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Intra-population variation and geographic correlation in *Canthon humectus hidalgoensis* using FTIR-ATR spectroscopy

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Abstract In some cases external morphology is not sufficient to discern between populations of a species, as occurs in the dung beetle *Canthon humectus hidalgoensis* Bates; and much less to determine phenotypic distances between them. FTIR-ATR spectroscopy show several advantages over other identification techniques (e.g. morphological, genetic, and cuticular hydrocarbons analysis) due to the non-invasive manner of the sample preparation, the relative speed of sample analysis and the low-cost of this technology. The infrared spectrum obtained is recognized to give a unique 'fingerprint' because vibrational spectra are specific and unique to the molecular nature of the sample. In our study, results showed that proteins, amino acids and aromatic ethers of insect exocuticle have promising discriminative power to discern between different populations of *C. h. hidalgoensis*. Furthermore, the correlation between geographic distances between populations and the chemical distances obtained by proteins + amino acids + aromatic ethers was statistically significant, showing that the spectral and spatial information available of the taxa together with appropriated chemometric methods may help to a better understanding of the identity, structure, dynamics and diversity of insect populations.

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Introduction

Theory of isolation by distance predicts an increase in genetic dissimilarity with geographic distance (Malécot 1991). Thus, polymorphisms should have a significant correlation between genetic and geographic distance and in the same terms with chemical distance, which can explain the existence of chemical diversity associated to different morphs (Kaltenpoth et al. 2007). Recently, taxonomy and species diversity has been studied using new methods for chemical identification and powerful mathematical tools that allow the characterisation of not only populations, but also insect colonies of the same population (Dronnet et al. 2006; Torres et al. 2007).

An ideal method to discriminate between populations should have several properties as requiring a minimum preparation of the samples (e.g. without reagents), quick analysis, non-invasive manner in sample preparation, be able to provide both qualitative and semiquantitative information, reproducibility and low cost (Santos et al. 2010; Singh et al. 2012). In order to obtain an unbiased, precise and statistically adequate method capable of estimating population differences we used here Fourier transform infrared-Attenuated total reflectance (FTIR-ATR) spectroscopy combined with powerful chemometric methods. FTIR-ATR spectroscopic techniques have several advantages over other identification techniques (e.g. morphological, genetic, and cuticular hydrocarbons analysis) due to the relative speed of sample analysis and the low-cost of the technology (Cole et al. 2003). Furthermore, the infrared spectrum (IR) of any sample or compound (e.g. proteins, carbohydrates, lipids, nucleic acids, etc.) is recognized to give a unique 'fingerprint' because vibrational spectra are molecule-specific and unique to the nature of the sample (Adt et al. 2006; Santos et al. 2010; Singh et al. 2012). Several

spectroscopic techniques have been employed to analyse bacterial strains (Samuels et al. 2008; Savic et al. 2008; Davis and Mauer 2010; Dziuba 2013), filamentous fungi identification (Santos et al. 2010), interspecific variation in plant leaves (Ribeiro da Luz 2006), inter- and intraspecific variation in wood composition (Hori and Sugiyama 2003; Zhou et al. 2011), molecular characterization (Barth 2000; Hayes et al. 2003; Oberg et al. 2004), characterization of chitin and insect gland secretions (Ammar et al. 2013; Davies et al. 2013) and intraspecific variation in insect cuticle and chorion composition and structure (Gibbs and Crowe 1991; Iconomidou et al. 2001; Junior et al. 2008; Srivastava et al. 2011).

As a model to study the potentiality of FTIR-ATR spectroscopic techniques in discriminating insect populations and in order to study distribution patterns, we used the dung beetle *Canthon humectus hidalgoensis* Bates (Coleoptera: Scarabaeinae). This subspecies is patchily distributed throughout the Mexican High Plateau, from the States of Guanajuato, Querétaro and Hidalgo to Durango and Chihuahua by the Center of Mexico; Tamaulipas, Nuevo León and Texas (Brownsville) by the East, being a well-known subspecies with high morphological and physiological diversity (Halffter 1961; Halffter et al. 2011; Verdú 2011). Actually, the existence of well-established populations seems to be limited by the presence of different kinds of livestock (sheep, cow and horse, mainly) inhabiting a mosaic of vegetation types composed by crassicaule and submountainous scrublands (Verdú et al. 2007; Halffter et al. 2011). The variation of several characters such as the dorsal surface coloration, body size, pronotum microsculpture, and aedeagal morphology has been used up to now to describe the dung beetle polymorphism in the complex *Canthon humectus* (Halffter et al. 2011). However, within this subspecies all the studied morphological characters presents critical limitations and are not sufficient to discern between subpopulations or at least to define distances between 'morphos' or 'ecotypes'. Until now, only some physiological characteristics related with chill tolerance variability have been well documented within and among populations of *C. h. hidalgoensis* (Verdú 2011).

In this study we examine the population discriminatory capacity of FTIR-ATR spectroscopic techniques on the individuals collected in four different localities of this dung beetle subspecies. Our main objective is thus to examine the possibilities of FTIR-ATR techniques when directed to discern among populations, but we also analyse whether the so obtained 'chemical similarity' between individuals is correlated with its geographic proximity. We report data about the chemical composition of the cuticle and experimental data showing that the IR spectra obtained for each individual of *C. humectus hidalgoensis* are 'fingersprints' capable of discriminating species populations and reflecting the geographic distances that isolated them.

Materials and methods

Study area and beetles collection

Beetles were collected at four sites (Fig. 1) on the Mexican High Plateau in the State of Hidalgo (Mexico) during 25–28 August 2008. The climate is semiarid with temperatures from 21 to 15 °C in average and average annual precipitation from 350 to 450 mm. Concretely, the vegetation of each site is characterized as follows: (1) Los Mármoles National Park (LM) is dominated by a mosaic of grasslands, xerophilous shrublands and patches of pine-oak forest, (2) Xhitá (XH) is characterized by a mountainous xerophilous landscape dominated by leguminosae trees and cactaceae, (3) Venta del Río (TL) which is dominated by crassicaule scrubland, and (4) Barranca de Metztitlán Biosphere Reserve (MZ), dominated by crassicaule and submountainous scrubland (Verdú 2011, for more details).

In the field, prior to transport to laboratory, all beetles were immediately acclimated at 10–15 °C (close to ambient temperature) in portable refrigerated chambers in order to avoid the high temperatures during the transport within the vehicle. In the laboratory, ten individuals collected in each surveyed site were frozen at −80 °C until sample preparation. To assure a common 'physical' state for all the individuals, thus allowing the comparison of measures between the populations, we selected only mature specimens according to cuticle deterioration of tibial and pronotum in conjunction with the hardness of the cuticle. As has been previously demonstrated, these morphological characters allow the identification of individuals of approximately the same age (Tyndale-Biscoe 1984).

Cuticle samples

As both the physical state of the individuals and the chemical and physical conditions of the environment have a notable effect on the infrared spectrum (Coates 2000), beetles were not treated with any chemical compound (preservatives, solvents, etc.). For FTIR-ATR measurements, thoracic cuticles were cut (4 × 4 mm) with fine needles and micro-forceps and washed several times in distilled water and sonicated to remove superficial residues. Finally beetles were then thoroughly air-dried at 30 °C.

FTIR-ATR absorption spectra were acquired by a FTIR IFS 66/S spectrometer (Bruker Optics, Germany), equipped with a DTGS detector coupled to an ATR accesory with a diamond crystal (Specac Golden Gate). The scanning range was from 600 to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1}. A pressure applicator with a torque knob between the cuticle sample and the diamond crystal ensured that the same pressure was applied for all measurements. Give that FTIR-ATR spectrometer have an IR penetration depth of 4 μm, band

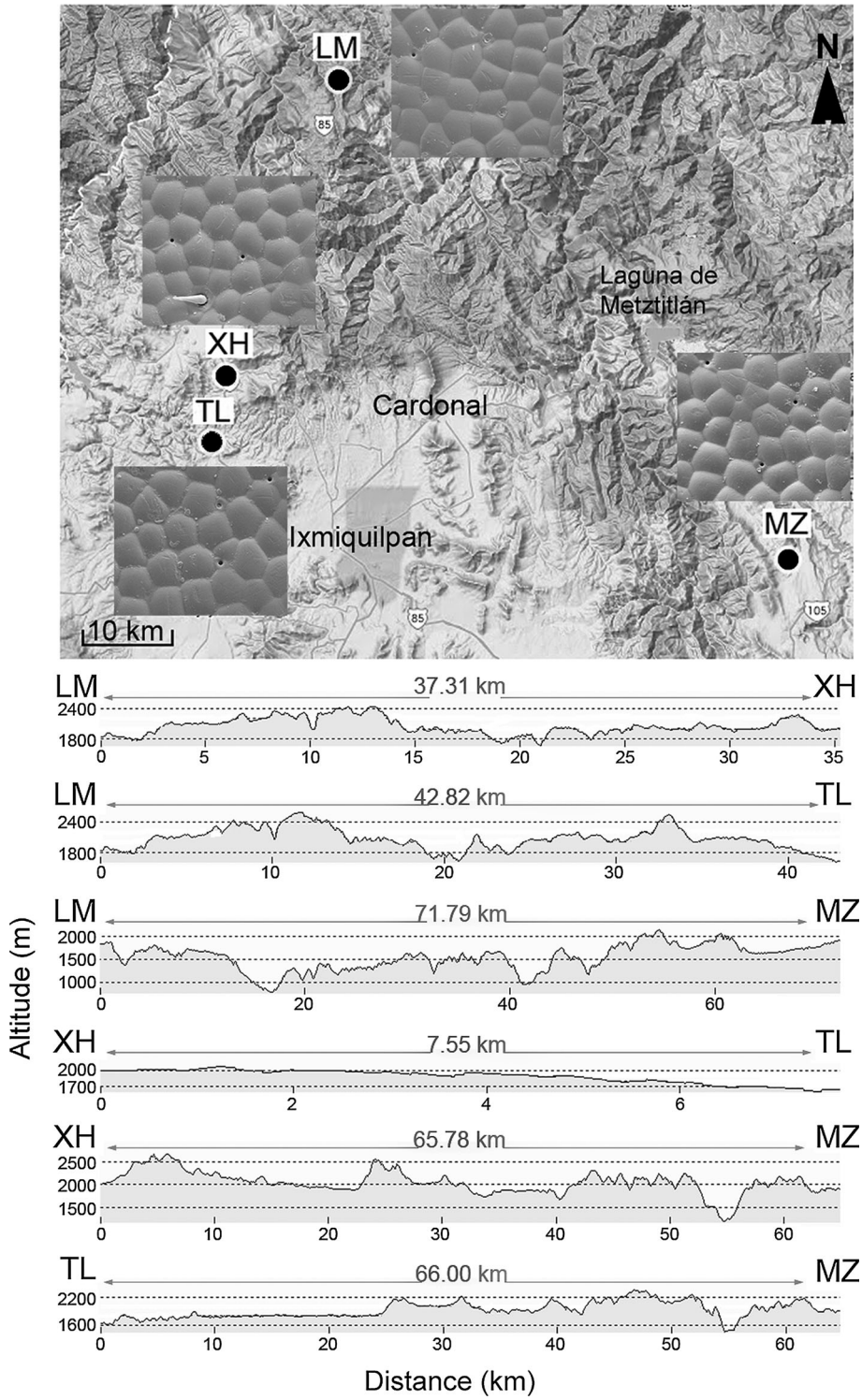

Fig. 1 Geographic location of *Canthon humectus hidalgoensis* populations, scanning electron micrographs of pronotum microsculpture (×1000) topographical profiles (fixed exaggeration = ×4) and distances between sites. *LM* Los Mármoles National Park, *MZ* Barranca de Metztitlán Biosphere Reserve, *TL* Venta del Río, *XH* Xhitá

assignments corresponds to epicuticle and exocuticle components. Ten replicas of each cuticle were prepared. For each cuticle sample, 64 scans were acquired and averaged. The so obtained IR spectra were pre-processing by using three different algorithms: vector normalization, auto baseline correction, and auto scale. Subsequently, major vibration band frequencies are selected considering both the second derivative spectra

and the absorbance spectra. Spekwin 32 software version 1.71.6.1 was used for analyses (Menges 2013).

Scanning electron microscope analysis

Pronotum microsculpture of *C. h. hidalgoensis* was studied using a scanning electron microscope (SEM). The thoracic cuticles were mounted on aluminium stubs with the dorsal surface upward and then sputter-coated with gold in a Balzers model SCD 004 E 5400 high resolution sputter 004. The structure of the thorax surface was examined qualitatively and photographed using an SEM (JEOL model JSM-840, operated at 10 kV).

Chemometric analysis

For multivariate analysis, we build a matrix including the spectral intensity of all the individual measurements (rows) for each one of the delimited chemical functional groups (columns). This matrix data was submitted to a Permutational multivariate analysis of variance (PERMANOVA) (Anderson et al. 2008) to estimate differences between the four populations according to the spectral intensity of each one of chemical functional groups. The Whittaker's index of association (Whittaker 1952), which is well adapted to relative abundance data (Legendre and Legendre 1998; Clarke and Gorley 2006) was used in these analyses. Post-hoc pairwise comparisons among sites were obtained calculating a pseudo-F statistic for each site and P values estimated by using a permutation procedure (999 iterations in this study) followed by a Bonferroni correction to the P values. For each analysis combining functional groups we created a new distance matrix based on Huygens' theorem (Clarke and Gorley 2006), which calculated the distance to centroids corresponding with the groups formed by the individuals of each site. PRIMER v.6 and PERMANOVA software was used for all analyses (Anderson et al. 2008). Non-metric multidimensional scaling (MDS) were use to construct a 'map' of the relationships between the distance matrices previously obtained.

From distance matrices obtained previously we analyse the relation between the geographic distances among sites and the 'distances' obtained from chemical data performing a pairwise Mantel test. Geographic distances were calculated measuring spatial distances between sites considering topographical profiles using Google Earth v. 7.1.2.2041 application (Google Inc. 2013). Topographical profiles were made using HeyWhatsThat Path profiler application (HeyWhatsThat Path Profiler 2014). A permutation test compared the original R (Pearson's correlation coefficient) to R computed in 10,000 random permutations and one-tailed P value is obtained. The results were plotted using non-metric MDS. PAST software v.2.17 was used for Mantel test and permutation analysis (Hammer et al. 2001).

Results and discussion

Pronotal microsculpture of *C. h. hidalgoensis*

We examined qualitatively ten dorsal surface of pronotum for each site using scanning electron micrographs magnified 1,000 times (Fig. 1). Pronotal microsculpture not varied between sites studied and only individuals from Metztitlán population showed a higher relief (convexity) in the granules that conforms pronotal microsculpture (Fig. 1). These morphological characters along with the morphology of aedeagus have been validated at subspecies and species level, for example to discern between *C. h. hidalgoensis* and *C. h. alvarengai* Halffter subspecies (Halffter 1961; Halffter et al. 2011), however, given the current data, external morphology is not sufficient to discern between populations of *C. h. hidalgoensis* and much less to determine distances between them.

ATR-FTIR spectra of cuticle samples

Ten individual FTIR-ATR spectra were obtained for each site. Figure 2 illustrates FTIR-ATR spectra averaged for each site and the IR vibration bands selected according to the second derivative of the spectrum and specialized bibliography (Table 1). Band assignments can be obtained from group frequency charts published in several general bibliographies and from more specialized references on the physical and chemical composition of insect cuticle that may be helpful in obtaining a correspondence between chemical functional groups and band frequencies. Cuticle hydrocarbons, lipids and proteins without chitin fibers are the main components of the epicuticle while the exocuticle contain chitin fibers

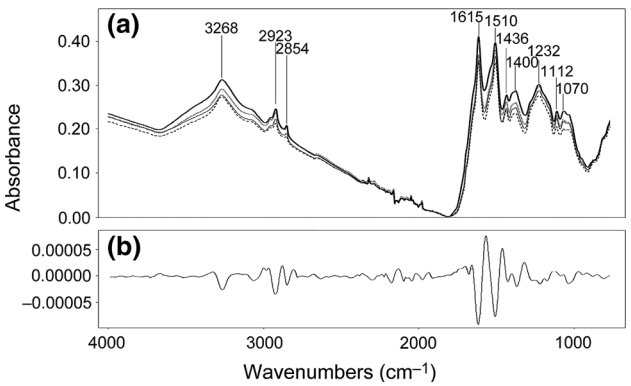

Fig. 2 FTIR-ATR spectra of thoracic cuticle of *Canthon humectus hidalgoensis*. **a** Absorbance spectra averaged ($n = 10$) for each one of the four considered survey sites (MZ Barranca de Metztitlán Biosphere Reserve: thickness solid line; TL Venta del Río: *dotted line*; XH Xhitá: *thin solid line*; and LM Los Mármoles: *dashed line*), **b** second derivative spectrum. See Table 1 for a description of chemical functional groups and their band assignment

Table 1 Assignment of the chemical functional groups associated with the main vibration bands of the infrared (IR) spectra observed in the thoracic cuticle of *Canthon humectus hidalgoensis*

Wavenumber (cm^{-1})	Origin	Functional groups	References	Assignment
3268	O–H N–H	Stretching OH symmetric NH stretching	Coates (2000), Socrates (2001)	Carbohydrate (chitin); amino acid
2923	$-CH_2$	Methylene C–H asymmetric stretching	Coates (2000), Socrates (2001)	Saturated hydrocarbons (alkanes)
2854	$-CH_3$	Methyl C–H asymmetric/symmetric stretching	Coates (2000), Gibbs (2002), Socrates (2001)	Saturated hydrocarbons (alkanes)
1615	C = C–C	Ring C–C stretch of phenyl	Barth (2000), Coates (2000), Iconomidou et al. (2001)	Amino acids (Tyr); Amide I band (β sheet)
1510	C = C–C	Aromatic ring stretch	Barth (2000), Coates (2000), Iconomidou et al. (2001), Srivastava (2011)	Amino acids (Trp, Tyr); Amide II band (β sheet)
1436	$-CH_3$	Asymmetric CH_3 bending of Methyl groups of lipids	Socrates (2001)	Unsaturated hydrocarbons (alkenes)
1400	$CH = CH_2$	C–H in plane asymmetric deformation vibration	Socrates (2001)	Unsaturated hydrocarbons (alkenes)
1232	C–N, C–O, C–C,	Mixture of several bending and stretching vibrations	Barth and Zscherp (2002), Srivastava (2011)	Amide III band (β sheet)
1112	$CH = CH_2$	C–H in plane asymmetric deformation vibration	Socrates (2001)	Unsaturated hydrocarbons (alkenes)
1070	C–O–C	Cyclic ethers, large rings, C–O stretch	Coates (2000)	Aromatic ethers

embedded in a proteinaceous matrix (Chen et al. 2002; Hamodrakas et al. 2002).

Table 1 list the assignment of chemical functional groups associated with major vibration bands observed in IR spectra of the thoracic cuticle of *Canthon h. hidalgoensis* (Fig. 2). Although in this type of samples the band assignment is tentative, the obtained band assignments seem to be congruent with the results provided by other studies about insect cuticle (Gibbs and Crowe 1991; Iconomidou et al. 2001; Srivastava et al. 2011). Thus, several vibration bands are assignable to typical side chain vibrations of amino acid residues, especially to those of v(C–C) aromatic ring-containing Tyrosine (Tyr) and Tryptophan (Trp) (e.g. Srivastava et al. 2011). In the Amide I region (1600–1700 cm^{-1}) a well-defined peak at 1615 cm^{-1} appear which is typical of the β-sheet structure of cuticle proteins (Barth 2000; Iconomidou et al. 2001). In the Amide II vibrational region (1400–1500 cm^{-1}), a v(C–O) strong band was observed at 1510 cm^{-1}, also probably associated with Trp–Tyr ring vibrations or β-sheet structure (Barth 2000; Iconomidou et al. 2001; Srivastava 2011). In the Amide III region (1230–1320 cm^{-1}) the 1232 cm^{-1} infrared band was tentatively assigned to β-sheet structure (Barth and Zscherp 2002; Srivastava 2011). On the other hand, in the region 2975–2840 cm^{-1}, two well-defined peaks was assigned to the asymmetric and symmetric CH ($-CH_2-$) stretching vibrations corresponding to saturated hydrocarbons (Socrates 2001) were observed at 2854 and 2923 cm^{-1}. Although the hydrocarbon components on the insect cuticle are usually complex (Blomquist 2010), aliphatic (alkanes) and unsaturated hydrocarbon (alkenes) vibrational bands could be assigned to the cuticle lipids responsible of cuticle water loss regulation (Singer 1998; Gibbs and Rajpurohit 2009). Mixtures of different alkanes and alkenes and their interactions in the insect cuticle has been implicated in the state for cuticle transpiration (Gibbs 1998; Gibbs and Rajpurohit 2009), chemical defence and communication (Carlson et al. 1971; Blomquist 2010). Interestingly, cuticle lipids can vary it composition and amount at inter- and intraspecific levels (Hadley 1994; Junior et al. 2008). For unsaturated hydrocarbons, in many cases, a close relation with chemical communication has been reported, having a diverse role as pheromones (e.g. Carlson et al. 1971; Darbro et al. 2005) or chemical cues (Martin et al. 2008).

Analysis of IR functional groups and discrimination of *C. h. hidalgoensis* populations

According to the nature of the observed chemical functional groups (see Coates 2000 for a revision) four different groupings were considered: (1) a general grouping comprising all delimited functional groups together, (2) aromatic functional groups corresponding to cuticle amino acids and proteins [vibrations bands (cm^{-1}): 1070, 1232, 1510 and 1615], (3) functional

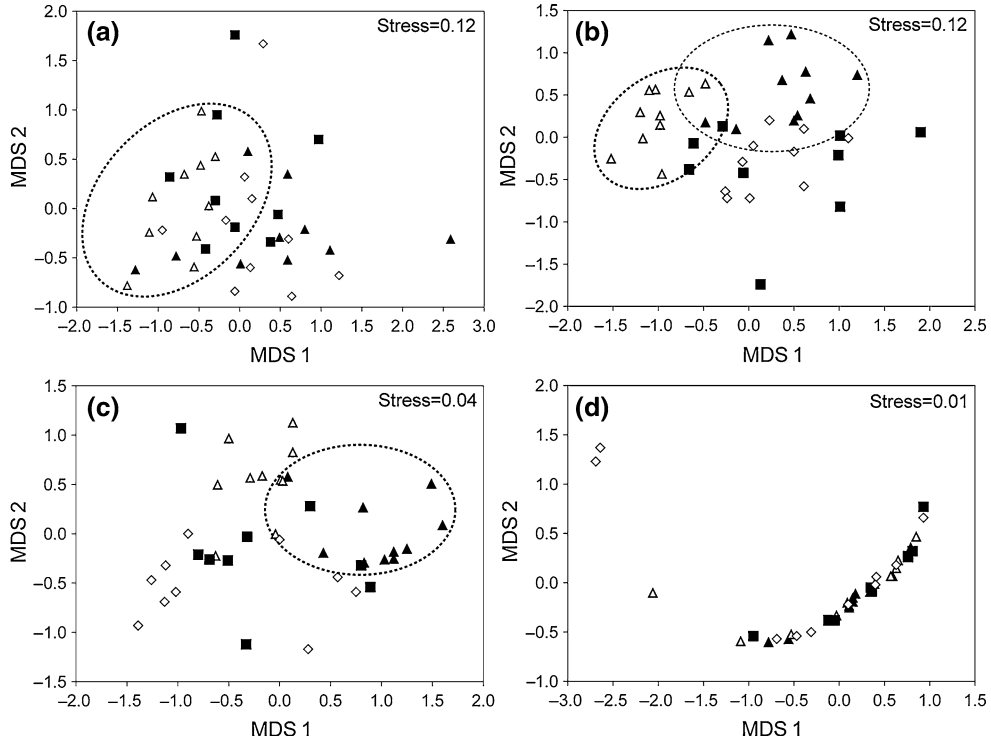

Fig. 3 Non-metric multidimensional scaling (MDS) and Permutational multivariate analysis of variance (PERMANOVA) for each vibrational band grouping: **a** all chemical functional groups included, **b** proteins + aminoacids + aromatic ethers group, **c** unsaturated hydrocarbons, and **d** saturated hydrocarbons. Sites showing statistical significant differences are highlighted in *circles*. Los Mármoles National Park (*black triangles*), Barranca de Metztitlán Biosphere Reserve (*white triangles*), Venta del Río (*black squares*), Xhitá (*white rhombouses*)

Table 2 Permutational multivariate analysis of variance (PERMANOVA) test showing differences between sites for each functional group (FG), and results of Mantel tests between the geographic distance matrix and each distance matrix obtained using Whittaker's index of association for each functional group

PERMANOVA test[a]	Functional group							
	All FGs		Proteins + amino acids + aromatic ethers		Unsaturated hydrocarbons		Saturated hydrocarbons	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*
LM–MZ	2.87	0.006	5.05	0.006	5.41	0.006	0.25	1.000
LM–TL	1.56	0.504	2.44	0.006	3.39	0.012	1.37	1.000
LM–XH	1.26	1.000	3.29	0.006	4.55	0.006	1.20	1.000
MZ–TL	2.25	0.036	3.65	0.006	1.57	0.540	1.23	1.000
MZ–XH	2.56	0.006	5.09	0.006	3.50	0.006	0.94	1.000
TL–XH	1.04	1.000	1.11	1.000	1.20	1.000	1.83	1.000
Mantel test (r_M)	0.69	0.046	0.86	0.042	0.59	0.956	–0.81	0.750

LM Los Mármoles National Park, *MZ* Barranca de Metztitlán Biosphere Reserve, *TL* Venta del Río, *XH* Xhitá
[a]Probabilities (*P*) are Bonferroni-corrected

groups corresponding to unsaturated hydrocarbons and ethers [vibration bands (cm^{-1}): 1112, 1400, and 1436], and (4) functional groups corresponding to saturated hydrocarbons [ν(C–H) bands (cm^{-1}): 2854 and 2923]. The analysis comprising all vibration bands showed a first approximation on the applied possibilities of this technique in order to explore intraspecific variations. Figure 3A illustrates that only those individuals belonging to the Barranca de Metztitlán site (MZ) seem to significantly differ from those all the other geographical sites in all pairwise comparisons (Table 2). The second analysis comprising cuticle amino acids and proteins showed the higher robustness to differentiate or identify intraspecific variations at population or subpopulation level. In this case, the individuals of MZ and LM sites showed significant differences between them

but also with respect to those collected in the other two sites; only TL and XH populations do not show statistically significant differences between them. In the case of unsaturated hydrocarbons only the individuals of LM site showed statistically significant differences with those all the other localities. Finally, saturated hydrocarbons not allow us to differentiate anyone of the considered populations (Table 2).

The results presented here showed that proteins, amino acids and aromatic ethers can provide a substantially higher between-population discrimination capacity that the other detected chemical functional groups observed in the cuticle of this insect species. Changes in exocuticle composition and structure attributed to proteinaceous matrix could be the responsible of the detected intraspecific variation of absorption features at 1070, 1232, 1510 and 1615 cm^{-1} vibrations bands. Unfortunately, to date there has been no systematic structural analysis of these cuticle proteins and their interaction with the chitin filaments (Iconomidou et al. 2001). However, our results here show conclusively for the first time that proteins, amino acids and aromatic ethers of the insect exocuticle have promising discriminative power at intraspecific level. In our case, FTIR-ATR analyses suggest that at least three different populations of *C. h. hidalgoensis* exist in the studied region and that, probably, the individuals inhabiting at XH and TL sites belong to the same population.

Correlations between spectrometric and geographic distances

Table 2 lists the Mantel correlation values between spectrometric distances considering each chemical functional groupings and the geographic distances between sites. The grouping formed by all functional groups, the proteins + amino acids + aromatic ethers group, and the unsaturated hydrocarbons, all presented high correlation positive values. However, only the correlation between geographic distances and the grouping formed by proteins + amino acids + aromatic ethers was statistically significant. Figure 4 resumes the relationship among geographic distances and observed chemical functional groups, showing that the structural components of exocuticle, such as proteins, were the more informative components. These proteins with β sheet conformation usually are the responsible to produce the matrix, with varying mechanical properties, interacting and stabilising with chitin microfibrils (see Andersen 1979; Hamodrakas et al. 2002; Vincent and Wegst 2004, for a review). The variations of cuticle, even at intraspecific level, would be related with the degree of sclerotization (hardening), which is determined largely by the cuticle proteins and regulated by cuticle protein genes (Charles 2010). From a genetic point of view, several studies provided evidence for allelic variation in cuticle proteins even in natural populations (see Willis 2010, for a revision and literature cited therein). Furthermore, insect cuticle proteins appear to be a source of adaptive evolution within genera (Futahashi 2008; Cornman 2009). Based on commented above, we suggest that distances obtained from IR spectra (Fig. 3b) could have a reliable relation with genetic distances between populations of *C. h. hidalgoensis*.

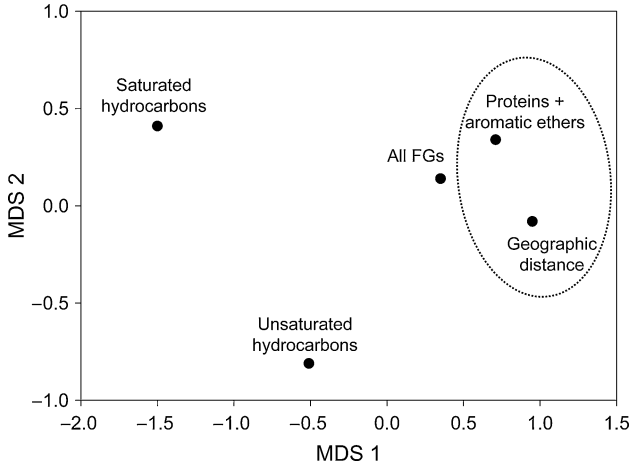

Fig. 4 Non-metric multidimensional scaling (MDS) showing an overall description of the similarity among the different observed chemical functional groups and geographic distance matrix. Functional groups showing statistical significant similarities with the geographic distance matrix are highlighted in circles (see Table 2 for Mantel statistics and probabilities) (FGs: all functional groups)

In conclusion, the spectral and spatial information available of *C. h. hidalgoensis* together with appropriated chemometric methods may help to a better understanding of the structure, dynamics and diversity of this taxa. Further microsatellite genotyping may help to corroborate the population discrimination provided by FTIR-ATR analyses. Finally, multidisciplinary experimental studies must elucidate the capacity of IR spectroscopic results in explaining biogeographic, genetic and physiologic clines across environmental gradients and its correlation with dung beetle diversity.

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References

Adt I, Toubas D, Pinon JM, Manfait M, Sockalingum GD (2006) FTIR spectroscopy as a potential tool to analyse structural modifications during morphogenesis of *Candida albicans*. Arch Microbiol 185:277–285

Ammar E-D, Alessandro R, Shatters RG Jr, Hall DG (2013) Behavioral, ultrastructural and chemical studies on the honeydew and waxy secretions by nymphs and adults of the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae). PLoS One 8:e64938

Andersen SO (1979) Biochemistry of insect cuticle. Ann Rev Entomol 24:29–61
Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA + for PRIMER: guide to software and statistical methods. PRIMER-E, Plymouth
Barth A (2000) The infrared absorption of amino acid side chains. Prog Biophys Mol Biol 74:141–173
Barth A, Zscherp C (2002) What vibrations tell us about proteins. Q Rev Biophys 35:369–430
Blomquist GJ (2010) Structure and analysis of insect hydrocarbons. In: Blomquist GJ, Bagnères AG (eds) Insect hydrocarbons. Cambridge University Press, Cambridge, pp 19–34
Carlson DA, Mayer MS, Silhacek DL, James JD, Beroza M, Bierl BA (1971) Sex attractant pheromone of the house fly: isolation, identification and synthesis. Science 174:76–78
Charles JP (2010) The regulation of expression of insect cuticle protein genes. Insect Biochem Mol Biol 40:205–213
Chen B, Peng X, Wang W, Zhang J, Zhang R (2002) Research on the microstructure of insect cuticle and the strength of a biomimetic preformed hole composite. Micron 33:571–574
Clarke KR, Gorley RM (2006) Change in marine communities: an approach to statistical analysis and interpretation. Primer-E Ltd, Plymouth
Coates J (2000) Interpretation of infrared spectra, a practical approach. In: Meyers RA (ed) Encyclopedia of analytical chemistry. John Wiley, Chichester, pp 10815–10837
Cole TJ, Ram MS, Dowell FE, Omwega CO, Overholt WA, Ramaswamy SB (2003) Near-infrared spectroscopic method to identify *Cotesia flavipes* and *Cotesia sesamiae* (Hymenoptera: Braconidae). Ann Entomol Soc Am 96:865–869
Cornman RS (2009) Molecular evolution of *Drosophila* cuticular protein genes. PLoS ONE 4:e8345
Darbro JM, Millar JG, McElfresh S, Mullens BA (2005) Survey of muscalure [(Z)-9-tricosene] on house flies (Diptera: Muscidae) from field populations in California. Environ Entomol 34:1418–1425
Davies GJG, Knight DP, Vollrath F (2013) Chitin in the silk gland ducts of the spider *Nephila edulis* and the silkworm *Bombyx mori*. PLoS ONE 8:e73225
Davis R, Mauer LJ (2010) Fourier transform infrared (FT-IR) spectroscopy: a rapid tool for detection and analysis of foodborne pathogenic bacteria. Appl Microbiol 1:1582–1594
Dronnet S, Lohou C, Christides J-Ph, Bagnères A-G (2006) Cuticular hydrocarbon composition reflects genetic relationship among colonies of the introduced termite *Reticulitermes santonensis* Feytaud. J Chem Ecol 32:1027–1042
Dziuba D (2013) Identification of Propionibacteria to the species level using Fourier transform infrared spectroscopy and artificial neural networks. Pol J Vet Sci 16:351–357
Futahashi R, Okamoto S, Kawasaki H, Zhong YS, Iwanaga M, Mita K, Fujiwara H (2008) Genome-wide identification of cuticular protein genes in the silkworm, *Bombyx mori*. Insect Biochem Mol Biol 38:1138–1146
Gibbs AG (1998) Waterproofing properties of cuticular lipids. Am Zool 38:471–482
Gibbs AG (2002) Lipid melting and cuticular permeability: new insights into an old problem. J Insect Physiol 48:391–400
Gibbs AG, Crowe JH (1991) Intra-individual variation in cuticular lipids studied using Fourier transform infrared spectroscopy. J Insect Physiol 37:743–748
Gibbs AG, Rajpurohit S (2009) Cuticular lipids and water balance. In: Blomquist GJ, Bagnères AG (eds) Insect Hydrocarbons. Cambridge University Press, Cambridge, pp 100–120
Google Inc. (2013) https://www.google.com/earth/
Hadley NF (1994) Water relations of terrestrial arthropods. Academic Press, San Diego
Halffter G (1961) Monografía de las especies norteamericanas del género *Canthon* Hoffsg. Ciencia (Mex.) 20:225–320
Halffter G, Halffter V, Martínez-Sánchez KM, Moreno CE, Sánchez-Rojas G (2011) Hybridization between subspecies of *Canton humectus* (say) (Coleoptera: Scarabaeidae). Col Bull 65:425–431
Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron 4:1–9
Hamodrakas SJ, Willis JH, Iconomidou VA (2002) A structural model of the chitin-binding domain of cuticle proteins. Insect Biochem Mol Biol 32:1577–1583
Hayes KD, Ozen BF, Nielsen SS, Mauer LJ (2003) FTIR determination of ligand-induced secondary and tertiary structural changes in bovine plasminogen. J Dairy Res 70:461–466
Hori R, Sugiyama J (2003) A combined FT-IR microscopy and principal component analysis on softwood cell walls. Carbohyd Polym 52:449–453
HeyWhatsThat Path Profiler (2014). http://www.heywhatsthat.com/profiler.html
Iconomidou VA, Chryssikos GD, Gionis V, Willis JH, Hamodrakas SJ (2001) "Soft"-cuticle protein secondary structure as revealed by FTRaman, ATR FT-IR and CD spectroscopy. Insect Biochem Mol Biol 31:877–885
Junior WA, Suárez YR, Izida T, Andrade LHC, Lima SM (2008) Intra-and interspecific variation of cuticular hydrocarbon composition in two *Ectatomma* species (Hymenoptera: Formicidae) based on Fourier transform infrared photoacoustic spectroscopy. Genet Mol Res 7:559–566
Kaltenpoth K, Kroiss J, Strohm E (2007) The odor of origin: kinship and geographical distance are reflected in the marking pheromone of male beewolves (*Philanthus triangulum* F., Hymenoptera, Crabronidae). BMC Ecol 7:11
Legendre P, Legendre L (1998) Numerical ecology. 2nd English edition. Elsevier, Amsterdam
Malécot G (1991) The Mathematics of Heredity. Freeman, San Francisco
Martin SJ, Vitikainen E, Helanterä H, Drijfhout FP (2008) Chemical basis of nest-mate discrimination in the ant *Formica exsecta*. P Roy Soc B 275:1271–1278
Menges F (2013) Spekwin32-optical spectroscopy software, Version 1.71.6.1, 2013, http://www.effemm2.de/spekwin/
Oberg KA, Ruysschaert JM, Goormaghtigh E (2004) The optimization of protein secondary structure determination with infrared and circular dichroism spectra. Eur J Biochem 271:2937–2948
Ribeiro da Luz B (2006) Attenuated total reflectance spectroscopy of plant leaves: a tool for ecological and botanical studies. New Phytol 172:305–318
Samuels AC, Snydera AP, St Amant D, Emge DK, Minter J, Campbell M, Tripathi A (2008) Classification of select category A and B bacteria by Fourier transform infrared spectroscopy. P SPIE 6954:695413-1
Santos C, Fraga ME, Kozakiewicz Z, Lima N (2010) Fourier transform infrared as a powerful technique for the identification and characterization of filamentous fungi and yeasts. Res Microbiol 161:168–175
Savic D, Jokovic J, Topisirovic L (2008) Multivariate statistical methods for discrimination of lactobacilli based on their FTIR spectra. Dairy Sci Technol 88:273–290
Singer TL (1998) Roles of hydrocarbons in the recognition systems of insects. Am Zool 38:394–405
Singh B, Gautam R, Kumar S, Kumar BNV, Nongthomba U, Nandi D, Mukherjee G, Santos V, Somasundaram K, Umapathy S (2012) Application of vibrational microspectroscopy to biology and medicine. Curr Sci India 102:232–244
Socrates G (2001) Infrared and Raman characteristic group frequencies: tables and charts. John Wiley
Srivastava AK, Iconomidou VA, Chryssikos GD, Gionis V, Kumara K, Hamodrakas SJ (2011) Secondary structure of chorion proteins of the Lepidoptera *Pericallia ricini* and *Ariadne merione* by ATR FT-IR and micro-Raman spectroscopy. Int J Biol Macromol 49:317–322
Torres CW, Brandt M, Tsutsui ND (2007) The role of cuticular hydrocarbons as chemical cues for nestmate recognition in the invasive Argentine ant (*Linepithema humile*). Insect Soc 54:363–373

Tyndale-Biscoe M (1984) Age-grading methods in adult insects: a review. Bull Entomol Res 74:341–377

Verdú JR (2011) Chill tolerance variability within and among populations in the dung beetle *Canthon humectus hidalgoensis* along an altitudinal gradient in the mexican semiarid high plateau. J Arid Environ 75:119–124

Verdú JR, Moreno CE, Sánchez-Rojas G, Numa C, Galante E, Halffter G (2007) Grazing promotes dung beetle diversity in the xeric landscape of a Mexican Biosphere Reserve. Biol Conserv 140:308–317

Vincent JFV, Wegst UGK (2004) Design and mechanical properties of insect cuticle. Arthropod Struct Dev 33:187–199

Whittaker RH (1952) A study of summer foliage insect communities in the Great Smoky Mountains. Ecol Monogr 22:1–44

Willis JH (2010) Structural cuticular proteins from arthropods: annotation, nomenclature, and sequence characteristics in the genomics era. Insect Biochem Mol Biol 40:189–204

Zhou G, Taylor G, Polle A (2011) FTIR-ATR-based prediction and modelling of lignin and energy contents reveals independent intra-specific variation of these traits in bioenergy poplars. Plant Method 7:9