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▶ **To cite this version:**

Kenzy I Peña-Carrillo, Chantal Poteaux, Chloé Leroy, Rubí N Meza-Lázaro, Jean-Paul Lachaud, et al.. Highly divergent cuticular hydrocarbon profiles in the cleptobiotic ants of the Ectatomma ruidum species complex. Chemoecology, In press, 10.1007/s00049-020-00334-0 . hal-03010578

HAL Id: hal-03010578
https://hal.science/hal-03010578

Submitted on 24 Nov 2020

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Highly divergent cuticular hydrocarbon profiles in the cleptobiotic ants of the *Ectatomma ruidum* species complex

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Abstract

In social insects, chemical communication is the main communication mode among colony members, which use the blends of cuticular hydrocarbons as recognition cues to discriminate between nestmates and non-nestmates and to prevent the exploitation of their nest resources by aliens. The aim of this study was to assess the variation of nestmate recognition cues in the ant *Ectatomma ruidum*, a species complex with a considerably conserved morphology and one of the few ant species where intraspecific thievery, a form of cleptoparasitism, has been reported. We analyzed the cuticular hydrocarbon profiles of ants collected from a number of geographically separated populations, and examined DNA sequence data to assess their species identity. We focused on one species of the complex, *E. ruidum* sp. 3-4, whose species delineation remains controversial. We documented that several quantitative and qualitative traits of the cuticular hydrocarbon profiles varied significantly between populations, indicating that this species harbors more cuticular chemical phenotypic diversity than expected within a single species. In particular, there was a striking divergence among populations in the proportion of methylalkanes, alkenes, alkadienes and odd-chain components, which likely play a major role in nestmate/non-nestmate discrimination, a process which might have been crucial in these cleptobiotic ants. Further investigations are needed to test the hypothesis that biotic pressures, such as the need to discriminate conspecific intruders and limit thievery, could have played an important role in promoting the evolutionary divergence between populations in this ant species complex.

Keywords: biotic interactions, chemical divergence, nestmate recognition cues, species complex, thievery.

Introduction

Recognition of group members has favored the evolution and maintenance of sociality (Hamilton 1987; d'Ettorre and Lenoir 2010). Albeit social insects communicate in different forms, chemical communication is the main communication mode among colony members, allowing them to exchange different kinds of information (Wyatt 2014; Leonhardt et al. 2016). Among the different contents that a chemical message can encode, social insects use the blend of hydrocarbons they bear on their cuticle as a chemical signature that conveys information about castes, task specialization and reproductive status (Blomquist and Bagnères 2010). Cuticular hydrocarbons also allow social insects to discriminate between nestmates and non-nestmates, and prevent exploitation of nest resources by aliens (van Zweden and d'Ettorre 2010; Leonhardt et al. 2016). Indeed, when social insects encounter another individual, the presence of sufficiently large differences between their cuticular hydrocarbon profiles can trigger aggressive behavioral responses and allows colony residents to reject intruders (Dani et al. 2001; Martin et al. 2008; van Zweden and d'Ettorre 2010).

In insects, cuticular hydrocarbon mixtures are usually composed of different hydrocarbon classes, such as linear alkanes (*n*-alkanes), alkanes with one or more methyl branches (mono-, di-, tri-, tetra-methylalkanes) and hydrocarbons with one or two unsaturations, alkenes and alkadienes (Blomquist and Bagnères 2010). Usually, quantitative variations of the same set of hydrocarbons discriminate between colonies (intraspecific differences), whereas different species have qualitatively different profiles, even among closely related taxa (Hölldobler and Michener 1980; van Zweden and d'Ettorre 2010). Cuticular hydrocarbons are typically species-specific and qualitatively stable across the entire species distribution range (Lockey and Metcalfe 1988; Drijfhout et al. 2009; Guillem et al. 2016). Consequently, they are considered as useful taxonomic tools for species delimitation (Bagnères and Wicker-Thomas 2010; Kather and Martin 2012). For instance, cuticular hydrocarbons allowed to separate *Myrmica sabuleti* from *M. scabrinodis*, two species that are morphologically extremely similar but differ chemically in the composition of their cuticular layers, in particular in the presence of specific methylalkanes, which thus can serve as accurate diagnostic characters (Guillem et al. 2012). Studies focused on species such as *Formica japonica* (Akino et al. 2002), *Neoponera* (=*Pachycondyla) villosa* (Lucas et al. 2002), *Tetramorium caespitum*/*impurum* complex (Schlick-Steiner et al. 2006), *Crematogaster levior* and *Camponotus femoratus* (Hartke et al. 2019) are other examples of ant taxa with confirmed cryptic diversity that was discovered after examining their cuticular hydrocarbon profiles. Cuticular hydrocarbon analyses have also recently highlighted that sympatric, cryptic species exist in *Cr. levior* and *Ca. femoratus*, two neotropical ant species that exhibit parabiotic association, a lifestyle which might have promoted chemical divergence (Hartke et al. 2019).

Ectatomma ruidum Roger is a mainly Neotropical, widely distributed ant taxon that is likely to include a cryptic species diversity, which only has been recently started to be unveiled. Two recent studies based on mitochondrial (mt) and nuclear DNA sequence data and morphological evidence revealed that this taxon actually represents a species complex (Aguilar-Velasco et al. 2016; Meza-Lázaro et al. 2018), though its species delineation still remains unclear. In the first of these studies, morphological data indicated the existence of four morphospecies, two of which apparently are widespread along the Neotropics (*E. ruidum* spp. 1 and 2), whereas the remaining two species (*E. ruidum* spp. 3 and 4) and a presumably hybrid population (*E. ruidum* sp. 2 x 3) are restricted to few localities in Oaxaca, southeast

Mexico. These results became more complicated with the subsequent discovery of extensive heteroplasmy (i.e. presence of multiple mtDNA haplotypes in a single organism; Magnacca and Brown 2010) in specimens of the morphospecies *E. ruidum* spp. 3, 4 and 2x3 which might represent a single species whose populations share heteroplasmy (Meza-Lázaro et al. 2018).

E. ruidum, as currently known, is unique in a behavioral trait named intraspecific cleptobiosis. Besides the "normal" foraging behavior, some colony members display an alternative, parasitic, foraging strategy that consists of visiting conspecific colonies to take food from inside the nest and bringing it back to their home colony (Breed et al. 1990; De Carli et al. 1998). During foraging, thief ants exhibit a peculiar behavior: unlike other foragers, during their travel home they do not always follow a narrow path to their colony, and often hide (Jandt et al. 2015). Thieves also differ from other foragers in their cuticular chemical profile, which is significantly poorer in hydrocarbons (Jeral et al. 1997), and have been proposed as a distinct caste of foragers (McGlynn et al. 2015). This behavior has been reported in different locations along the distribution range of *E. ruidum* (De Carli et al. 1998, Breed et al. 1999), but its relation with the taxonomic complexity of the species remains unclear.

The intriguing complexity for species identification within the *E. ruidum* complex, coupled with its cleptobiotic behavior make it an ideal model system to study variation in recognition cues, as parasitic relationships have been identified as potential evolutionary drivers of polymorphism in these cues (Crozier 1986). These results might also contribute to future integrative taxonomy approaches where data from multiple sources (i.e. morphology, genetic analyses, ecological niche modeling, chemical analyses, etc.) are useful to identify or define species boundaries (Heethoff et al. 2011; Steiner et al. 2018). Here we compared the cuticular hydrocarbon profiles within the *E. ruidum* complex. Specifically, we tested for the intraspecific variation of the cuticular hydrocarbon profiles between populations of *E. ruidum* sp. 3-4 from southeast Mexico, whose boundaries remain controversial (Aguilar-Velasco et al. 2016; Meza-Lázaro et al. 2018).

Material and methods

Study model

Ectatomma ruidum is an earth-dwelling ant that occurs in a wide range of habitats in Neotropics, including plantations and damp forests, from sea level up to 1600 m (McGlynn et al. 2015; Aguilar-Velasco et al. 2016). Typically, *E. ruidum* nests have a single entrance and contain between 50 and 200 individuals (Lachaud 1985) and their densities can be very high (up to 11200 nests per hectare, Schatz and Lachaud 2008). These ants have a very generalist diet and the foraging activity is mainly diurnal (Lachaud 1990). In general, colonies are monogynous but in some regions they are facultatively polygynous with size-dimorphic queens (macro and microgynes) (Lachaud et al. 1999).

Collection of ants

We collected *E. ruidum* colonies in the populations Cozoaltepec, Huaxpaltepec, Punto 3, Río Grande and Santo Domingo de Morelos along a transect of 150 km across the Pacific coastal zone in the state of Oaxaca, México, in order to increase the sampling area covered by Aguilar-Velasco et al. (2016) (populations Coyula, Mazunte, Piedras Negras, Puerto Escondido). We also collected ants in two additional populations, one 500 km far from Coyula, Oaxaca to the south Pacific coast in Cantón Leoncillos, Chiapas, Mexico, and the remaining one in Cali, Colombia (Fig. 1 and Table 1); the ants from Cali, Canton Leoncillos, Coyula, Mazunte, Piedras Negras and Puerto Escondido belong to the same sampling as that in Aguilar-Velasco et al. (2016) and Meza-Lázaro et al. (2018). Colonies from the same population were at least 2 m distant from each other. In Oaxaca and Chiapas, Mexico, the climate is hot and semi-humid to semi-dry with tropical deciduous and semi-deciduous forests (Rzedowski 2006). Ants in Mexico were collected in plantations or forest, whereas in Colombia, they were collected in the urban area.

Species identification

We identified the ants from Cozoaltepec, Huaxpaltepec, Punto 3, Río Grande, and Santo Domingo de Morelos based on the diagnostic morphological features of the species *E. ruidum* mentioned by Kugler and Brown's (1982) and Arias-Penna's (2008) keys to species of *Ectatomma*. For their molecular characterization, we sequenced the barcoding region, which is a fragment of the cytochrome oxidase I (COI) mtDNA gene. We also included in the molecular analyses previously published sequences of ants from Cali, Cantón Leoncillos, Coyula, Mazunte, Puerto Escondido and Piedras Negras (Aguilar-Velasco et al. 2016; Meza-Lázaro et al. 2018). All newly generated sequences were obtained following the laboratory procedures, primers and PCR programs mentioned by Aguilar-Velasco et al. (2016) (Table 1). The specimens that were sequenced in the current study were deposited in the National Collection of Insects (CNIN) at the National Autonomous University of Mexico (UNAM), Mexico, and the National Natural History Museum (MNHN) in Paris, France.

To confirm the taxonomical identification, we conducted a Maximum Likelihood (ML) analysis using the program RAxML version 8 (Stamatakis 2014) to reconstruct the phylogenetic relations among representative specimens (workers) of all the examined populations of the *E. ruidum* complex. We used the GTRCAT model of sequence evolution and partitions based on codon position, and bootstrap support values were generated with 1000 rapid bootstrap replicates. ML analyses were carried out in the CIPRES Science

Gateway v. 3.3 (Miller et al. 2010) portal. In case it was not possible to assign ants to any of the previously proposed morphospecies we used the putative species assignation of Meza-Lázaro et al. (2018) based on mitogenome information. In Meza-Lázaro et al. (2018), all the seven individuals of *E. ruidum* spp. 3 and 4 that were examined were heteroplasmic with highly divergent mitochondrial haplotypes (1.88 – 5.81 % polymorphic sites spread across their genomes).

Fig. 1 Map showing the examined populations of the *E. ruidum* species complex

Chemical analyses

A total of 130 foragers collected from the foraging area were used for the chemical analyses (five ants per colony, with the exception of Huaxpaltepec with nine ants per colony, and Santo Domingo de Morelos, with six ants in one of the colonies) (Table 1). Ants were killed by freezing them in individual tubes at -20 °C after their arrival to the laboratory. The extracts of their cuticular hydrocarbons were obtained by dipping each ant separately in 100 µl of pentane supplemented with an internal standard (C_{18} at 5ng/µl) for 20 min. The extracts were then dried and re-diluted in 20 µl of pentane before use.

We injected 2 µl of the extract in a 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with an HP-5MS capillary column (30 m × 250 µm × 0.25 µm) and a splitless injector, coupled to a 5975C Mass Spectrometer (Agilent Technologies) with 70 eV electron impact ionization. The carrier gas was helium at a constant flow of 1 ml/min. The temperature program was as follows: an initial hold at 70°C for 1 min, then 70–200°C at 30°C/min, then 200–300°C at 3°C/min, then 300–320°C at 20°C/min, then hold at 320°C for 3 min. Peaks areas were integrated with the MSD ChemStation software E.02.01.1177 (Agilent Technologies).

Hydrocarbons were identified based on diagnostic ions and mass spectra and compared with entries of the mass spectral library in NIST 2014 to known standards. We calculated relative abundances of hydrocarbons as ion count proportions with respect to total ion-count in the chromatogram. Thirty-one peaks were not identified because at trace levels. These unknown hydrocarbons were used for the visual representation of proportions of different classes of cuticular hydrocarbons, but were excluded from the statistical analyses.

Statistical analyses

In general, the chemical profiles of the ants of the *E. ruidum* species complex consisted of large numbers of very small peaks between 23 and 37 carbon atoms. Individuals from each population had both shared and population-specific hydrocarbons, which were absent in individuals from the other populations. The high number of hydrocarbons (i.e., variables) and the high number of zeros in the whole dataset of the relative abundances (population-specific hydrocarbons absent from most populations) made most statistical analyses inappropriate. Moreover, both qualitative and quantitative data are relevant when comparing the chemical profiles of different species, especially if information (e.g., colony identity) is mainly encoded in quantitative traits (Menzel et al. 2017a). Therefore, we built a simplified chemical profile of each ant which on one side reduced the number of variables for the analyses, and on the other included quantitative compositional data. For this, we grouped the hydrocarbons with the same carbon-chain length (i.e., same number of carbon atoms) by class as described by Elmes et al. (2002). For each chain length between 23 and 37 carbon atoms, we calculated the total percentage of *n*-alkanes, alkenes, alkadienes, methylalkanes and dimethylalkanes. The simplified chemical profiles contained 53 variables with a maximum of 5 variables per chain length (e.g., for the chain length of 25 carbon atoms, we calculated the percentage of *n*-C25, C25:1, C25:2, methyl-C25, dimethyl-C25 and so on for any chain length). We used the simplified chemical profiles for all statistical and exploratory analyses.

We made detailed statistical analyses of the *E. ruidum* sp. 3-4 from Mazunte, Puerto Escondido and Piedras Negras (Oaxaca, n = 40 ants, with at least two colonies per

population). In order to investigate the amount of chemical variation among these ants we analyzed nine different traits in their cuticular chemical profiles: 1-2) NMDS scores (axis 1 and 2); 3) median chain length; 4-8) proportion of hydrocarbon classes and 9) overall proportion of hydrocarbons with an odd number of carbon atoms.

The nine traits were calculated as follows.

The NMDS scores were obtained by performing a non-parametric, descriptive analysis running Nonmetric Multidimensional Scaling (NMDS). We ran a NMDS whose scores were used to perform a further analysis (see below). In this analysis, we used the Bray-Curtis distance measures of the relative abundance of the (untransformed) simplified ant chemical profiles. We calculated the median chain length by computing the percentage of hydrocarbons for each chain length and we identified the median chain length as the lowest chain length where the cumulative sum of percentages was ≥ 50% (Menzel and Schmitt 2012; Menzel et al. 2017a; Menzel F. pers. comm.). We also determined the proportion of odd-chain (vs even) hydrocarbons in the chemical profile. Finally, we summarized the variation in the chemical profile of *E. ruidum* sp. 3-4 by entering the following traits in a Principal Component Analysis (PCA, variables standardized via correlation matrix, Varimax rotation): NMDS scores for axes 1 and 2, median chain length, proportion of the five hydrocarbon classes and overall proportion of odd-chain hydrocarbons.

We tested for differences in each chemical trait separately and in the PCs summarizing the *E. ruidum* sp. 3-4 ant chemical variation by running Generalized Linear Mixed Models (GLMMs) for normally distributed data (identity link), where traits were the response variables, population was the fixed factor and colony the random factor to account for the non-independence of data from ants of the same origin.

We performed a second NMDS on the complete dataset (n = 130 ants from the 11 populations) to explore the amount of chemical variation within the whole *E. ruidum* species complex (Bray-Curtis distance measures of the relative abundance of the simplified cuticular profiles). The small number of colonies sampled per population did not allow for statistical analyses on this data set.

All statistical analyses were conducted with the software IBM SPSS version 23. The NMDS was performed in PAST (Paleontological Statistics, version 3.25) (Hammer et al. 2001). If not stated otherwise, descriptive values are given as mean ± standard errors.

Results

Species assignation

The phylogram derived from the ML analysis is given in Supplementary Material 1. Based on the clades that were recovered and on our morphological examination, we could not assign ant workers from Cozoaltepec, Punto 3 and Río Grande neither to *E. ruidum* sp. 3 nor sp.4. We therefore consider all populations from Oaxaca belonging to *E. ruidum* sp. 3-4 as a single putative species. Ants from Santo Domingo de Morelos were assigned to *E. ruidum* sp. 2. We could not assign the ants from Huaxpaltepec to any of the previously proposed putative species based on molecular data and thus we provisionally considered them as E. *ruidum* sp. 5. For the rest of populations, species identification is available in Table 1.

Table 1 Collection sites of the *Ectatomma ruidum* ants.

Species		Locality	State, Country	Coordinates	Number of workers used in chemical analysis (number of colonies, sampling**)	Gen Bank accession number	References
1		Cantón Leoncillos	Chiapas, Mexico	14°45'59.00"N/ 92°24'12.00"W	20 (4, original sampling)	KU570636-39	Aguilar-Velasco et al. 2016
2		Cali	Cali, Colombia	3° 22’ 39"N/ 76°31’52”W	10 (2, original sampling)	MG870224	Meza-Lázaro et al. 2018
		Coyula	Oaxaca, Mexico	15°45'2.85"N/ 96°17'51.05"W	10 (2, original sampling)	MN957982-85	Aguilar-Velasco et al. 2016
		Santo Domingo de Morelos	Oaxaca, Mexico	15°50'34.00"N/ 96°40'11.00"W	11 (2, new sampling)	MN848418-19	This paper
3-4	3*	Puerto Escondido	Oaxaca, Mexico	15°56'24.00"N / 97° 4'15.60"W	20 (4, original sampling)	KU570668	Meza-Lázaro et al. 2018/ *Aguilar-Velasco et al. 2016
	4*	Mazunte	Oaxaca, Mexico	15°41'7.44"N/ 96°33'42.48"W	10 (2, original sampling)	KU570662-64	Meza-Lázaro et al. 2018/ *Aguilar-Velasco et al. 2016
		Piedras Negras	Oaxaca, Mexico	15°43'30.00"N/ 96°39'36.00"W	10 (2, original sampling)	MG870246	
		Cozoaltepec	Oaxaca, Mexico	15°45'44.80"N/ 96°42'41.01"W	10 (1, new sampling)	MN848420-21	This paper
		Punto 3	Oaxaca, Mexico	15°57'24.84"N/ 97°11'46.32"W	10 (1, new sampling)	MN848417	This paper
		Río Grande	Oaxaca, Mexico	16° 1'8.76"N/ 97°26'26.16"W	10 (1, new sampling)	MN848415-16	This paper
5		Huaxpaltepec	Oaxaca, Mexico	16°20'26.00"N/ 97°57'34.00"W	9 (1, new sampling)	MN848413-14	This paper

**Sampling: original sampling means that the ants used for the analyses of the cuticular hydrocarbons were studied by Aguilar-Velasco et al. 2016 and or Meza-Lázaro et al. 2018; new sampling refers to the newly collected ants.

Chemical profile of the ants *E. ruidum* sp. 3-4 and median chain length

The chemical profiles of *E. ruidum* sp. 3-4 from Mazunte, Piedras Negras and Puerto Escondido comprised 75, 74 and 61 peaks, respectively, and they were separated in the NMDS plot (Fig. 2). Ants from Piedras Negras had variable and mainly negative values in NMDS axis 1, while those from Puerto Escondido only had positive values. While the ants from Mazunte had intermediate values in NMDS axis 1, they were fairly well differentiated from the others in the NMDS axis 2 (Fig. 2).

The median chain length of the cuticular hydrocarbons varied from 26 to 30 carbon atoms.

Fig. 2 Two-dimensional Nonmetric Multidimensional Scaling ordination of the chemical profiles of *E. ruidum* sp. 3-4

Variation in the classes of hydrocarbons within *E. ruidum* sp. 3-4

The differences in the proportions of alkadienes, alkenes and methyl-branched alkanes were highly significant among the populations Mazunte, Puerto Escondido and Piedras Negras (alkadienes $F_{2,37} = 3.798$, $P = 0.032$; alkenes: $F_{2,37} = 21.628$, $P < 0.0001$; methyl-branched alkanes $F_{2,37} = 18.960$, $P < 0.0001$) (Fig. 3). In contrast, the proportions of linear alkanes and dimethyl branched alkanes did not differ significantly (linear alkanes $F_{2,37} = 1.308$, $P = 0.283$; dimethyl branched alkanes $F_{2,37} = 1.810$, $P = 0.178$).

All ants had chemical profiles extremely rich in odd-chain hydrocarbons (grand mean: 89.5% ± 0.3; Piedras Negras: 92.5 ± 0.5, Puerto Escondido: 89.5 ± 0.2, and Mazunte: 86.8 ± 0.6), but their proportions significantly differed by population ($F_{2,37} = 30.166$, $P < 0.0001$) (Fig. 3). There were significant differences between populations in the proportions of odd-chain methylalkanes ($F_{2,37} = 18.960$, $P < 0.000$), alkenes ($F_{2,37} = 21.628$, $P < 0.000$), alkadienes ($F_{2,37} = 3.798$, $P = 0.032$). There were no significant differences in the proportions of odd-chain dimethylalkanes ($F_{2,37} = 1.810$, $P = 0.178$) and linear alkanes ($F_{2,37} = 1.308$, $P = 0.283$).

Fig. 3 Mean proportions of: a) cuticular hydrocarbons and b) odd-chain hydrocarbons of ants *E. ruidum* sp. 3-4 by class

PCA on the chemical traits within *E. ruidum* sp. 3-4

The variation of the chemical profiles of the ants assigned to *E. ruidum* sp. 3-4 was summarized in two principal components that together explained 76.7% of the total variance. PC1 values (56.6 % variance explained) discriminated among the three populations (GLMM, $F_{2,37} = 10.649$, $P < 0.0001$). At one extreme of PC1 variation, the ants from Piedras Negras presented the highest proportions of alkenes and alkadienes (Table 2), whereas on the other extreme, ants from Puerto Escondido and Mazunte presented the highest values of NMDS axis 1 and the highest proportions of methylalkanes and dimethylalkanes (Table 2). With respect to PC2 (20.1 % variance explained), the ant profiles significantly differed by population ($F_{2,37} = 29.032$, $P < 0.0001$), with those from Mazunte having higher NMDS axis 2 scores and larger proportion of *n*-alkanes than those from Piedras Negras and Puerto Escondido (Table 2, Fig. 4).

Table. 2 Scores of the PCA factor loadings on the nine chemical traits (in bold only r > 0.700). The peaks are sorted by loading size. High loadings indicated that the trait was highly correlated with the PC.

Rotated Component Matrix	Principal Component	
	1	2
NMDS axis1 scores	**0.971**	-0.071
Proportion of methylalkanes	**0.962**	-0.102
Proportion of alkenes	**- 0.961**	-0.126
Proportion of alkadienes	**- 0.827**	-0.179
Proportion of dimethylalkanes	**0.808**	-0.058
Median chain length	0.654	0.456
NMDS axis 2 scores	-0.068	**0.840**
Proportion of *n*-alkanes	-0.214	**0.705**
Proportion of odd chain hydrocarbons	-0.589	-0.690

Fig. 4 The PCA's plot of the nine traits describing the cuticular hydrocarbon profiles of ants of *E. ruidum* sp. 3-4

Exploratory analysis of the chemical profiles in the *E. ruidum* species complex

The chemical profiles of the ants of *E. ruidum* sp. 3-4 had 64 peaks in Cozoaltepec and 58 in Punto 3 and Rio Grande. Ants of *E. ruidum* sp. 2 from Cali, Colombia, and Coyula and Santo Domingo de Morelos, Mexico, had 53, 50 and 42 hydrocarbon peaks, respectively, whereas the ones assigned to *E. ruidum* sp. 1 from Cantón Leoncillos, had 46 peaks. There were 55 peaks in the chemical profiles of the ants *E. ruidum* sp. 5 from Huaxpaltepec. Overall, we identified 12 alkadienes, 23 alkenes, 10 *n*-alkanes, 37 methylalkanes, 50 dimethylalkanes (Supplementary material 2). Including 31 peaks that could not be identified (unknown hydrocarbons), we had a total of 163 hydrocarbon peaks in the whole data set of *E. ruidum*.

The NMDS analysis performed on the simplified chemical profiles of the whole data set suggested that extensive variation might exist both within and between the putative species (*E. ruidum* sp. 1, sp. 2, sp. 3-4 and sp. 5) that have been proposed for the complex (Fig. 5), though no statistical analysis could be done due to the limited sample size. With respect to *E. ruidum* sp. 3-4, ants from Río Grande and Cozoaltepec appear as chemically distant from those of Mazunte, Puerto Escondido, Piedras Negras and Punto 3 (high negative values in NMDS axis 2). Within the *E. ruidum* sp. 2 samples, those from Santo Domingo de Morelos may differ from those from Coyula and Cali on the NMDS axis 1, while ants from Cali may differ from the ants of Santo Domingo de Morelos and Coyula on the NMDS axis 2.

Fig. 5 Two-dimensional Nonmetric Multidimensional Scaling ordination of the chemical profiles of the *E. ruidum* species complex

Discussion

The current results show striking divergences in quantitative and qualitative components in the chemical profiles of *E. ruidum* sp. 3-4 ant populations. Such chemical divergence largely exceeds the current taxonomic delimitation based on molecular data (Meza-Lázaro et al. 2018), indicates high intraspecific diversity within *E. ruidum* sp. 3-4 and highlights the need for integrative taxonomic studies. The chemical variation observed among populations of *E. ruidum* sp. 3-4 was particularly evident in traits involving branched and unsaturated hydrocarbons.

Within species, differences in chemical profiles are typically correlated to geographical distances. For example, distant populations (> 300 km) of the social wasp *Polistes biglumis* have more divergent chemical profiles than close ones (a few tens of km), although differences were typically quantitative (Bonelli et al. 2015). Our results, however, suggest that the divergence in the chemical profiles of *E. ruidum* sp. 3-4 might not be fully explained by geographical distance. The ants from Mazunte and Piedras Negras inhabit close geographic areas (< 10 km of distance), and were considered as a single species based on mtDNA genome data (Meza-Lázaro et al. 2018), but differences in their chemical profiles exceeds the variation usually found at the intraspecific level. Therefore, factors other than geographical distance are likely to be the drivers of chemical divergence, although conclusive evidence will need extensive population sampling and focused experiments.

Cuticular hydrocarbons are typically species-specific (Lockey and Metcalfe 1988; Drijfhout et al. 2009; Guillem et al. 2016). The statistically different chemical profiles within the ants from the three populations of *E. ruidum* sp. 3-4 therefore support the existence of cryptic diversity in the complex. Similar examples of several chemotypes identified within single putative species were found in eusocial insects as the orchid bees of the genus *Euglossa* (Pokorny et al. 2014), as well as the *Formica japonica* (Akino et al. 2002) and *Crematogaster levior* and *Camponotus femoratus* (Hartke et al. 2019) ants, where variation in the quantities of branched hydrocarbons and unsaturated compounds unveiled hidden cryptic diversity. High intraspecific diversity was also found in the ant *Formica archboldi,* where different populations from Florida, USA, varied considerably in the abundance of methyl-branched cuticular compounds, matching the chemical profiles of different, sympatric species of *Odontomachus* ants more closely than those of conspecific populations (Smith 2019), but genetic distance among *F. archboldi* ant populations is unknown.

Overall, the cuticular traits related to branched alkanes, alkenes and alkadienes were the most variable in *E. ruidum* sp. 3-4 ants. These classes of compounds likely play a major role in the recognition processes in social insects, and especially in nestmate-non-nestmate discrimination (Dani et al. 2001; Châline et al. 2005; Lorenzi et al. 2011). For instance, Dani et al. (2001) showed that the supplementation of linear alkanes on alive social wasps of *Polistes dominulus* did not have aggression-eliciting effects on nestmates, whereas alkenes and methyl-branched alkanes did. Linear alkanes and alkenes had similar effects in *Formica exsecta* ants (Martin et al. 2008), where C25:1-supplemented ants were attacked by nestmates more than *n*-C_{25}-supplemented ones. Linear alkanes are likely to serve mainly as waterproofing functions (Wagner et al. 2001; d'Ettorre and Lenoir 2010; van Zweden and d'Ettorre 2010), even if not exclusively (e.g. Lorenzi et al. 2004). Although sampling from a larger number of colonies would have increased the statistical power of our analyses, our

current results on *E. ruidum* sp. 3-4 show that traits related to branched alkanes, alkenes and alkadienes distinguish the three different populations, whereas linear alkanes play a minor role. While *E. ruidum* sp. 3-4 ants have relatively similar external morphologies, they have highly diversified chemical recognition cues, and our preliminary analyses suggest that the same might be true for other taxa within the *E. ruidum* complex.

Environmental factors are known to affect the expression of cuticular hydrocarbons. For instance, diet changes in *Linepithema humile* and *Acromyrmex subterraneus subterraneus* ants altered chemical profiles and affected nestmate recognition (Buczkowski et al. 2005; Richard et al. 2004). On the other hand, in burying beetles (*Nicrophorus vespilloides*), differences in diet highlighted the importance of cuticular hydrocarbons as encoders of nutritional condition and other physiological states (Steiger et al. 2007). Also, abiotic factors such as air temperature and humidity affected the cuticular lipid layer of the fly *Drosophila melanogaster* (Rajpurohit et al. 2016). Although focused studies would be necessary to test the effect of abiotic factors on the cuticular chemical variation of *E. ruidum* sp. 3-4 ants, at present, there is no indication that the diet varies significantly among the populations under scrutiny. With respect to physical climatic parameters, our data suggest that linear alkanes (the likely most important waterproofing component of cuticular hydrocarbons) do not contribute significantly to the variation between populations. However, proportion of alkenes and methyl branched alkanes can also be affected by physical environmental conditions (Menzel et al. 2017b; Sprenger and Menzel 2020) making the link between function and hydrocarbon class less clear-cut.

Interspecific interactions have also been addressed as potentially relevant forces in the evolutionary change of cuticular hydrocarbons, both in quantitative and qualitative aspects. For example, living in association with other species (parabiosis) and not climatic factors may have promoted the evolution of chemical profiles characterized by longer hydrocarbons and higher proportions of branched alkenes and alkadienes among the ants of the genus *Camponotus* and *Crematogaster* (Menzel and Schmitt 2012; but see Sprenger et al. 2019). Similarly, the proportion of branched hydrocarbons was larger in two populations of *Polistes biglumis* social wasps that were infested by *Polistes atrimandibularis* social parasites than in a parasite-free population, while local climatic factors did not explain this divergence (Lorenzi et al. 2014). On a qualitative perspective, *Formica fusca and Temnothorax longispinosus* ant colonies from parasitized populations had higher cue diversity than non-parasitized ones (Martin et al. 2011; Jongepier and Foitzik 2016).

Ants of the *E. ruidum* complex discriminate nestmates from non-nestmates, although at different extents depending on geographic location (Breed et al. 1999). However, to our knowledge no study has tested which hydrocarbons (or which hydrocarbon classes) act as recognition cues. If branched alkanes, alkenes and alkadienes are the most important recognition cues in this ant complex, as it occurs in other social insects (van Zweden and d'Ettorre 2010), the need to distinguish between nestmates and non-nestmates may have been one of the most important pressures for the divergence of chemical cues among its species. Further studies and extended sampling are needed to test the link between local biotic and abiotic factors and CHC variation in this group.

The colonies of *E. ruidum* reach extremely high densities (Pratt 1989; Schatz and Lachaud 2008) and these ants exhibit intraspecific thievery, where specialized individuals consistently

"forage" by stealing food from neighboring colonies (Breed et al. 1990; De Carli et al. 1998; McGlynn et al. 2015). Although in some populations these ants have a relatively permissive non-nestmate acceptance threshold (Breed et al. 1990; De Carli et al. 1998; Jandt et al. 2015), high nest density and thievery might have promoted stricter acceptance thresholds in other populations. Intruder acceptance thresholds are usually plastic and are adjusted to local food availability (d'Ettorre et al. 2004; Jandt et al. 2015), as well as thievery frequency in *E. ruidum* ants (Guénard and McGlynn 2013).

E. ruidum species complex has a broad Neotropical distribution range but to our knowledge *E. ruidum* sp. 3-4 ants are found only in a relatively small geographic area in the state of Oaxaca. The distribution of the *E. ruidum* complex of closely related species (Aguilar et al. 2016 and Meza-Lazaro et al. 2018) might reflect a mosaic sympatry (*sensu* Mallet et al. 2009) and be the result of non-allopatric speciation events originating from polymorphism in recognition cues. Intraspecific parasitic interactions favor genetic divergence (e.g. Savolainen and Vepsäläinen 2003) and the maintenance of variation in recognition cues (Crozier 1986). *E. ruidum* ants exhibit cleptobiosis (Breed et al. 1990; De Carli et al. 1998), which is a form of parasitic interactions (Breed et al. 2012; Guénard and McGlynn 2013). For the foregoing reasons, it is suggestive to draw an evolutionary scenario where high colony density and high risk of colony intrusion by intraspecific thief ants (cleptobiosis) may have promoted polymorphism in recognition cues (via disruptive selection) and this in turn may have favored genetic divergence within the species complex. This hypothesis needs to be tested in future studies exploring the association between thievery, intruder acceptance threshold and variation in cuticular hydrocarbons in different populations to explore the role of recognition cues in the speciation process.

Acknowledgments

This study is part of the PhD project of KIPC supported by a CONACyT-French government scholarship and partially funded by a grant given by DGAPA UNAM (Proyecto PAPIIT no. IN201119) and CONACyT (Programa Ciencia de Frontera convocatoria 2019, project No. 58548) to AZR and by a grant given by the Ecos-Nord-CONACYT Program (proyecto no.M12A01) to AZR and CP. Thanks to Jovanna Jasso, Sian Gadelha, Jorge Guitérrez, Carlos Santamaria and Gabriela Pérez-Lachaud for their help during the field trips in Oaxaca, México. We also thank two anonymous referees for valuable feedback on previous versions of the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare no conflict of interest.

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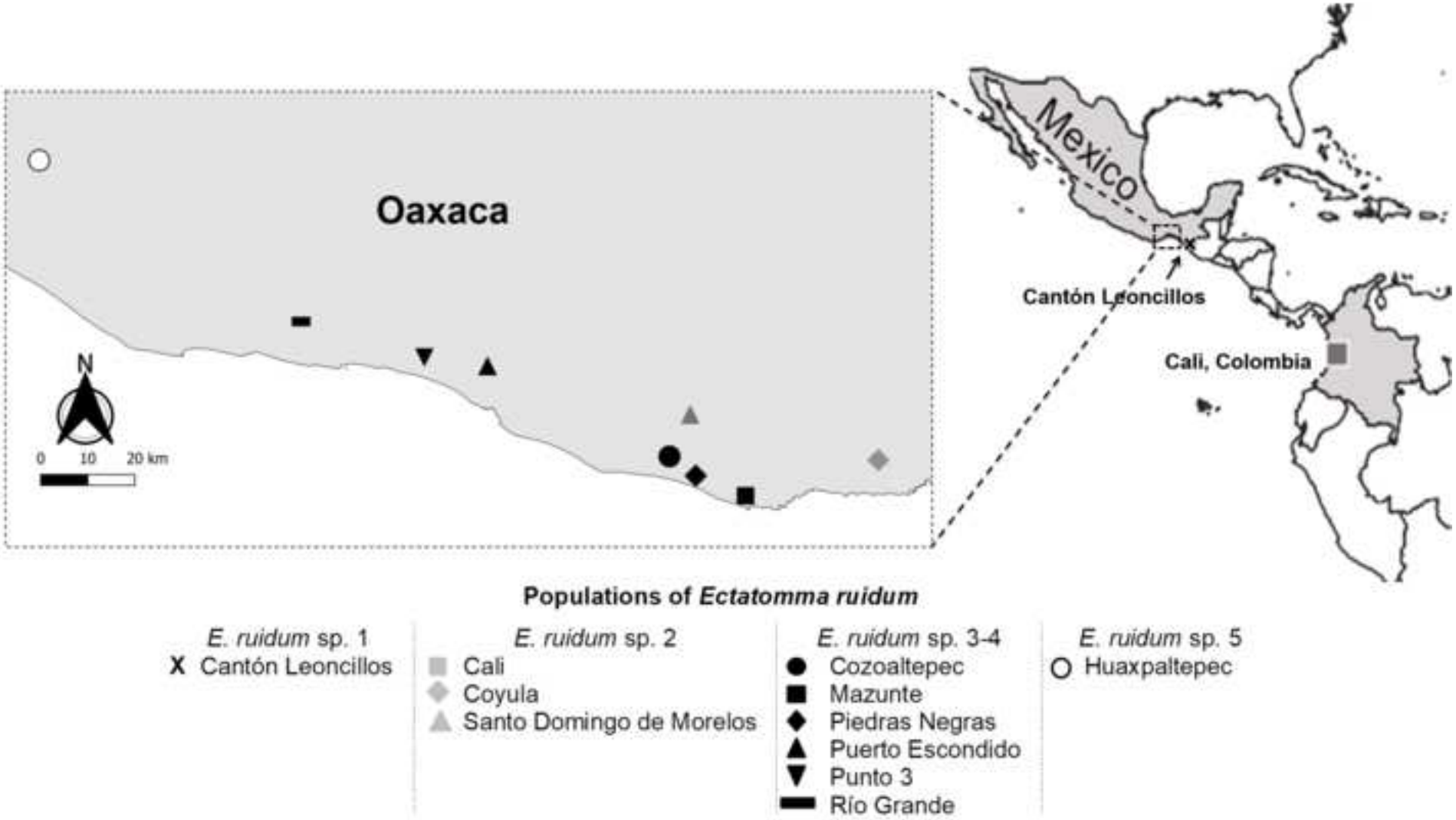
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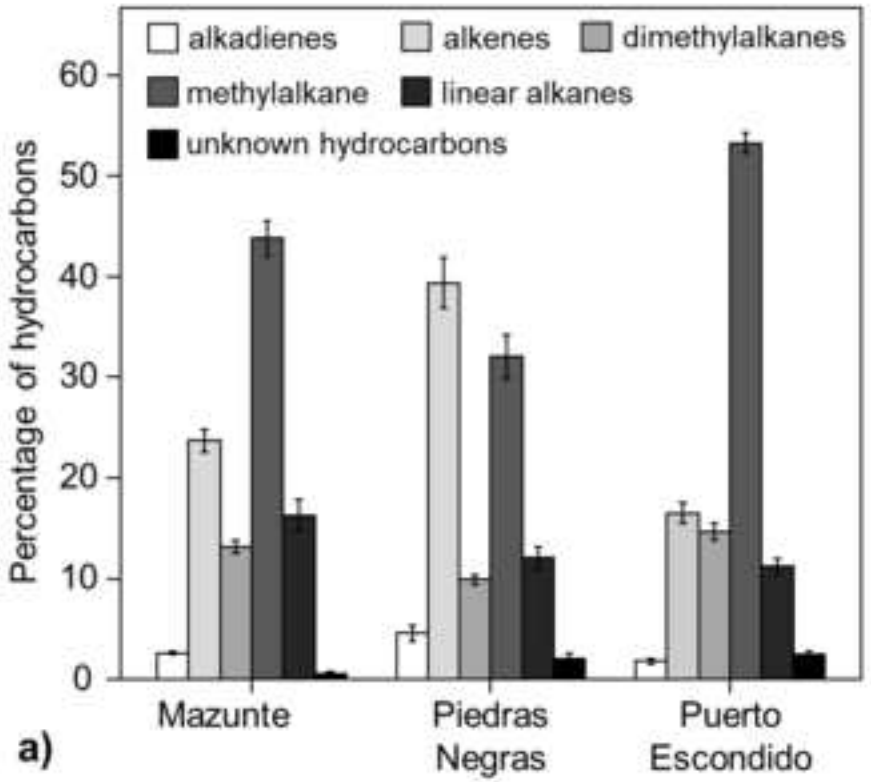
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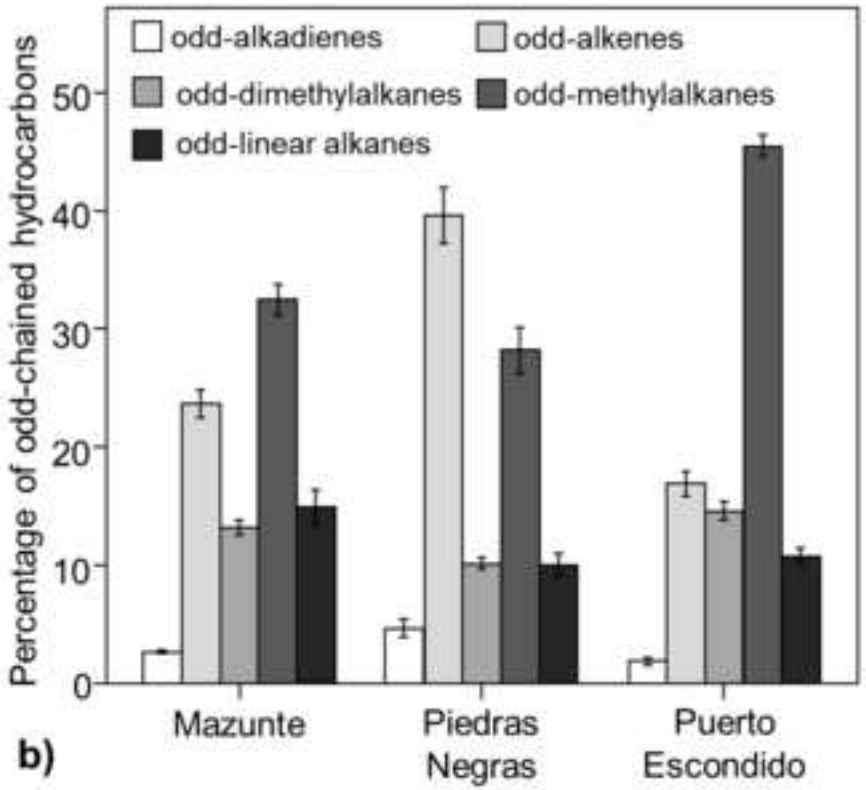
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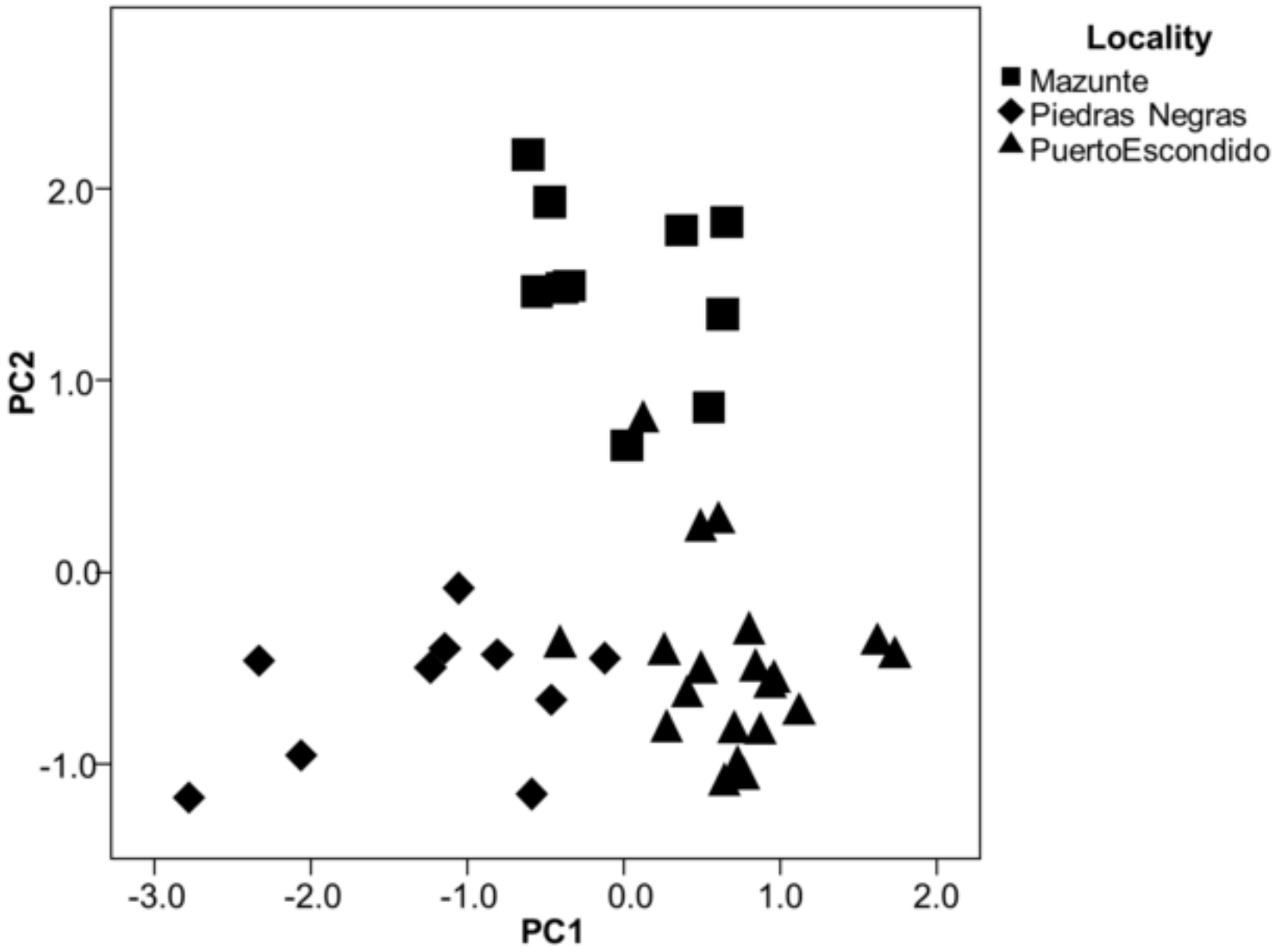
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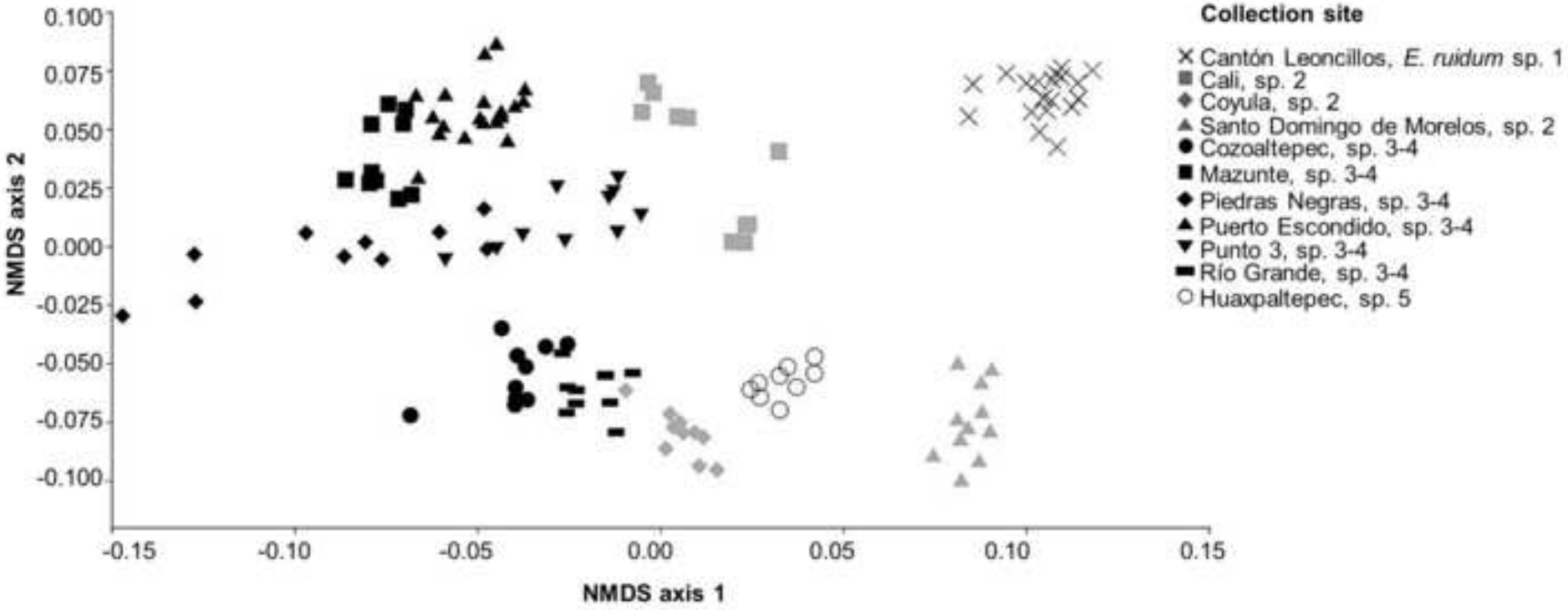
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Figure

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