only has an importance in fundamental research but also has an applied significance. Precise taxonomic knowledge of both pest insects and their natural enemies is essential for the establishment of a biological control program. The ecological data gathered during this work brought some light on the taxonomy and ecology of bruchid beetles as well as of potential parasitoids for the control of bruchid infestations in Mexico and other countries in Central and South America. Bruchid beetles as seed pests have a close and intimate relationship with their host plant. Nevertheless, plants do not affect the ecological distribution of bruchid beetles of the genus *Acanthoscelides* and *Zabrotes* in the same manner. While host plant does not dictate the distribution of the *Acanthoscelides* species, the species of the genus *Zabrotes* displayed strong host plant association.

While a good taxonomic database has a basal role in the comprehension of multitrophic interactions, knowledge of the nature and strength of the interactions among organisms is crucial for a better understanding of the incredible diversity of nature. In the Origin of Species, Darwin first emphasized the importance of both the diversity of species and the diversity of interactions between species in the evolution of biological diversity in his most famous paragraph, "The Entangled Bank" (Darwin, 1859).

Future directions

Interactions among species are the driving forces in shaping biodiversity. Unlike species number, the number of interactions among them is infinite. While answering some questions on this tritrophic system comprising beans, bruchids and their parasitoids, many unresolved questions remain still open, among these:

1. *Is there inter-specific competition among the* Horismenus *species?*

How do the three *Horismenus* species coexist and maintain themselves on a limited resource? Did they evolve dispersal ability to colonize empty niches or did they develop defence mechanisms to impose themselves in a niche?

2. *Is the parasitoid's performance affected by the host plant?*

Do the different bean species differentially affect the performance of the *Horismenus* parasitoids? Is *H. depressus* adapted to the cyanogenic compounds present in *P. lunatus*? Is *H. missouriensis* adapted to P. coccineus? Are the host races of *H. butcheri* adapted to their host plants?

3. *Is the parasitoid's performance affected by the host plant?*

Do the different bruchid beetle species differentially affect the performance of the *Horismenus* parasitoids? Are there differences in the suitability of the various bruchid species for parasitoid performances?

4. *Is* P. vulgaris *an hybrid zone between* Horismenus *populations on* P. lunatus *and* P. coccineus*?*

The ecological differentiation among the *Horismenus* species also characterizes them as different taxa, but might also provide an opportunity for hybridisation. *Horismenus depressus* is the only *Horismenus* species found on *P. lunatus* between sea level and 1200 m, and *H. missouriensis* is the only species found on *P. coccineus* between 1700 and 2500 m, but they can be found in sympatry on *P. vulgaris* at altitudes between 1200 and 2100 m (Aebi et al. 2004, see chapter five). The host plant *P. vulgaris* may therefore represent a hybrid zone in which both species can meet and hybridize.

5. *Do symbiotic micro organisms play a role in the genetic differentiation of* Horismenus *species?*

To what extent have symbiotic organisms such as *Wolbachia*, played a role in the genetic differentiation and further speciation events in this complex of parasitoid species? *Wolbachia* are maternally-transmitted alpha-proteobacteria that infect the reproductive tissues of many arthropods. The bacteria often manipulate the reproductive system of their host in various manners. These include the conversion of males into females (feminization), the killing of males, the induction of parthenogenesis and the induction of cytoplasmic incompatibility. The latter may act as a catalyst of speciation mechanisms due to the reduction of gene flow between populations infected with different strains of *Wolbachia* or between infected and non-infected populations.

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CHAPTER two

Horismenus species (Hymenoptera: Eulophidae) in a bruchid beetle parasitoid guild, including the description of a new species.

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Horismenus species (Hymenoptera: Eulophidae) in a bruchid beetle parasitoid guild, including the description of a new species

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Abstract

Four species of *Horismenus* (Hymenoptera: Eulophidae) associated with *Acanthoscelides* spp. (Coleoptera: Bruchidae), three of which are also associated with *Phaseolus* spp., are treated. One of the species, *H. butcheri*, is described as new and the remaining three species are redescribed. All four species are diagnosed in a key. A lectotype is designated for *Holcopelte productus* Ashmead.

Key words: *Horismenus,* Eulophidae, *Acanthoscelides*, Bruchidae, species description, revision, *Phaseolus*

Introduction

This taxonomic work was triggered by an ongoing project studying the effects of plant variability on host-parasitoid interactions and consequences for the genetic population structure of these organisms. Female parasitoids are known to rely on volatile chemical cues emanating from the plant on which their host feeds to localise their hosts (Turlings & Wäckers 2004). While the parasitoid's host-location behaviour and performance can clearly be enhanced by the use of these chemical cues, the impact of plant features such as allelochemistry, nutritional quality or morphology can also alter the parasitoid reproductive success (Barbosa and Benrey 1998; Turlings and Benrey 1998; Karban and Huc 1999). The potential impact of plants on the parasitoids reproductive success raises the question whether plant quality could influence the genetic population structure of the parasitoid populations. A previous study has shown that the performance (parasitism rate, development time and sex ratio) and host-location behaviour of a parasitoid attacking bruchid beetles that feed on the seeds of the genus *Phaseolus* are greatly affected by the plant species and variety on which the bruchid host feeds (Benrey *et al.* 1998).

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For this study, wild bean samples (*Phaseolus vulgaris, P. lunatus* and *P. coccineus*) were collected during three consecutive years (2001-2003, between December and April) in Mexico. The samples (from a total of 49 populations) were found to be infested with bruchid beetles of two genera: *Acanthoscelides* (99.7%) and *Zabrotes* (0.3%) (Coleoptera: Bruchidae). Members of a parasitoid guild comprising the following families were reared from this material: Eulophidae (*Horismenus* spp., 59.1%), Eupelmidae (*Eupelmus* sp., 16.8%), Eurytomidae (*Chryseida* sp. 13.4%), Braconidae (*Stenocorse bruchivora,* 7.7%), Torymidae (*Microdontomerus* sp., 2.9%) and Pteromalidae (*Dinarmus* sp., 0.1%). The genus *Horismenus* was the most abundant parasitoid group and was selected for the second author's population genetic project.

Parasitoids often display plant specificity (Godfray 1994). As preliminary genetic data on a pool of undetermined *Horismenus* individuals showed that specimens originating from different *Phaseolus* species were highly genetically differentiated, we suspected that our initial sample contained several species. In order to avoid misinterpretation of further genetic results it was therefore necessary to ascertain the species identity of the specimens in our samples.

The new species *Horismenus butcheri* turned out to be important in understanding the impact of plant variability on the genetic structuring of parasitoid populations as it is the only species displaying plant-associated genetic differentiation and potential host-race formation (Aebi *et al.* unpublished data).

Beans are of great nutritional value in Central and South America. Bruchid beetles cause enormous economic losses to cultivated beans, 35% in Mexico and Central America, 7.4% in Colombia and 13% in Brazil (van Schoonhoven & Cardona 1986). As parasitoids of the genus *Horismenus* are the most abundant group in this system they might be good candidates for storage pest control. Indeed, several studies have documented the efficacy of endemic chalcidoid parasitoids in reducing storage bruchid infestations in Africa (Leveque *et al.* 1993; Sanon *et al.* 1998; Sanon *et al.* 1999; Van Huis *et al.* 2002). A recent study by Schmale *et al.* (2002) in Columbia showed that while *H. ashmeadii* Dalla Torre attacks bruchids (*A. obtectus*) in the field, it was unable to develop on bruchids under stored conditions (prior to this study the identity and nomenclature of some of the species included here has been very unclear and it is probable that the name *ashmeadii* has been wrongly interpreted in the past, see below “Remarks” under *H. productus*). However, we reared large numbers of *H. missouriensis* from cultivated beans collected on a monthly basis between December and February 2002 in various local markets. The continuance of parasitoid emergences during a period of 3 months confirmed that *H. missouriensis* can be successfully maintained under storage conditions. *H. missouriensis* was the only parasitoid reared from cultivated beans infested with the same bruchid genus as the wild beans. The fact that this parasitoid is also the most common *Horismenus* species found on wild bean samples, suggests that it most likely attacks cultivated beans in the field from where it gets transported by humans into storage facilities. The great species richness of this bruchid

Horismenus parasitoid complex highlights the importance of precise taxonomic identification of these and other beneficial insects for the success of biological control programs.

The genus *Horismenus* is predominantly a New World group, with its main distribution in the Neotropical region. Currently there are 53 species from the Americas (ten from the Nearctic, 39 from the Neotropics, and four from both regions), and one species from Europe. The species are parasitoids or hyperparasitoids on a variety of hosts, most commonly on larvae of Coleoptera, Diptera and Lepidoptera (LaSalle & Schauff 1995). Even though *Horismenus* is one of the most frequently encountered groups of Eulophidae in the Neotropical region very little is known of the genus from this biogeographical region. The majority of the species remain undescribed and the identities of many of the about 50 already described species are unclear due to poor original descriptions, missing type specimens, lack of revisions, etc.

FIGURE 1. *Horismenus missouriensis*, habitus, male.

Abbreviations of morphological terms

HE, height of eye; HW, height of forewing; LG, length of gaster; LM, length of marginal vein in forewing; LW, length of forewing; MM, length of mesosoma; MS, length of malar space; OOL, shortest distance between one posterior ocellus and adjacent eye; PM, length of postmarginal vein in forewing; POL, distance between posterior ocelli; POO, distance between posterior ocelli and occipital margin; ST, length of stigmal vein in forewing; WH, width of head; WM, width of mouth opening; WT, width of thorax across “shoulders”. See Hansson (2002) for illustrations of these terms.

Acronyms of museums

BMNH, Natural History Museum, London, England; CH, collection of Christer Hansson; CNC, Canadian National Collection of Insects, Ottawa, Canada; CNIN, Colección Nacional de Insectos, Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Mexico City, México; MHNN, Museum d’Histoire Naturelle de Neuchâtel, Switzerland; USNM, United States Natural History Museum, Smithsonian Institution, Washington, D.C, USA.

Key to *Horismenus* species from *Phaseolus* spp. in Mexico

1 Scutellum flat, posteromedian part of scutellum smooth and shiny (Figs 12–13) .... ***depressus* Gahan** (female, male)

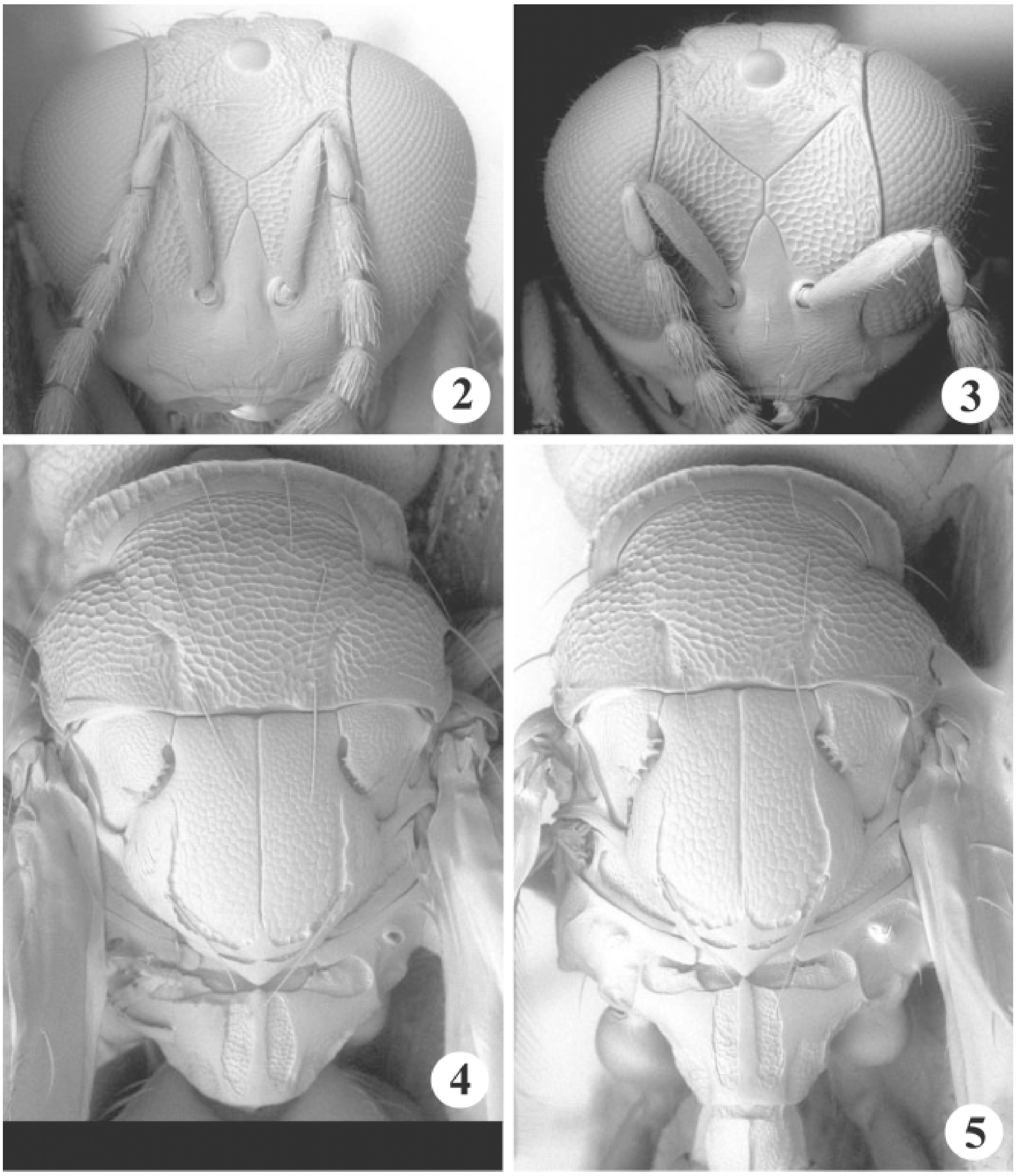
– Scutellum distinctly convex, posteromedian part of scutellum reticulate (Figs 4–5, 16–17) 2

2 Funicular segments longer (Figs 20–21), first funicular segment 3.5X as long as wide in female, 2.5–2.8X as long as wide in male; female gaster long, ratio length of mesosoma/length of gaster = 0.8–0.9 and with apex more pointed (Fig. 6) .... ***butcheri* sp.nov.** (female, male)

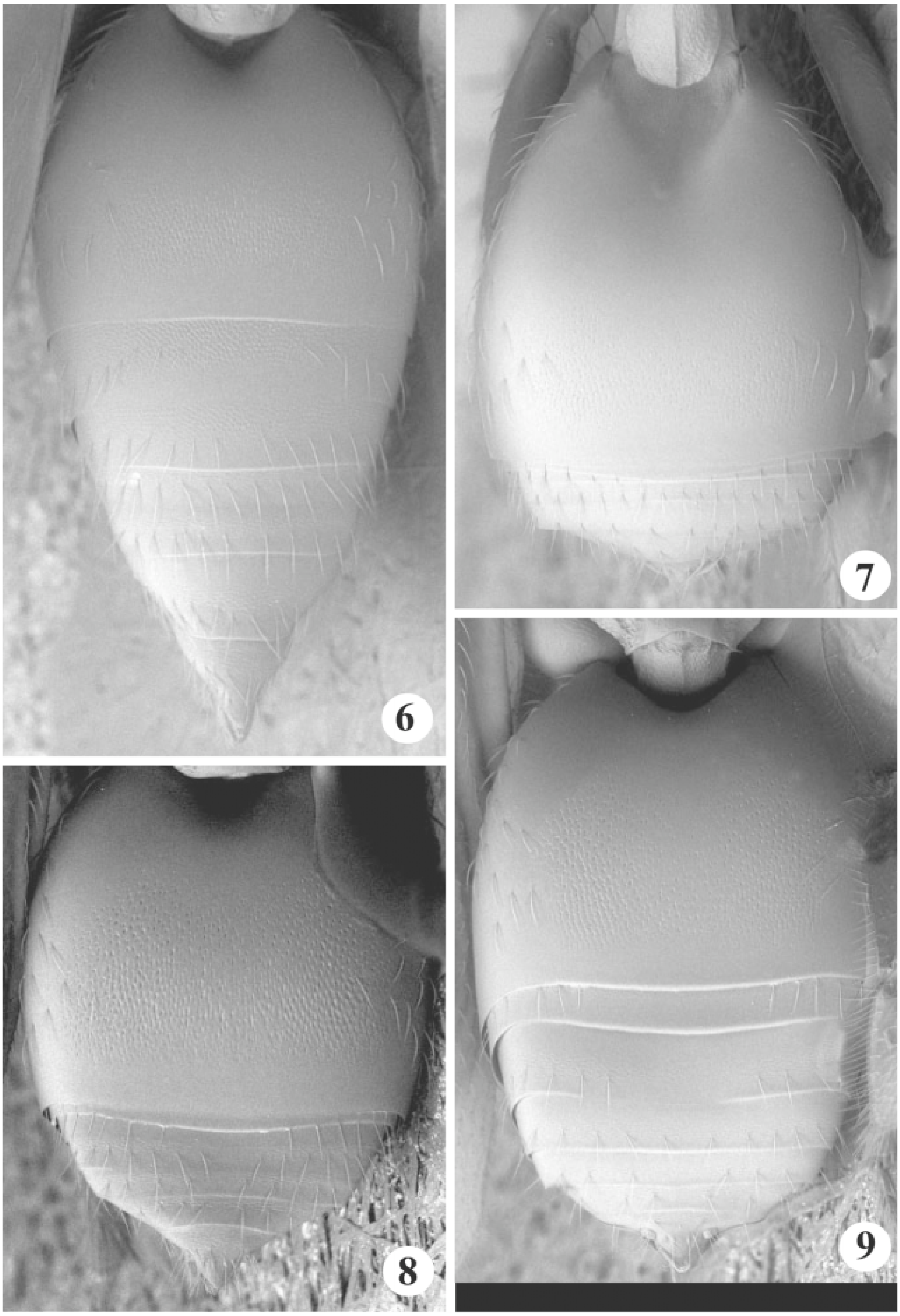
– Funicular segments short and stout (Figs 24–26), female with first funicular segment at most 1.8X as long as wide, male with first funicular segment 1.9X as long as wide (appearance of flagellum in male *productus* not known); female gaster short, ratio length of mesosoma/length of gaster = 1.1–1.8 and with apex more rounded (Fig. 18)33

3 Frons metallic bluish-green in female, metallic purple in male; female with first funicular segment 0.8X as long as second funicular segment (Fig. 24); female gaster longer than in alternate, ratio length of mesosoma/length of gaster = 1.1 .... ***missouriensis* (Ashmead)** (female, male)

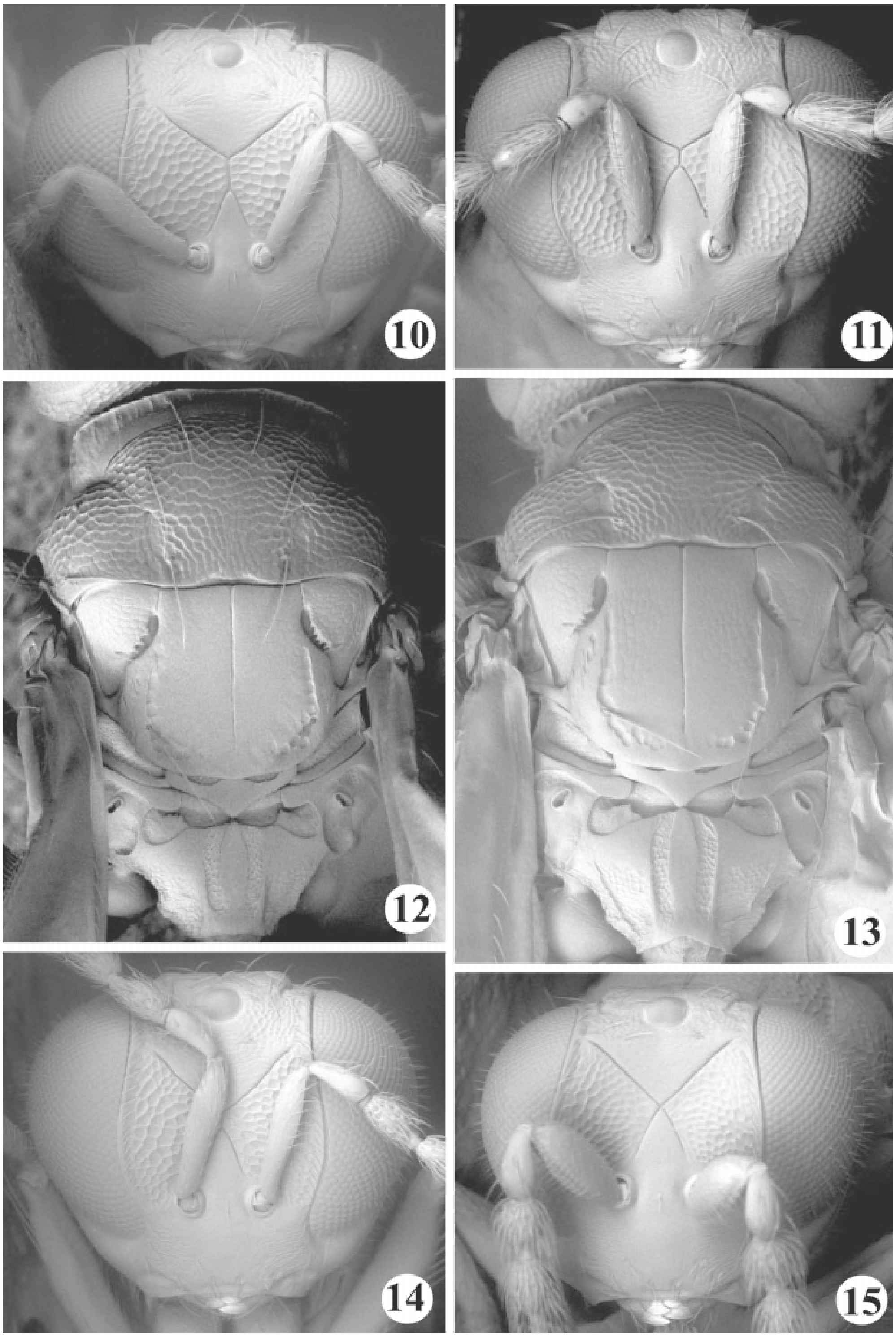
– Frons metallic purple in female, golden-green in male; female with first and second funicular segments equally long (Fig. 26); female gaster short, ratio length of mesosoma/length of gaster = 1.8 .... ***productus* (Ashmead)** (female, male)



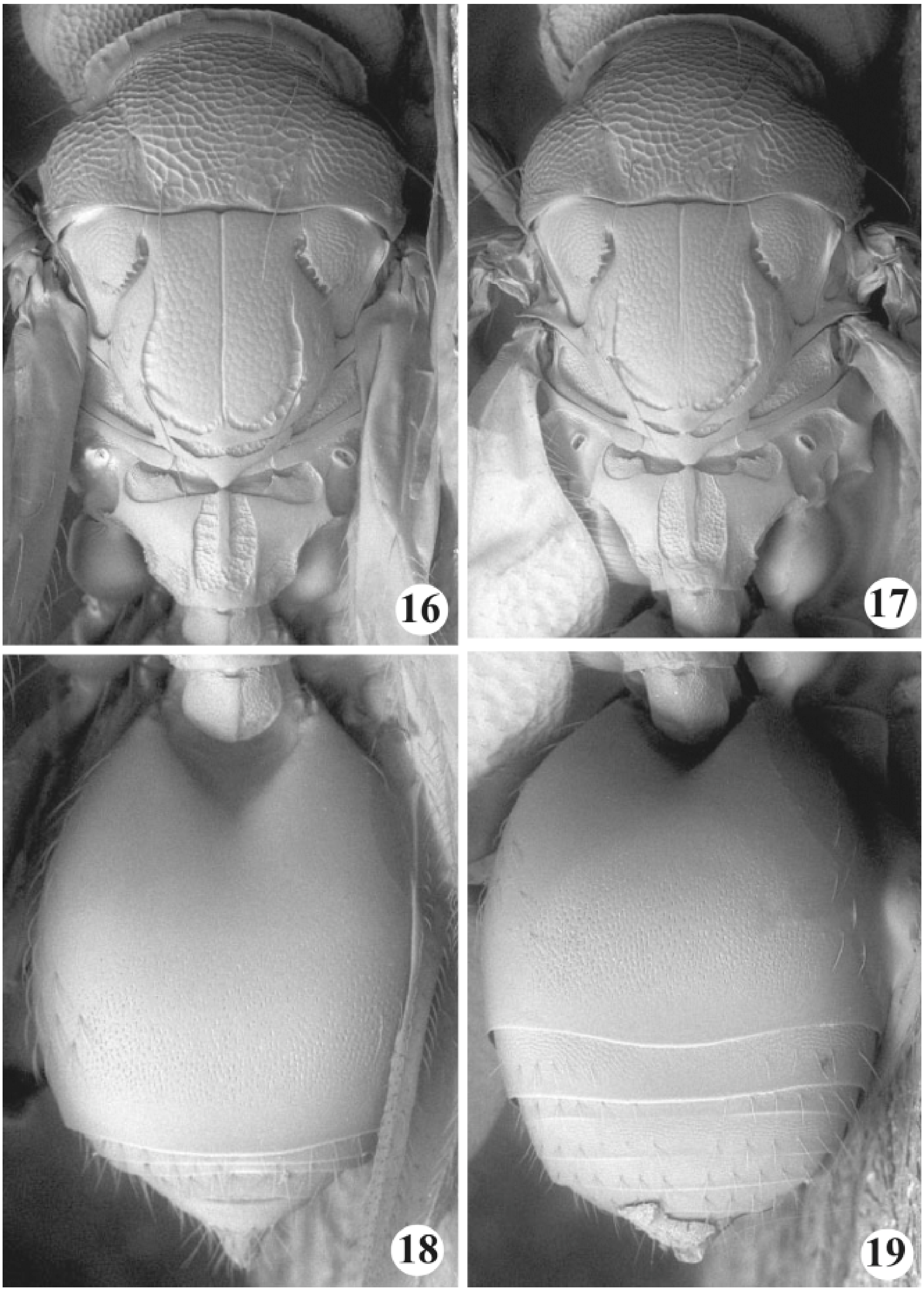
FIGURES 2–5. *Horismenus butcheri*. 2. Head frontal, female. 3. Head frontal, male. 4. Thoracic dorsum, female. 5. Thoracic dorsum, male.



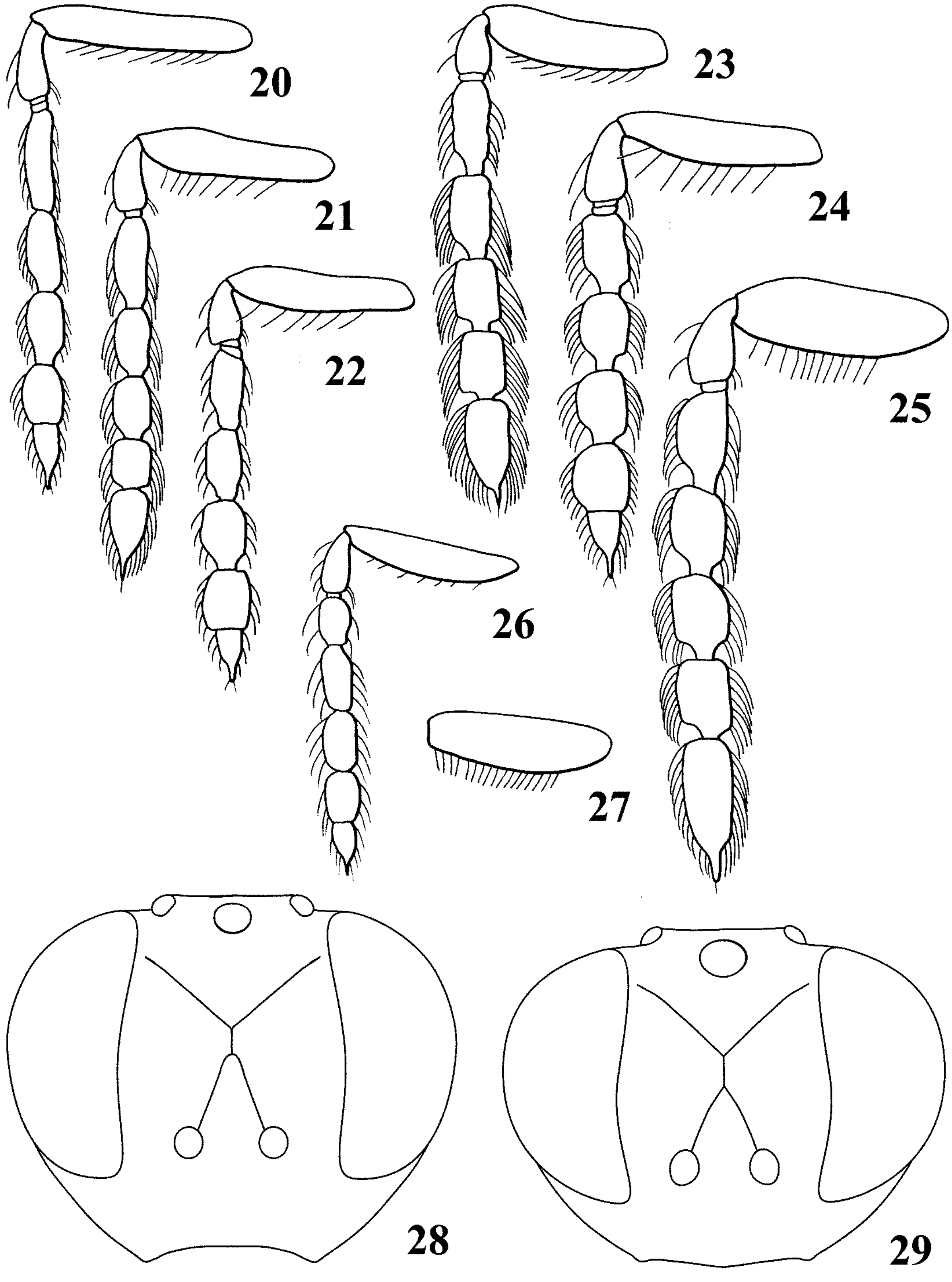
FIGURES 6–9. *Horismenus* spp., gaster dorsal. 6. *H. butcheri*, female. 7. *H. butcheri*, male. 8. *H. depressus*, female. 9. *H. depressus*, male.



FIGURES 10–15. *Horismenus* spp. 10. *H*. *depressus*, head frontal, female. 11. *H*. *depressus*, head frontal, male. 12. *H*. *depressus*, thoracic dorsum, female. 13. *H*. *depressus*, thoracic dorsum, male. 14. *H*. *missouriensis*, head frontal, female. 15. *H*. *missouriensis*, head frontal, male.



FIGURES 16–19. *Horismenus missouriensis*. 16. Thoracic dorsum, female. 17. Thoracic dorsum, male. 18. Gaster dorsal, female. 19. Gaster dorsal, male.



FIGURES 20–29. *Horismenus* spp. 20–26. Antenna lateral. 20. *H. butcheri*, female. 21. *H. butcheri*, male. 22. *H. depressus*, female. 23. *H. depressus*, male. 24. *H. missouriensis*, female. 25. *H. missouriensis*, male. 26. *H. productus*, female. 27. *H. productus*, scape lateral, male. 28. *H. productus*, head frontal, female. 29. *H. productus*, head frontal, male.

Horismenus butcheri **sp.nov. Hansson & Aebi**

Figs 2–7, 20–21

Diagnosis. Female gaster elongate (MM/LG = 0.8–0.9) (Fig. 6); female funicular segments long (Fig. 20), e.g. first funicular segment 3.5X as long as wide; male scape narrow, 4.5X as long as wide (Fig. 21).

Description. Length of body female 2.6–2.8 mm, male 2.2–2.6 mm.

Scape yellowish-white with apical 1/4 metallic bluish-green; pedicel and flagellum metallic bluish-green. Frons golden-green in female, metallic purple in male. Vertex metallic bluish-green in female, golden-green with purple tinges in male. Mesosoma metallic bluish-green with purple tinges to golden-green, propodeum metallic bluish-green to golden-green with sunken and reticulate parts dull golden-purple. Coxae metallic bluish-green; femora, tibiae and tarsi yellowish-white. Wings hyaline. Petiole dull golden-purple. First gastral tergite metallic bluish-green in anterior 1/3, dull golden-purple in posterior 2/3.

Antennae as in Figs 20–21. Frons with strong small-meshed reticulation, reticulation weaker above frontal suture, interscrobal area and clypeal region smooth and shiny; frontal suture V-shaped, terminating before reaching eyes; antennal scrobes join below frontal suture. Vertex with strong, engraved and small-meshed reticulation; with a median groove in posterior half, groove continues down on occiput. Occipital margin rounded. Ratios of HE/MS/WM female 3.6/1.0/2.3, male 4.3/1.0/2.8; POL/OOL/POO 6.0/1.3/1.0; WH/WT 1.1.

Mesoscutum with strong large-meshed reticulation; notauli indistinct and wide in posterior 1/3. Scutellum with strong and engraved reticulation. Dorsellum smooth, with two large foveas anterolaterally, foveas reticulate. Coxae with weak reticulation. Fore wing speculum open below. Propodeum smooth and shiny, with sunken parts reticulate; propodeal callus with two setae. Petiole 1.1X as long as wide in female, 1.4X as long as wide in male. Ratios of LW/LM/HW 1.8/1.1/1.0; PM/ST 0.9.

First tergite smooth and shiny in anterior 1/3, posterior 2/3 with micropunctures. Ratios of MM/LG female 0.8–0.9, male 1.0–1.1.

Distribution. Mexico, USA (New Mexico).

Hosts. *Acanthoscelides* sp. on *Phaseolus vulgaris* L. and *Bruchus amicus* Horn, both hosts are Bruchidae (Coleoptera).

Material examined. Holotype female labeled “Mexico: Est. Mexico, Temascaltepec, 100º02´ W, 19º02´ N, 1750m, 21.xii.2001–2.iii.2002, A. Aebi, ex *Acanthoscelides* sp. on *Phaseolus vulgaris*” (BMNH). Paratypes: 3 females, 4 males with same label data as holotype (BMNH, USNM); 13 females 12 males from same locality as holotype but collected 5.i–20.ii.2003 (CH, CNIN, MHNN); 2 females, 1 male from same locality as holotype but collected 27.i–21.ii.2001 and from 1700m altitude (CNC); 3 females, 4 males from same locality as holotype but collected 16.xii.2001–3.ii.2002 and from 1700m altitude (BMNH, CNC); 5 females, 5 males “Mexico: Est. Mexico, Valle de Bravo, 100º09´ W, 18º56´ N, 1950m, 1.i–15.ii.2002, A. Aebi, ex *Acanthoscelides* sp. on *Phaseolus vulgaris*" (CH, CNC, USNM). Non-type material: a female included in the type material of *Horismenus productus* (Ashmead) (see below) from *Bruchus amicus* in New Mexico (USNM) is conspecific with *butcheri*. This specimen is not included in the type material because it is damaged.

Etymology. This species is named in honour of Dr. Robert D.J. Butcher, for guidance and support throughout the dissertation research of Alexandre Aebi.

Horismenus depressus **Gahan**

Figs 8–13, 22–23

Horismenus depressus Gahan, 1930:8. Holotype female in USNM, not examined.

Diagnosis. Scutellum flat and with posteromedian part smooth and shiny (Figs 12–13); female gaster short (MM/LG = 1.1) and ovate (Fig. 8); female funicular segments short and stout (Fig. 22), e.g. first funicular segment 2.4X as long as wide; male scape 3.7X as long as wide (Fig. 23).

Description (of Mexican specimens). Length of body female 2.0–2.1 mm, male 1.9–2.0 mm.

Scape yellowish-white with apical 1/3 metallic bluish-green; pedicel and flagellum metallic bluish-green. Frons metallic bluish-green in female, metallic purple in male. Vertex metallic bluish-green in female, golden-green in male. Mesosoma metallic bluish-green with purple tinges, propodeum metallic bluish-green with sunken and reticulate parts dull golden-purple. Coxae metallic bluish-green; femora, tibiae and tarsi yellowish-white. Wings hyaline. Petiole dull golden-purple. First gastral tergite metallic bluish-green in anterior 1/3, dull golden-purple in posterior 2/3.

Antennae as in Figs 22–23. Frons with strong small-meshed reticulation, reticulation weaker above frontal suture, interscrobal area smooth and shiny, clypeal region with weak transverse meshes; frontal suture V-shaped, terminating before reaching eyes; antennal scrobes join below frontal suture. Vertex with strong, engraved and small-meshed reticulation; with a median groove in posterior half, groove continues down on occiput. Occipital margin rounded. Ratios of HE/MS/WM female 3.6/1.0/2.2, male 3.2/1.0/2.0; POL/OOL/POO 5.7/1.3/1.0; WH/WT 1.1.

Mesoscutum with strong large-meshed reticulation; notauli indistinct and wide in posterior 1/3. Scutellum flat, with weak and engraved reticulation, partly to predominantly smooth. Dorsellum smooth, with two large foveas anterolaterally, foveas reticulate. Coxae smooth and shiny. Fore wing speculum open below. Propodeum smooth and shiny with sunken parts reticulate; propodeal callus with 2–3 setae. Petiole 1.1X as long as wide in female, 1.4X as long as wide in male. Ratios of LW/LM/HW 1.7/1.0/1.0; PM/ST 0.8.

First tergite smooth and shiny in anterior 1/3, posterior 2/3 with micropunctures. Ratios of MM/LG female 1.1, male 1.2.

Distribution. Mexico (De Santis 1989) and the U.S.A. (California (Gahan 1930), Texas (Burks 1971)).

Hosts. *Acanthoscelides* sp. on *Phaseolus* spp. (new record), *A. obtectus* (Say) (De Santis 1989), "*Bruchus* sp." in *Acacia* seeds (Burks 1971), *Stator pruininus* (Horn) from seeds of *Acacia* and *Olneya* (Gahan 1930). All hosts are Bruchidae (Coleoptera).

Material examined. 22 females, 21 males from Mexico (Mexico, Michoacan, Morelos) from *Acanthoscelides* sp. on *Phaseolus vulgaris* (CH).

Horismenus missouriensis **(Ashmead)**

Figs 1, 14–19, 24–25

Holcopelte missouriensis Ashmead, 1888:101. Lectotype female in USNM, designated by Burks (1971), examined.

Holcopelte popenoei Ashhmead, 1888:101. Synonymized by Girault (1934).

Horismenus missouriensis (Ashmead), Schmiedeknecht (1909).

Diagnosis. Female gaster short (MM/LG = 1.1) and ovate (Fig. 18); female funicular segments short and stout (Fig. 24), e.g. first funicular segment 1.7X as long as wide; male scape swollen (Fig. 25), 2.8X as long as wide.

Description (of Mexican specimens). Length of body female 2.2–2.5 mm, male 2.0–2.2 mm.

Female scape yellowish-white with apical tip dark brown, male scape with basal half yellowish-white and apical half metallic bluish-purple; pedicel and flagellum metallic bluish-green in female, metallic bluish-purple in male. Frons metallic bluish-green in female, metallic purple in male. Vertex metallic bluish-green in female, golden-green in male. Mesosoma metallic bluish-green with purple tinges, propodeum metallic bluish-green with sunken and reticulate parts dull golden-purple. Coxae metallic bluish-green; femora, tibiae and tarsi yellowish-white. Wings hyaline. Petiole dull golden-purple. First gastral tergite metallic bluish-green in anterior 1/3, dull golden-purple in posterior 2/3.

Antennae as in Figs 24–25. Frons with strong small-meshed reticulation, reticulation weaker above frontal suture, interscrobal area smooth and shiny, clypeal region with weak transverse meshes; frontal suture V-shaped and complete; antennal scrobes join below frontal suture. Vertex with weak, engraved and small-meshed reticulation; with a median groove in posterior half, groove continues down on occiput. Occipital margin rounded. Ratios of HE/MS/WM female 4.2/1.0/2.5, male 2.9/1.0/2.4; POL/OOL/POO 3.2/1.0/1.0; WH/WT 1.0.

Mesoscutum with strong large-meshed reticulation; notauli distinct and more or less wide in posterior 1/3. Scutellum with strong and engraved reticulation. Dorsellum smooth, with two large foveas anterolaterally, foveas reticulate. Coxae predominantly smooth and shiny with very weak reticulation at base. Fore wing speculum open below. Propodeum smooth and shiny with sunken parts reticulate; propodeal callus with two setae. Petiole

1.1X as long as wide in female, 2.1X as long as wide in male. Ratios of LW/LM/HW 1.8/1.1/1.0; PM/ST 1.0.

First tergite smooth and shiny in anterior 1/3, posterior 2/3 microreticulate with isodiametric meshes and with micropunctures, or predominantly with micropunctures. Ratios of MM/LG female 1.1, male 1.4.

Distribution. Brazil (De Santis 1980, Sari et al. 2002), Mexico (new record), U.S.A. (Ashmead 1888) (from New York and southward (Burks 1979)).

Hosts. *Acanthoscelides* sp. on *Phaseolus* spp. (new record), *A. floridae* Horn (Brett 1946), *A. submuticus* (Sharp) (Peck 1963), *Amblycerus robiniae* (Fabricius) (Peck 1951), *Ctenocolum crotonae* (Fåhraeus) (Sari et al. 2002), *Gibbobruchus* sp. (Burks 1971). All hosts are Bruchidae (Coleoptera).

Material examined. 25 females, 21 males from Mexico (Distrito Federal, Mexico, Michoacan, Morelos) from *Acanthoscelides* sp./*Zabrotes* sp. on *Phaseolus vulgaris* and *P. coccineus* (CH).

***Horismenus productus* (Ashmead)**

Figs 26–29

Holcopelte productus Ashmead, 1894a:342. Lectotype female in USNM, here designated, examined.
Horismenus productus (Ashmead), Schmiedeknecht (1909).

Diagnosis. Female gaster short (MM/LG = 1.8) and round; female funicular segments short and stout (Fig. 26), e.g. first funicular segment 1.5X as long as wide; male scape swollen (Fig. 27), 3.2X as long as wide.

Description. Length of body female 1.8 mm, male 1.6 mm.

Scape yellowish-brown, pedicel and flagellum dark brown with metallic tinges. Frons metallic purple in female, golden-green in male. Vertex golden-green. Mesosoma golden-green, propodeum golden-green tinged with blue. Coxae golden-purple; femora, tibiae and tarsi yellowish-white. Wings hyaline. Petiole dull golden-purple. First gastral tergite golden-green in anterior half, dull golden-purple in posterior half.

Female antenna as in Fig. 26, male scape as in Fig. 27 (flagellum missing in male antenna). Frons with strong small-meshed reticulation, reticulation weaker above frontal suture, interscrobal area and clypeal region smooth and shiny; frontal suture V-shaped and complete; antennal scrobes join below frontal suture. Vertex with weak small-meshed reticulation; with a median groove in posterior half, groove continues down on occiput. Occipital margin rounded. Ratios of HE/MS/WM female 3.1/1.0/1.8, male 3.8/1.0/2.2; POL/OOL/POO 2.3/1.0/1.3; WH/WT 1.2.

Mesoscutum with strong large-meshed reticulation. Scutellum with strong and engraved reticulation. Dorsellum smooth, with two large foveas anterolaterally. Coxae predominantly smooth and shiny with very weak reticulation at base. Fore wing speculum open below. Propodeal callus with two setae. Ratios of LW/LM/HW 1.8/1.1/1.0; PM/ST 1.4.

First tergite smooth and shiny in anterior half, posterior half microreticulate with elongate meshes, remaining tergites hidden in first tergite. Ratios of MM/LG female 1.8, male 2.0.

Hosts. *Bruchus amicus* Horn (Ashmead 1894a). Host records published after the original description remain uncertain due to the difficulties of species identification and the confused nomenclature associated with this species.

Distribution. USA (New Mexico).

Material examined. Lectotype female “N. Mex.", “Type No. 2144, U.S.N.M.” (USNM). Paralectotypes: 3 females, 1 male with same label data as lectotype (USNM). The lectotype and the paralectotypes are designated here (see below under “Remarks”).

Remarks. The type material of *Horismenus productus* Ashmead (1894a) consists of five specimens on three pins, two females, a male and a female, and a female. The single female, the male and the female on the same pin, and one of the females (the smaller female) on the pin with two females, are conspecific. The larger female on the pin with the two females belongs to a different species. The original description of *productus* is brief and not especially informative, but from the size range of the specimens given in the description (2–2.6 mm) it is obvious that *productus* was described from all specimens mentioned here. Possibly more specimens were included in the original description, specimens that subsequently have been lost – Ashmead states “several specimens” in the description. Since there are two species in the type material of *productus*, the question is which species shall bear the name “productus”. The description is of no help here so either one will do. We choose the species represented by most specimens, and also represented by both sexes, to carry the name *productus*. To maintain the nomenclatural stability we select the (smaller) female on the pin with the two females as lectotype for *Horismenus productus*. The large female on the same pin as the lectotype belongs to *H. butcheri* which is described above. The remaining type specimens mentioned here are designated as paralectotypes.

Ashmead described another species with the same name (*productus*), and during the same year (1894b), but this latter species was from St. Vincent. The “St. Vincent-species” has subsequently been renamed, “*ashmeadii*” (Dalla Torre 1898), and is not conspecific with *productus* from New Mexico (which retains the name *productus*). Both species were originally described in genus *Holcopelte*, but both have subsequently been transferred to *Horismenus*.

The gastral tergites and sternites 2–7 have been retracted into the first tergite in all type specimens. The single male has lost the flagellum on both antennae.

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CHAPTER three

Microsatellite markers in a complex of *Horismenus sp.* (Hymenoptera : Eulophidae), parasitoids of bruchid beetles.

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PRIMER NOTE

Microsatellite markers in a complex of *Horismenus* sp. (Hymenoptera: Eulophidae), parasitoids of bruchid beetles

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Abstract

Parasitoids of the genus *Horismenus* (Hymenoptera: Eulophidae) are the main natural enemies of bruchid beetles that feed on several species of *Phaseolus* beans. Samples of *Horismenus depressus*, *H. missouriensis* and *H. butcheri* were collected from seeds of three *Phaseolus* species throughout Mexico to examine the impact of plant variability on the genetic structure of parasitoid populations. For this purpose, six microsatellite loci were isolated and characterized. These loci are of great interest in understanding the taxonomy of the genus *Horismenus*, the most important Eulophid genus in the Neotropics.

Keywords: beans, Eulophidae, *Horismenus*, microsatellite, parasitoid, population structure

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Host location by parasitoids and their reproductive success can be greatly influenced by the plant species on which the host feeds. Various studies show that features of plants, such as allelochemistry, nutritional quality and morphology, can affect natural enemies directly or indirectly (Barbosa & Benrey 1998; Benrey *et al*. 1998; Turlings & Benrey 1998). As yet, little is known about how these plant effects influence the genetic population structure of the parasitoids.

Bruchid beetles that feed on seeds of the genus *Phaseolus* are attacked by several species of hymenopteran parasitoids. We use this system to investigate the role of plants on the genetic population structure of a complex of parasitoids. For this purpose, we collected individuals of the genus *Horismenus*, the most important parasitoid in this system, from bruchid-infested beans (*P. vulgaris*, *P. coccineus* and *P. lunatus*) throughout Mexico. Preliminary genetic data suggested the presence of various species in the original sample. Based on morphological traits, three species were identified: *Horismenus depressus* Gahan, *H. missouriensis* Ashmead and *H. butcheri* Hansson and Aebi (Hansson *et al*. 2004).

Total genomic DNA was extracted using a Puregen™ DNA isolation kit (Gentra Systems) from a pool of 136 individuals (0.135 g of fresh material) of the three species, collected in Tepoztlan, Tejupilco and Temascaltepec (Mexico State). Microsatellite-enriched libraries were built following Billotte *et al*. (1999): DNA was digested to completion with *Rsa*I (Eurogentec) and 500–1000 bp DNA fragments were selected after resolving on TAE-0.8% (w/v) agarose gel and isolated using an extraction kit (Promega). The enrichment step was pursued as described in Kijas *et al*. (1994) and Edwards *et al*. (1996). The enriched microsatellite partial library was then constructed by ligating the polymerase chain reaction (PCR) products into pGEM-T (Promega), following the manufacturer's recommendations. Epicurian-coli XL1-Blue MRF′ supercompetent cells (Stratagene) were used for the transformation of the cloned DNA fragments. Following standard blue–white selection on Xgal/IPTG/ampicilin plates, 384 white transformant clones were transferred on Hybond-N+ nylon membranes (Amersham) and hybridized using ^{32}P-labelled oligoprobes $(CT)_{15}$ and $(GT)_{15}$. After hybridization, the filters were washed twice with 4× SSC (10 min, 57 °C) and then with 0.1× SSC/10% (w/v) SDS (10 min, 40 °C). Of these clones, 223 gave a strong positive signal, of which 48 inserted DNA fragments were sequenced. Fourteen primer pairs were designed of which six gave satisfactory amplification patterns (i.e. PCR products of the predicted size, and supernumerary bands of low intensity).

PCR amplifications were performed in a final volume of 5 µL containing 1 µL of extracted DNA (2 ng/µL), 2.5 µL of HotStarTaq Master Mix (Qiagen), 0.25 µL of 10 µm reverse

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Table 1 Polymorphic microsatellite loci for *Horismenus depressus*

Locus	Motif	Genbank Accession no.	Primer sequences (5′–3′)	Size range (bp)	T_a (°C)	Atila (n = 18) Na	Atila (n = 18) H_E	Atila (n = 18) H_O	Temascaltepec (n = 9) Na	Temascaltepec (n = 9) H_E	Temascaltepec (n = 9) H_O
Ho4b	$(GT)_9$	AY166613	F: CATCGAAAGGGATATGCGCACG R: CTATACAAAGCTCCATTCACTCG	126–140	61	6	0.60	0.72	6	0.64	0.33*
Ho8b	$(CA)_6TA(CA)_{16}$	AY655757	F: CTTAAAACTCTACAATGGCGTCTTT R: GATAAAGTACAGATTTCGCGC	164–298	58	9	0.71	0.44*	10	0.91	0.56*
Ho10b	$(CT)_{11/5}$	AY166616	F: GCATAGAGTCGCGGAATCG R: CCACTCGAAATACTTGTAAC	154–174	63	8	0.62	0.61	5	0.78	0.78
Ho16	$(GA)_{49}$	AY166618	F: TCTGAACTTGCATTGTCATG R: GCAAAATTGCGTTTCGTCTG	170–238	57	15	0.85	0.78	7	0.43	0.44
Ho6b	$(GA)_{22}$	AY166614	F: CGTTATGCGCATACGCTGGGT R: CAACACAAGACAACGCAGCTCCG	163–181	65	6	0.62	0.50	7	0.86	0.67
Ho9b	$(CT)_9$	AY655758	F: TGTGCGTGGTATATGGCTCAC R: AGGACGATCGATTCCGCGAC	95–113	60	5	0.68	0.50*	4	0.73	0.11*

T_a: annealing temperature, Na: number of alleles detected, H_E: expected heterozygosity under Hardy–Weinberg equilibrium, H_O: observed heterozygosity, n: number of individuals tested. *Significant deviation from H_E ($P < 0.05$).

Table 2 Polymorphic microsatellite loci for *Horismenus missouriensis* and *H. butcheri*

	Horismenus missouriensis							*Horismenus butcheri*						
		Malinalco (n = 16)			Temascaltepec (n = 11)				Malinalco (n = 23)			Temascaltepec (n = 18)		
Locus	T_a (°C)	Na	H_E	H_O	Na	H_E	H_O	T_a (°C)	Na	H_E	H_O	Na	H_E	H_O
Ho4b	61	9	0.71	0.44*	4	0.68	0.55*	61	—	—	—	—	—	—
Ho8b	58	19	0.85	0.75	8	0.73	0.73	58	17	0.58	0.48	5	0.38	0.22*
Ho10b	60	6	0.72	0.38*	3	0.41	0.09*	62	8	0.59	0.52	7	0.51	0.44
Ho16	57	15	0.83	0.75	12	0.78	0.73	57	19	0.79	0.83	19	0.75	0.77
Ho6b	65	7	0.71	0.50*	5	0.46	0.45	65	11	0.71	0.39*	2	0.06	0.06
Ho9b	60	8	0.78	0.31	5	0.67	0.64	60	10	0.83	0.74	6	0.45	0.39

Abbreviations as in Table 1.

and forward primer (5′ IRD-700/800 modified) and 1 µL of double-distilled H_2O. PCRs were performed on a Biometra® T gradient thermocycler using the following cycling conditions: initial denaturing step of 95 °C for 15 min followed by 40 cycles of: 95 °C (30 s), T_a (30 s), 72 °C (40 s); final elongation at 72 °C (10 min). PCR products were mixed with 2.5 µL of stop solution [95% (v/v) desionized formamide, 50 mm EDTA/10 mm NaOH/0.1% (w/v) bromophenol blue/0.1% (w/v) xylene cyanol green] and denatured at 94 °C for 2 min, prior to electrophoresis on a denaturing 7.4 m urea –6.5% (w/v) polyacrylamide gel (Sequagel XR, National Diagnostics) on a Li-Cor DNA Analyser. Isolated bands were visualized and analysed using saga ir^2 software, version 2.2.2.

The degree of polymorphism at the six loci was tested for each *Horismenus* species using females from two different populations. We calculated expected and observed heterozygosities, estimated heterozygote deficit using Hardy–Weinberg exact tests and checked for linkage desequilibrium (genepop 3.3; Raymond & Rousset 1995).

For *H. depressus*, all loci were polymorphic, with 4–15 alleles per population (Table 1). A significant deficit of heterozygotes was observed for three loci (Ho8b and Ho9b in both populations and Ho4b in Temascaltepec), suggesting the presence of null alleles. Eight of the 15 possible pairs of loci were out of linkage disequilibrium in Atila, whereas none was out of linkage disequilibrium in Temascaltepec. The inconsistency between sites suggests a Wahlund effect in Atila, a large population growing on *P. vulgaris* with the peculiarity of being close to populations of parasitoids growing on *P. lunatus*, thereby potentially creating substructure in the population.

We found 3–19 alleles per locus in *H. missouriensis* (Table 2). A significant deficit of heterozygotes was observed for five loci (Ho4b, Ho10b in both populations and Ho8b, Ho6b, Ho9b in Malinalco), suggesting the presence of null alleles. No linkage-disequilibrium was detected.

We found 2–19 alleles in H. butcheri (Table 2). A significant deficit of heterozygotes was seen for two loci (Ho6b in Malinalco and Ho8b in Temascaltepec) suggesting the presence of null alleles. No linkage disequilibrium was detected.

PCR conditions were optimized for each species separately (Tables 1 and 2). All loci except for Ho4b in H. butcheri showed a high degree of cross-amplification, providing useful tools for population genetics studies in this Horismenus complex. The genus Horismenus is one of the largest in the Eulophidae in the Neotropics but the majority of species remain undescribed (Hansson et al. 2004). These microsatellites provide valuable tools for a better knowledge of the Neotropical Horismenus.

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CHAPTER four

Molecular differentiation and phylogenetic relationships among *Horismenus spp.* (Hymenoptera: Eulophidae) attacking bruchid beetles in Mexico.

Molecular Differentiation and Phylogenetic Relationships among *Horismenus spp.* (Hymenoptera: Eulophidae) Attacking Bruchid Beetles in Mexico

Aebi A, Hansson C, Mansion G and Benrey B.

Abstract

A new complex of parasitoids of the genus *Horismenus* (Hymenoptera: Eulophidae), attacking bruchid beetles on beans of the genus *Phaseolus* was recently described and revised on the basis of morphological traits. The present study describes their differentiation at nuclear DNA (microsatellites) and mitochondrial DNA (Cytochrome oxydase I) loci. The molecular data supports the treatment of the three species as different species.

Key words: *Horismenus*, microsatellite, mt-DNA, phylogeny, genetic distance.

Introduction

Parasitoids of the super-family Chalcidoidea present a large range of morphological and ecological diversity, even within genera. Within the genus *Horismenus* (Eulophidae), for example, we can find parasitoids or hyperparasitoids on a variety of terrestrial hosts, most commonly on larvae of Coleoptera, Diptera and Lepidoptera (LaSalle and Schauff 1995), but also one species that attacks the pre-pupa stage of Psephenidae beetles under water (Burks, 1968). The genus *Horismenus* is the most abundant Eulophid in Mexico and Central America, but the majority of the species remain unknown (Hansson, personal communication). Very recently, a complex of three *Horismenus* parasitoids was described, comprising *H. depressus* Gahan, *H.*

missouriensis Ashmead and *H. butcheri* Hansson and Aebi, attacking bruchids of the genus *Acanthoscelides* and *Zabrotes* on *Phaseolus* beans. Among these, *H. butcheri* was determined as a new species.

The three species are morphologically very similar and differ only in a few characters. The differences stand in the shape and ornamentation of the scutelum, shape and length of the first funicular segment, shape of the gaster and coloration of the frons (Hansson et al. 2004). A recent ecological analysis revealed that the distribution of *H. depressus* and *H. missouriensis* is dictated by altitude, which leads to specific host plant associations with *H. depressus* specializing on *P. lunatus*, a bean growing at low altitudes, and *H. missouriensis* specializing on *P. coccineus*, growing at high altitudes. In contrast, the distribution of *H. butcheri* is strongly associated with its host plant (Aebi et al. 2004). Despite these differences in altitudinal range and host plant associations, the distribution of these closely related species broadly overlaps. In this study, we used microsatellite and mt-DNA data to determine the degree of genetic similarity between *H. missourensis*, *H. depressus* and *H. butcheri*, to confirm the status of the third as a new and distinct species and to provide additional characters that can be used to differentiate them. The phylogenetic relationship among the three species is also described.

Materials and Methods

Sampling procedure

Wild bean samples of *Phaseolus vulgaris, P. coccineus and P. lunatus* were collected between January 2001 and February 2003 in Mexico from five pure plant populations (only one species of *Phaseolus* present): ATI, TEPI, SUL, TEJ and VDB, and from three mixed plant populations (more than one *Phaseolus* species): SJS, MAL and TEM. Cultivated bean samples were bought directly from farmers between January 2002 and February 2003 from three localities: COAT, SSI and TZIN. The populations comprise the following host-plant combinations: wild *P. vulgaris*, wild *P. lunatus*, wild *P. coccineus*, wild *P. vulgaris* + *P. lunatus*, wild *P. vulgaris* + *P. coccineus* and cultivated *P. vulgaris*. The collected bean pod samples were placed in climate chambers (temperature: 25°C (day), 20°C (night), 70% RH, LD 16:8h photoperiod) and the emerging adults removed and counted every two to three days and stored in absolute ethanol at –20°C. The parasitoid specimens were identified using a taxonomic key based on morphology (Hansson et al. 2004).

Microsatellite genotyping

A total of 216 females (118 *H. depressus*, 44 *H. missouriensis* and 54 *H. butcheri*) were genotyped using the 6 polymorphic microsatellite loci described in (Aebi et al. 2004) and locus Ho17 (F: CATCGAAAGGGATATGCGCACG and R: GAAGAGAAGCTATACAGGCAC). Total genomic DNA from each individual was extracted using a Puregene™ DNA isolation kit, Tissue kit (Gentra systems, Minneapolis, USA). PCR were performed following Aebi et al. (2004) and the products deposited for electrophoresis on a denaturing 7.4M urea – 6.5% (w/v) polyacrylamide gel (Sequagel XR, National Diagnostics) resolved on a Li-Cor DNA Analyser. Isolated bands were visualised and analysed using Saga IR^2 software, version 2.2.2.

Statistical analyses

To verify molecular differentiation of the three *Horismenus* species, a principal coordinate analysis and a Mantel test (999 permutations) were performed using the R4 package (Casgrain and Legendre 1997) on a matrix of allele data from the seven polymorphic microsatellite loci.

To assess the level of hybridization between *H. depressus, H. missouriensis* and *H. butcheri*, the number of discrete clusters of genotypes in the entire dataset was determined using the program STRUCTURE (Pritchard et al. 2000). The model used by STRUCTURE attempts to split a multilocus dataset into a specific number of genotype groupings. Each individual is assigned to a group by

Markov Chain Monte Carlo simulations under the assumptions of Hardy-Weinberg and linkage equilibrium. To assess the number of groups, the simulation was run several times with the number of groups (K) varying from 1 to 12. The posterior probability Pr (K|X), calculated for each run from the estimated Ln probability of data with Baye's Rule was compared to determine the optimal number of groups matching the actual genetic data. A burnin period of 5000 was used. Three replicates for each K were performed to ensure convergence in estimated parameter values. Although STRUCTURE usually uses only genetic information to learn about population structure, we used the prior population information (i.e. population name) ancestry model to assist the clustering.

Mitochondrial DNA haplotypes

Data generation

Sequence data of the cytochrome oxydase I gene were generated for 18 individuals (4 *H. depressus*, 8 *H. missouriensis* and 6 *H. butcheri*), from various populations on their natural host plant. Initial amplification of the cytochrome oxydase I region was performed using the primers Cox-one 1L (CAACATTTATTTTGATTTTTTGG) and Cox-one 1R (TCCATTGCACTAATCTGCCATATTA) (Lopez-Vaamonde et al. 2001). Amplifications were made in 3 x 25µl reaction volume containing 1x PCR buffer 2mM MgCl2, 250 mM DNTPs, 0.625 units *Taq* polymerase (Promega), 0.4 µM of each primer and 1 µl of DNA template. The PCR cycle consisted of one cycle at 94°C for 3 min, 40 cycles at 94°C for 30 s, 47°C for 30 s, 72°C for 1 min, and one cycle at 72°C for 10 min. PCR products were brought together and purified with minicolumns (Microcon YM-30) and ligated into PGEM-t easy vector system (Promega). After electroporation, transformed bacteria (DH10-B *E. coli*) were plated on LB-ampicilin-Xgal-IPTG agar and incubated at 37°C. Presence of an insert in positive clones was ensured by direct PCR on single colonies (after reinoculation) using M13 and M13-rev primers. Positive clones were inoculated in 3 ml LB-ampicilin. Recombined plasmids were extracted by resuspension of the bacteria in (25 mM Tris-HCl pH 8.0; 50 mM Glucose; 10 mM EDTA pH 8.0), (0.2 N NaOH; 1% SDS) and (3 M KOAc; 5 M glacial acetic acid) followed by centrifugation at 12000 g for five minutes. DNA was extracted from the supernatant with chloroform (1:2) prior to precipitation in ethanol 70% (v/v) (final concentration). Following centrifugation for 10 minutes at 12000 g, the pellet was washed three times in 70% (v/v) ethanol and resuspended in 50 l TE pH 8.9 with RNAse (20 g/ml). Restriction digest with NCO I and SAL I (Promega) following the manufacturer's recommendations was performed to ensure the presence of the insert prior to sequencing with M13 and M13 rev using an Amersham Biosciences Thermo Sequenase™* Primer Cycle Sequencing Kit. Sequencing PCR was performed in a 6.4 µl reaction volume containing 1.2 µl of each DNTP, and 1.2 µl of a mix containing 4µl of DNA, 1 µl of each primer and 1µl of miliQ water. PCR products were deposited for electrophoresis on a denaturing 7.4M urea – 6% (w/v) polyacrylamide gel (Sequagel *XR*, National Diagnostics) on a Li-Cor DNA Analyser. Isolated bands were visualised and analysed using e-Sequ Version 2.0.

Statistical analysis

Forward and reverse sequences were aligned using ClustalW (Thompson et al. 1994) and further adjusted by sequential pairwise comparison. A phylogenetic tree was reconstructed by distance methods using uncorrected distance under version 4.0b10 of PAUP (Swofford 2002). A UPGMA analysis was conducted for the set of sequences. Heuristic searches were performed under the parsimony criterion. Branch support was performed using the bootstrap method under the parsimony optimal criterion. A total of 100 replicates were performed. Homoplasy measures were computed using the consistency index (CI), excluding all uninformative characters, and the retention index (RI).

Results

Microsatellite genotyping.

The first two axes of the PCA represent 11.97% of the variance (Eigenvalue 1 = 7.09 and eigenvalue 2 = 4.88) (Fig. 1). Because of the low percentage of the variance explained, care should be taken in the interpretation of the data. Nevertheless, the cluster presented in figure 1 shows that the three *Horismenus* species are differentiated along the first axis of the plot. Mantel correlation between allelic distribution and species affiliation was positive (Mantel r = 0.367) and significantly different from zero (P value = 0.001) confirming allelic differentiation among the three *Horismenus* species.

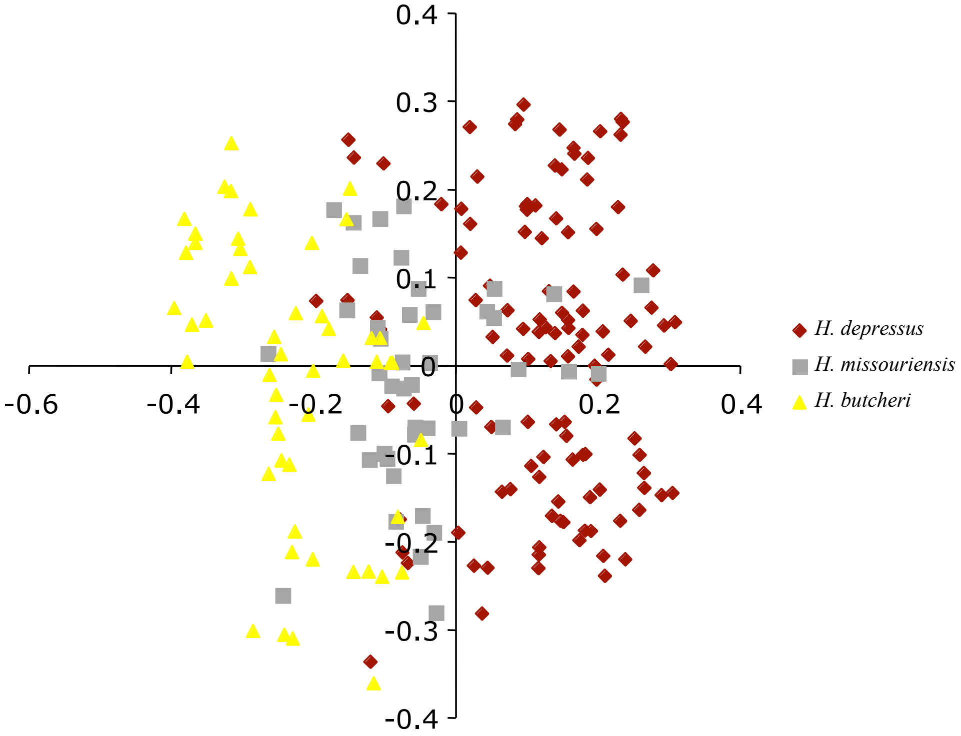

Figure 1. Principal coordinate analysis based on an allelic matrix from 7 polymorphic microsatellite loci, for individuals from *H. depressus*, *H. missouriensis* and *H. butcheri*. (Axis 1: Eigenvalue = 7.09 and axis 2: eigenvalue = 4.88).

The analysis using STRUCTURE gave strong evidence of the existence of five genetic pools within the sample (Table 1). *Horismenus depressus* and *H. missouriensis* form two distinct genetic pools. Two further genetic pools consist of individuals of *H. butcheri*, which can be explained by the strong population structuring found for this species (Fig. 2) (see chapter 5). A fifth genetic pool consists of a few individuals scattered throughout the sample. The three species are therefore distinct, but close examination of the bar-plot (Fig. 2) shows that some individuals are miss-assigned. This is probably due to identification errors, but could also suggest some amount of hybridization between species.

K	ln Pr(X|K)	Pr(K|X)
1	-7091	~ 0
2	-6530	~ 0
3	-6352	~ 0
4	-6217	~ 0
5	-6192	~ 1
6	-6229	~ 0
7	-6401	~ 0
8	-6381	~ 0
9	-6697	~ 0
10	-6329	~ 0
11	-6441	~ 0
12	-7409	~ 0

Table 1. Conditional and posterior probabilities associated with each pool number (K) for *H. butcheri*. Ln Pr(X|K) is the estimated conditional probability of observing the data under a model with the specified number of pools. Pr(K|X) is the posterior probability of each pool number given the genotypic information.

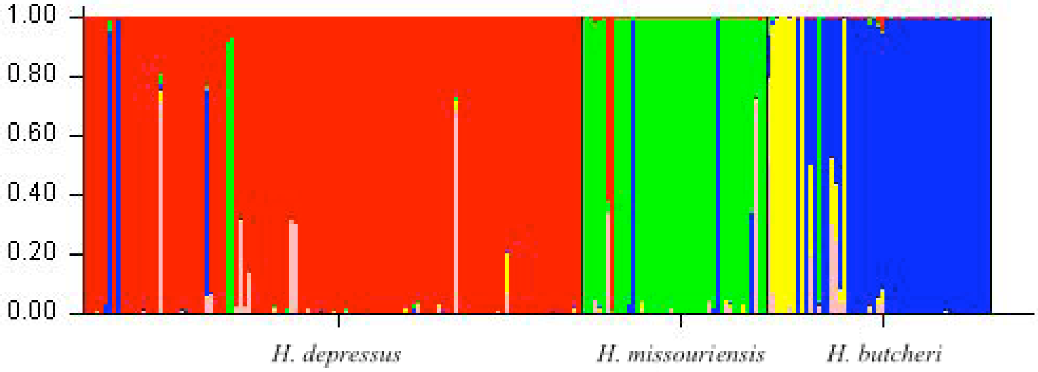

Figure 2. Results of a Bayesian cluster analysis for *H. depressus* (118 individuals), *H. missouriensis* (44 individuals) and *H. butcheri* (54 individuals). Each individual included in the analysis is represented by a vertical line, partitioned into coloured segments that represent the individual's probability of belonging to each of the K genetic clusters.

Phylogeny

Cytochrome oxydase I

Analysis of the cytochrome oxydase I matrix revealed 149 parsimony-informative characters out of a total of 654 characters. The heuristic search resulted in 16 most parsimonious (MP) trees of 41373 steps, filtering under parsimony criterion retained two trees (one is shown in Fig. 3) with a CI of 0.811 and a RI of 0.866. Bootstrap values for node support are indicated next to the branches.

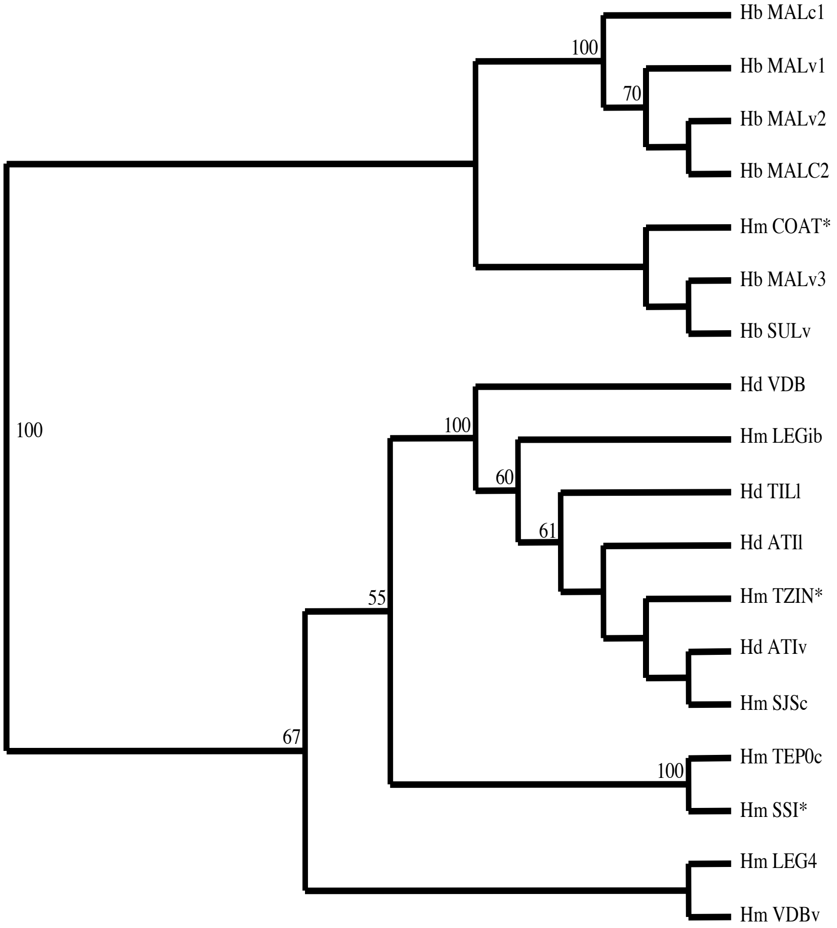

Figure 3. UPGMA tree obtained from the Cytochrome oxydase I data set. Bootstrap values >50% are given above the branches. Hb = *H. butcheri*, Hm = *H. missouriensis*, Hd = *H. depressus*, v = *P. vulgaris*, c = *P. coccineus*, l = *P. lunatus*, * stands for cultivated bean populations.

The tree obtained is well resolved with good branch support. Examination of the tree shows *H.*

butcheri as a well-differentiated monophyletic group (Fig. 1) with 5 distinct haplotypes confirming its species status. One individual determined as *H. missouriensis* clustered with the *H. butcheri* group, possibly due to an identification error. In contrast, *H. missouriensis* and *H. depressus* did not cluster into well-differentiated clades. No further genetic differentiation was detected at the intraspecific level suggesting that no cryptic species were present in our sample.

Discussion

This study supports treatment of *H. butcheri* as a new distinct species. Almost all individuals morphologically diagnosed as *H. butcheri* were unambiguously genotyped as a distinct species at the seven polymorphic loci. In addition the tree obtained from mt-DNA showed that *H. butcheri* clustered in a well-differentiated clade. These results suggest that *H. butcheri* rarely or never hybridizes with *H. missouriensis* and *H. depressus* in nature despite their overlapping ecological range and similar morphology.

Both molecular markers, the nuclear microsatellites and the mt-DNA haplotype sequences, support the existence of *Horismenus depressus*, *H. missouriensis* and *H. butcheri* as differentiated species. The data obtained with the microsatellites suggest that these species are closely related as they share some alleles. Nevertheless, private alleles were detected for each species (see chapter 5). The analysis of population structure revealed the existence of five genetic pools among which the *Horismenus* species formed well-differentiated groups. There was no strong evidence for hybridization between species but miss-assigned individuals might represent hybrids. Mt-DNA analysis failed to separate *H. depressus* and *H. missouriensis* into well differentiated clades. This result may be due to ancestral polymorphism retained by these very closely related species or could be the result of historical introgressions between *H. depressus* and *H. missouriensis*. Separating the confounding effects of long-term population history from actual gene flow can be difficult. A combination of nuclear and mitochondrial data enabled us to discriminate the role of present gene flow and historical events. Indeed, while our mitochondrial dataset show genetic similarity between *H. depressus* and *H. missouriensis*, our nuclear data show that contemporary gene flow is rare between these two species. Laboratory cross-mating experiments would enable us to investigate the possibility of hybridization and the viability of such descent.

Overall, these results confirm the treatment of *H. depressus*, *H. missouriensis* and *H. butcheri* as different species, so that in this system molecules and morphology are in broad agreement.

The ecological differentiation among the *Horismenus* species also characterizes them as different taxa, but might also provide an opportunity for hybridisation. *Horismenus depressus* is the only *Horismenus* species found on *P. lunatus* between sea level and 1200 m, and *H. missouriensis* is the only species found on *P. coccineus* between 1700 and 2500 m, but they can be found in sympatry on *P. vulgaris* at altitudes between 1200 and 2100 m. The host plant *P. vulgaris* may therefore represent a hybrid zone in which both species can meet and hybridize. The rarity of hybrids may therefore be due to natural selection against hybrids or simply a low level of hybridisation, perhaps due to pheromonal or behavioural differences.

Bruchid beetles are among the most important pests of stored beans worldwide (van Schoonhoven and Cardona, 1986). As parasitoids of the genus *Horismenus* are the most abundant group that attack these bruchids in Mexico, they might be good candidates for storage pest control. Their use as biological control agents has been limited by a lack of knowledge of their biology. This study is a first contribution towards a complete understanding of the evolutionary relationship among these key parasitoids in their native range.

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CHAPTER five

Plants as mediators of host race formation at the third trophic level.

Plants as Mediators of Host Race Formation at the Third Trophic Level

Alexandre Aebi, Nadir Alvarez and Betty Benrey

Abstract

Due to their long evolutionary history and their complex interactions, tri-trophic systems provide ideal models for the study of host-race formation and sympatric speciation. Parasitoids may follow two possible routes to sympatric speciation, as they may shift between two host species or between host-plant species attacked by the same herbivore. We investigated host race formation in a newly described *Horismenus* complex (Hymenoptera: Eulophidae), comprising *H. depressus, H. missouriensis* and *H. butcheri*, that attack two bruchid beetles of the genus *Acanthoscelides* (Coleoptera: Bruchidae), on three bean species of the genus *Phaseolus* (Leguminosae: Papilionidae) in Mexico. A multidisciplinary approach comprising an ecological description, behavioural analyses and a genetic population structure analysis lead to the following results.

1. Plants shape the community structure of this parasitoid complex. Despite their close taxonomic position, the three *Horismenus* species have specific ecological requirements allowing them to occupy different ecological niches scattered along an altitudinal gradient. Host-plant association and altitude are the main factors that best describe their ecological distribution.
2. Geographic isolation, bruchid host species and bean species differentially affect the genetic population structure of these parasitoid species. *H. depressus* and *H. missouriensis* showed no population structure due to their high dispersal abilities. Finally, *H. butcheri* revealed host plant mediated genetic differentiation, with moderate to high levels of genetic differentiation between parasitoids from distant populations and between groups of parasitoids on different host plants within populations.
3. All parasitoid species display host-plant fidelity. In an olfactometer assay in which parasitoids were allowed to choose between their plant of origin and alternative plants, all *Horismenus* species preferred their plant of origin rather than an alternative plant species or the

control.

4. Ecological and genetic analyses on the bruchid beetles attacked by these parasitoids revealed no patterns of host-plant specialization and no genetic differentiation among groups of bruchids from different bean species.

Together, these results show that host-plants can strongly affect the genetic population structure of parasitoids. This would be the first study to show evidence of a potential process of plant-mediated host race formation at the third-trophic level.

Key words host race, population structure, tritrophic interactions, *Horismenus*, bruchids, beans, microsatellites

Introduction

Sympatric speciation has become accepted as an important evolutionary process by an increasing number of ecologists in the past decade. Insects dominate the world's fauna and have evolved an incredible array of life history strategies enabling them to colonize very different habitats, but mechanisms leading to sympatric speciation in the Insecta have only been acknowledged in a very limited number of ecological situations (Drès and Mallet 2002). The only published examples of sympatric speciation via host-race formation have been described in phytophagous insect taxa (Berlocher and Feder 2002; Craig et al. 1997; Drès and Mallet 2002; Emelianov et al. 2001; Emelianov et al. 2003). The mechanism by which a host shift may lead to sympatric speciation was first suggested by Bush (1969) who proposed that frequent host shifts of herbivorous insects could lead to assortative mating within sub-populations on different hosts and hence to genetic differentiation, and ultimately to speciation. In this context, plant traits such as secondary chemistry may play a key role in this process, as host shifts are most common for insects feeding on similarly defended or closely related plants (Bernays and Graham 1988; Jaenike 1990).

One of the current challenges in the study of sympatric speciation is to evaluate its frequency in nature (Beebe et al. 2001). The tritrophic interactions between plants, herbivores and their natural enemies may represent a powerful driver of sympatric speciation; while herbivores and plants have an intimate and unambiguous trophic relationship, the natural enemies of herbivores such as parasitoids are also dependent on plant traits for their reproductive success (Turlings and Benrey, 1998) and therefore have the capacity to undergo host-race formation on different plant species. Parasitoids of herbivores are known to display a high degree of plant specialization (Godfray 1994). To find their often specialized and concealed hosts, parasitic wasps rely on volatile cues emanating from the plant to find the microenvironment where their hosts live (Turlings & Wäckers 2004). The learning ability of parasitoids enables them to enhance further their host-location ability (Fukushima et al. 2002; Tumlinson et al. 1993; Turlings and Benrey 1998;

Turlings et al. 1993; Vet 1999). Moreover, plant features can directly or indirectly affect parasitoid performance. While plant morphological traits such as trichomes can interfere directly with host-location behaviour (Lovinger et al. 2000), other traits such as allelochemistry or nutritional quality are known to affect parasitoid reproductive success indirectly by altering the quality of their host (Barbosa and Benrey 1998; Karban and Huc 1999; Ode et al. 2004; Turlings and Benrey 1998).

Parasitoids may follow two possible routes to sympatric speciation. They may shift between two host species, or they may also skip a trophic level and shift between two host-plant species attacked by the same herbivore (Askew 1968). The latter possibility remains unexplored, but the former has received comparatively little attention. Only two studies have investigated whether natural enemies display sequential radiation in response to host race formation in their host or prey. Both examples involve *Eurosta solidaginis*, a tephritid fly that induces the formation of galls on two closely related species of *Solidago* (Compositae). Two host races of *E. solidaginis* have diverged on *S. altisima* and *S. gigantea* (Abrahamson et al. 2003). *Eurytoma gigantea* (Hymenoptera: Eurytomidae) is a specialist parasitoid attacking *E. solidaginis* but attempts to demonstrate diversification at the third trophic level as a consequence of diversification in their fly hosts have failed (Cronin and Abrahamson 2001). In contrast, *Mordellistema convicta* (Coleoptera: Mordellidae), a beetle feeding on *E. solidaginis* gall tissues and occasionally on the larvae or pupae, showed evidence of host race formation at the third trophic level (Abrahamson et al. 2003; Eubanks et al. 2003).

Among chalcidoid Hymenoptera, sympatric speciation may have played a key role in the incredible diversity of this group as more than 2000 species have been described from the nearctic region. The frequent assemblages consisting of closely related or sibling species described in many chalcidoid families support the idea that this group is in a state of active evolution (Askew 1968). Indeed, whereas allopatric speciation could be doubted because of their high dispersal ability, sympatric speciation might well be favoured by various characteristics of their biology. As most chalcidoids are generalists, adaptation to a new environment is thought to rely more on the host-location behaviour of the female than on the nutritional needs of the larvae. A mutation in female chalcidoid host location behaviour and host or host plant acceptance could be passed on rapidly to the next generation by the interaction of reproductive and genetic traits such as inbreeding and haplodiploidy (Askew 1968).

The intricate biology of parasitoids and plants provides new ecological conditions, which could lead to sympatric speciation via host race formation. In the current study, we used a combination of an ecological, a behavioural and a population genetics approach to investigate the role of plants on the genetic population structure and host race formation in a newly described *Horismenus* complex (Hymenoptera: Eulophidae), comprising *H. depressus* (Gahan), *H. butcheri* (Hansson & Aebi) and *H. missouriensis* (Ashmead), that attacks two bruchid beetles of the genus *Acanthoscelides* (Coleoptera: Bruchidae), on three bean species of the genus *Phaseolus* (Leguminosae: Papilionidae) in Mexico (Hansson et al. 2004).

Specifically, we addressed the following questions:

1. Do plants shape the community structure of this parasitoid complex?
2. Do these parasitoids display host plant fidelity?
3. What is the relative impact of geographic isolation, host variability and host plant variability on the genetic differentiation of *Horismenus* species?

Material and methods

Sampling procedure

Genetic analysis: Between January and April 2001 wild beans of *Phaseolus vulgaris* and *P. coccineus* were collected in Mexico from five allopatric populations: ATI, TEPI, SUL, TEJ and VDB, and from two sympatric populations: MAL and TEM (Figure 1).

Ecological description: Between 2001 and 2003 (from December to April), we collected parasitoids from localities presenting the following wild host-plant combinations: i) *P. lunatus*, ii) *P. lunatus* and *P. vulgaris*, iii) *P. vulgaris*, iv) *P. vulgaris* and *P. coccineus* and v) *P. coccineus* in the following populations: VDB, SBO, TLA, TEM, SAG, TEJ, SUL, MAL, TEP0, TEPI-TEPV, SJS, YAU, AHU, ATI, ATIH1, ATIH2 and TIL (Fig. 1). The collected bean pod samples (known number of pods) were placed in climate chambers (temperature: 25°C (day), 20°C (night), 70% RH, LD 16:8h photoperiod) and the emerging adults were removed and counted every two to three days and stored in absolute ethanol at –20°C. The parasitoid specimens were identified using a taxonomic key based on morphology (Hansson et al. 2004).

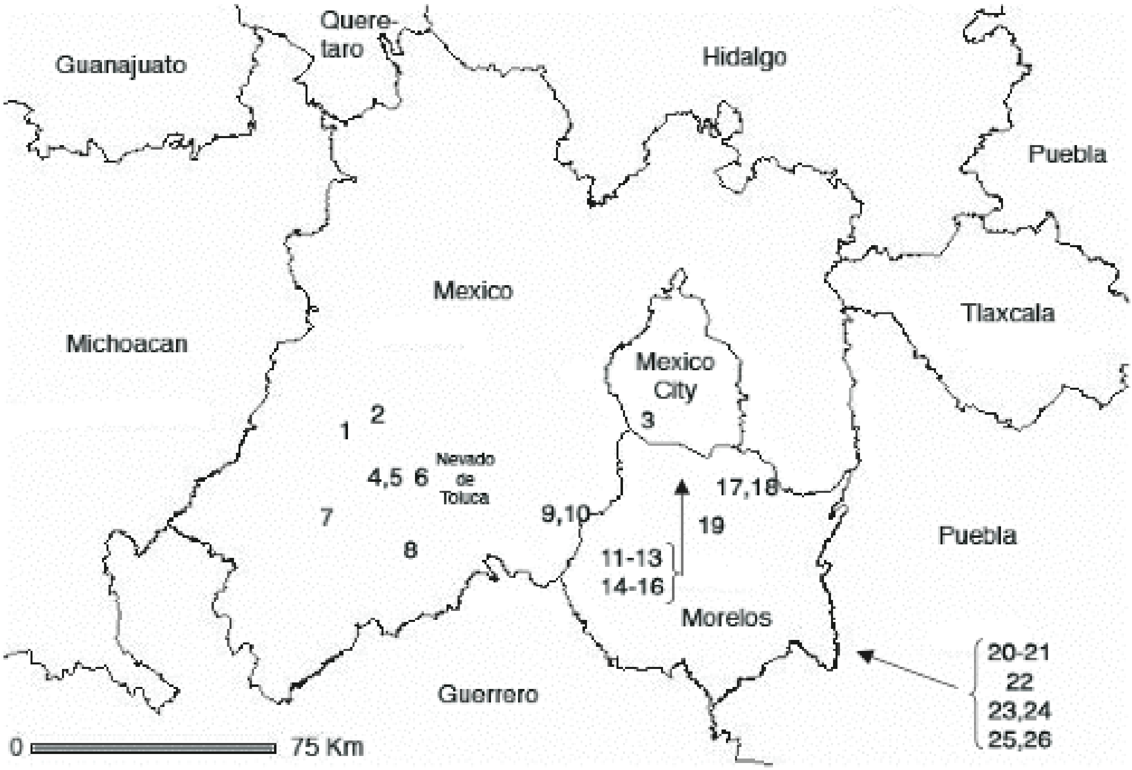

Figure 1. Collection sites. Numbers separated by a "," represent sympatric bean populations. Numbers separated by a "-" represent close but geographically distinct populations. v = P. vulgaris, c = P. coccineus and l = P. lunatus. 1v: Valle de Bravo (VDB), 2c: San Bartolo (SBO), 3c: Tlalpan (TLA), 4v,5c: Temascaltepec (TEMv, TEMc), 6v: San Andres de los Gammas (SAG), 7v: Tejupilco (TEJ), 8v: Sultepec (SUL) 9v,10c: Malinalco (MALv, MALc), 11-13v: Tepoztlan I-III (TEPI-TEPIII), 14-16c: Tepoztlan 0, IV and V (TEP0, TEPIV-TEPV), 17v,18c: San Jose de Los Laureles (SJSv, SJSc), 19v: Yautepec (YAU), 20-21v: Ahuehueyo (AHU), Atila (ATI), 22v: Tilapa (TIL), 23v,24l: Atila, hot-spot 1 (ATIH1), 25v,26l: Atila, hot-spot 2 (ATIH2).

Ecological description

In order to explain the environmental requisites of the three *Horismenus* species, a redundancy analysis (RDA) was performed using counts of *H. depressus*, *H. missouriensis* and *H. butcheri* as species data and altitude, annual mean temperature (obtained from INEGI, Instituto Nacional de Estadistica Geografia e Informatica, Mexico), plant of origin and bruchid infestation rate (where B = number of bruchids and P = number of pods), as environmental variables. The data were log-transformed to meet model assumptions. A Monte Carlo simulation (999 permutations under the reduced model) was used to test the significance of each axis of the RDA. Forward selection procedure was used to determine the relative importance and significance of each environmental variable. To evaluate the amount of variance explained by the variables included in the RDA, a PCA (principal component analysis) on the species data was performed. All multivariate analyses were performed with CANOCO 4.5 (ter Braak and Smilauer 2002)

Behavioural analysis

The degree of odour mediated host-plant fidelity was tested for each *Horismenus* species separately in a four-chamber olfactometer. The apparatus, inspired by Steidle and Scholler (1997), consisted of a cylinder (1.8 cm high, 14 cm) separated into four odour source chambers by vertical walls, covered by a removable walking arena (1.8 cm high, 14 cm). Openings (3.8 cm) were drilled on the floor of the walking arena above each chamber. The surface of the walking arena was covered with textile gauze kept in place by a rim of iron (2 mm). A plastic cover was used to close the apparatus. The olfactometer was placed in a white bucket to avoid visual orientation by the wasps. No airflow was generated. Odour sources consisted of 0.5g of cut bean pods from: the plant species of origin of the wasp (PO), the same plant species but collected from a different population (SPDP), the alternative plant species (AP) and a control (CO) consisting of an empty chamber. Wild wasps emerged from samples of the following populations were used: MAL, TLA, ATIH1, SBO, SJS, SJS, TEM, TEJ, TEP0. Prior to the trial the wasps were kept under similar light, temperature and relative humidity conditions as the experimental setup. Due to their small size and the difficulty of manipulating live parasitoids without harming them, species identity of each wasp was determined after testing. Each individual was placed in the centre of the arena and its behaviour recorded using a VCR camera for 13 min. To avoid any positional bias the olfactometer was rotated by 90° after each trial. To remove possible pheromone traces left by previously tested individuals, the textile gauze covering the walking arena was changed every 10 trials. Total time (walking and resting) in each zone was measured from the videos and recorded using the computer software The Observer version 3.0 (Noldus 1988). A Friedman two-way analysis of variance was used to test for differences in the time spent in the four sectors. In the case of significant differences, a Multiple Wilcoxon test was used to determine which sectors differed from each other. No significant difference between the time spent on PO (plant of origin) and SPDP (same plant but from a different population) was found for all three species. Therefore, the time spent on these sectors was summed and divided by two. The new variable was named PO'.

Population genetic structure

Data generation

To assess the role of geographic distance, host beetle specialization and host plant specialization on the genetic population structure of the three *Horismenus* species, a total of 216 females (118 *H. depressus*, 44 *H. missouriensis* and 54 *H. butcheri*) were genotyped using the 6 polymorphic microsatellite loci described in Aebi et al. (2004) and a newly developed locus Ho17 (F: CATCGAAAGGGATATGCGCACG and R: GAAGAGAAGCTATACAGGCAC). Total genomic DNA from each individual was extracted using a Puregene™ DNA isolation kit, Tissue kit (Gentra systems, Minneapolis, USA). PCR were performed following Aebi et al. (2004) and the products deposited for electrophoresis on a denaturing 7.4M urea – 6.5% (w/v) polyacrylamide gel (Sequagel XR, National Diagnostics) resolved on a Li-Cor DNA Analyser. Isolated bands were visualised and analysed using Saga IR^2 software, version 2.2.2.

Microsatellite data analysis

Allele frequencies, allelic richness, expected and observed heterozygosities were calculated for each population of the three parasitoid species. Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were calculated to assess whether the genotypic data fulfilled the assumptions of STRUCTURE (Pritchard et al. 2000). Calculations were performed with GENEPOP (Raymond and Rousset 1995) and FSTAT (Goudet 2001).

Analysis of population structure

Pairwise Fst values between populations within each *Horismenus* species were calculated following Weir and Cockerham (1984). To assess the role of geographic distance on the population structure of each species, isolation by distance analysis was performed. Population differentiation was tested for paired populations across all loci following Weir and Cockerham (Θ) and Fisher's method with the program FSTAT (Goudet 2001). Actual gene flow (Nm) among populations within each species was estimated with the private allele method using GENEPOP (Raymond and Rousset 1995). The number of discrete clusters of genotypes in each *Horismenus* species was determined using the program STRUCTURE (Pritchard et al. 2000). The model used by STRUCTURE attempts to split a multilocus dataset into a specific number of genotype groupings. Each individual is assigned to a group by Markov Chain Monte Carlo simulations under the assumptions of Hardy-Weinberg and linkage equilibrium. To assess the number of groups, the simulation was run several times with the number of groups (K) varying from 1 to 12 for *H. depressus* and *H. missouriensis* and 1 to 10 for *H. butcheri*. The posterior probability Pr (K|X), calculated for each run from the estimated Ln probability of data with Baye's Rule was compared to determine the optimal number of groups matching the actual genetic data. A burnin period of 5000 was used for *H. depressus* and *H. missouriensis* while a burnin period of 20000 was used for *H. butcheri* to reach sufficient sensitivity to investigate within population structure. Three replicates for each K were performed to ensure convergence in estimated parameter values. Although STRUCTURE usually uses only genetic information to learn about population structure, we used the prior population information (i.e. population name) ancestry model to assist the clustering.

Determinants of population structure

To assess the relative impact of geographic isolation, host beetle species and host plant variability on the population structure of *H. depressus*, *H. missouriensis* and *H. butcheri*, we used STRUCTURE to estimate the number of genetic pools within populations where at least two host plants are sympatric.

Under the assumptions that the two bruchid species, *A. obtectus* and *A. obvelatus* are present in each genotyped population we built the following hypotheses. For simplification, we considered geographic isolation, host-beetle species and host-plant variability as unique determinants of population structure even if, due to the complexity of the biological system, a combination of factors may be responsible for patterns of population structure.

Null hypothesis: No population structure. Number of genetic pools (K) is described by $K = 1$, the individuals collected from different populations constitute a single genetic pool. There is high gene flow between populations. No population structure is detected within populations.

Hypothesis 1: Geographic isolation as the unique determinant of population structure. Number of genetic pools is described by $1 < K < N$ (N = number of populations). Some population structure is detected among populations. No substructure is detected within populations.

Hypothesis 2: Host beetle as the unique determinant of population structure. Number of genetic pools is described by $K = 2$, through all populations. Population structure is detected within populations.

Hypothesis 3: Host-plant as unique determinant of population structure. Number of genetic pools is described by $K = N * 2$, in sympatric populations (N = number of sympatric populations). Population structure is detected within populations.

Results

Ecological description

The RDA analysis revealed that the first canonical axis (eigenvalue = 0.173, F = 12.579, P = 0.008) of the RDA, as well as the remaining axes (Trace = 0.215, F = 2.742, P = 0.004), were significant and therefore suitable for the interpretation of the dataset (Fig. 2). The distribution of *H. depressus* was positively correlated with high mean annual temperature and the presence of *P. vulgaris* and *P. lunatus,* and negatively correlated with high altitude, *P. coccineus* and infestation rate. The distribution of *H. missouriensis* was positively correlated with high altitude, *P. coccineus* and infestation rate, and negatively correlated with mean annual temperature and *P. lunatus*. No correlation was found between this species and *P. vulgaris*. The distribution of *H. butcheri* was positively correlated with *P. vulgaris* and mean annual temperature, and negatively correlated with high altitude, *P. coccineus* and infestation rate. The forward selection followed by a Monte-Carlo permutation test revealed that mean annual temperature is the best explanatory variable of the relative abundance of each of the *Horismenus* species (F = 10.99, P = 0.002), followed by bruchid infestation rate (F = 3.3, P = 0.03). All the other variables were not significant. Altitude was ranked in the last position as an artefact of the calculation method,

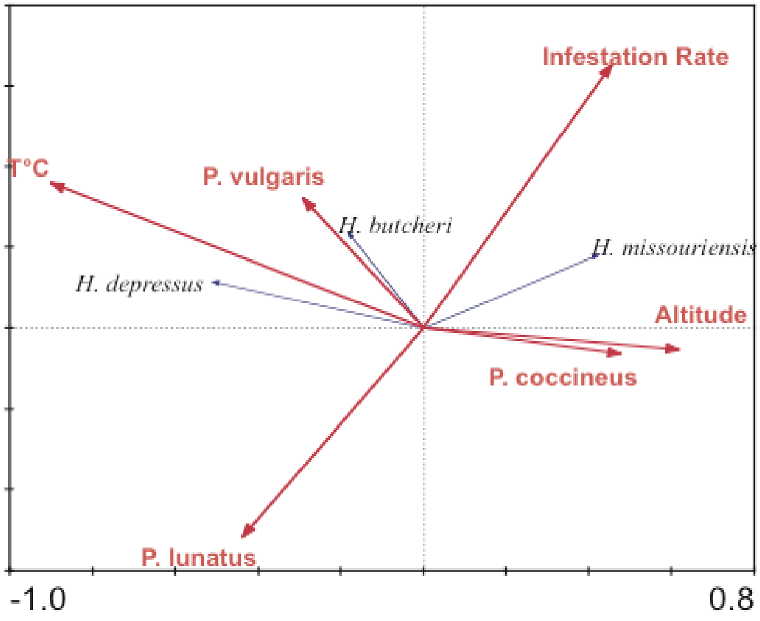

Figure 2. Distribution of *H. depressus*, *H. missouriensis* and *H. butcheri* based on altitude, mean annual temperature (T°C), infestation rate and host-plant (P. *vulgaris, P. lunatus* or *P. coccineus*) in the ordination biplot of an RDA. Axis 1 (Eigenvalue = 0.173) and axis 2 (Eigenvalue = 0.032) are presented.

because of its high negative correlation with mean annual temperature.

To evaluate the importance of the measured environmental variables on the distribution of these *Horismenus* species we also performed a PCA on the species data matrix. The results showed that the two axes of the PCA describe 88% of the variance (sum of eigenvalues = 0.881), whereas the RDA analysis comparing the species data matrix with the environmental variable matrix only explained 20% of the variance. This result suggests that other unmeasured variables have an impact on the distribution and abundance of these *Horismenus* species.

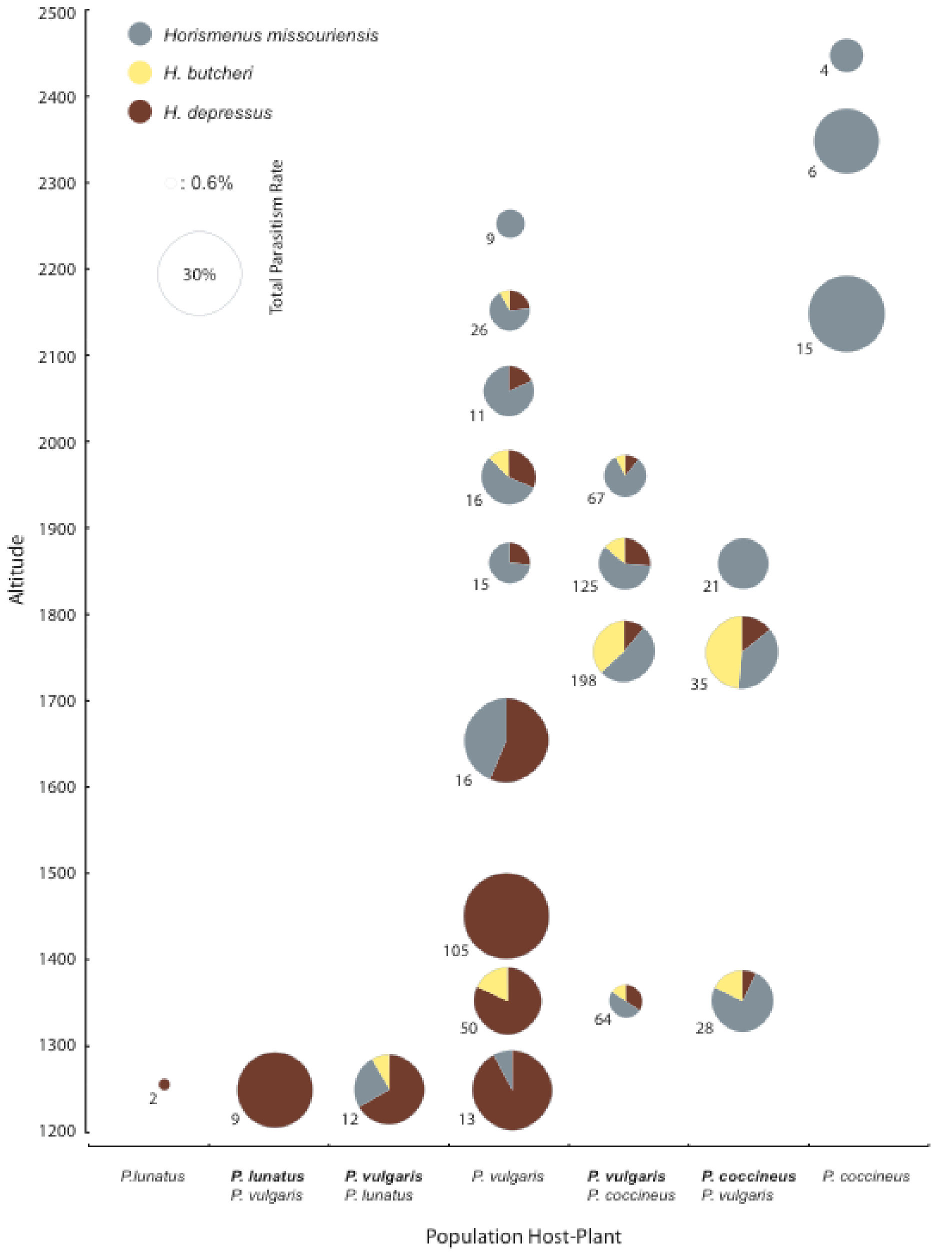

Figure 3. Distribution of *H. depressus, H. missouriensis* and *H. butcheri* in relation to their host-plant and to the altitude. The diameter of each pie chart is proportional to the total parasitism rate. Numbers = number of parasitoids collected.

Although the parasitoids *H. depressus, H. missouriensis* and *H. butcheri* were found in sympatry, they differ in their host plant association and altitudinal range (fig. 3). *Horismenus depressus* was the only species found on *P. lunatus* but it was also reared from many samples of *P. vulgaris*. It was the most abundant *Horismenus* species found in populations below 1700 m. Similarly, whereas *H. missouriensis* was the only species found in pure *P. coccineus* populations, it was also reared from samples of *P. vulgaris*, and was the most abundant *Horismenus* species found in populations above 1800 m. Finally, *H. butcheri* displayed a more strict host plant association, as it was only reared from *P. vulgaris*, except for a few accounts on *P. coccineus* in populations where this bean is mixed with *P. vulgaris*.

Behavioural analysis

Parasitoids placed in the olfactometer displayed walking, resting and grooming behaviour. When leaving a sector with an apparently attractive odour source, some parasitoids increased their turning rate and often returned to the sector. All *Horismenus* species displayed host fidelity, as they were preferentially attracted to their plant of origin (Fig. 4). We found significant differences in the time spent by *H. depressus* (males), *H. missouriensis* (males and females) and *H. butcheri* (males) in the different sectors (Table 1). Results from the multiple comparison Wilcoxon tests revealed that all these groups spent significantly more time on the sector containing the plant of origin (PO’) than in the sectors containing the alternative plant or the control. In each case, no significant difference was found between the time spent on the alternative plant and on the control. Due to very small sample sizes no statistical tests were performed for *H. butcheri* females, but the time spent in the PO' sector suggests a similar result.

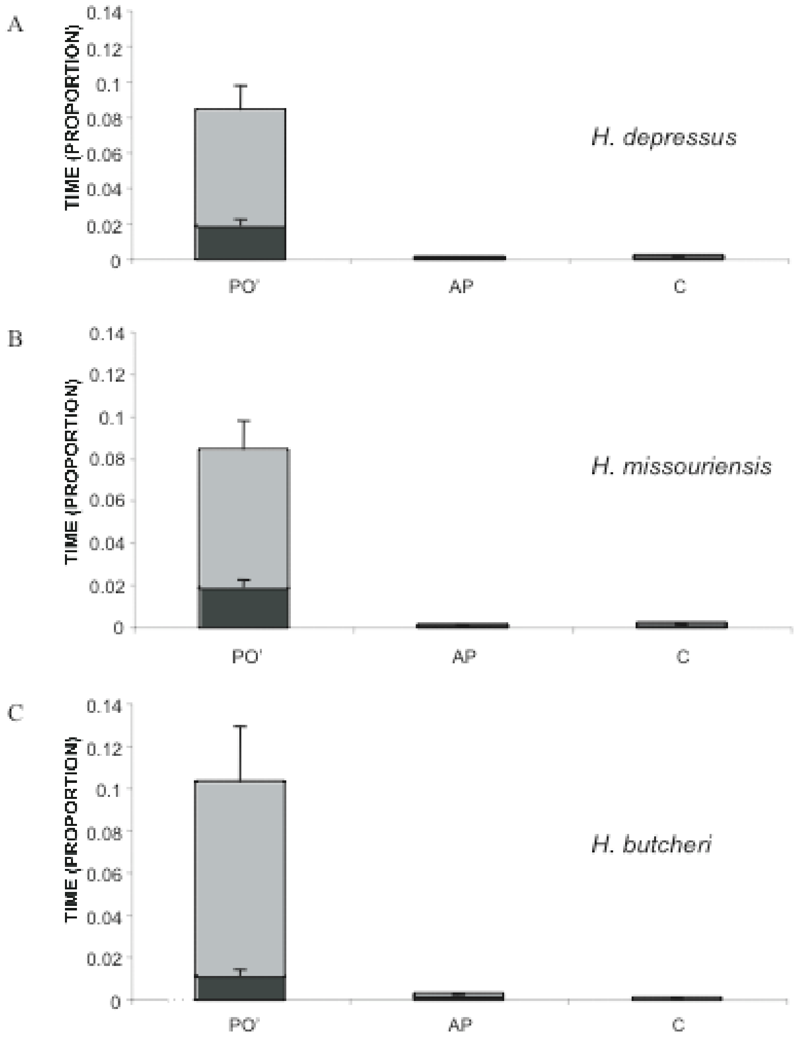

Figure. 4. Total time (mean ± SE) spent by A. *H. depressus*, B. *H. missouriensis* and C. *H. butcheri* on sectors of a four chamber olfactometer containing their plant of origin (PO'), the alternative plant species (AP) and a control (CO). Resting time is plotted in grey and walking time in black

Table 1. Behavioural analysis. P-values of the Friedman two-way analyses of variance and the multiple comparison Wilcoxon tests to compare the total (walking + resting) time spent by *Horismenus depressus, H. missouriensis* and *H. butcheri* males (m) and females (f) on the sectors containing their plant of origin (PO'), an alternative plant species (AP) and the control (C). N = number of individuals tested. * = significant p-values

	Sex	N	Friedman test	Wilcoxon test		
				PO'-AP	PO'-C	AP-C
H. depressus	m	10	0.003*	0.008*	0.015*	0.917
	f	9	0.062	-	-	-
H. missouriensis	m	40	<0.001*	<0.001*	<0.001*	0.894
	f	28	<0.001*	<0.001*	<0.001*	0.670
H. butcheri	m	8	0.002*	0.018*	0.018*	0.172
	f	2	-	-	-	-

Population genetic structure

Microsatellite data analysis

Allele frequencies, allelic richness, expected and observed heterozygosities, and deviations from Hardy-Weinberg equilibrium (HWE) are presented in appendices 1 to 3.

H. depressus

The seven polymorphic microsatellites generated a total of 108 alleles with very variable frequencies across populations. Allelic richness at each locus among all populations ranged from 1 to 3.715. Out of the 56 population-locus combinations, 17 showed a significant deficit in heterozygotes, probably due to the presence of null alleles.

A high level of linkage disequilibrium was found in MALv (10 pairs of loci from the 21 possible pairs) and TEJ (14 pairs of loci from the 21 possible pairs). Across all populations, 11 pairs of loci from the 21 possible pairs were found to be in highly significant ($p < 0.001$) linkage disequilibrium and 2 pairs in significant ($p < 0.05$) linkage disequilibrium. These results are probably due to severe bottlenecks caused by two-year cycles in host-plant abundance, causing dramatic bruchid and parasitoid population size reduction, observed in most of the sampled localities. Special care should therefore be taken in the interpretation of results generated with STRUCTURE.

H. missouriensis

The seven polymorphic microsatellites generated a total of 90 alleles with very variable frequencies across populations. Allelic richness at each locus among all populations ranged from 1 to 2. Among the 56 population-locus combinations, 8 showed a significant deficit in heterozygotes, again probably due to the presence of null alleles.

Across all populations, only 2 pairs of loci from the 21 possible pairs were found to be in slight but significant ($p < 0.05$) linkage disequilibrium, enabling us to trust the results generated with STRUCTURE.

H. butcheri

The seven polymorphic microsatellites generated a total of 94 alleles with very variable frequencies across populations. Allelic richness at each locus among all populations ranged from 1 to 1.966. Among the 56 population-locus combinations, eight showed a significant deficit in heterozygotes, probably due to the presence of null alleles.

Across all populations, no pairs of loci from the 21 possible pairs were found to be in linkage disequilibrium, enabling us to trust the results generated with STRUCTURE.

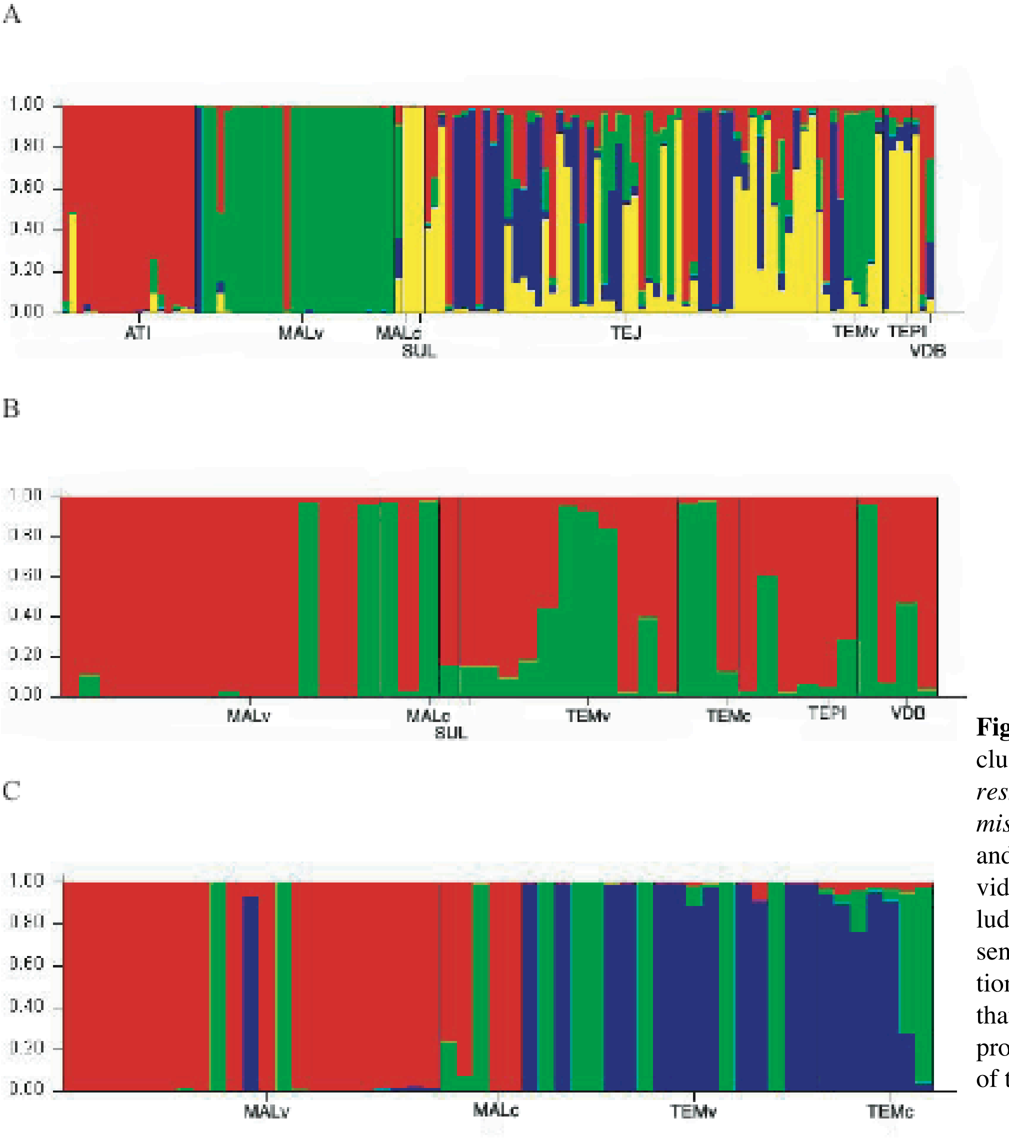

Figure 5. Results of a Bayesian cluster analysis for A. *H. depressus* (118 individuals), B. *H. missouriensis* (44 individuals) and C. *H. butcheri* (54 individuals). Each individual included in the analysis is represented by a vertical line, partitioned into colored segments that represent the individual's probability of belonging to each of the K genetic clusters

Analysis of population structure

H. depressus

The pairwise Fst analysis (Table 2) and the isolation by distance results (slope = -84.2, intercept = 93.1, *P* value = 0.0001) did not reveal any structure among the *H. depressus* populations separated by 20 to 170 Km. No genetic differentiation among populations was detected with Θ = 0.037, following Weir and Cockerham (1984). The analysis of genetic differentiation (Table 2) following Fisher's method revealed some genetic isolation of the ATI and MALv populations. Indeed, significant ($P < 0.05$) genetic differentiation was found between ATI and the populations: MALv and TEPI, and between MALv and the populations: ATI, SULI, TEJ, TEMv and TEPI. The low population structuring was confirmed by an overall high gene flow estimate (Nm = 2) calculated with the private allele method.

Table 2. Pairwise Fst values between *H. depressus* populations (lower triangle) and genotypic differentiation for each population pair across all loci following Fisher's method (upper triangle). * = $P < 0.05$, ** = $P < 0.001$

	ATI	MALv	SUL	TEJ	TEMv	TEPI	VDB
ATI	-	0.00**	0.104	0.111	0.095	0.011*	0.124
MALv	0.0491	-	0.024*	0.00**	0.003*	0.00**	0.236
SUL	0.0450	0.0454	-	0.068	0.811	0.018*	0.822
TEJ	0.0010	0.0651	0.0453	-	0.243	0.145	0.221
TEMv	0.0114	0.0242	-0.0017	0.0041	-	0.343	0.906
TEPI	0.0570	0.1325	0.1737	0.0217	0.0553	-	0.167
VDB	0.0372	0.0356	0.0437	0.0378	0.0097	0.1598	-

The genotype pool number estimation obtained with structure provided strong evidence (Pr(K|X) = ~ 1) of division of the genotypes obtained by screening individuals from 8 distinct populations into four groups (Table 3, Fig 5). An analysis of the populations constituting these four groups revealed that ATI, MALv, SULI and TEPI to a lesser extent represent well-defined entities, while the individuals from the remaining populations present mixed genotypes from all four groups. Although these results should be interpreted with care for the above-mentioned reasons, this pattern is consistent with the results obtained by other estimates of population structure. Furthermore, these results are consistent with the spatial distribution of these populations (Fig. 1) as the Nevado de Toluca, a volcano peaking at 4578m, separates the genotyped populations into two distinct groups with VDB, TEJ, TEM, TEJ SULI to the west and ATI, MALv and TEPI to the east.

K	ln Pr(X|K)	Pr(K|X)
1	-3524	~ 0
2	-3448	~ 0
3	-3417	~ 0
4	-3362	~ 1
5	-3443	~ 0
6	-3622	~ 0
7	-3669	~ 0
8	-3705	~ 0
9	-3400	~ 0
10	-3676	~ 0
11	-3667	~ 0
12	-4551	~ 0

Table 3. Conditional and posterior probabilities associated with each pool number (K) for *H. depressus*. Ln Pr(X|K) is the estimated conditional probability of observing the data under a model with the specified number of pools. Pr(K|X) is the posterior probability of each pool number given the genotypic information.

H. missouriensis

The pairwise Fst analysis (Table 4) and the isolation by distance results (slope = 39.01, intercept = 49.4, *P* value = 0.072) did not reveal any structure among the genotyped *H. missouriensis* populations distant from 20 to 100 Km. No genetic differentiation among populations was detected with $\Theta = 0.026$, following Weir and Cockerham (1984). The analysis of genetic differentiation (Table 3) following Fisher's method only reveals significant ($P < 0.05$) genetic differentiation between MALv and TEPI and between TEMv and TEPI. The apparent low population structuring was confirmed by a very high overall gene flow estimate (Nm = 4) calculated with the private allele method.

Table 4. Pairwise Fst values between *H. missouriensis* populations (lower triangle) and genotypic differentiation for each population pair across all loci following Fisher's method (upper triangle). * = $P < 0.05$, ** = $P < 0.001$

	MALv	MALc	TEMv	TEMc	TEPI	VDB
MALv	-	0.988	0.102	0.901	0.014*	0.064
MALc	-0.0239	-	0.095	0.992	0.747	0.889
TEMv	0.0250	0.0483	-	0.840	0.039*	0.175
TEMc	0.0431	0.0118	-0.0063	-	0.695	0.901
TEPI	0.0345	0.0685	0.0488	0.0587	-	0.442
VDB	0.0372	0.0284	0.0161	0.0883	0.0355	-

The genotype pool number estimation obtained with structure provided weak evidence (Pr(K|X) = 0.73) of division of the genotypes into two groups (Table 5, Fig 5). An analysis of the populations constituting these two groups reveals that MALv represents a well-differentiated group whereas the remaining populations are constituted of individuals with mixed genotypes. This pattern is consistent with the results obtained with other estimates of population structure and with the data obtained for *H. depressus,* and strongly suggests high gene flow among *H. missouriensis* populations.

K	ln Pr(X|K)	Pr(K|X)
1	-1357	~ 0
2	-1278	0.73
3	-1311	~ 0
4	-1279	0.27
5	-1381	~ 0
6	-1341	~ 0
7	-1386	~ 0
8	-1360	~ 0
9	-1352	~ 0
10	-1387	~ 0
11	-1461	~ 0
12	-1403	~ 0

Table 5. Conditional and posterior probabilities associated with each pool number (K) for *H. missouriensis*. Ln Pr(X|K) is the estimated conditional probability of observing the data under a model with the specified number of pools. Pr(K|X) is the posterior probability of each pool number given the genotypic information.

H. butcheri

Due to the low number of analyzed populations, we did not perform isolation by distance test. Nevertheless, the pairwise Fst analysis (Table 6) shows high genetic differentiation between all population pairs. High genetic differentiation among populations was detected with $\Theta = 0.257$, following Weir and Cockerham (1984). The analysis of genetic differentiation (Table 6) following Fisher's method revealed a significant ($p<0.01$) genetic differentiation between populations and between parasitoids originating from different host-plants within populations. The high population structuring was confirmed by an overall low gene flow estimate (Nm = 1) calculated with the private allele method.

Table 6. Pairwise Fst values between *H. butcheri* populations (lower triangle) and genotypic differentiation for each population pair across all loci following Fisher's method (upper triangle). * = $P < 0.05$, ** = $P < 0.001$

	MALv	MALc	TEMv	TEMc
MALv	-	0.00**	0.00**	0.00**
MALc	0.1896	-	0.005*	0.00**
TEMv	0.2899	0.1416	-	0.00**
TEMc	0.3233	0.3076	0.1424	-

The genotype pool number estimation obtained with STRUCTURE provided strong evidence (Pr(K|X) = ~ 1) of division of the genotypes into three groups (Table 7, Fig 5). Parasitoids originating from Malinalco (MALv and MALc) are genetically differentiated from the individuals collected in Temascaltepec (TEMv and TEMc), the third genetic pool consisting of individuals scattered throughout the populations.

K	ln Pr(X|K)	Pr(K|X)
1	-1411	~ 0
2	-1267	~ 0
3	-1204	~ 1
4	-1246	~ 0
5	-1220	~ 0
6	-1301	~ 0
7	-1265	~ 0
8	-1264	~ 0
9	-1228	~ 0
10	-1274	~ 0

Table 7. Conditional and posterior probabilities associated with each pool number (K) for *H. butcheri*. Ln Pr(X|K) is the estimated conditional probability of observing the data under a model with the specified number of pools. Pr(K|X) is the posterior probability of each pool number given the genotypic information.

Determinants of population structure

H. depressus

Because of the scarcity of individuals collected on *P. coccineus* in sympatric populations we were not able to assess the role of host plant variability on the population genetic structure for *H. depressus*. Nevertheless, the results of the STRUCTURE analysis with K estimated at 4 show that geographic isolation (Hypothesis 1) seemed to be the only factor affecting the population structure of this parasitoid species. The fact that no substructure was detected within the well-differentiated populations (ATI and MALv) suggests no genetic differentiation between groups of parasitoids on the two bruchid species.

H. missouriensis

For *H. missouriensis* the results even if not very strong may suggest host-beetle induced genetic differentiation as K was estimated as 2. Despite a division of individuals into two genetic pools, a further examination of the population structure within two large populations (MALv and TEMv) resulted in an estimated number of genetic pools of K = 1. No detection of further within-population genetic differentiation is probably due to high gene flow between the two gene pools.

H. butcheri

In the case of *H. butcheri*, results strongly support the presence of three genetic pools. A close examination of the results revealed that one genetic pool was constituted of individuals collected in Malinalco (MALv, on *P. vulgaris* and MALc on *P. coccineus*), and a second of the individuals collected in Temascaltepec (TEMv, on *P. vulgaris* and TEMc on *P. coccineus*). The third genetic pool consisted of individuals scattered through all four populations. A closer analysis of the population structure within the subpopulations on *P. vulgaris* (MALv and TEMv) strongly supported the presence of K = 2 genetic pools within each population, perhaps suggesting host-beetle induced genetic differentiation or the presence of two generations in the sample. In contrast, an analysis of the population structure within the subpopulation on *P. coccineus* (MALc and TEMc) strongly supports the presence of a single genetic pool within TEMc and suggested no further genetic differentiation within MALc (data not shown). These results suggest a shift from a group within the population from *P. vulgaris* to *P. coccineus*. A close examination of the barplot (Fig 5) reveals some gene flow between sub-populations present on the two plant species in both populations, as well as between the two populations.

Discussion

Do plants shape the community structure of this parasitoid complex?

Although the three bean species collected for this study can be found in sympatry, they differ greatly in their normal habitat characteristics. The wild relative of the Lima bean, *P. lunatus*, grows between sea level and 1600 m in deciduous tropical forests in moist areas. The ancestor of the common black bean, *P. vulgaris*, has a wider altitudinal range and can be encountered between 50 and 3000 m, but especially between 1500 to 1900 m in dry deciduous forests (Delgado-Salinas et al. 1988). Finally, the ancestor of the ayocote, *P. coccineus*, is restricted to habitats between 1400 and 2800 m in pine-oak forests (Freytag and Debouck 2002). Similarly, despite their close taxonomic position, the three *Horismenus* species have specific ecological requirements allowing them to take advantage of different ecological niches scattered along the altitudinal gradient. The wide altitudinal range of the *Horismenus* species brings them into contact with all three bean species. Yet their distributions differ, with *H. depressus* being the most abundant parasitoid below 1700 m, and *H. missouriensis* the most abundant above 1700 m. Additionally, plant features surely have an impact on the *Horismenus* distribution, as different plant species from a given altitude do not share the same parasitoid load. Indeed, *P. lunatus* (at low altitudes) only carries *H. depressus*, and *P. coccineus* (at high altitudes) only carries *H. missouriensis*, while all three parasitoid species can be found on *P. vulgaris* throughout the entire altitudinal range. Finally, *H. butcheri* is the only species displaying strict host plant specialization as it was only collected on *P. vulgaris* seeds, with the notable exception of samples collected on *P. coccineus* in sympatry with *P. vulgaris*. The occurrence of *H. butcheri* on these *P. coccineus* samples might be explained by the frequent introgression occurring between *P. vulgaris* and *P. coccineus* (Llaca et al. 1994; Wall and Wall 1975) Genetic studies show that *P. vulgaris* and *P. coccineus* are close relatives (Caicedo et al. 1999; Piñero and Eguiarte 1988), and they are often found in sympatry. In addition, *P. vulgaris* is autogamous, while *P. coccineus* is allogamous, thereby allowing transfer of pollen produced by *P. vulgaris* to *P. coccineus* via bees and other pollinators (Escalante et al. 1994). Thus, the existence of common traits between both plants enabled *H. butcheri* to use the hybridized *P. coccineus* as an alternative host plant.

Do these parasitoids display host-plant fidelity?

To localize their often-concealed hosts, parasitoids are known to rely on cues emanating from plants on which their hosts feed rather than from the hosts themselves (Dicke et al. 2003; Turlings and Wäckers 2004; Turlings et al. 1993; Vet et al. 1995). Various studies have demonstrated that chalcidoid parasitoids such as the ones studied here, also rely on volatile cues emanating from their host plants to localize their hosts including the bruchid parasitoids *Uscana* *lariophaga* (Trichogrammatidae) (van Huis et al. 1994), *Eupelmus vuilleti* (Eupelmidae) (Cortesero et al. 1993), and *Dinarmus basalis* (Pteromalidae) (Aebi, unpublished data). Parasitoids in the family Eulophidae, to which *Horismenus* belongs, are known to exhibit host plant specificity (Askew and Ruse 1974).

The olfactometer assays revealed that all three *Horismenus* species are more attracted to the odours emanating from their host plant of origin rather than to the odours from alternative plant species. This could be a genetically based preference, but could also be a conditioned response, as the wasps spend their entire immature life stages within bean seeds and are therefore prone to display some preference for the bean to which they have been exposed during their development (Cortesero and Monge 1994).

Host-plant fidelity most likely plays a key role in the genetic differentiation between sub-populations. This could be further reinforced if plant cues are also used to find mates. Host-plant recognition and preference displayed by both sexes may increase mating rates in such a complex environment and favour the selection of specific gene combinations (Craig et al. 1993; Rice 1984; Rice 1987).

What is the relative impact of geographic isolation, host variability and host-plant variability on the genetic population structure of Horismenus species?

Population structure at neutral genetic markers arises as a consequence of reduced gene flow between groups of individuals, and subsequent processes such as natural selection, genetic drift, inbreeding, and assortative mating within these groups. Geographic isolation is the most frequent cause of gene flow reduction. But, population structure may also arise without geographic isolation by various means. When groups of individuals specialize on different substrates, assortative mating may act as an efficient barrier to gene flow between sub-populations. The results of our analysis on population structure gave contrasting results for the three *Horismenus* species. The high dispersal ability of *H. depressus* must have a homogenizing effect among the screened populations, and only allowed some structuring between populations that are separated by a large distance or by a physical barrier such as a 4578 m mountain. No signature of host-beetle mediated genetic differentiation was detected for *H. depressus*. Host-plant mediated genetic differentiation could not be tested for this parasitoid species between *P. vulgaris* and *P. coccineus* because of low sample sizes. Nevertheless, the strong association of this species with *P. lunatus* and its ability to use *P. vulgaris* as an alternative plant in sympatry warrants further genetic analyses on this system.

H. missourensis was the only species collected on cultivated seeds and has the capacity to develop under storage conditions (Hansson et al. 2004). The extremely high dispersal ability of this species and the fact that it is a highly generalist parasitoid may explain its unique ability to use cultivated beans. Once it has colonized fields of cultivated beans, dispersal may be enhanced through bean trade among farmers. The results on the determinants of the genetic structure for this species suggested the existence of two distinct genetic pools scattered over the entire range (in both sympatric and allopatric populations). Although not strong, this result may correspond to two genetic strains perhaps specialized on *A. obtectus* and *A. obvelatus*. Although we do not have direct evidence for a parasitoid preference on the two bruchid species, a number of factors could explain a beetle-mediated genetic differentiation. Despite *A. obtectus* and *A. obvelatus* coexist in sympatry, their relative abundance varies greatly through the season (Alvarez et al. 2004). While *A. obvelatus* is univoltine, with a defined reproductive diapause that enables it to colonize wild beans at the beginning of the fruiting season, *A. obtectus* is multivoltine, overwinters in storage conditions and its abundance gradually builds up throughout the season (Alvarez et al. 2004). Such differences in the temporal abundance of these two bruchid species may result in different encounter rates for early and late season parasitoids that could eventually specialize on one of the two species and remain reproductively isolated. In a similar manner, allochronic reproductive isolation was demonstrated to partially reduce gene flow between two *Eurosta solidagnis* host races (Craig et al. 1993). Alternatively, Eulophids are known to be bivoltine (Bryan, 1983), therefore, the pattern observed may be due to the presence of individuals from both generations in the sample.

Finally, *H. butcheri* was the only parasitoid species for which host plant mediated genetic differentiation was detected. The level of genetic differentiation was similar between populations and between host plants within populations. The genotypes of the individuals collected from two pairs of sympatric bean populations clustered into 3 distinct genetic pools partially verifying hypotheses 2 and 3. A primary differentiation was detected between the individuals collected in the two localities. The third genetic pool consisted of individuals scattered through all populations. Further analyses of the data for individuals collected on the two *Phaseolus* species revealed that each *P. vulgaris* sub-population was actually composed of two genetic pools. The *P. coccineus* sub-population showed no further genetic population structure. These results strongly suggest a shift of part of the population growing on *P. vulgaris* to *P. coccineus* and a subsequent genetic differentiation triggered by assortative mating. Many insect species mate on their host-plant (Landolt and Phillips 1997). We conducted some preliminary experiments to ascertain the role of plants on the mating behaviour of these *Horismenus* species and found that mating rates increased in the presence of plant parts (pods). While no matings were observed (10 pairs) without plant parts, 5 pairs (out of 14) mated several times in the presence of pods suggesting that mating readily occurs on the host plant. Furthermore, to verify the existence of host races rather than sibling species on the two plant species, we compared the morphology and determined the amount of gene flow between *H. butcheri* on *P. vulgaris* and *H. butcheri* on *P. coccineus*. No morphological differences, but significant levels of gene flow, were detected between the two *H. butcheri* groups as revealed by the STRUCTURE analysis, indicating that they are indeed host races and not sibling species (Drès and Mallet 2002).

To further support host plant mediated genetic differentiation in the parasitoid *H. butcheri*, the extent of host plant mediated genetic differentiation has to be evaluated for each bruchid beetle. Despite their distinct altitudinal range, *A. obtectus* is only found between 1200 and 1900 m, and *A. obvelatus* between 1700 and 2400 m, both species can be found in sympatry (Alvarez et al. 2004; Pichard et al. 1991). In mixed plant populations, the relative abundances of *A. obtectus* and *A. obvelatus* are similar showing that host plant does not affect their ecological distribution and none of them are specialized on one plant species (Alvarez et al. 2004). Two separate studies using allozyme electrophoresis (Gonzalez-Rodriguez et al. 2000) and microsatellite markers (Alvarez et al. 2003), showed that host-plants have no effect on the genetic population structure of both *A. obtectus* and *A. obvelatus* collected on wild and cultivated seeds of *P. vulgaris* and *P. coccineus*. When individuals of *A. obvelatus* from four mixed population with *P. coccineus* and *P. vulgaris* were genotyped, no genetic differentiation was detected between groups of individuals from different host-plants as Fst values (calculated following Weir and Cockerham) ranged between 0.008 and 0.0183. Because *A. obvelatus* is more common than *A. obtectus* in wild bean populations (Alvarez, 2004), low sample size of *A. obtectus* prevent us from estimating levels of genetic differentiation between groups of individuals on different host plants. Nevertheless, a genetic analysis on *A. obtectus* populations shows a similar pattern as the one observed for *A. obvelatus* (Alvarez et al. in prep*)*, with no evidence for host-plant mediated genetic differentiation. Therefore, for *H. butcheri*, the two host races associated to the two bean species, *P. vulgaris* and *P. coccineus*, must be the result of host-plant mediated genetic differentiation at the third trophic level and not via the bruchid host.

Conclusion

Despite being closely related and having similar feeding habits, *H. depressus, H. missouriensis* and *H. butcheri* differ greatly in their ecological distribution. The interplay of strict host plant specialization, host plant fidelity and host-plant induced genetic differentiation found for *H. butcheri* strongly supports the idea of host race formation at the plant level in this species. Other studies have proposed the existence of tree-associated forms in another eulophid species (Askew and Coshan 1973; Rott and Godfray 2000). However, this work represents the first study of host race formation at the third trophic level, including ecological and genetic data. Eulophids, with their assemblages of closely related species and their host-plant induced community structure, provide ideal models for the study of new sympatric speciation scenarios via host-race formation.

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Appendix 1. Microsatellite allele frequencies for all *H. depressus* populations. Number in brackets = sample size. *He* = Expected heterozygosities, *Ho* = observed heterozygosities, *A* = allelic richness. * = significant heterozygote deficit.

Locus	Allele	ATI (18)	MALv (27)	MALc (1)	SULI (3)	TEJ (53)	TEMv (9)	TEPI (4)	VDB (3)
Ho4b	122	-	-	-	-	0.01	-	-	-
	124	0.071	-	-	-	0.04	-	-	-
	126	0.286	0.288	-	0.167	0.18	0.214	0.125	0.5
	128	0.071	0.173	0.5	-	0.24	0.357	0.5	-
	130	0.179	0.077	0.5	0.5	0.11	0.071	0.125	-
	132	0.357	0.365	-	0.333	0.29	0.214	0.25	0.5
	134	0.036	0.038	-	-	0.1	0.071	-	-
	136	-	0.019	-	-	0.03	0.071	-	-
	140	-	0.038	-	-	-	-	-	-
	He	0.11	0.20	0.01	0.02	0.41	0.06	0.03	0.01
	Ho	0.13	0.18*	0.01	0.01	0.39	0.03*	0.03	0.02
	A	2.864	2.806	-	2.6	3.014	3.072	2.771	2
Ho8b	162	0.033	-	-	-	-	-	0.25	-
	164	-	-	-	-	-	0.111	-	-
	168	0.267	0.02	-	0.167	0.181	-	-	0.333
	170	-	0.06	-	0.333	0.053	0.278	-	0.667
	172	-	-	-	-	0.074	-	-	-
	174	0.033	0.02	-	-	0.021	0.111	0.125	-
	176	0.067	0.1	-	-	-	0.111	-	-
	178	0.067	0.04	-	0.333	0.064	0.056	-	-
	180	0.133	0.18	0.5	-	0.149	0.056	-	-
	182	0.133	0.22	-	0.167	0.032	0.056	0.125	-
	184	0.233	0.2	-	-	0.202	0.056	0.125	-
	186	0.033	0.12	-	-	0.16	0.111	0.25	-
	188	-	-	-	-	-	-	0.125	-
	190	-	-	0.5	-	-	-	-	-
	192	-	0.04	-	-	-	-	-	-
	196	-	-	-	-	0.021	-	-	-
	212	-	-	-	-	0.021	-	-	-
	214	-	-	-	-	0.021	-	-	-
	298	-	-	-	-	-	0.056	-	-
	He	0.13	0.22	0.01	0.03	0.41	0.08	0.04	0.02
	Ho	0.08*	0.03*	0.01	0.01	0.17*	0.05*	0.02*	0
	A	3.225	3.266	-	3.2	3.31	3.498	3.571	1.933
Ho10b	146	-	-	-	-	0.01	-	-	-
	148	0.036	-	-	-	0.01	-	-	-
	150	-	-	-	-	0.01	-	-	-
	152	0.036	-	-	0.167	0.01	-	-	-
	154	0.071	0.077	-	0.333	0.26	0.167	0.125	0.167
	156	0.214	0.269	0.5	0.167	0.16	0.389	-	0.333
	158	0.393	0.288	0.5	-	0.27	0.222	0.5	0.5
	160	0.107	0.154	-	0.333	0.15	0.167	-	-
	162	0.036	-	-	-	0.02	0.056	0.25	-
	164	-	0.038	-	-	0.01	-	0.125	-
	166	-	0.038	-	-	-	-	-	-
	168	-	-	-	-	0.01	-	-	-
	174	0.107	0.135	-	-	0.08	-	-	-

	He	0.11	0.21	0.01	0.03	0.41	0.07	0.03	0.02
	Ho	0.11	0.20	0.01	0.01	0.36	0.07	0.04	0.03
	A	2.989	3.012	-	3.2	3.027	2.895	2.771	2.6
Ho16	170	-	0.022	-	-	-	-	-	-
	172	0.094	-	-	-	0.076	-	-	-
	174	-	-	-	-	0.022	-	-	-
	176	-	-	-	-	0.022	0.125	-	-
	178	-	0.065	-	-	0.065	-	-	-
	180	-	0.022	-	-	0.022	0.125	-	-
	182	-	0.043	-	-	0.011	0.125	-	-
	184	-	-	-	0.333	0.043	-	0.333	0.167
	186	-	-	-	-	0.022	-	-	-
	188	-	0.022	0.5	-	0.011	-	-	-
	190	-	0.087	0.5	-	0.054	-	-	-
	192	0.063	0.022	-	-	0.043	-	-	-
	194	-	-	-	0.167	-	-	0.167	-
	196	0.031	0.13	-	-	0.022	-	0.167	0.167
	198	0.094	0.043	-	-	0.033	0.125	-	-
	200	0.063	-	-	-	0.076	-	-	-
	202	0.094	-	-	-	0.011	0.125	-	-
	204	-	0.087	-	-	0.054	-	-	-
	206	0.063	0.022	-	0.167	0.087	-	0.167	-
	208	0.031	0.283	-	-	0.054	-	-	0.167
	210	0.031	-	-	-	0.043	0.25	-	-
	212	0.094	0.043	-	-	0.022	-	-	-
	214	0.094	0.022	-	-	0.054	-	-	0.167
	216	-	0.022	-	-	0.033	-	-	-
	218	0.094	0.022	-	-	-	-	-	-
	220	0.094	0.022	-	-	0.022	-	-	-
	222	-	-	-	-	0.022	-	-	-
	224	-	-	-	0.167	0.011	-	0.167	-
	226	-	-	-	0.167	-	-	-	-
	230	-	0.022	-	-	0.011	-	-	-
	232	0.031	-	-	-	0.011	-	-	-
	234	-	-	-	-	0.011	-	-	-
	238	-	-	-	-	-	0.125	-	-
	240	-	-	-	-	0.011	-	-	-
	246	0.031	-	-	-	-	-	-	0.167
	248	-	-	-	-	0.022	-	-	-
	250	-	-	-	-	-	-	-	0.167
	He	0.15	0.21	0.01	0.03	0.44	0.04	0.03	0.03
	Ho	0.14	0.17*	0.01	0.03	0.40*	0.04	0.03	0.03
	A	3.715	3.435	-	3.6	3.78	3.786	3.6	4
Ho17	222	-	-	-	-	0.041	-	-	-
	226	-	-	-	-	0.122	0.167	0.125	0.167
	230	0.346	0.442	-	0.167	0.316	0.444	0.625	0.333
	234	0.5	0.077	1	0.5	0.418	0.333	0.25	0.167
	238	0.154	0.404	-	0.333	0.031	0.056	-	0.333
	242	-	0.038	-	-	0.071	-	-	-
	246	-	0.038	-	-	-	-	-	-
	He	0.08	0.17	-	0.02	0.34	0.06	0.02	0.03
	Ho	0.07	0.12*	-	0.01	0.29*	0.06	0.03	0.03
	A	2.304	2.376	-	2.6	2.625	2.546	2.286	3.2

Ho6b	163	-	0.038	-	-	-	-	-	-
	165	-	0.019	-	-	-	-	-	-
	169	0.033	-	-	-	0.049	0.056	-	-
	171	0.033	0.096	-	-	0.069	0.111	-	-
	173	0.133	0.038	0.5	-	0.049	0.111	-	-
	175	0.233	0.231	-	0.667	0.167	0.167	-	0.5
	177	0.433	0.25	-	0.333	0.5	0.333	1	0.5
	179	0.133	0.327	-	-	0.098	0.111	-	-
	181	-	-	-	-	0.039	0.111	-	-
	183	-	-	0.5	-	-	-	-	-
	189	-	-	-	-	0.01	-	-	-
	193	-	-	-	-	0.02	-	-	-
	He	0.11	0.20	0.01	0.02	0.36	0.08	-	0.02
	Ho	0.09	0.17*	0.01	0	0.16*	0.06	-	0.01
	A	2.766	2.882	-	1.933	2.694	3.235	1	2
Ho9b	191	-	-	-	-	0.038	-	-	-
	195	0.029	0.019	-	-	0.019	-	-	-
	197	0.441	0.077	0.5	0.167	0.528	0.278	0.75	0.25
	199	0.206	0.5	0.5	0.833	0.226	0.444	-	-
	201	0.088	0.231	-	-	0.113	0.111	-	0.75
	203	0.235	-	-	-	0.075	0.167	0.25	-
	205	-	0.077	-	-	-	-	-	-
	207	-	0.019	-	-	-	-	-	-
	209	-	0.019	-	-	-	-	-	-
	211	-	0.019	-	-	-	-	-	-
	213	-	0.038	-	-	-	-	-	-
	He	0.12	0.18	0.01	0.01	0.36	0.07	0.02	0.01
	Ho	0.09*	0.13*	0.01	0.01	0.15*	0.01*	0.02	0.01
	A	2.655	2.627	-	1.667	2.479	2.657	1.786	2

Appendix 2. Microsatellite allele frequencies for all *H. missouriensis* populations. Number in brackets = sample size. *He* = Expected heterozygosities, *Ho* = observed heterozygosities, *A* = allelic richness. * = significant heterozygote deficit.

Locus	Allele	MALv (16)	MALc (3)	SULI (1)	TEMv (11)	TEMc (3)	TEPI (6)	VDB (4)
Ho4b	124	0.036	-	-	-	-	-	-
	126	0.036	-	-	-	-	-	-
	128	0.25	0.25	1	0.3	-	0.6	0.5
	130	0.357	-	-	0.25	-	-	-
	132	0.036	0.25	-	0.35	1	0.2	0.375
	134	0.071	0.25	-	-	-	-	0.125
	136	0.143	0.25	-	0.1	-	-	-
	138	0.036	-	-	-	-	-	-
	140	-	-	-	-	-	0.1	-
	142	-	-	-	-	-	0.1	-
	148	0.036	-	-	-	-	-	-
	He	0.11	0.02	-	0.08	-	0.03	0.03
	Ho	0.07*	0.02	-	0.06*	-	0.03	0.01
	A	1.807	2	1	1.753	1	1.644	1.786
Ho8b	178	0.036	-	-	0.111	-	-	0.167
	180	0.036	-	-	-	-	-	-
	182	0.071	-	-	-	-	0.1	-
	184	0.071	-	-	-	-	-	0.167
	186	0.071	-	-	-	-	-	0.167
	190	0.036	-	-	-	-	-	-
	192	0.036	-	-	-	-	-	-
	194	0.107	0.667	-	0.222	-	-	0.333
	196	0.036	-	0.5	0.056	0.5	-	-
	198	0.071	-	-	-	-	-	0.167
	200	0.036	-	-	0.111	-	0.1	-
	204	0.036	-	0.5	0.222	-	0.1	-
	206	0.071	-	-	-	-	0.1	-
	208	0.036	-	-	0.111	-	0.1	-
	210	0.036	-	-	0.111	-	-	-
	212	0.071	-	-	-	-	-	-
	214	-	-	-	0.056	-	-	-
	216	0.071	0.167	-	-	-	-	-
	218	0.036	-	-	-	-	0.1	-
	222	-	0.167	-	-	-	0.1	-
	224	-	-	-	-	-	0.1	-
	228	-	-	-	-	-	0.1	-
	232	0.036	-	-	-	0.5	0.1	-
	He	0.14	0.02	0.01	0.08	0.01	0.05	0.03
	Ho	0.12	0.02	0.01	0.08	0.01	0.05	0.02
	A	1.974	1.6	2	1.895	2	2	1.933
Ho10b	152	0.033	-	-	-	-	0.667	-
	154	0.167	-	0.5	0.143	-	-	-
	156	0.333	0.667	-	-	-	-	-
	158	0.333	0.333	0.5	0.5	1	0.333	0.25
	160	0.1	-	-	0.357	-	-	0.5
	168	-	-	-	-	-	-	0.25
	174	0.033	-	-	-	-	-	-
	He	0.11	0.02	0.01	0.04	-	0.02	0.02

	Ho	0.06*	0	0.01	0.01*	-	0	0.02
	A	1.763	1.533	2	1.648	1	1.533	1.833
Ho16	152	-	-	-	-	-	-	0.167
	154	0.143	0.25	0.5	-	-	0.083	-
	156	0.107	0.5	-	0.167	0.75	-	0.167
	158	0.071	-	-	-	-	-	-
	160	-	-	-	0.111	-	0.083	-
	166	0.036	-	-	0.056	-	0.083	-
	170	0.036	-	-	0.111	-	0.167	-
	174	0.071	-	0.5	-	-	-	-
	176	-	-	-	-	-	0.083	-
	178	0.036	-	-	0.111	-	-	0.167
	182	0.036	-	-	0.056	-	-	0.167
	184	-	-	-	0.056	-	-	0.167
	186	-	-	-	-	-	0.083	-
	188	-	-	-	0.056	-	-	-
	190	0.107	-	-	-	-	-	-
	192	-	-	-	0.056	-	-	0.167
	194	-	-	-	0.056	-	0.333	-
	202	0.107	-	-	-	0.25	-	-
	204	0.071	0.25	-	0.111	-	-	-
	210	0.036	-	-	-	-	-	-
	212	0.036	-	-	-	-	-	-
	214	0.071	-	-	0.056	-	-	-
	220	0.036	-	-	-	-	-	-
	226	-	-	-	-	-	0.083	-
	He	0.13	0.02	0.01	0.09	0.01	0.05	0.03
	Ho	0.12	0.01	0.01	0.08	0.01	0.04	0.03
	A	1.95	1.833	2	1.954	1.5	1.894	2
Ho17	230	0.167	-	-	0.222	-	0.25	-
	234	0.267	0.25	1	0.389	0.5	0.333	1
	238	0.333	0.75	-	0.333	0.5	-	-
	242	0.233	-	-	0.056	-	0.333	-
	246	-	-	-	-	-	0.083	-
	He	0.11	0.01	-	0.07	0.01	0.05	-
	Ho	0.12	0.01	-	0.05	0.01	0.02*	-
	A	1.761	1.5	1	1.725	2	1.773	1
Ho6b	163	0.033	-	-	-	-	-	-
	167	0.033	-	-	-	-	0.083	-
	169	-	-	-	-	-	-	0.25
	173	0.067	-	-	0.05	-	0.167	-
	175	0.167	0.333	-	0.1	-	-	0.375
	177	0.4	0.333	0.5	0.7	1	0.417	0.25
	179	0.267	0.333	-	0.05	-	0.333	-
	181	-	-	0.5	0.1	-	-	-
	185	0.033	-	-	-	-	-	-
	191	-	-	-	-	-	-	0.125
	He	0.11	0.02	0.01	0.05	-	0.04	0.03
	Ho	0.08*	0	0.01	0.05	-	0.01*	0.02
	A	1.759	1.8	2	1.511	1	1.742	1.821
Ho9b	197	0.067	-	-	-	-	-	-
	201	0.1	-	-	-	-	-	-

203	0.033	-	-	0.4	-	0.167	-
205	0.067	0.333	-	-	-	0.167	-
207	-	-	-	-	-	0.083	-
209	0.2	0.333	1	0.2	-	0.25	0.5
211	0.333	0.167	-	0.3	1	0.25	0.5
213	0.167	-	-	0.05	-	0.083	-
215	-	-	-	0.05	-	-	-
217	0.033	0.167	-	-	-	-	-
He	0.12	0.03	-	0.07	-	0.05	0.01
Ho	0.05*	0.02	-	0.07	-	0.04	0
A	1.828	1.867	1	1.742	1	1.879	1.667

Appendix 3. Microsatellite allele frequencies for all *H. butcheri* populations. Number in brackets = sample size. *He* = Expected heterozygosities, *Ho* = observed heterozygosities, *A* = allelic richness. * = significant heterozygote deficit.

Locus	Allele	MALv (n= 23)	MALc (n=5)	TEMv (n=18)	TEMc (n=7)
Ho4b	124	-	0.5	-	-
	128	1	-	-	-
	130	-	0.5	1	1
	He	-	0.01	-	-
	Ho	-	0.02	-	-
	A	1	1.667	1	1
Ho8b	174	-	-	-	0.6
	180	0.036	-	-	-
	182	0.143	-	0.188	-
	184	-	-	0.188	0.1
	186	0.107	0.4	0.25	-
	188	-	0.4	0.188	-
	190	-	-	0.188	0.3
	192	0.036	-	-	-
	196	0.071	-	-	-
	198	0.036	-	-	-
	200	0.036	-	-	-
	202	-	0.1	-	-
	210	0.036	-	-	-
	212	0.036	-	-	-
	214	0.071	-	-	-
	220	0.036	-	-	-
	232	0.071	-	-	-
	240	0.071	-	-	-
	242	0.071	-	-	-
	244	0.036	-	-	-
	260	0.071	-	-	-
	262	0.036	-	-	-
	He	0.13	0.04	0.07	0.03
	Ho	0,11	0.05	0.04*	0.01*
	A	1.96	1.733	1.85	1.6
Ho10b	146	0.029	-	-	-
	148	0.147	-	-	-
	150	0.382	0.6	-	-
	152	0.147	-	0.091	0.25
	154	0.029	0.2	0.182	0.583
	156	-	0.2	0.227	-
	158	0.029	-	0.045	-
	160	0.059	-	0.318	-
	162	0.176	-	0.091	0.167
	174	-	-	0.045	-
	He	0.14	0.03	0.09	0.04
	Ho	0.12	0*	0.08	0.03
	A	1.797	1.622	1.831	1.621
Ho16	154	0.026	-	-	0.5
	156	-	-	-	-
	158	0.026	-	-	-

	160	0.053	-	-	-
	162	0.079	-	-	-
	164	-	-	0.107	-
	166	-	0.1	-	-
	168	0.132	-	-	-
	170	0.079	-	0.036	-
	172	0.079	-	-	-
	174	0.105	-	0.071	-
	176	0.053	-	0.036	-
	178	0.053	-	0.071	-
	180	0.026	0.3	-	-
	182	0.026	-	0.071	-
	184	0.026	0.1	0.036	-
	188	0.026	-	-	-
	190	0.026	-	0.036	-
	192	-	0.2	0.036	-
	194	-	0.2	-	-
	196	-	-	0.143	0.25
	198	-	-	0.071	0.125
	200	-	-	0.036	-
	202	-	0.1	0.036	0.125
	204	0.026	-	0.036	-
	206	-	-	0.036	-
	208	0.105	-	-	-
	210	0.026	-	0.036	-
	214	-	-	0.036	-
	216	0.026	-	-	-
	224	-	-	0.036	-
	228	-	-	0.036	-
	He	0.18	0.04	0.14	0.03
	Ho	0.19	0.05	0.14	0.03
	A	1.952	1.889	1.966	1.75
Ho17	230	0.025	-	-	-
	234	0.35	0.5	0.136	-
	238	0.075	0.1	0.636	1
	242	0.225	-	0.136	-
	246	0.325	0.4	0.091	-
	He	0.15	0.03	0.06	-
	Ho	0.12*	0.05	0.02*	-
	A	1.733	1.644	1.576	1
Ho6b	161	0.053	-	0.029	-
	163	0.053	0.8	0.971	0.6
	167	0.184	-	-	-
	177	0.132	-	-	-
	179	0.289	0.1	-	0.4
	181	0.026	-	-	-
	183	0.053	-	-	-
	185	0.105	-	-	-
	187	0.026	-	-	-
	189	-	0.1	-	-
	195	0.053	-	-	-
	197	0.026	-	-	-
	He	0.16	0.02	0.01	0.03
	Ho	0.09*	0.01	0.01	0.04

	A	1.863	1.378	1.059	1.533
Ho9b	199	0.023	-	-	-
	201	0.114	-	-	-
	203	0.136	0.1	0.071	-
	205	0.273	0.5	0.643	0.583
	207	0.136	0.3	0.143	-
	209	0.068	-	0.071	0.417
	211	0.091	0.1	-	-
	213	0.068	-	0.036	-
	217	0.068	-	-	-
	223	0.023	-	0.036	-
	He	0.19	0.04	0.08	0.03
	Ho	0.17	0.02	0.07	0.05
	A	1.872	1.711	1.574	1.53

CHAPTER six

Bruchids and parasitoids from wild and cultivated beans: An ecological and genetic approach.

Bruchids and Parasitoids from Wild and Cultivated Beans: An Ecological and Genetic Approach.

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Abstract

Bruchid beetles are important pests of field and stored beans all around the world and cause enormous economical losses in Mexico and Central America. Parasitoids have been successfully used to suppress bruchid beetles infestation in Africa, but few studies have assessed their potential to reduce seed damage in the New World and no successful biological control programs have been implemented. The latter is partly due to the poor knowledge of the biology and ecology of bruchids and their parasitoids in this region of the world. In the present study, we examined the distribution and host-plant use of two sibling species of bruchids in the genus *Zabrotes* (*Z. subfasciatus* and *Z. sylvestris*) that attack the seeds of *Phaseolus* beans in Mexico. Results showed that *Zabrotes subfasciatus* and *Z. sylvestris* have clearly differentiated ecological niches. *Zabrotes subfasciatus* is most common at low altitudes on *P. lunatus*, whereas *Z. sylvestris* is more common at high altitudes on *P. vulgaris*. From a complex of three parasitoid species in the genus *Horismenus*, only one of them, *Horismenus missouriensis* (Eulophidae), attacked these beetles on both wild and cultivated beans. For this species we estimated the genetic distance between populations, using mitochondrial DNA. No genetic differentiation was found among groups of *H. missouriensis* collected from wild and cultivated bean species and one other legume, implying that this parasitoid has a good dispersal ability, which results in high levels of gene flow among populations. These results are discussed in the context of the potential use of this generalist parasitoid as a biological control agent of bruchid pests in Mexico.

Key words: *Horismenus*, *Zabrotes*, ecological distribution, biological control, sibling species, wild and cultivated beans, genetic distance.

Introduction

Bruchid beetles (Coleoptera: Bruchidae) are important pests of field and stored beans all around the world. In the American continent great economic losses have been attributed to bruchid damage (35% losses in Mexico and Central America, 13% losses in Brazil and 7.4% losses in Colombia; van Schoonhoven and Cardona, 1986). But even though bean consumption provides the major protein source in the diet of inhabitants of many Latin America countries (Broughton et al. 2003), little attention has been paid to hymenopteran parasitoids as control agents in this region of the world. Various studies have characterized the use of endemic parasitoids in augmentative and conservation biological control of bruchid populations in Africa (Dugravot et al. 2002; Ketoh et al. 2002; Leveque et al. 1993; Sanon et al. 1998; Sanon et al. 1999; van Huis et al. 2002). For instance, efficient biological control of the bruchid *Callosobruchus maculatus* that causes substantial losses in storage of cowpea, *Vigna unguiculata* in Burkina Faso was obtained with *Dinarmus basalis* an endemic parasitoid of the family Pteromalidae that attacks *C. maculatus* in cowpea fields (Sanon et al. 1998). Although naturally present in granaries, the low number of parasitoids at the beginning of the season was not sufficient to reduce the *C. maculatus* populations that build up through the multiple generations of this multivoltine species (Sanon et al. 1998). Introduction of *D. basalis*, at the beginning of the season significantly reduced seed weight loss to less than 10% of the seed weight (Sanon et al. 1998). This study showed that augmentative introduction of endemic natural enemies successfully reduces bruchid infestation. In Colombia, the potential use of parasitoids as classical biological control agents has been assessed for three species of non native parasitoids, *D. basalis* Aschmead, *Anisopteromalus calandrae* Howard (Pteromalidae) and *Heterospilus prosopidis* Viereck (Braconidae) (Schmale et al. 2001). Among these, *D. basalis* was considered the most suitable due to its high offspring production and high longevity enabling it to suppress bruchid infestations more efficiently than the other two species. Surprisingly, only one study has surveyed native parasitoid species for augmentative or conservation biological control of bruchids in Latin America. In a study conducted also in Colombia, Schmale et al. (2002) showed that only one parasitoid species, *Horismenus sp.* Ashmead (Eulophidae), attacked *Acanthoscelides obtectus* (Say) in storage conditions. However, they demonstrated that this species (whose taxonomic position remains unclear) was not able to get established. A correct identification of the natural enemies associated to a potential pest, is one of the keys for the success in the exploration and importation phases of a biological control program, as well as for their subsequent establishment in the new environment (Rosen 1986). For example, *Aphytis* parasitoids attacking the California red scale *Aonidiella aurantii* (Homoptera: Diaspididae) in the Orient were misidentified as *A. chrysomphali*, an inefficient parasitoid already present in California and therefore ignored over half a century as potential biological control agents. Once recognized as different species, *A. lingnanensis* and *A. melinus* were successfully introduced in many countries (Rosen 1986). Similarly, the mass production of indigenous species relies heavily on a correct identification, since cultures may become contaminated by undesirable and ineffective species (Rosen and DeBach 1973). For example mass cultures of *Encarsia perniociosi* (Hymenoptera: Aphelinidae) a parasitoid used in Europe against the San Jose Scale *Quadraspidiotus perniciosus* were invaded and overtaken by a related ineffective species *E. fasciata*, which had a greater competitive ability under storage conditions (Rosen and DeBach 1973).

The recognition of different biological races within a species is as important as the identification of different parasitoid species (Rosen 1986). Because host-races have different ecological requirements (Drès and Mallet 2002), the lack of recognition of different host-races could lead to unsuccessful biological control programs (De Bach 1969). For instance, the introduction of two ecotypes of *Tryoxys pallidus* (Hymenoptera: Aphidiidae) from France and Iran were necessary to control the walnut aphid *Chromaphis juglandicola* (Homoptera: Aphididae) along the coast and in the central and northern part of California. The French ecotype was not able to establish itself in the dryer inland environment while the Iran ecotypes were better adapted to such environmental conditions (Van den Bosh et al. 1979).

Beans of the genus *Phaseolus* have been domesticated for thousands of years (Kaplan 1971).

Phaseolus vulgaris, P. coccineus and *P. lunatus* are among the species of beans that have been domesticated in Mexico, were one of the center of origins of this genus has been identified (Freytag and Debouck 2002; Delgado-Salinas et al. 1988), and where the greatest variety of both wild and cultivated beans exists. In this region, these three *Phaseolus* species are infested by sibling bruchid beetles species of the genera *Acanthoscelides* and *Zabrotes*. Heavy bruchid infestations of bean stocks force farmers to sell their products directly after the harvest, when market prices are very low. This practice inflicts an economic loss of 35% in Mexico (van Schoonhoven and Cardona, 1986). Until now, the control of these pests has relied mostly on insecticide use, which not only can have negative health consequences, but also represent high economic costs for small-scale farmers. One of the main reasons for the lack of use of biological control against bruchid pests in Mexico and Central America is the poor knowledge on the ecology and biology of both, the beetles and the natural enemies that attack them in field and storage conditions.

Recently, a detailed description on the ecology of three species of *Acanthoscelides*, one of the two major genera that attack wild and cultivated beans in Mexico was conducted (Alvarez et al. 2004a,b). The three *Acanthoscelides* species, *A. obtectus* Say, *A. obvelatus* Bridwell and *A. argillaceus* Sharp can be found in sympatry although they have very distinct ecological requirements (Alvarez et al. 2004b). While altitudinal range and bean domestication status rule the distribution of the sibling species *A. obtectus* and *A. obvelatus*, host plant specialization dictates the distribution of *A. argillaceus* (Alvarez et al. 2004b). Information on the ecological distribution and factors influencing the niche segregation of the other most abundant genus, *Zabrotes*, does not exist.

In contrast to the genus *Acanthoscelides*, which has a cosmopolitan distribution, the genus *Zabrotes* is more restricted to Mexico and Central America (Johnson 1983). Precise knowledge on the interaction with its host plants and associated parasitoids is of great value because of the long coevolutionary process they may have undergone. The existence of two sibling species *Z. subfasciatus* and *Z. sylvestris* attacking beans in Mexico was recently discovered (Romero and

Johnson 1999) and their sibling species status was further suggested by the absence of gene flow between them (Gonzalez-Rodriguez et al. 2002).

The parasitoid guild that attacks species in the genus *Acanthoscelides* and *Zabrotes* on wild and cultivated beans was recently described (Hansson et al. 2004). Among various families, the genus *Horismenus* represents the most abundant parasitoid in that system. A taxonomic survey revealed that three species: *H. depressus* Gahan, *H. missouriensis* Ashmead and *H. butcheri* Hansson & Aebi (Hansson et al. 2004) attack bruchid beetles on *P. lunatus, P. vulgaris* and *P. coccineus*. Despite the fact that the three *Horismenus* species can be found in sympatry, they have very distinct environmental requirements, as altitude and host-plant association are the major factors affecting their distribution (Aebi et al. 2004). *Horismenus depressus* is the most common species collected below 1700 m and represents the only species collected on *P. lunatus*, a bean restricted to low altitudes (from sea level to 1700 m). Similarly, *H. missouriensis* is the most common *Horismenus* found above 1700 m, and was the only species collected on *P. coccineus*, a bean restricted to high altitudes (1400 to 3000 m). In contrast, *H. butcheri* is specialized on *P. vulgaris* and has a wide altitudinal range. Recently, two host-plant races specialized on *P. vulgaris* and *P. coccineus* were described within *H. butcheri* (Aebi et al. 2004). While all three species were reared on wild beans, *H. missouriensis* is the only one found on cultivated *P. vulgaris* beans and able to maintain itself in storage conditions (Hansson et al. 2004). We do not know however, if individuals of *H. missouriensis* on wild and cultivated beans belong to one or two distinct genetic groups.

The recent acquired information on the taxonomy and ecology of species within the genera *Acanthoscelides*, and *Horismenus* (Aebi et al. 2004; Alvarez et al. 2004b; Hansson et al. 2004) will be crucial for any future control of these bruchid pests. Nevertheless, a more complete understanding on the nature of the interactions between cultivated beans, all of their bruchid pests and their parasitoids is essential in order to implement successful biological control programs on this complex tri-trophic system. In this study, by using an ecological and genetic approach we aimed to answer the following questions: 1) which ecological factors determine the distribution and host-plant association of the two sibling species in the genus *Zabrotes*: *Z. subfasciatus* and *Zabrotes sylvestris*. Specifically, we determined the effect of altitude and host-plant on the ecological distribution of these two species, and 2) is *H. missourensis* an appropriate parasitoid to control the bruchid species that attack these beans? Among the three known species of *Horismenus* that parasitize these beetles, *H. missourensis* was chosen for this study because it was the only species found in cultivated beans. In addition, because it was also found on wild beans, we wanted to determine whether individuals on these two bean varieties (wild and cultivated) were genetically different.

Material and Methods

Sampling

Wild bean samples of the genus *Phaseolus* were collected between January 2001 and February 2003. Cultivated bean samples were bought directly from farmers on a monthly basis between January 2002 and February 2003. A total of 66 bean populations were sampled along an altitudinal gradient ranging from sea level to 2890 m (Table 1). The populations comprise the following host-plant combinations: wild *P. vulgaris*, wild *P. lunatus*, wild *P. coccineus*, wild *P. vulgaris* + *P. lunatus*, wild *P. vulgaris* + *P. coccineus* and cultivated *P. vulgaris*. When encountered in the proximity of *Phaseolus* populations, seeds of other legume species were collected and identified. Labelled seed samples were brought to the laboratory for incubation in natural conditions, in a shielded space. At emergence, bruchids and parasitoids were placed in 100% ethanol for further taxonomic determination, DNA extraction and genetic analysis.

Table 1. Population collected for this study. v = *P. vulgaris*, l = *P. lunatus*, c = *P. coccineus*, p = *P. polyanthus*, * indicates cultivated beans.

Population	Code	Host-plant species	Longitude (°W)	Latitude (°N)	Altitude (m)
Copandaro	COPI	v	101°45’35.4”	19°26’24.1”	2087
Erongaricuaro	ERO	v	101°42’32.8”	19°35’56.3”	2072
San Andres de los Gabeles	SAG	v	99°57’01.5”	19°02’19.5”	2280
San Francisco Periban	SFP	v	102°24’29.0”	19°32’31.4”	1620
San Isidro cerca Coeneo	SICC	v	101°34’23.9”	19°50’56.6”	2040
Santa Ana	SAN	v	101°39’39.6”	19°31’86.6”	2255
Sultepec I	SUL	v	99°59’20.9”	18°51’07.8”	2164
Sultepec II	SUL2	v	99°58’04.1”	18°50’44.0”	2200
Tejupilco	TEJ	v	100°09’00.1”	18°55’51.2”	1400
Tepoztlan I	TEPI	v	99°07’15.7”	18°59’36.3”	1931

Table 1, continued

Tepoztlan II	TEPII	v	99°07’19.2”	18°58’14.9”	1700
Tepoztlan III	TEPIII	v	99°07’19.2”	18°58’14.9”	1700
Tlayacapan	TLAY	v	99°03’24.4”	18°57’20.0”	1750
Valle de Bravo	VDB	v	100°07’05.1”	19°13’56.8”	1918
Yautepec	YAU	v	99°04’43.8”	18°57’36.4”	1475
Anenecuilco	ANE	l	98°59’37.0”	18°47’84.9”	1396
Ayala	AYA	l	98°59’10.4”	18°45’24.8”	1215
Elabillal	ELA	l	102°21’74.7”	18°00’44.4”	28
Hollo del culo del mundo	OCM	l	98°33’74.9”	18°37’79.7”	1384
Mazunte	MAZ	l	96°28’00.0”	16°47'00.0”	20
Playa Azul	PLAYA	l	102°21’24.0”	17°59’34.8”	21
San Juan Bosco	SJB	l	102°18’14.6”	18°04’60.0”	194
Tetecala	TET	l	99°30’28.7”	18°57’20.0”	2044
Tilapa	TIL	l	99°33’44.7”	18°35’83.9”	1339
Yautepec	YAUL	l	99°02’39.8”	18°55’19.3”	1373
Huitzilac	HUI	c	99°16’42.8”	19°01’60.4”	2548
Ixtlahuaca	IXT	c	100°09’05.1”	18°55’83.4”	1468
San Bartolo	SBO	c	100°03’20.8”	19°14’31.7”	2320
San Lorenzo	SLO	c	99°28’93.4”	19°07’91.3”	2868
San Pedro Techuchulco	SPT	c	99°31’04.3”	19°07’21.2”	2844
San Pedro Texcalyacac	SPTX	c	99°30’06.7”	19°07’57.0”	2886
Tenango	TEN	c	99°36’07.1”	19°06’38.7”	2768
Tepoztlan 0	TEPO	c	99°06’53.3”	19°00’26.6”	2292
Tepoztlan IV	TEPIV	c	99°06’29.4”	19°01’10.6”	2547
Tepoztlan V	TEPV	c	99°05’66.8”	19°01’37.7”	2663
Tepoztlan VI	TEPVI	c	99°05’00.0”	19°01’0.00”	2692
Tepoztlan VII	TEPVII	c	99°05’36.2”	19°01’38.9”	2722
Tlalpan	TLA	c	99°12’04.3”	19°17’50.3”	2403
Ahuehueyo	AHU	v+l	98°34’21.1”	18°36’57.6”	1366
Atila	ATI	v+l	98°33’75.3”	18°36’63.9”	1381
Santo Tomas	STO	v+l	100°16’90.1”	19°10’35.3”	1150
Copandaro III	COPIII	v+c	101°46’11.8”	19°26’26.1”	2117
Malinalco	MAL	v+c	99°30’08.9”	18°57’13.2”	1935
San José de los Laureles	SJS	v+c	99°00’05.0”	18°58’49.7”	1855
Temascaltepec	TEM	v+c	100°02’44.2”	19°02’35.9”	1734
Arocutin	ARO*	v	101°41’37.7’’	19°33’22.5’’	2060
Coatepec Harinas	COAT*	v	99°52’59.9”	18°52’59.9”	2100
Coeneo	COE*	v	101°34’59.3”	19°49’13.9”	2100
Coinzio	COI*	v	101°16’28.2’’	19°38’25.0’’	1905
Copandaro campo	COP*	v	101°46’27.3’’	19°26’36.2’’	2120
Napizarro	NAP*	v	101°41’33.6’’	19°35’50.5’’	2060
Ocumicho	OCU*	v	102°13’11.8’’	19°47’46.1’’	2045
San Antonio	SAT*	v	101°17’33.1’’	19°38’49.9’’	1930
San Bartolo	SBO*	v	100°03’34.8’’	19°14’29.8’’	2310
San Francisco Periban	SFPII*	v	102°24’28.4”	19°32’32.4”	1800
San Francisco Periban	SFPI*	v	102°24’27.5’’	19°32’33.5’’	1850
San Gabriel	SGA*	v	100°07’28.1”	19°15’35.8”	2292
San Ildefonso	SILD*	v, c	100°08’56.9’’	19°22’19.8’’	2400
San Jose de los Laureles	SJS*	v	98°59’35.0’’	18°58’48.1’’	1800
San Pedro de Tejalpa	SPTJ*	v	99°36’00.0”	18°52’59.9”	1750
San Simon	SSI*	v	100°00’25.8”	19°01’27.4”	2135
Santa Lucia	STLU*	v	100°00’03.7”	18°52’12.5”	1790
Santa Maria	STM*	v	99°33’42.7”	18°49’03.6”	2000
Tejupilco	TEJ*	v	100°09’00.3”	18°55’50.0”	1400
Tequesquipan	TEQ*	v, c	99°56’33.1”	19°03’09.2”	2300
Tzintzuntzan	TZIN*	v	101°34’41.5”	19°37’43.9”	1980

Influence of altitude and host-plant species on the distribution of Zabrotes spp.

Upon emergence, the *Zabrotes* individuals collected on the different host-plant species were determined using a taxonomic key based on genitalia morphology (Romero and Johnson 1999). To assess the role of altitude and host-plant species on the ecological distribution of *Z. subfasciatus* and *Z. sylvestris*, non-parametric statistical analyses (Wilcoxon/Kruskal-Wallis) rank tests were performed. Since plant distribution is highly correlated with altitude and since no *Zabrotes* individuals were found on *P. coccineus*, a Pearson test was performed to evaluate the repartition of *Z. subfasciatus* and *Z. sylvestris* on *P. vulgaris* and *P. lunatus*.

Genetic distance among H. missouriensis from different plant origins.

We compared the genetic identity of individuals of *H. missouriensis* collected on wild and cultivated beans from different populations. Forward and reverse mitochondrial DNA sequences were generated for single individuals obtained from 3 populations of cultivated *P. vulgaris*, 1 population of wild *P. vulgaris*, 2 populations of wild *P. coccineus* and for comparison with parasitoids emerging from an alternative host plant attacked by an *Acanthoscelides* species (Hetz and Johnson, 1988), we also examined individuals obtained from 2 populations of *Leucaena sp.* A legume that was relatively abundant in some of the localities and whose seeds were infested by bruchids and *H. missourensis.*

Total genomic DNA from each individual was extracted using a Puregene™ DNA isolation kit, Tissue kit (Gentra systems, Minneapolis, USA) with modifications to take into account the small size of the samples. Initial amplification of the Cytochrome oxydase I region (1000 bp) was performed using the primers Cox-one 1L (CAACATTTATTTTGATTTTTTGG) and Cox-one 1R (TCCATTGCACTAATCTGCCATATTA) (Lopez-Vaamonde et al. 2001). Amplifications were made in 3 x 25μl reaction volume containing 1x PCR buffer 2mM MgCl2, 250 mM DNTPs, 0.625 units *Taq* polymerase (Promega), 0.4 μM of each primer and 1 μl of DNA template. The PCR cycle consisted of one cycle at 94°C for 3 min, 40 cycles at 94°C for 30 s, 47°C for 30 s, 72°C for 1 min, and one cycle at 72°C for 10 min. PCR products were brought together and purified with minicolumns (Microcon YM-30) and ligated into PGEM-t easy vector system (Promega). After electroporation, transformed bacteria (DH10-B *E. coli*) were plated on LB-ampicilin-Xgal-IPTG agar and incubated at 37°C. Presence of an insert in positive clones was ensured by direct PCR on single colonies (after reinoculation) using M13 and M13-rev primers. Positive clones were inoculated in 3 ml LB-ampicilin. Recombined plasmids were extracted by resuspension of the bacteria in (25 mM Tris-HCl pH 8.0; 50 mM Glucose; 10 mM EDTA pH 8.0), (0.2 N NaOH; 1% SDS) and (3 M KOAc; 5 M glacial acetic acid) followed by centrifugation for12000 g for five minutes. DNA was extracted from the supernatant with chlorophorm (1:2) prior to precipitation in ethanol 70% (v/v) (final concentration). Following centrifugation for 10 minutes at 12000 g, the pellet was washed thrice in 70% (v/v) ethanol and resuspended in 50 l TE pH 8.9 with RNAse (20 g/ml). Restriction digest with NCO I and SAL I (Promega) following manufacturers recommendations, was performed to ensure the presence of the insert prior to sequencing with M13 and M13 rev using an Amersham biosciences Thermo Sequenase™* Primer Cycle Sequencing Kit. Sequencing PCR was performed in a 2.4 µl reaction volume containing 1.2 µl of each DNTP, and 1.2 µl of a mix containing 4µl of DNA, 1 µl of each primer and 1µl of miliQ water. PCR products were deposited for electrophoresis on a denaturing 7.4M urea – 6% (w/v) polyacrylamide gel (Sequagel *XR*, National Diagnostics) on a Li-Cor DNA Analyser. Isolated bands were visualised and analysed using e-Sequ Version 2.0.

M13 and M13 rev sequences were aligned using ClustalW (Thompson et al. 1994) and further adjusted by sequential pairwise comparison. A phylogenetic tree was reconstructed by distance methods using uncorrected distance under version 4.0b10 of PAUP (Swofford 2002). A UPGMA analysis was conducted for the set of sequences. Heuristic searches were performed under the parsimony criterion. Branch support was performed using the bootstrap method under the parsimony optimal criterion. A total of 100 replicates were performed. Homoplasy measures were computed using the consistency index (CI), excluding all uninformative characters, and the retention index (RI).

Results

Influence of altitude and host-plant species on the distribution of Zabrotes spp.

Counts of *Zabrotes* on the different bean species sampled revealed that *P. lunatus* is the bean species harbouring the major number of *Zabrotes* bruchid beetles while only a few individuals were reared from *P. vulgaris* seeds and no *Zabrotes* emerged from *P. coccineus*. The identification of the *Zabrotes* species collected on *P. lunatus* and *P. vulgaris* along an altitudinal gradient revealed a clear altitude and host-plant segregation pattern. Most *Z. subfasciatus* were collected from sea level to 1000 m, on *P. lunatus* with some individuals collected on *P. vulgaris*, while all *Z. sylvestris* were collected from 1200 m to 2000 m on *P. vulgaris*. The Wilkoxon/Kruskal-Wallis test revealed a significant difference in altitude repartition of *Z. subfasciatus* and *Z. sylvestris* (p<0.0001) (Fig. 1).

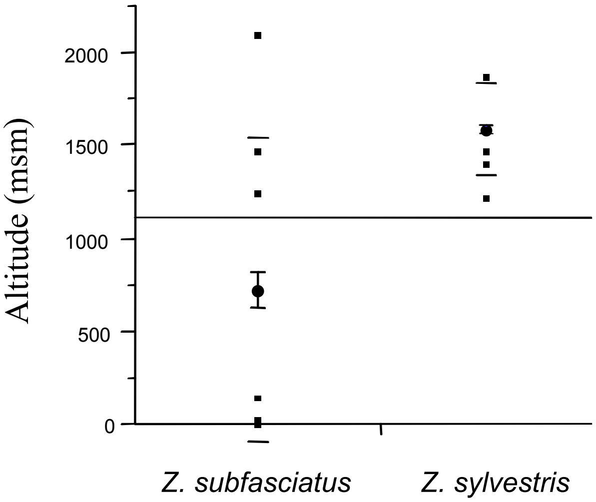

Figure 1. Altitudinal distribution of *Z. subfasciatus* and *Z. sylvestris*. Wilkoxon/Kruskal-Wallis test (p<0.0001).

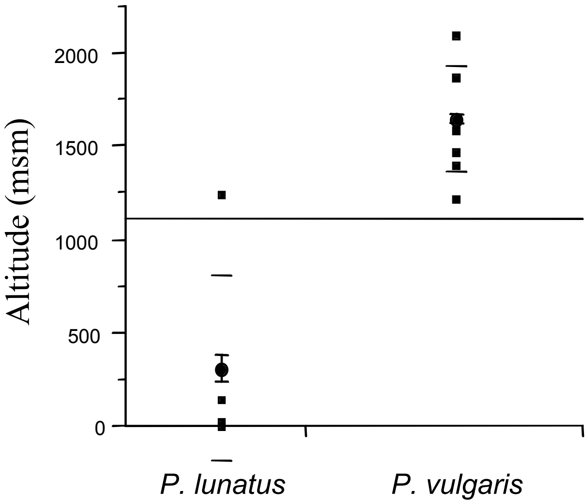

Figure 2. Altitudinal distribution of *P. vulgaris* and *P. lunatus*. Wilkoxon/Kruskal-Wallis test (p<0.0001).

Similarly, the Wilkoxon/Kruskal-Wallis test revealed a significant difference in altitude repartition of *P. vulgaris* and *P. coccineus* (p<0.0001) (Fig. 2). The Pearson test revealed a significant host-plant association (p<0.0001) of *Z. subfasciatus* on *P. lunatus* and *Z. sylvestris* on *P. vulgaris* (Fig. 3).

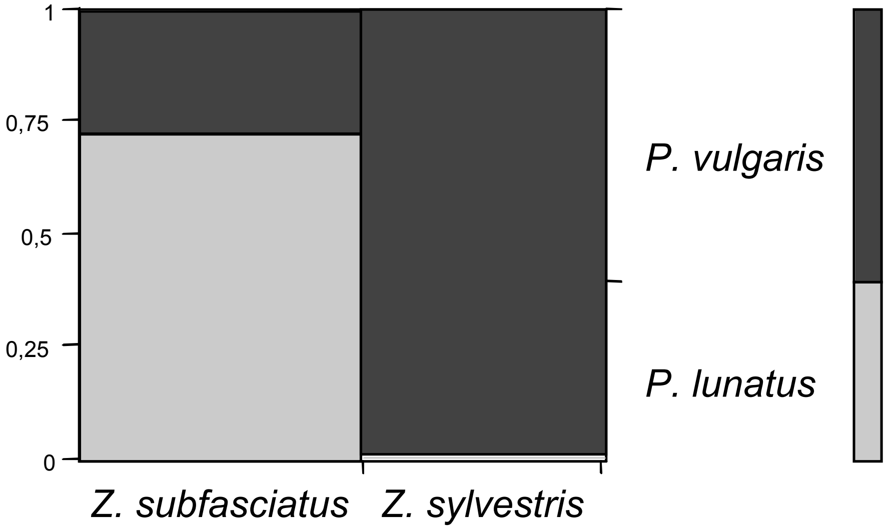

Figure 3. Host plant association of *Z. subfasciatus* and *Z. sylvestris* on *P. vulgaris* and *P. lunatus*. Pearson (p<0.0001).

Genetic distance among H. missouriensis from different plant origins

Analysis of the cytochrome oxydase I matrix revealed 65 parsimony-informative characters out of a total of 823 characters. The heuristic search resulted in 4 most parsimonious (MP) trees of 578 steps, filtering under parsimony criterion retained 1 tree (shown in Fig. 4) with a CI of 0.8832 and a RI of 0.7745. Bootstrap values for node support are indicated next to the branches. The tree obtained is well resolved with good branch support. Examination of the tree revealed that *H. missouriensis* individuals originating from the different host plant categories (wild *P. vulgaris*, wild *P. coccineus*, cultivated *P. vulgaris* and alternative host plant) are not clustered in different clades showing no genetic differentiation due to host-plant specialization.

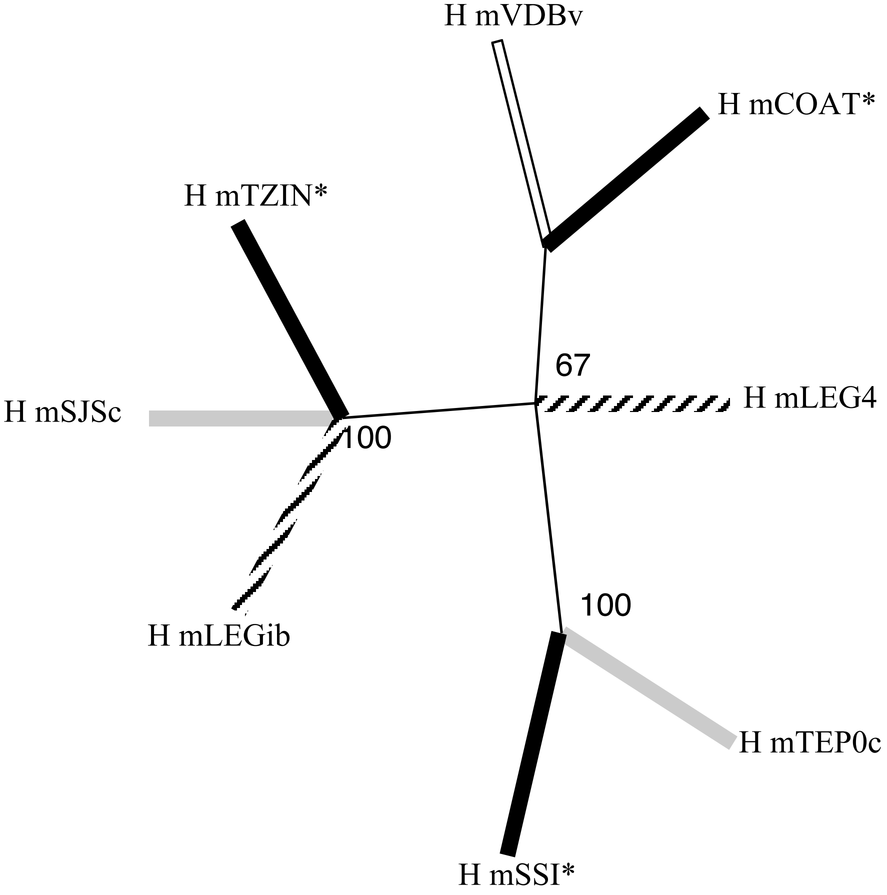

Figure 4. Phylogenetic tree of single *H. missouriensis* individuals collected on cultivated *P. vulgaris*, wild *P. vulgaris*, wild *P. coccineus* and *Leucaena sp*. Bootstrap values > 50% are shown.

Discussion

Influence of altitude and host-plant species on the distribution of Zabrotes spp.

Zabrotes subfasciatus and *Z. sylvestris* have very well differentiated ecological niches. The cosmopolitan *Z. subfasciatus* is more common at low altitudes on *P. lunatus* while the Mexican univoltin *Z. sylvestris* is more common at high altitudes on *P. vulgaris*. While altitude and bean domestication status (wild versus cultivated) explain the ecological distribution of the sibling *Acanthoscelides* species (Alvarez, 2004b) host-plant species seem to be the most important factor determining the distribution of *Z. subfasciatus* and *Z. sylvestris*. This great host-plant specialization can be explained by the bean phylogeny and specific secondary chemistry. Phaseolus *vulgaris* and *P. coccineus* both belong to the *P. vulgaris* group and are very close relatives whereas *P. lunatus* belongs to the *P. lunatus* group (Caicedo et al. 1999; Piñero and Eguiarte 1988). A striking characteristic of *P. lunatus* is its very high concentration of cyanogenic compounds found in the seed coat that are liberated subsequently to ingestion from the hydrolysis of glucoside linmarin (Janzen, 1977). Other toxic compounds such as Phaseolin (vicilin) polypeptides that have been isolated from the seed coat of *P. lunatus* are known to affect development of *Callosobruchus maculatus*, the cowpea weevil (Moraes, 2000). *Phaseolus lunatus*, the ancestor of lima bean is a new world legume that was domesticated in the New World (Kaplan 1971). The fact that *Z. subfasciatus* attacks wild and cultivated *P. lunatus* suggests that through their long evolutionary history, this bruchid species has evolved resistance to the chemical defence of the plant. Thus, adaptation to host-plant secondary chemistry played a key role in the niche differentiation between *Z. subfasciatus* and *Z. sylvestris*. Another bruchid species, *A. argillaceus* has evolved ability to overcome *P. lunatus* chemical defences and uses its seeds as only food source (Janzen, 1977).

Genetic distance among H. missouriensis from different plant origins.

The analysis on the genetic similarity between *Horismenus missouriensis* individuals collected from wild and cultivated *P. vulgaris*, wild *P. coccineus* and one of its alternative host plant of the genus *Leucaena* showed no genetic differentiation among wasps from these host plant populations. The capacity of *H. missouriensis* to utilize such different host plants suggests that this parasitoid is a generalist. In a review of the hymenopteran parasitoids of bruchid beetles in North and Central America, Hetz and Johnson (1988), listed 37 genera of host plants (mostly in the family Leguminosae) and 12 genera of bruchid hosts from which *H. missouriensis* was reared, confirming its very broad host and host plant range. Additionally, *H. missouriensis* was reported on *Lonchocarpus muehlbergianus* (Leguminosae) seeds attacked by the bruchid of the genera *Amblycerus, Gibbobruchus* and *Sennius* (Sari et al. 2002). We argue that the ability of *H.*

missouriensis to switch between various host plants enabled it to use cultivated beans despite the harsh storage conditions. The other two parasitoid species of the genus *Horismenus* of this system present more restricted host ranges. Apart from beans of the genus *Phaseolus*, *H. depressus* has only been reported on *Acacia* (Burks 1971) and *Olneya* (Gahan 1930). In an even more restricted manner, *H. butcheri* uses a single host plant, *P. vulgaris* and is the only parasitoid of this complex to show strict host plant specialization (Aebi et al. 2004). Generalist and specialist parasitoids are subjected to different selection pressures and may therefore display different behaviour when faced with the same type of hosts. Generalists will therefore be more prone to adapt to a new host. Recently, Campan and Benrey (2004) investigated plant mediated host suitability on the behaviour and performance of a specialist and a generalist parasitoid. They found that the oviposition response of the specialist *Stenocorse bruchivora* (Hymenoptera: Braconidae) depended on the type of seeds from which it was reared and exposed to during experimental trials. In contrast, the generalist *Dinarmus basalis* (Hymenoptera: Pteromalidae) was willing to parasitize hosts on plants that had not had encountered before showing the flexibility of generalists parasitoid to use alternative host plants.

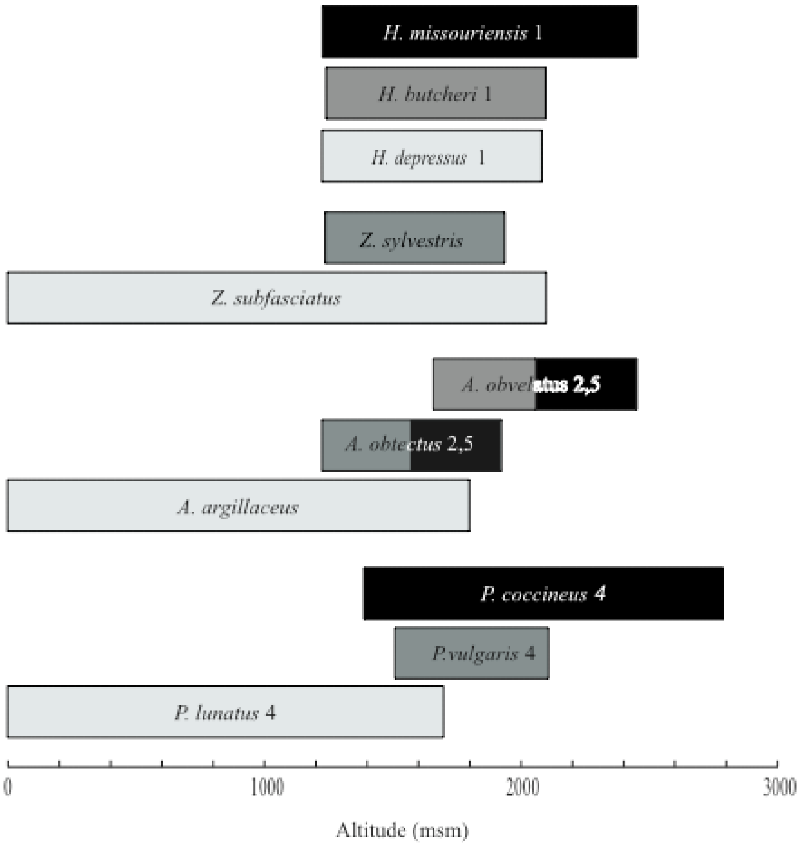

Figure 5. Altitudinal distribution of *Phaseolus spp.*, *Acanthoscelides spp.*, *Zabrotes spp*. and *Horismenus spp*. in Mexico. 1: (Aebi et al. 2004), 2: (Alvarez et al. 2004b), 3: (Delgado-Salinas et al. 1988), 4: (Pichard et al. 1991), 5: (Freytag and Debouck 2002). Bar colors reflect the interactions among trophic levels.

Among biological control practitioners, a controversy exists on whether to introduce exotic parasitoids for the biological control of crop pests because of the risk of parasitization and extinction of non-target organisms (Simberloff and Stiling 1996; Willianson 1996; Elton 2000). Extinction of non-target organisms because of exotic parasitoids is thought to be due to the transmission of pathogens depressing the immunological system of the host (Beckage 1985; Bouletreau 1986; Kraaijeveld et al. 1998; Godfray 2000) or to the lack of defence of the host that has not coevolved with its introduced enemy (Hokkanen and Pimentel 1989). Additionally, only 10% of exotic control agents are able to adapt themselves to the specific environmental conditions in which their hosts live and among them just a few successfully control the pest (Quicke 1997; Mills 2000; Hastings 2000). For these reasons, it is argued that native parasitoids should be favoured in the establishment of biological control programs.

Despite their arduous identification due to their very small size, members of the Chalcidoidea superfamily are the most important parasitoid used in biological control programs (Noyes, 1978: LaSalle 1993). Within this group, Eulophidae are known to be the most successful family in biological control programs (Schauff et al. 1997). The information obtained from this study on the nature and type of interactions among beans of the genus *Phaseolus*, their bruchids and associated parasitoids in Mexico (Fig. 5) should be considered in the development of effective biological control program against these bruchid pests.

Acknowledgments

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ANNEX

Isolation and characterization of polymorphic microsatellite markers in *Zabrotes subfasciatus* Boheman (Coleoptera: Bruchidae).
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PRIMER NOTE

Isolation and characterization of polymorphic microsatellite markers in *Zabrotes subfasciatus* Boheman (Coleoptera: Bruchidae)

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Abstract

Bruchid beetles of the genus *Zabrotes* (Coleoptera: Bruchidae) are important worldwide pests of legume seeds. Samples of *Zabrotes subfasciatus* and *Z. sylvestris* were collected from seeds of two *Phaseolus* species throughout Mexico to assess the role of host plant variation and plant domestication on the evolution of host use in this species. For this purpose six polymorphic microsatellite loci were isolated and characterized for the bruchid *Z. subfasciatus*. Cross-species amplification tests were performed on *Z. sylvestris* and revealed that three loci amplified successfully and were polymorphic in this closely related species.

Keywords: bean, Bruchidae, domestication, microsatellite, population structure, *Zabrotes*

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Bruchid beetles in the genus *Zabrotes* oviposit and feed on several legume species (Romero & Johnson 1999) and are considered a major pest of field crops and stored beans worldwide (Leroi *et al*. 1990). *Zabrotes subfasciatus* Boheman (Coleoptera: Bruchidae) specializes on lima bean (*Phaseolus lunatus*) and common black bean (*P. vulgaris*) but is also able to feed on seeds of other Fabaceae genera, such as *Vicia* (Sànchez 1992) and *Vigna* (Hills 1990). Originating from the New World (Credland & Dendy 1992), this species now has a cosmopolitan distribution mainly due to trading of the cultivated seeds which it attacks. Recently, a new species of the same genus has been described, *Z. sylvestris* (Romero & Johnson 1999), which is only distinguishable from *Z. subfasciatus* by examination of the genitalia. A study using allozyme markers investigated the degree of genetic similarity between *Z. subfasciatus* and *Z. sylvestris* (Gonzalez-Rodriguez *et al*. 2002). These authors found genetic evidence that these two bruchid species are sibling species as no gene flow was detected between them. Great genetic differentiation was detected among *Z. subfasciatus* populations but the low number of polymorphic allozyme loci (three out of six) may have led to bias estimates (Gonzalez-Rodriguez *et al*. 2002). The genetic differentiation among *Z. sylvestris* populations could not be evaluated as only one allozyme locus revealed polymorphism. Therefore, we isolated microsatellite loci from *Z. subfasciatus* and tested for cross-species amplification in *Z. sylvestris* to further investigate the degree of genetic variation among populations and to determine the role of host plant variation and plant domestication on the evolution of these species.

Total genomic DNA was extracted using a Puregene™ DNA isolation kit (Gentra Systems) from individuals of *Z. subfasciatus* collected in Tepoztlan, Tejupilco, Temascaltepec and Malinalco (Mexico State). Microsatellite-enriched libraries were built following Billotte *et al*. (1999): DNA was digested to completion with *Rsa*I (Eurogentec) and 500–1000-bp DNA fragments were selected after resolving on TAE 0.8% (w/v) agarose gel and isolated using an extraction kit (Promega). The enrichment step was as described in Kijas *et al*. (1994) and Edwards *et al*. (1996). The enriched microsatellite partial library was then constructed by ligating the PCR products into pGEM-T (Promega) according to the manufacturer's recommendations. Epicurian-coli XL1-Blue MRF′ supercompetent cells (Stratagene) were used for the transformation of the cloned

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Table 1 Polymorphic microsatellite loci for *Zabrotes subfasciatus*

						Tejupilco (n = 10)			Mazunte (n = 10)		
Locus	Motif	GenBank Accession no.	Primer sequences (5′–3′)	Size range (bp)	T_a (°C)	n_a	H_E	H_O	n_a	H_E	H_O
Zs2	$(CA)_{11}C$	AY684292	F: CACAAGTGGTGTGAAAACGG R: AGTTCACTTAGCAGTTGCCC	252–278	66	4	0.61	0.20*	7	0.85	0.30*
Zs6	$(AC)_{10}$	AY684293	F: AGTCATCTTGCAGATAAGC R: ATCAGATCAGTTTCATCCG	248–270	49	6	0.84	0.50*	5	0.81	0.10*
Zs8	$(CA)_{19}C$	AY684294	F: GAAAACAGTTTCTCAATGGTG R: TGCAGAATGGCTACGATGG	214–332	59	5	0.82	0.40*	7	0.69	0.30*
Zs15	$(GT)_6TT(GT)_{14}$	AY684295	F: TCACCACGATTAGAATGTCC R: TAGCTCTTTGGCTACTTCGC	216–270	55	4	0.61	0.50	12	0.77	0.70*
Zs16	$(AC)_8$	AY684296	F: CAAACTTAGACAAATAGGCAG R: AGATGGTAAGCAAACTCAGCA	228–242	59	5	0.63	0.40*	2	0.19	0.00
Zs17	$(AC)_{11}A$	AY684297	F: ACAACCAATGCCCATTGGC R: GAACCTGTAGTAGAACGTCA	190–242	55	5	0.78	0.70	11	0.92	0.40*

T_a, annealing temperature; n_a, number of alleles detected; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; H_O, observed heterozygosity; n, number of individuals tested.
*Significant deviation from H_E ($P < 0.05$).

Table 2 Polymorphic microsatellite loci for *Zabrotes sylvestris*

		San José de los Laureles (n = 10)			Ahuehueyo (n = 10)		
Locus	T_a (°C)	n_a	H_E	H_O	n_a	H_E	H_O
Zs2	66	2	0.19	0.00	2	0.27	0.10
Zs6	49	5	0.75	0.60	5	0.78	0.50*
Zs8	59	—	—	—	—	—	—
Zs15	55	—	—	—	—	—	—
Zs16	59	5	0.66	0.40	2	0.53	0.20
Zs17	55	—	—	—	—	—	—

Abbreviations as in Table 1.

DNA fragments. Following standard blue–white selection on X-Gal/IPTG/ampicillin plates, 184 white transformant clones were transferred on Hybond-N + nylon membranes (Amersham) and hybridized using the ^{32}P-labelled oligoprobes $(CT)_{15}$ and $(GT)_{15}$. After hybridization, the filters were washed twice with 4 × SSC (10 min, 57 °C) and then with 0.1 × SSC/10% (w/v) sodium dodecyl sulphate (10 min, 40 °C). Of these clones, 74 gave a strong positive signal of which 41 inserted DNA fragments were sequenced. Seventeen primer pairs were designed of which six gave satisfactory amplification patterns (i.e. PCR products of the predicted size and supernumerary bands of low intensity).

PCR amplifications were performed in a final volume of 5 μL which contained 1 μL of extracted DNA (2 ng/μL), 2.5 μL of HotStarTaq Master Mix (Qiagen), 0.25 μL of 10 μm reverse and forward primer (5′ IRD-700/800 modified) and 1 μL of double-distilled H_2O. PCRs were performed on a Biometra® T gradient thermocycler using the following cycling conditions: an initial denaturing step of 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, annealing temperature for 30 s and 72 °C for 40 s, and a final elongation at 72 °C for 10 min. PCR products were mixed with 2.5 μL of stop solution [95% (v/v) desionized formamide, 50 mm EDTA/10 mm NaOH/0.1% (w/v) bromophenol blue/0.1% (w/v) xylene cyanol green] and denatured at 94 °C for 2 min prior to electrophoresis on a denaturing 7.4 m urea–6.5% (w/v) polyacrylamide gel (Sequagel XR; National Diagnostics) on a Li-Cor DNA Analyser. Isolated bands were visualized and analysed using saga IR^2 software (version 2.2.2).

The degree of polymorphism at the six loci was tested for *Z. subfasciatus* using females from two populations: Tejupilco (*P. vulgaris*) and Mazunte (*P. lunatus*). We calculated expected and observed heterozygosities, estimated heterozygote deficit using Hardy–Weinberg exact tests and checked for linkage disequilibrium (genepop 3.3 package; Raymond & Rousset 1995).

All loci were polymorphic with two to 12 alleles per population and observed heterozygosities ranging from 0.00 to 0.70 (Table 1). A significant deficit of heterozygotes was observed for all loci (Zs2, Zs6, Zs8 and Zs16 in Tejupilco and Zs2, Zs6, Zs8, Zs15 and Zs17 in Mazunte), suggesting the presence of null alleles. No linkage disequilibrium was detected for either population.

Cross-species amplification tests on *Z. sylvestris*, using similar PCR conditions, on females from two populations (San José de los Laureles and Ahuehueyo on *P. vulgaris*) showed that three loci (Zs2, Zs6 and Zs16) out of six amplified consistently and were polymorphic (Table 2). Two to five loci were detected and observed heterozygosities ranged from 0.00 to 0.60. One locus (Zs6) showed a heterozygote deficit suggesting the presence of null alleles. No linkage disequilibrium was detected.

Due to probable high null allele occurrence, these loci are not appropriate for studies of breeding systems. Nevertheless, these six novel polymorphic markers should enable us to estimate genetic diversity at these loci among and within populations to obtain a detailed picture of the population structure of *Z. subfasciatus* in relation to host plant variability and bean domestication status.

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