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The potential of native parasitoids for the control of Mexican bean beetles: A genetic and ecological approach

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ABSTRACT

Bruchid beetles in the genus *Zabrotes* are important pests of field and stored beans all around the world and cause enormous economical losses in Mexico and Central America. Native parasitoids have been successfully used to suppress infestations by bruchid beetles 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, mainly due to the poor knowledge on their biology, systematics and ecology in this region. In this study, we used molecular tools to describe a new complex of three parasitoid species of bruchid beetles in the genus *Horismenus*, and investigated the level of gene flow and presence of ecotypes in this complex. We also examined the specific association between species of *Horismenus* and two sibling species of *Zabrotes* beetles, in order to evaluate their potential as biological control agents. Microsatellite data support the previous morphological description of three species, *H. butcheri, H. missouriensis* and *H. depressus*, but suggest some gene flow between *H. missouriensis* and *H. depressus*. Host-plant is shown to be the most important factor determining the ecological distribution of the two *Zabrotes* species, whereas altitude explains most of the distribution of the three *Horismenus* species. These results complement our understanding of this tritrophic system, providing a solid base for a potential biological control program using native parasitoids.

1. Introduction

Two factors have been identified as key elements for a successful biological control programs. First, a correct identification of the natural enemies associated with a pest will provide valuable information on their ecological requirements. Second, a precise understanding of the interactions between key parasitoids and their host in their natural environment is needed prior to the development of a biological control program (Aebi et al., 2007; DeBach, 1969; Rosen and DeBach, 1973; Van den Bosh et al., 1979). Indeed, the selection, collection, rearing and release of appropriate natural enemies can only be achieved on the base of a sound taxonomy and a good ecological comprehension of the system. While an ecological approach remains crucial and largely acknowledged, molecular tools have proved to be very useful in detecting cryptic species or ecotypes (Challis et al., 2007; Drès and Mallet, 2002; Yara et al., 2000).

Bruchid beetles (Coleoptera: Bruchidae) are important pests of field and stored beans all around the world. Great economic losses have been attributed to bruchid damage with ca. 35% in Mexico and Central America, 13% in Brazil and 7.4% in Colombia (van Schoonhoven and Cardona, 1986). In Africa, various studies have shown the use of parasitoids in augmentative and conservative biological control of bruchid populations (Dugravot et al., 2002; Ketoh et al., 2002; Leveque et al., 1993; Sanon et al., 1998, 1999; van Huis et al., 2002). For example, efficient biological control of the bruchid *Callosobruchus maculatus* that causes substantial losses in storage of cowpea *Vigna unguiculata*, in Burkina Faso, was obtained by rearing and releasing the native parasitoid *Dinarmus basalis* (Pteromalidae) (Sanon et al., 1998). In Central and South America beans are the main source of protein in the human diet (Broughton et al., 2003). Yet, little attention has been paid to hymenopteran parasitoids as bio-control agents in this region of the world. While a successful use of exotic parasitoids (*D. basalis* Aschmead, *Anisopteromalus calandrae* Howard and *Heterospilus*

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prosopidis Viereck) as biological agents against bruchids was demonstrated in Colombia (Schmale et al., 2001), only one native parasitoid species, *Horismenus* sp. (Eulophidae), has been studied for augmentative or conservation biological control (Schmale et al., 2002). Natural occurrence of *Horismenus* in granaries suggests that it could be used in a conservation or augmentation biological control programs. However, its precise taxonomy remains unknown and detailed information on its biology and the specific interaction with its bruchid hosts are lacking.

Parasitoid species can show specialization to their hosts, to the host-plants their host use or to particular habitat characteristics (for example elevation). The goal of this study was to use a molecular and ecological approach to elucidate the taxonomical status and the relationship of three sibling species in the genus *Horismenus* that attack bruchid beetles in *Phaseolus* beans in Mexico. In addition, by elucidating the ecological requirements of the parasitoids and their hosts, we aimed to determine the potential of these parasitoids in the biological control of bruchid beetles.

The parasitoid guild that attacks bruchid species on wild and cultivated beans in Mexico was recently described (Hansson et al., 2004). Among these, species in the genus *Horismenus* represent the most abundant parasitoids. A taxonomic survey based on morphological traits revealed that three species, *Horismenus depressus* Gahan, *Horismenus missouriensis* Ashmead and *Horismenus butcheri* Hansson & Aebi, attack bruchid beetles on *Phaseolus lunatus, P. vulgaris* and *P. coccineus* (Hansson et al., 2004). The three *Horismenus* species vary greatly in their host-range and host-plant use. *Horismenus missouriensis* is the only species reared from cultivated *P. vulgaris* beans infested with *Acanthoscelides* sp. and found in storage conditions (Hansson et al., 2004). It appears to be a generalist and has been reared from 15 genera of bruchid hosts on 38 genera of host-plants (mostly in the family Leguminosae), collected in the field (Hetz and Johnson, 1988; Sari et al., 2002). In contrast *H. depressus*, besides being found in seeds of *P. vulgaris* and *P. lunatus* parasitizing bruchids in the genera *Acanthoscelides* and *Zabrotes* has only been reported on two other legumes, *Acacia* (Burks, 1971) and *Olneya* (Gahan, 1930). Finally, *H. butcheri* a newly described species (Hansson et al., 2004), has only been reared from wild seeds of *P. vulgaris* and *P. coccineus*.

The most important pests of bean seeds in Mexico are bruchids in the genera *Zabrotes* and *Acanthoscelides*. Recent studies have examined the ecology of two sibling species in the genus *Acanthoscelides*, *A. obtectus* Say, and *A. obvelatus* Bridwell in Mexico (Alvarez et al., 2006). Altitudinal range and bean domestication status (wild vs. domesticated) are the main factors that determine their distribution (Alvarez et al., 2006). In contrast to the genus *Acanthoscelides*, which has a cosmopolitan distribution, the origin and distribution of the genus *Zabrotes* is restricted to Mexico and Central America (Johnson, 1983), and more recently has been found in some countries in Africa (Wortmann et al., 1998). However, detailed information on the ecological factors that dictate the distribution and the niche segregation of species within the genus does not exist. The existence of two sibling species, *Z. subfasciatus* and *Z. sylvestris* attacking beans (*P. vulgaris* and *P. lunatus*) 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). More precise knowledge on the interaction of these two sibling species with their host-plants and associated parasitoids should be of great value because of the long co-evolutionary process they might have undergone. Indeed, the potential of native parasitoids such as the three *Horismenus* species as biological control agents may be greater if they share a common evolutionary history with the target hosts.

Here, we use a genetic and ecological approach, to answer the following questions: (i) Does molecular data (microsatellites and mt-DNA) support the newly described *Horismenus* complex? (ii) Is there evidence for cryptic species or ecotypes among the three species? And, (iii) what are the cological factors determining the distribution of *Horismenus* spp. and *Zabrotes* spp? To date precise ecological information exists only for *Acanthoscelides* beetles. Solving this final question is crucial if we want to determine the potential of these parasitoids for the biological control of *Zabrotes* and *Acanthoscelides* beetles.

2. Materials and methods

2.1. Sampling procedure

2.1.1. Beans

Wild bean samples of *Phaseolus* were collected between January 2001 and February 2003. Cultivated beans of known origin were bought directly from farmers on a monthly basis between January and April in 2002 and 2003. In addition, a total of 66 bean populations were sampled along an altitudinal gradient ranging from sea-level to 2890 m (Table 1) throughout six states in central Mexico. 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. Labeled seed samples were brought to the laboratory for incubation in natural conditions, in a shielded space.

2.1.2. Bruchid beetles

A total of 209 individuals of *Zabrotes* sp. were reared and identified using a taxonomic key based on genitalia morphology (Romero and Johnson, 1999). To determine the ecological factors that influence the distribution of the two *Zabrotes* species we recorded: bean species of origin, host-plant composition in the sampled population and altitude. At emergence, bruchids were placed in 100% ethanol and stored at −20 °C for genetic analysis.

2.1.3. Parasitoids

A total of 216 females of *Horismenus* (118 *H. depressus*, 44 *H. missouriensis* and 54 *H. butcheri*) were reared and identified using a taxonomic key based on morphological traits (Hansson et al., 2004). To determine the relative importance of several biotic and abiotic traits, we recorded, from the population of origin: altitude, annual mean temperature (obtained from INEGI, Instituto Nacional de Estadistica Geografia e Informatica, Mexico), seed of origin (bean species) and total bruchid infestation rate $I = B/P$ (where B, number of bruchids and P, number of pods). After emergence, individuals were placed in 100% ethanol and stored at −20 °C for genetic analyses.

2.2. Molecular analysis of the Horismenus complex

2.2.1. Microsatellite genotyping

All Horismenus specimens were genotyped using 7 polymorphic microsatellite loci (6 from Aebi et al., 2004a, plus 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. (2004a), and the products deposited for electrophoresis on a denaturing 7.4 M urea—6.5% (w/v) polyacrylamide gel (Sequagel XR, National Diagnostics) resolved on a Li-Cor DNA Analyzer. Iso-

Table 1
Population collected for this study

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
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	1	98°59′37.0″	18°47′84.9″	1396
Ayala	AYA	1	98°59′10.4″	18°45′24.8″	1215
Elabillal	ELA	1	102°21′74.7″	18°00′44.4″	28
Hollo del culo del mundo	OCM	1	98°33′74.9″	18°37′79.7″	1384
Mazunte	MAZ	1	96°28′00.0″	16°47′00.0″	20
Playa Azul	PLAYA	1	102°21′24.0″	17°59′34.8″	21
San Juan Bosco	SJB	1	102°18′14.6″	18°04′60.0″	194
Tetecala	TET	1	99°30′28.7″	18°57′20.0″	2044
Tilapa	TIL	1	99°33′44.7″	18°35′83.9″	1339
Yautepec	YAUL	1	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 Techuchulco	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 + 1	98°34′21.1″	18°36′57.6″	1366
Atila	ATI	v + 1	98°33′75.3″	18°36′63.9″	1381
Santo Tomas	STO	v + 1	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 Jose de los Laureles	SJS	v + c	99°00′05.0″	18°58′49.7″	1855
Temascal tepee	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

v, *P. vulgaris*; 1, *P. lunatus*; c, *P. coccineus*; p, *P. polyanthus*.
* Indicates cultivated beans.

lated bands were visualized and analyzed using Saga IR^2 software, version 2.2.2.

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

To establish the difference among individuals identified as *H. depressus, H. missouriensis* and *H. butcheri*, at microsatellite loci, 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 to ensure convergence of likelihood values. Three replicates for each K were performed to ensure convergence in estimated parameter values.

2.2.2. Mitochondrial DNA haplotypes

Sequence data of the cytochrome oxydase I gene were generated for 18 individuals of *Horismenus* (4 *H. depressus*, 8 *H. missouriensis* and 6 *H. butcheri*), from various populations on their natural host-plants. Initial amplification of the cytochrome oxydase I region was performed using the primers Cox-one 1L (CAACATTTATTTTGATTTTTTGG) and Cox-one 1R (TCCATTGCAC TAATCTGCCATATTA) (Lopez-Vaamonde et al., 2001). Amplifications were made in 3×25 µl reaction volume containing $1\times$ PCR buffer, 2 mM $MgCl_2$, 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 re-inoculation) 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 min 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 4 µl of DNA, 1 µl of each primer and 1 µl of miliQ water. PCR products were deposited for electrophoresis on a denaturing 7.4 M urea—6% (w/v) polyacrylamide gel (Sequagel *XR*, National Diagnostics) on a Li-Cor DNA Analyzer. Isolated bands were visualized and analyzed using e-Sequ Version 2.0.

Forward and reverse sequences were aligned using ClustalW (Thompson et al., 1994) and further adjusted by sequential pairwise comparison. Sequence data were transformed in a distance matrix by using the uncorrected distance option available in the version 4.0b10 of PAUP (Swofford, 2002). The distance matrix was then used to produce a phenogram via the neighbor joining clustering method implemented in PAUP. Bootstrap values for branches support were calculated in Paup.

2.3. Association between Horismenus and Zabrotes: ecological factors that determine the distribution of Horismenus spp. and Zabrotes spp.

To determine the environments in which bruchid and parasitoids may coexist, we determined the influence of environmental factors on the probability of occurrence of different *Zabrotes* and *Horismenus* species. The role of altitude and bean species on the ecological distribution of *Z. subfasciatus* and *Z. sylvestris* were analyzed with non-parametric statistical analyses (Wilcoxon/Kruskal–Wallis, Rank Sums). Multiple comparisons between the two *Zabrotes* species and (1) altitude distribution and (2) bean species were performed. To evaluate the repartition of *Z. subfasciatus* and *Z. sylvestris* on their potential host-plant species Pearson tests were performed.

For the three *Horismenus* species, a redundancy analysis (RDA) was performed using counts of *H. butcheri, H. depressus* and *H. missouriensis* and the following environmental variables: altitude, annual mean temperature of the locality of collection, plant of origin and bruchid infestation rate. 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) was performed using only counts of *H. butcheri, H. missouriensis* and *H. depressus*. All multivariate analyses were performed with CANOCO 4.5 (ter Braak and Smilauer, 2002).

3. Results

3.1. Does molecular data support the newly described Horismenus complex?

Results from a principal component analysis show that the three *Horismenus* species are molecularly different. The first two axes of the PCA represent 11.97% of the variance (Eigen-

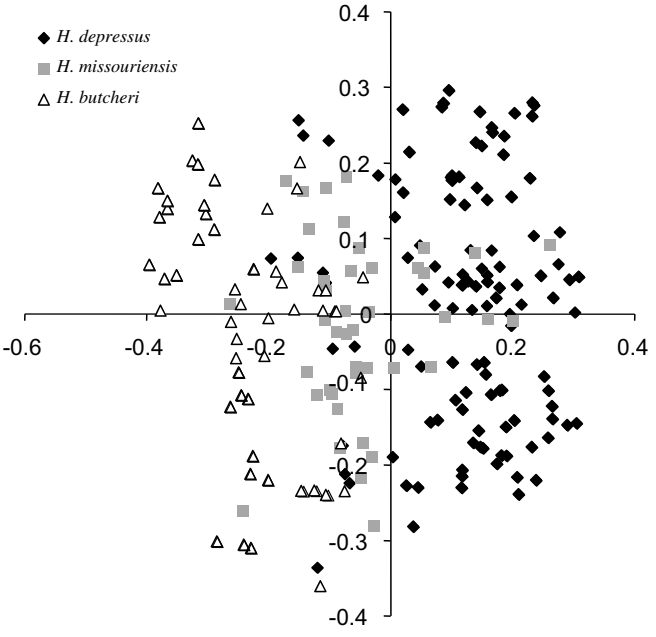

Fig. 1. Principal coordinate analysis based on an allelic matrix from 7 polymorphic microsatellite loci, for individuals from *H. depressus*, *H. missouriensis* and *H. butcheri*. (Axis 1: Eigenvalue = 7.09 and axis 2: eigenvalue = 4.88).

value 1 = 7.09 and eigenvalue 2 = 4.88) (Fig. 1). Because of the low percentage of the variance explained, the following data must be treated with caution. Nevertheless, the cluster presented in Fig. 1, shows that the three *Horismenus* species are differentiated along the first axis of the plot. A 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.

The analysis using the program STRUCTURE that blindly allocates individuals to different genetic pools without prior information about their taxonomic identity, gave strong evidence of the existence of five genetic pools within the sample. The posterior probability Pr (K/5) = 1 while all others were near zero. *Horismenus depressus* and *H. missouriensis* form two distinct genetic pools. *Horismenus butcheri* forms two further genetic pools. 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 (2 for *H. depressus*, 2 for *H. missouriensis* and 1 for *H. butcheri*) are miss-assigned on the basis of their genotype.

Analysis of the cytochrome oxydase I matrix (Accession numbers: EU435156–EU435173) revealed 149 parsimony-informative characters out of a total of 654 characters (22.7%). The NJ tree obtained is well resolved with good branch support (51–100). Topological examination of the tree shows *H. butcheri* as a well-differentiated cluster (Fig. 3) comprising six distinct haplotypes. One individual determined as *H. missouriensis* clustered with the *H. butcheri* group. In contrast, *H. missouriensis* and *H. depressus* did not form well-differentiated groups.

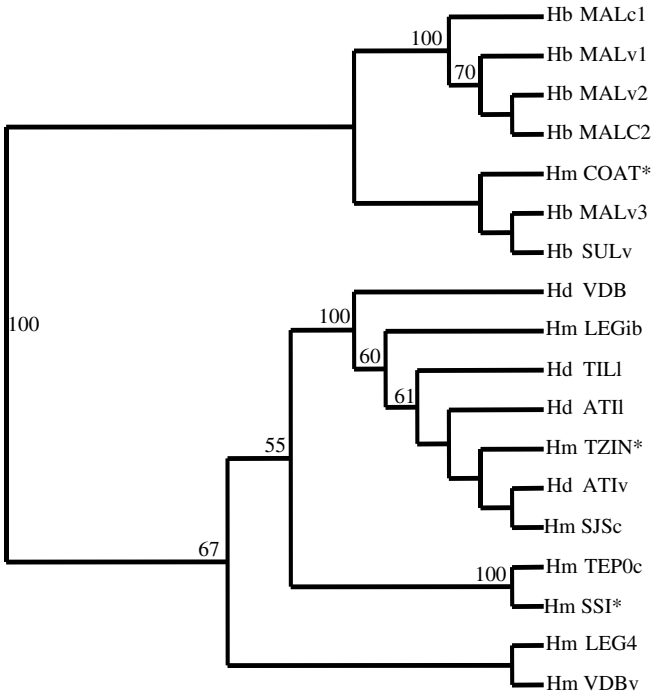

Fig. 3. Neighbour joining tree obtained from the Cytochrome oxydase I data set. Bootstrap values >50% are given above the branches. Hb, *H. butcheri*; Hm, *H. missouriensis*; Hd, *H. depressus*; v, *P. vulgaris*; c, *P. coccineus*; l, *P. lunatus*. LEGib and LEG4 are host-plant of the genus Leucaena. *Stands for cultivated bean populations.

3.2. Is there evidence of cryptic species or ecotypes among the three Horismenus species?

The absence of a well-defined cluster independent of *H. depressus, H. missouriensis* and *H. butcheri* in the PCA (Fig. 1), suggests an absence of cryptic species or ecotypes within this parasitoid complex.

The analysis using STRUCTURE (Fig. 2) shows no further genetic differentiation within the *H. depressus* and *H. missouriensis* samples. On the other hand, two genetically distinct genetic pools were detected in *H. butcheri*. A close examination of the output result files revealed that the individuals from these distinct genetic pools were reared from two different host-plants, *P. vulgaris* (41 individuals) and *P. coccineus* (12 individuals). The NJ tree (Fig. 3), obtained from the COI haplotype dataset does not show evidence for the presence of cryptic species or ecotypes among the three *Horismenus* species.

3.3. Association between Horismenus and Zabrotes: ecological factors that determine the distribution of Horismenus spp. and Zabrotes spp

Counts of *Zabrotes* on the different bean species sampled revealed that *P. lunatus* harbors the major number of *Zabrotes* beetles, while only a few individuals were reared from seeds of *P. vulgaris* 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, as 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* ($P < 0.0001$).

Similarly, the Wilkoxon/Kruskal–Wallis test revealed a significant difference in altitude repartition of *P. lunatus* and *P. vulgaris*

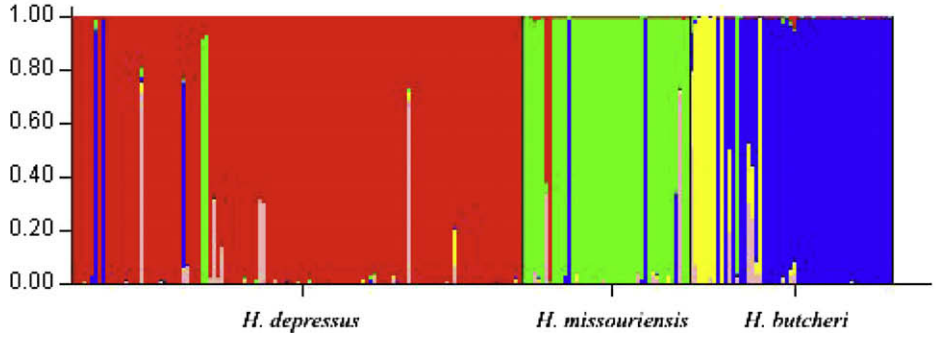

Fig. 2. Results of a Bayesian cluster analysis for *H. depressus* (118 individuals), *H. missouriensis* (44 individuals) and *H. butcheri* (54 individuals). Each individual included in the analysis is represented by a vertical line. Each color represents a genetic cluster. The vertical lines, partitioned into colored segments, represent the individual's probability of belonging to each of the K genetic clusters. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

($P < 0.0001$). The Pearson test revealed a significant host-plant association ($P < 0.0001$) of *Z. subfasciatus* on *P. lunatus* and *Z. sylvestris* on *P. vulgaris*. *Zabrotes subfasciatus* was found on *P. lunatus* (74%) and *P. vulgaris* (26%), and *Z. sylvestris* was only found on *P. vulgaris*.

Results from the redundancy analysis performed on the ecological data gathered on the three *Horismenus* species are summarized in Fig. 4. The 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. 4). The distribution of *H. depressus* was positively correlated with mean annual temperature and the presence of *P. vulgaris* and *P. lunatus*, and negatively correlated with altitude, the presence of *P. coccineus* and bruchid infestation rate. This was 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) (Fig. 4). The distribution of *H. missouriensis* was positively correlated with altitude, the presence of *P. coccineus* and infestation rate, and negatively correlated with mean annual temperature and the presence of *P. lunatus*. No correlation was found between this species and the presence of *P. vulgaris*. *Horismenus missouriensis* is most common above 1700 m (Fig. 4). Finally, the distribution of *H. butcheri* was positively correlated with *P. vulgaris* and mean annual temperature, and negatively correlated with high altitude, the presence of *P. coccineus* and infestation rate. It is found along a wide altitudinal range (1200–2200 m) (Fig. 4). The forward selection procedure 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 artifact of the calculation method, because of its high negative correlation with mean annual temperature. Nevertheless, the descriptive analysis shown in Fig. 5 shows the important role of altitude in this biological system. 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.

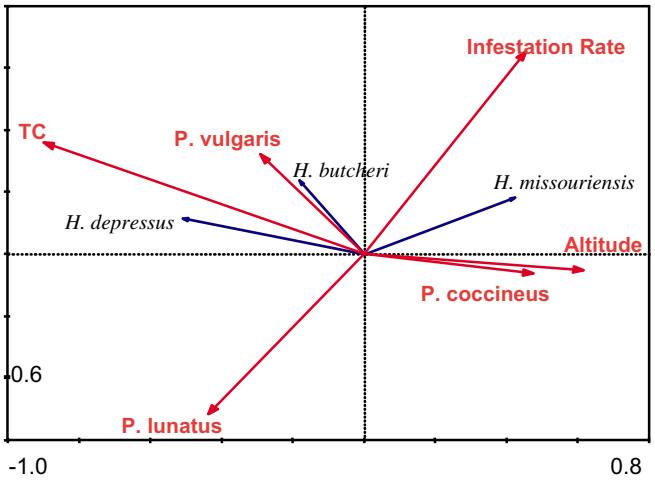

Fig. 4. Distribution of *H. depressus*, *H. missouriensis* and *H. butcheri* based on altitude, mean annual temperature (T °C), infestation rate and host-plant (*P. vulgaris*, *P. lunatus* or *P. coccineus*) in the ordination biplot of an RDA. Axis 1 (Eigenvalue = 0.173) and axis 2 (Eigenvalue = 0.032) are presented.

4. Discussion

4.1. Does molecular data support the newly described Horismenus complex?

In this study, most of the individuals morphologically referred to as *H. butcheri* formed a strongly supported genetic cluster (Figs. 1 and 2), which further supports a specific ranking for the recently described *H. butcheri*. The data obtained with the microsatellites suggest that the three species are closely related as they share some alleles, although private alleles were detected for each species (Aebi, 2004). The analysis of population structure revealed the existence of five genetic pools among which the three *Horismenus* species formed well-supported genetic pools. The analysis showed that the specimens identified as *H. butcheri* belong to two genetic pools corresponding to specimens collected on two different *Phaseolus* species (see below). The fifth genetic pool is scattered over the three species and concern a small number of individuals (9 individuals have a posterior probability >20% to belong to these genetic pool). Neither population of origin nor host-plant of origin can explain the existence of this genetic pool. Potential hybrids among this species complex or miss-identifications could explain this fifth genetic pool.

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, to incomplete lineage sorting 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 (Caterino et al., 2000). A combination of nuclear and mitochondrial data can help to discriminate the role of present gene flow and historical events in the population structure of a species. In our case, while the mitochondrial dataset show genetic similarity between *H. depressus* and *H. missouriensis*, the nuclear data show that contemporary gene flow is rare between these two species. Overall, molecules validate *H. butcheri* as a new species but do not fully support *H. missouriensis* and *H. depressus* as good species. Further research such as laboratory cross-mating experiments would enable us to clarify this situation.

4.2. Is there evidence of cryptic species or ecotypes among the three Horismenus species?

Cryptic species are very common among insects (Challis et al., 2007). In our study no evidence for cryptic species was found but we analyzed a small proportion of the known *Horismenus* species and within every species, we mostly focused on individuals reared from *Phaseolus* beans. Analyzing many specimens reared from a wide variety of hosts, host-plants and geographic locations would certainly improve our understanding of the potential diversity within these newly described parasitoid species. Especially for *H. missouriensis*, one could wonder if it is a genuine generalist or if this species actually consist of a complex of cryptic species specialized on a subset of its host spectrum or restricted to specific ecological conditions. A recent study on Costa Rican Eulophids found that *H. missouriensis* can be found from sea-level up to 1500 m. Morphological analysis showed no differences between the Mexican and Costa Rican populations (Hansson, personal communication). This unusually large altitudinal range may be explained by the presence of cryptic or sibling species (with narrower altitudinal ranges) within *H. missouriensis*.

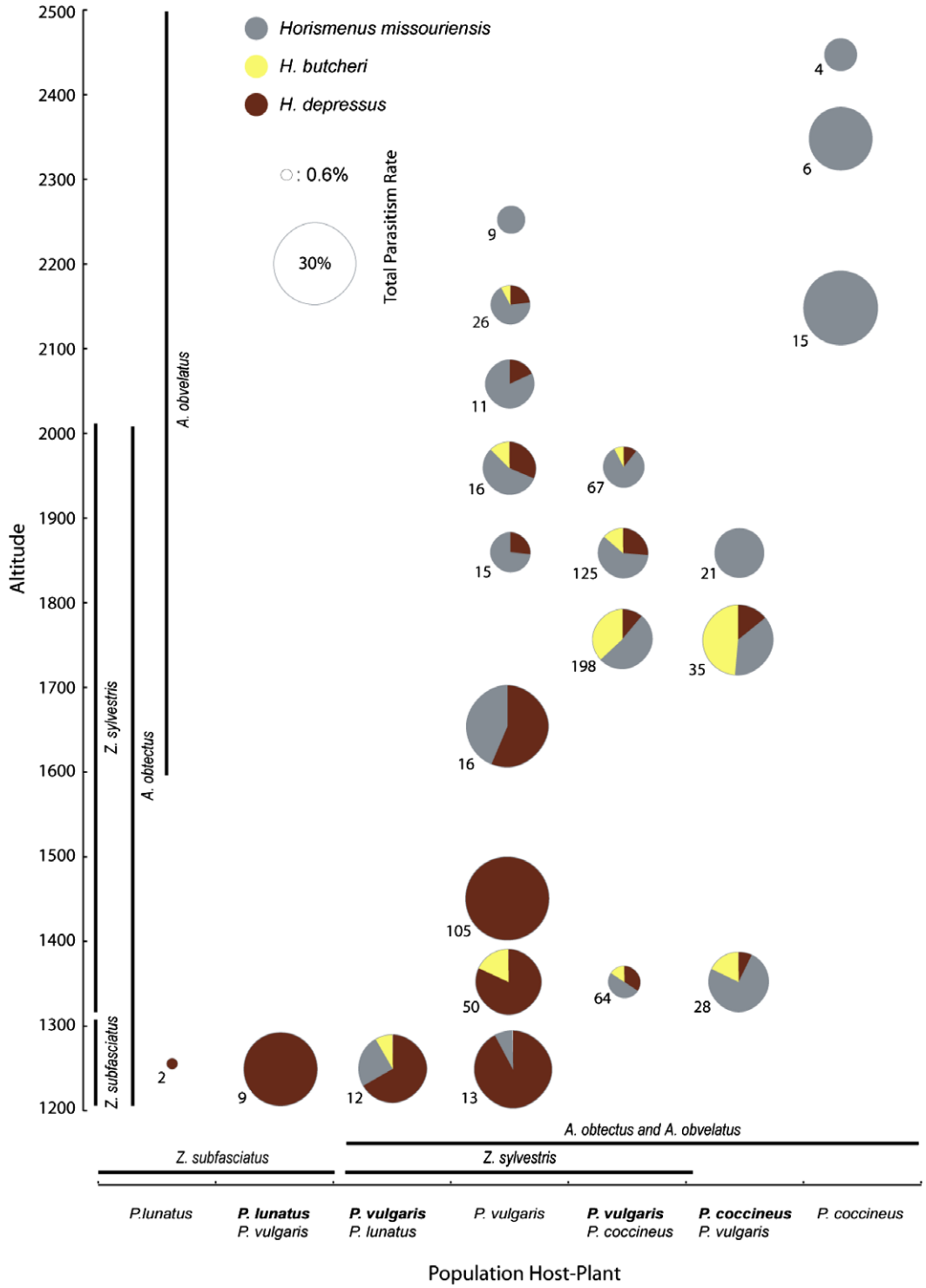

Fig. 5. Distribution of *H. depressus*, *H. missouriensis* and *H. butcheri* in relation to their host-plant and to the altitude. The diameter of each pie chart is proportional to the total parasitism rate. Numbers, number of parasitoids collected. Horizontal bars represent the host-plant association of *Zabrotes* and *Acanthoscelides* bruchid species. Vertical bars represent the altitudinal distribution of *Zabrotes* and *Acanthoscelides* bruchid species. For host-plant sympatric populations, the host-plant in bold is the one from which *Horismenus* spp. Emerged.

No convincing examples of ecotypes among parasitoid species are known (Drès and Mallet, 2002). In the case of *H. butcheri*, genetic differentiation between specimens reared from *P. vulgaris* and specimens reared from *P. coccineus* was detected. While this result might be an evidence of ecotypes specialized on their host-plants, this pattern could also be explained by assortative mating or may be just an artifact due to small sample size. To further investigate the potential presence of ecotypes within *H. butcheri*, further analysis with a much larger sample size is needed.

4.3. Association between Horismenus and Zabrotes

Our results suggest that *Zabrotes subfasciatus* and *Z. sylvestris* have very well-differentiated ecological niches. In Mexico, *Z. sub-*

fasciatus is more widely distributed and more common at low altitudes on *P. lunatus* compared to the univoltine *Z. sylvestris* with a more restricted distribution and found only at high altitudes on *P. vulgaris*. While altitude and bean domestication status (wild versus cultivated) might explain the ecological distribution of the sibling *Acanthoscelides* species (Alvarez et al., 2006), host-plant species seem to be the most important factor determining the distribution of *Z. subfasciatus* and *Z. sylvestris*. This host-plant preference could be explained by the bean specific secondary chemistry. 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 detrimental for insects 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 et al., 2000). The fact that *Z. subfasciatus* attacks wild and cultivated *P. lunatus* may suggests that through their long evolutionary history, this bruchid species could have evolved resistance to the chemical defense of the plant. Another bruchid species, *A. argillaceus* has evolved ability to overcome *P. lunatus* chemical defenses and uses its seeds as only food source (Janzen, 1977). Finally, while the effects of host-plants and altitude are difficult to disentangle as host-plant's distribution is dictated by altitude, both factors seem to have a great impact on the distribution of these *Zabrotes* species. A recent study on the phylogeography of *Zabrotes* in Mexico (Zryd, 2008), showed that *Z. sylvestris* was found in several populations of wild *P. lunatus* and at lower altitudes that we report in the present study. It may be that our sampling missed some of those populations, but also, and most likely, that this species has been expanding its distribution. The study of Zryd was conducted 5 years after our sampling. One of the reasons why bruchid beetles are important pests of seeds in the field and in storage conditions, is because of their great ability to adapt to new and changing environments. We have witnessed a similar process with its related genus *Acanthoscelides*, which has a cosmopolitan distribution and has become a pest in many countries of the old and new world. Although at present, *Z. sylvestris* is not an important pest since it has been found mostly in wild bean populations, its recent expansion on *P. lunatus* suggests that it may have the potential to invade and get established in cultivated populations at lower altitudes and become a problematic pest.

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 (Fig. 5). The wide altitudinal range of the *Horismenus* species brings them into contact with all three-bean species and in particular with *P. lunatus* and *P. vulgaris* harboring *Z. subfasciatus* and *Z. sylvestris*. 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 (Fig. 5).

For the first time, ecological information for *Zabrotes* spp. and *Acanthoscelides* spp. the two most abundant bruchid beetles attacking *Phaseolus* beans in Mexico, and their *Horismenus* parasitoid complex is available. As altitude was shown to be the most important factor influencing the ecological distribution of all three parasitoid species, these species most likely attack bruchid species from both genera (*Zabrotes* and *Acanthoscelides*). Further study using molecular barcode consisting of bruchid specific microsatellite markers (Aebi et al., 2004b; Alvarez et al., 2003) would certainly help to shed light on this question.

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). Given the unambiguous taxonomic status of the three *Horismenus* species, 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 should be considered in the development of effective biological control program against these bruchid pests.

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