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Titre :

THE ONGOING SPECIATION IN A NEOTROPICAL ANT SPECIES COMPLEX

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"It may be said that natural selection is daily and hourly scrutinising, throughout the world, every variation, even the slightest; rejecting that which is bad, preserving and adding up all that is good; silently and insensibly working, whenever and wherever opportunity offers, at the improvement of each organic being in relation to its organic and inorganic conditions of life."

Charles Darwin, 1859

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RÉSUMÉ

L'identification des espèces est essentielle pour bien décrire et comprendre la biodiversité. Certaines espèces ayant pu se diversifier sans changements dans leur morphologie, l'utilisation simultanée de différents outils taxonomiques est fortement recommandée pour les identifier. L'un des avantages de l'utilisation de ces différents outils est d'obtenir en même temps des informations sur les traits qui ont été impliqués dans la diversification des espèces. Dans cette étude, je me suis intéressée à la variation observée chez l'espèce *Ectatomma ruidum* comme étant à l'origine de différents taxons. *E. ruidum* est une fourmi largement répandue dans les régions néotropicales et des études antérieures, basées sur des séquences mitochondriales, ont proposé que l'espèce comprendrait au moins quatre taxons différents. La distribution géographique des différentes espèces potentielles montre que certaines d'entre elles sont limitées à de petites zones, sans aucune barrière géographique apparente séparant les populations, ce qui soulève la question des mécanismes évolutifs qui auraient entrainé leur divergence. En analysant des indices de reconnaissance, des signaux acoustiques ainsi que leurs paramètres morphologiques, et des séquences d'ADN (fragment du gène mitochondrial COI, et 3RAD et UCE), j'apporte des arguments soutenant la séparation de la plupart des espèces précédemment proposées. En outre, la combinaison des informations phénotypiques et génétiques a montré que les indices de reconnaissance ont pu jouer un rôle très important dans la diversification de ce complexe d'espèces. Dans l'ensemble, cette étude amène des preuves en faveur de l'utilisation d'une approche multi-traits pour la délimitation d'espèces génétiquement proches.

Mots clés: Fourmis, *Ectatomma ruidum*, hydrocarbones cuticulaires, signaux acoustiques, spéciation

ABSTRACT

To describe and understand biodiversity, the identification of species is essential. Because some species diversify without revealing any morphologic change, the use of different taxonomic tools is highly recommended. Among the advantages of employing different traits for species classification, one of the most remarkable is that at the same time we obtain information about which traits have been involved in the diversification of species. In this study I investigated the variation observed in the ant species *Ectatomma ruidum* as an evidence of different taxa. *E. ruidum* is a widely distributed ant from the Neotropics and in previous studies based on mitochondrial sequences the species was proposed to include at least four different taxa. The geographic distribution patterns of the putative species shows that some of them are restricted to small areas, without any apparent geographic barrier separating populations, which raised the question about which mechanisms separated them. By analyzing recognition cues, acoustic signals, morphological acoustic traits and DNA sequences (mitochondrial DNA COI gene, 3RAD and UCE) I provide evidence supporting the separation for most of the previously proposed species. Additionally, the combination of phenotypic and genetic information unveiled that recognition cues may have had a very important role in the diversification of the species complex. Overall, this study adds evidence in favor of the use of a multi trait approach for the delimitation of closely related species.

Keywords: Ants, *Ectatomma ruidum*, cuticular hydrocarbons, acoustic signals, speciation.

PREFACE

The chapters of this thesis constitute a set of one published article in a peer-reviewed journal, one article *in press* and another one in preparation.

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Peña-Carrillo KI, Lorenzi MC, Brault M, Devienne P, Lachaud JP, Pavan G, Poteaux C. A new putative species in the *Ectatomma ruidum* complex (Formicidae: Ectatomminae) produces a species-specific distress call. Submitted to Bioacoustics.

Meza-Lázaro RN, **Peña-Carrillo KI**, Poteaux C, Lorenzi MC, Bayona-Vásquez NJ, Branstetter MG, Zaldívar-Riverón A (in preparation). Species diversification of the Neotropical ant species complex *Ectatomma ruidum* (Ectatomminae) based on genome-wide and cuticular chemical cues.

Posters at meetings

Peña-Carrillo K.I., Poteaux C., Leroy C., Lachaud J.P, Zaldívar-Riverón A., Lorenzi M.C. Extreme divergence in cuticular hydrocarbons profiles in the species complex *Ectatomma ruidum* (Roger). Journées du GdR MediatEC 2019. Lille, France, Octobre 29-31th, 2019.

Peña-Carrillo K.I., Poteaux C., Leroy C., Lachaud J.P, Zaldívar-Riverón A., Lorenzi M.C. Extreme divergence in cuticular hydrocarbons profiles in the species complex *Ectatomma ruidum* (Roger). International Student Course in Behavioural Biology, organized by Institut Francilien d'Ethologie, IFE - Villetaneuse - Université Paris 13. September 19, 20st, 2019.

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Peña-Carrillo, K.I., Pavan G., Meza-Lazaro, R., Zaldívar-Riverón, A., Lachaud J.P., Lachaud, G.P., Poteaux, C. Use of acoustics for species delimitation in a Neotropical ant species. European Conference of Tropical Ecology, Paris, France. March 26-29th, 2018.

1.

Introduction

I.1. Species concept, speciation

The fundamental unit that all studies about evolutionary biology have in common is the species, and although a plethora of publications about how to define a species exists, a universal concept is still lacking (Butlin et al. 2012). Any change in a determined species concept will affect research fields as taxonomy, biogeography, ecology, biological conservation and macroevolution (Agapow et al. 2004). But, regardless these problems, when studying the process of speciation, evolutionary biologists need to keep in mind an objective criterion for species delimitation (Butlin et al. 2012).

Different concepts of species exist, i.e., *ecological species concept* which emphasizes on ecological differentiation; *evolutionary species concept* that refers to separately evolving and independent lineages; *phylogenetic species concept* which includes three distinct classes of species definitions associated with the taxonomic concepts derived from phylogenetic systematics or cladistics, and gives emphasis to cladogenesis and monophyly (de Queiroz 1998; de Queiroz 2007; Hong 2020), among others. With the aim to provide an alternative and overcome current difficulties about how to define species, de Queiroz (2007) proposed the *unified species concept* which equates species with separately evolving metapopulation lineages, where metapopulation refers to an inclusive population made up of connected subpopulations. Despite some scientist agree that populations are a relevant aspect of species, others consider they are not sufficient for a species concept, and new species definition are added, like, the gen-morph species concept recently proposed by Hong (2020), that define species of outbreeding organisms.

In the case of sexually reproductive eukaryotes, up to now the most widely adopted species definitions have been the *unified species concept* (de Queiroz 2007) and the *biological species concept*. The latter define species as groups of interbreeding natural populations that are reproductively isolated from other such groups (*sensus* Mayr 1996). According to this concept, speciation, the joint evolution of two or more groups (Coyne and Orr 2004), implies the evolution of reproductive isolating mechanisms that prevent gene exchange between newly arising taxa, in a process involving many complex mechanisms, including ecology, behavior and interactions between multi-locus genotypes (Turelli et al. 2001). In this thesis, the species definition I adopted is the *biological species concept*. Particularly, I agree with the view of Coyne and Orr (2004) about the *biological species concept*, for which species are characterized by substantial but not necessarily complete reproductive isolation. I will also adopt Coyne and Orr's (2004) view that speciation involves acquiring reproductive barriers, in a process that yields intermediate stages when species status is difficult to assign.

Among the diverse mechanisms driving the build-up reproductive isolation, the following ones have been identified: a) natural selection such as disruptive selection, directional selection, uniform directional selection (Lowry and Hopkins 2013); b) reproductive isolating barriers as premating, post-mating, prezygotic and postzygotic isolation barriers, reinforcement (Table 1); c) sexual selection through mechanisms like: *1. Sensory drive*, where particular features of communication signals are preferred by individuals because some aspects ot their sensory world promted their preferences, i.e. snapper fishes are adapted to perceive better the wavelength of light in deep waters than closely related species of snapper that live in shallow estuaries (Lowry and Hopkins 2013); *2. Good genes*, which means that individuals from a population carry alleles that confer them a higher fitness in comparison to those that do not carry them, i.e. genes to resist diseases (Lowry and Hopkins 2013); *3. Fisherian Runaway selection*, where males display exaggerated traits as a response to female preferences, even if the traits imply a high fitness cost, i.e., the peacock tail (Lowry and Hopkins 2013); *4. sexual conflict*, which occurs when the genetic interests of males and females diverge, leading to distinct roles, but traits favored in one sex can be costly to the other (Chapman et al. 2003), i.e., male water striders benefit by mating with multiple females, but because females benefit the most by mating only once, females evolved traits to resist frequent mating; these sex differences generate continual evolutionary change; d) hybridization: adaptive introgression (when the fitness of a species (its gene pool) is increased by the incorporation of the gene pool from a foreigner species) or allopolyploidization may accelerate speciation (Abbot et al. 2013; Bugarella et al. 2019); e) genetic drift and chance events (Butlin et al. 2012): during divergence, drift and selection could work simultaneously and/ or interact (Templeton 2008; Sobel et al. 2010 in Sobel et al. 2010). Genetic drift can lead to shifts in allelic frequencies due to reduction of population size, resulting in fast divergence between populations. Also, speciation by founder effects is theoretically possible (Gavrilets and Hastings 1996), where colonization by very few individuals promote the evolution of new species by favoring rapid genetic changes (Moya et al. 1995), as happens in invasive species (Hedrick 2013).

One of the challenges for the biological species concept concerns *allopatric groups*. It is hard to know if two geographically distant (allopatric) groups would merge into a single species if they become sympatric (secondary contact) (Hendry 2009). However, because many allopatric populations exchange migrants (they are not completely isolated), their ability to interbreed with local individuals may help to solve their species status (Coyne and Orr 2004). The other challenge is *hybridization and introgression*; according to the view of Coyne and Orr (2004), a low frequency of gene exchange is not an issue because taxa showing unbridled gene exchange are rare. Other factors that faint hybridization as a big challenge for their view of the biological species concept are: hybridization resulting from human disturbance of habitats (not common under natural conditions), the possibility of hybridization as a transient phase of evolution (because intermediates will disappear after reproductive isolation is attained), as well as, the confusion of geographic variants with hybrids. Nonetheless, recent studies have shown that hybridization is more frequent, but according to Schumer et al. (2014) species will remain genetically differentiated due to hybrid incompatibilities even in species without complete reproductive isolation.

I.2. Species delimitation: integrative taxonomy

Species are framed by speciation events (Bock 2004), and for the process of speciation, properties as morphological distinguishability, reproductive isolation and monophyly remain the best lines of evidence to identify lineage divergence (de Queiroz 2005, 2007). But, the evolution of such properties does not have a sequential order and can arise at different times (de Queiroz 2005, 2007). The terms *species concept* and *species taxon* are sometimes confused (Bock 2004; Fiser et al. 2018), but while the former treats species as evolutionary units, the latter has a practical value, referring to an elementary unit differentiated by delimitation methods (Fiser et al. 2018). In other words, species taxa are recognized and delimited with descriptions that allow the identification of other individuals as members of the same *species taxon* (Bock 2004); species taxa should be purely considered as scientific hypotheses of separately evolving entities (Hey et al. 2003; de Queiroz 2007).

To describe and understand biodiversity, species taxa identification is a fundamental step (Schlick-Steiner et al. 2010), and the discipline in charge of the characterization, classification and naming of taxa (even if extinct) is *taxonomy* (Schlick-Steiner 2010; Padial et al. 2010). Traditionally, taxon identification has been based on morphological diagnoses (Jinbo et al. 2011), but, this morphology-based taxonomy presents some disadvantages, like: it only discriminates morphospecies (Dayrat 2005), only experts and trained personal are able to identify taxa (Jinbo et al. 2011), and in many cases speciation is not accompanied by morphological changes (Brickford et al. 2007). In the last century, the growing concern over threats to biodiversity fostered an emphasis on species delimitation and a desire to describe accurately as many species as possible before they disappear (Wiens 2007). To reach this goal, different tools have been proposed, among them DNA barcoding as it is a reliable, cost-effective and an easy molecular identification tool suitable for many metazoan taxa (Hebert and Gregory 2005). However, similarly to morphology based identification, taxonomy based on DNA face limitations; for example, when species taxa have very recent origins and still sharing alleles, it is difficult to differentiate them (Tautz et al. 2005).

To solve taxonomic limitations linked to the use of a single type of character (i.e. mainly morphological), integrative taxonomy was proposed as an approach combining data obtained from different disciplines to delimit species, i.e., phylogeography, population genetics, ecology, behavioral biology (Dyrat 2005; Will et al. 2005). According to Schlick-Steiner et al. (2010), at least three conclusive disciplines (defined arbitrary) should place all specimens into species in the same way, where the term "conclusive discipline" refers to a delimitation hypothesis resulted as significant, when more than one data set of a specific discipline was analyzed. Besides identification, the use of different disciplines could help scientists to understand evolutionary processes behind the taxa of study; also, integrative taxonomy provides more rigorous delimitation which results in better biodiversity inventories. For instance, the discovery of cryptic species has increased our appreciation of biodiversity.

Cryptic species are defined as two or more distinct species that were earlier classified as one because they were indistinguishable, at least morphologically (Brickford et al. 2007). They have been identified in most animal phyla (Pérez-Ponce de León and Poulin 2016; Pfenninger and

Schwenk 2007) and despite being quite common, they are poorly considered in testing ecological and evolutionary theories (Beheregaray and Caccone 2007). Because cryptic species lack of determinant morphological characters, to study their DNA variation has been a useful approach to uncover them (Jörger and Schrödl 2013). Different hypotheses have been involved to explain the origin of cryptic species, among them: a) recent divergence: cryptic species are expected to have diverged recently, therefore, morphological differentiation is not evident yet (Egea et al. 2016). Put differerently, if the morphological differentiation depends mainly on time since their divergence, its absence may indicate these taxa are relatively young (however, according to Fiser et al. 2018, recent divergence could explain only a fraction of cryptic species); b) phylogenetic niche conservatism: this hypothesis postulates that morphological differentiation and niche evolution are constrained by selection. This prompted some lineages to retain their ecological niches over time, as for instance, different species of whale lice (*Cyamus*) living on distinct parts of a whale's body (Kaliszewska et al. 2005); c) morphological convergence: morphological similarity evolved among distantly related species in response to similar selective pressures (Fiser et al. 2018), examples of this exist among amphipod cryptic species of the same complex that independently colonized freshwater-semiterrestrial, subterranean habitats (Yang and Li 2013; Villacorta et al. 2008). d) morphological stasis, when environmental conditions impose stabilizing selection on morphology, reducing or preventing morphological changes (Brickford et al. 2007).

Integrative taxonomy has successfully helped to describe new species and delimitate boundaries of cryptic species, as for example in ants of the *Tetramorium caespitum* complex (Wagner et al. 2017). However, according to Pante et al. (2015), despite the use of integrative taxonomy has increased since its formal introduction in 2005 (Dayrat 2005; Will et al. 2005), when new species are discovered, a high proportion of them is not systematically described and they remain unnamed. Contrary to the the barcode approach, integrative taxonomy did not accelerate the rate of species descriptions between the years 2006 and 2013 (Pante et al. 2015). Some of the reasons for this include: authors do not describe species in the article where the species were delimited; the pressure to write more articles leads to publish studies in different sets of journals instead of a single synthetic publication; taxonomists refuse to describe species without morphological differences, lack of experience to describe species, or due to the lack of comparisons with type materials, among others. In this study, I combined data from different sources such as genetic sequences and traits from the communication system of the model species (recognition cues, acoustic signals and morphometry of acoustic organ).

Table 1. Classification of reproductive isolating barriers. Modified from Coyne and Orr (2004).

I. Premating isolating barriers. Isolating barriers that impede gene-flow before transfer of sperm to members of other species.

a. Behavioral isolation (also called "ethological" or "sexual" isolation). Includes all differences that lead to a lack of cross-attraction between members of different species, preventing them from initiating courtship or copulation.

b. Ecological isolation. Isolating barriers based primarily on differences in species' ecology, i.e. barriers that are direct byproducts of adaptation to the local environment.
   1. Habitat isolation. Species have genetic or biological propensities to occupy different habitats when they occur in same general area, thus preventing or limiting gene exchange through spatial separation during the breeding season. This isolation can be caused by differential adaptation, differential preference, competition, or combination of these factors.
   2. Temporal (allochronic) isolation. Gene flow between sympatric taxa is impeded because they breed at different times.

c. Mechanical isolation. Inhibition of normal copulation or pollination between two species due to incompatibility of their reproductive structures. This incompatibility can result from lack of mechanical fit between male and female genitalia (structural isolation) or the failure if heterospecific genitalia to provide proper stimulation for mating (tactile isolation).

d. Mating system "isolation". The evolution of partial or complete self-fertilization (autogamy) or the asexual production of offspring (apomixis) that can result in the creation of a new taxon or set of lineages.

II. Postmating, prezygotic isolating barriers. Isolating barriers that act after sperm transfer but before fertilization.

a. Copulatory behavioral isolation. Behavior of an individual during copulation is insufficient to allow normal fertilization.

b. Gametic isolation. Transferred gametes cannot effect fertilization.
   1. Non competitive gametic isolation. Intrinsic problems with transfer, storage, or fertilization of heterospecific gametes in single fertilization between members of different species.
   2. Competitive gametic isolation (conspecific sperm preference). Heterospecific gametes are not properly transferred, stored, or used in fertilization only when competing with conspecific gametes.
      a. Postzygotic isolating barriers (hybrid sterility and inviability).
      b. Extrinsic. Postzygotic isolation depends in the environment, either biotic or abiotic.
      c. Ecological inviability. Hybrids develop normally but suffer lower viability because they cannot find an appropriate ecological niche.
      d. Behavioral sterility. Hybrids have normal gametogenesis but are less fertile than parental species because they cannot obtain mates. Most often, hybrids have intermediate phenotypes or courtship behaviors that make them unattractive.
      e. Intrinsic. Postzygotic isolation reflects a developmental problem in hybrids that is relatively independent of the environment.
      f. Hybrid inviability. Hybrids suffer developmental difficulties causing full or partial lethality.
      g. Hybrid sterility.
      h. Physiological sterility. Hybrids suffer problems in the development of the reproductive system or gametes.
      i. Behavioral sterility. Hybrids suffer neurological or physiological lesions that render them incapable of successful courtship.
      j. Hybryd breakdown. Inviability or sterility observed in hydrbids in the F2 generation of interspecific or intersubspecific crosses, while F1 hybrids are viable and fully fertile (Oka et al. 2004)

I.3. Communication and its role in speciation

Organisms can alter the behavior of another organism in its proximity in many ways, and two types of sensory interactions can be distinguished. When the perceiver responds to cues associated with another organism, the interaction is described as an *indicator system*, and when the perceiver responds to a signal, the interaction is described as *communication* (Ruxton and Schaefer 2011). In this respect, cues and signals can be differentiated by the response they trigger in animal sensory interactions. *Cues* are generated inadvertently or for purposes other than communication, they are unintentionally presented by an emitter and used by a receiver to infer information about the emitter (Bradbury and Vehrencamp 2011; Leonhardt et al. 2016). *Signals* are stimuli produced by a sender and monitored by a receiver; the aim of which is to provide information to another animal (Bradbury and Vehrencamp 2011).

Without any communication means, social behavior is hardly conceived, as it is the transmission of information without any link to social interactions. Therefore, communication is fundamental for social systems (Reznikova 2017; Luhman 1982), and its evolution is subjected to natural selection (Wiley 1983). Communication depends on the changes in the fitness of the sender and the receiver of a signal, that is, a change in the rate at which genes influencing an individual's actions spread in the population. An individual should produce and respond to signals that increase its fitness (Wiley 1983).

Chemical senses were probably among the first senses to have evolved and all life forms are able to perceive them (Wyatt 2014a). Communication signals evolve to influence the action of other individuals (Bradbury and Vehrencamp 2011), in the sender-precursor model of signal evolution (Bradbury and Vehrencamp 2011; Figure 1), signals evolved from molecules released by an "originator animal or emitter". Firstly, in the "ancestral" phase of the evolution, an individual acts as the receiver of the molecules (or single molecule) released by the emitter, but it lacks special adaptations to receive the molecule(s) beyond detection. Secondly, in the "perception" phase, there is a change in the receiver and an association between the molecule(s) and a condition of the emitter (chemical and release remaining unchanged) exists. In this phase the molecule(s) becomes a cue and receivers must be able to perceive or evolve receptors for the cue and gain benefits from being able to do so. Thirdly, a further step leading to bilateral benefits to both sender and receiver could occur if there is a selective advantage to the sender, which leads to the ritualization of the cue that is now transformed into a signal, to maximize information transfer. In this phase true communication occurs (Wyatt 2014b; Leonhardt 2016).

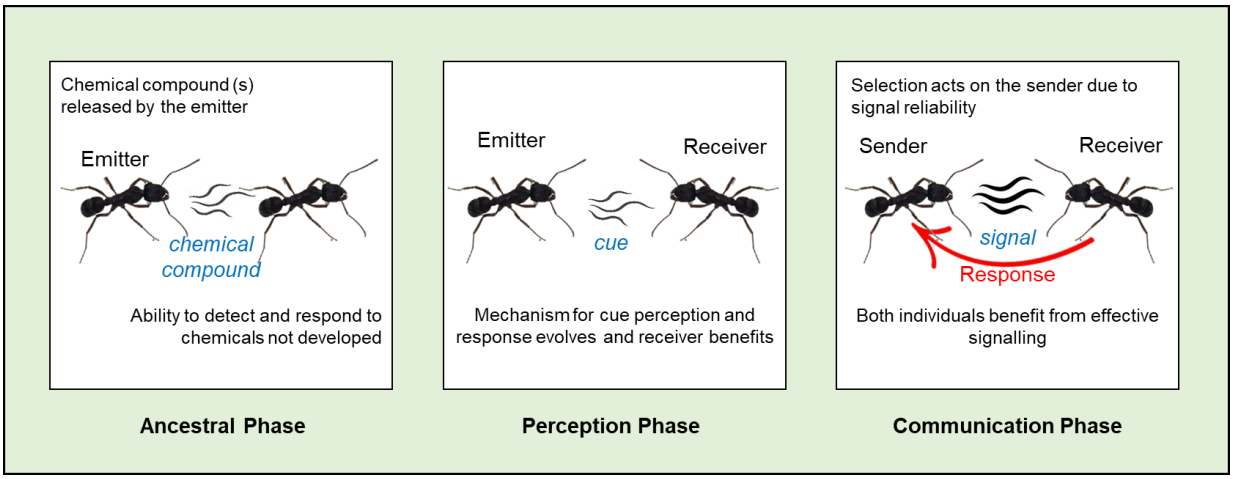


Figure 1. The sender-precursor model of signal evolution. In the ancestral phase an individual produces and emits chemicals that are not perceived by a receiver. Next, in the perception phase, a change in a receiver allows the individual to perceive the chemical cue and incorporate the information into a decision rule and a response; the receiver gets a fitness advantage. Finally, true communication occurs if the sender experiences a selective advantage that leads to the ritualization of the cue now transformed into a signal, maximizing information transfer and benefiting both individuals, leading to true communication. Adapted from Leonhardt et al. (2016).

In social insects, chemical communication is the main communication mode (Wyatt 2014b, Leonhardt et al. 2016). It has a significant role in the evolution of unicoloniality (Helanterä and Ratnieks 2009) and provides insects with information about important aspects for colony life, such as location of food resources (Farina et al. 2007), identity of colony members (van Zweden and d'Ettorre 2010) or health condition (Beani et al. 2019). Nonetheless, other modes of communication such as acoustic/ vibrational, visual and tactile ones exist in social insects and contribute to the complexity of their social life (Richard and Hunt 2013; Wyatt 2014b).

The most studied functions of communication signals in social insects are a) fertility signaling by chemical signals such as pheromones and visual signals (Hölldobler and Wilson 1990; Keller and Nonacs 1993; Tannure-Nascimento et al. 2008; Oi et al. 2015); b) nestmate recognition through chemical cues as cuticular hydrocarbons; c) recruitment to food sources by means of tactile, chemical signals such as pheromones or both (Leonhardt et al. 2016) and d) alarm signaling via chemical signals such as pheromones (Hölldobler and Wilson 1990; Verheggen et al. 2010), acoustic signals (Hölldobler 1999; Nehring et al. 2013) and substrate-borne vibrations (Virant-Doberlet and Cockl 2004).

Communication is also fundamental for reproduction. As pre-mating isolating mechanisms, signals are essential in mate recognition and thus play a vital role in the divergence of populations and their eventual reproductive isolation (Zuk and Tinghitella 2008); in other words, signals have a key role in speciation (West-Eberhard 1983). Signals to recognize siblings can also arise from adaptation to specific ecological niches or drift (Leal and Losos 2010). Because behavior is often an evolved and heritable trait, communication behavior should be adaptive and related species should perform communication tasks in similar ways (Bradbury

and Vehrencamp 2011). Taxonomic groups with high abilities to diversify their species-recognition signals are more likely to speciate and are probably those that diverged to the greatest extent (Leal and Losos 2010).

Cuticular hydrocarbons

Insects are covered with a thin epicuticular layer of wax that consists of lipids, including hydrocarbons, alcohols, fatty acids, waxes, glycerides, phospholipids and glycolipids (Gibbs and Crockett 1998). In general, hydrocarbons are universally present in the epicuticular lipid layer of insects (Martin and Drijhout 2009a; Blomquist and Bagnères 2010) and consist of organic molecules of carbon and hydrogen. They are produced by specialized cells (oenocytes) (Chung and Carrol, 2015) and those that are transferred to the cuticule are referred to as cuticular hydrocarbons (Kaib et al. 1991). All cuticular hydrocarbons have the same general structure and typically consist of long chains of carbon atoms (Blomquist and Bagnères 2010).

The three major classes of cuticular hydrocarbons are: *n*-alkanes (saturated molecules, linear alkanes), methyl-branched components (saturated) and olfeins (unsaturated) (Drijfhout et al. 2009; Blomquist 2010a). Linear alkanes are saturated carbons joined by single bonds (Drijfhout et al. 2009) whose chain range generally extends from 21 to 33 carbons; nonetheless, some studies showed that more than 70 carbon atoms can be found in the cuticular layers of some insects (Cvačka et al. 2007). In insects, the odd-numbered *n*-alkanes predominate due to their formation from mostly two carbon units followed by a decarboxylation (Blomquist 2010b). Olfeins have one (alkenes or monoenes), two (alkadiene or dienes) or three (alkatrienes or trienes) double bonds along the chain, and they exist in two isometric forms, the cis-form (Z-alkenes) and the trans-form (E-alkenes), whose structures appear to be important in determining functions (Drijfhout et al. 2009). In insects, difficulties in determining the position and stereochemistry of the double bonds of cuticular hydrocarbons have sometimes rendered their characterization challenging, especially when they are found as minor components. The third major class of saturated hydrocarbons corresponds to methyl-branched hydrocarbons. They have one or more methyl groups (CH_3) attached to one or more carbon atoms at the end or the middle of the chain; examples of methyl-branched alkanes can be monomethylalkanes, dimethylalkanes, tri and tetramethlyalkanes. Together with chain length, the presence and position of methyl groups and/or double bonds have a major impact on the physical properties of the compounds (Drijfhout et al. 2009) (see below).

Diverse studies indicate that oenocytes are the major site of hydrocarbon synthesis, including the three main classes, n-alkanes, methylalkanes and olfeins (Lokey 1988). Oenocytes are cells associated with the epidermal layer or the peripheral fat bodies beneath the epidermis, modified for lipid metabolism; their anatomic location varies among insect species and developmental stages (Lokey 1988; Bagnères and Blomquist 2010). Insects synthesize hydrocarbons by elongating fatty acyl-CoAs to produce long-chain fatty acids that are later converted into hydrocarbons by loss of their carboxyl group (Nelson and Blomquist 1995; Howard and Blomquist 2005). After their synthesis, hydrocarbons are shuttled via the hemolymph by lipophorin, but the mechanism of hydrocarbons up take to lipophorin, their deposition to epidermal cells and transit across cuticle to the surface or specific glands of insects have not

yet been completely elucidated (Bagnères and Blomquist 2010). The biosynthetic pathway of hydrocarbons involves the synthesis of medium-chain fatty acids by a fatty acid synthetase (FAS), their desaturation by desaturases followed by their elongation to very-long-chain fatty acids by elongases and their later decarboxylation to hydrocarbons (Wicker-Thomas and Chertemps 2010) mediated by cytochrome P450 (Chung and Carroll 2015). Key enzymes in the cuticular hydrocarbon variation are fatty acid synthases, elongases, desaturases, reductases and a P450 decarbonylase (Falcón et al. 2014).

Studies about the origin of cuticular hydrocarbons using *Drosophila* species as models of study, have shown that cuticular hydrocarbon production is controlled by genetic factors (Holze et al. 2020), and that different genetic mechanisms promote their convergent evolution (Ferveur 2005). A research work based on pharaoh ant *Monomorium pharaonis* also documented that they heritable and shaped by natural selection (Walsh et al. 2020). Moreover, several studies have started to unveil the contribution of a number of genes to variations in cuticular hydrocarbon composition (Holze et al. 2020). Insects generally synthesize most of the components of their cuticular hydrocarbon profiles (Blomquist and Bagnères 2010) but hydrocarbons from different environmental sources can be incorporated into their cuticular profiles, i.e., from diet and from resins used for nest building (Blomquist 2010b; Leonhardt et al. 2011, 2013). In comparison with solitary insects, social insects are expected to have a more complex cuticular hydrocarbon genetic architecture, because the individual odour is affected by that of the other colony members and depends on the collective genetic composition of the colony. That is, within-colony genetic variance exists (derived from variation between individuals, i.e., queen, workers), and it affects the phenotype of individuals in a colony (Linksvayer 2006, 2015). Cuticular hydrocarbons can be socially transferred to nestmates by trophallaxis (transfer of liquid food among individual), allogrooming (van Zweden and d'Ettorre 2010).

Cuticular hydrocarbons have a dual function as they serve both as protection from physical agents and as communication means (Blomquist and Bagnères 2010; Chung and Carroll 2015). They play a major role in protection against desiccation: they control the trans-cuticular flux of water on insects' cuticle by restricting the amount of water loss and preventing insects' dehydration (Drijfhout et al. 2009). The melting point of hydrocarbons increases with chain length, and by introducing a double bond into the molecule (i.e. alkenes) or a methyl group in the carbon chain (branched hydrocarbons), the boiling point and volatility of hydrocarbons are also affected. For example, chains of *n*-alkanes longer than 16 carbon atoms are wax-like solids at room temperature with a melting point of around 50°C. When they are mixed with alkenes and methyl-branched alkanes, their melting point decreases and allows insects to keep their cuticle flexible and regulate the permeability of their cuticle in wide range of ambient temperatures (Morgan 2004; Drijfhout et al. 2009; Gibbs and Rajpurohit 2010).

Besides waterproofing properties, cuticular hydrocarbons also act as a barrier against microbes (Howard and Blomquist 2005) and in social insects they encode information about tasks within the colony, information about castes, and are involved in advertising reproductive status (i.e., species-specific sex pheromones) and allowing nestmate recognition (Drijfhout et al. 2009; Blomquist and Bagnères 2010; van Zweden and d'Ettorre 2010). For instance, nestmate

recognition was tested by exposing captive colonies of harvester ants (*Pogonomyrmex barbatus*) to glass blocks covered with cuticular extracts from nestmate and non-nestmates workers. As a result, blocks coated with cuticular extracts from non-nestmates elicited higher levels of aggression than those coated with nestmates' cuticular extracts, thus demonstrating that harvester ants can use cuticular hydrocarbon composition for nestmate recognition (Wagner et al. 2000). Additionally, cuticular hydrocarbons in ant footprints' serve as indirect cues for taking decisions about their movement direction, i.e., in an experimental arena, foragers of *Lasius niger* avoided footprints of non-nestmate conspecifics that could represent potential unknown opponents (Würst and Menzel 2016).

Hydrocarbons like alkenes, monomethyl and dimethyl alkanes are expected to play an important role in chemical communication because they can be distinguished by the position of the double bond or methyl groups, while linear alkanes can only be distinguished based on their chain length (Walsh et al. 2020). Within colonies, cuticular hydrocarbons can encode information like, age, social environment and caste (Sprenger and Menzel 2020). For example, in the cuticular profile of *Formica exsecta* ants, the linear alkane part of the chemical profile was found to vary among individuals depending on task (forager vs non-forager) (Martin and Drijfhout 2009). In *Pogonomyrmex* ants, the behavior of scouts elicited foragers to wait for their return before starting harvesting seeds and tests showed that the behavioral response was triggered only by scout cuticular hydrocarbons (Greene and Gordon 2003). Between colonies, cuticular hydrocarbons information is important for nestmate/ non-nestmate discrimination and this role has been documented in many social insect taxa (van Zweden and d'Ettorre 2010; Sprenger and Menzel 2020). Branched alkanes and alkenes have been especially linked to this role (Dani et al. 2001; Châline et al. 2005; Lorenzi et al. 2011). While it is generally unclear which compounds, or which part of the profile encodes colony identity information (van Zweden & d'Ettorre 2010; Ferguson et al. 2020; Martin et al. 2008), (z)-9-alkenes may be directly involved in nestmate recognition in *Formica exsecta* (Martin and Drijfhout 2009).

Cuticular hydrocarbons represent discrete and constant characters that are heritable and shaped by natural selection (Drijfhout et al. 2009; Walsh et al. 2020). In addition, because typically the same species possess the same qualitative composition across populations (van Zweden and d'Ettorre 2010), cuticular hydrocarbons have been used as taxonomic tools (chemotaxonomy). For example, the study of cuticular hydrocarbons in the termites *Reticulitermes santonensis* and *R. flavipes* allowed researchers to suggest the synonymy between the two species and to identify the recent origin of the European populations from the North-American *R. flavipes* ones. This termite was introduced into Europe during commercial exchanges (presumably of wood) occurring between the two continents during the 19th century; in this case, the origin of colonization in France has been identified in the harbour of La Rochelle (Bagnères et al. 1990; Austin et al. 2005; Dronnet *et al*. 2005). As another example, differences in hydrocarbon profiles of honeybees enabled researchers to discriminate the aggressive Africanized bee *Apis mellifera scutellata* from the European honey bee *A. mellifera mellifera* (reviewed by Bagnères and Wicker-Thomas 2010).

After the introduction of molecular techniques in taxonomic studies, the number of publications based on chemical identifications has dropped (Bagnères and Wicker-Thomas 2010). However,

nowadays it is widely recognized that the best way to delimit species is by integrating different lines of evidence (Pante et al. 2015). In this view, cuticular hydrocarbons have been added to the taxonomy toolkit of scientists and combined with data from different sources (i.e., morphology, behavior, genetics, etc.); they have been employed to delimit closely related taxa such as ants of the *Cataghlyphis bicolor* group, *Cataglyphis niger* complex, *Crematogaser levior and Camponotus femoratus* (Eyer et al. 2017; Brodetzi et al. 2019; Hartke et al. 2019).

Acoustic signals

Besides chemical communication, which represents the main communication mode of insects, acoustic and vibrational signals are also employed to communicate (Bradbury and Vehrencamp 2011; Wyatt 2014).

Sound is a radiating disturbance in the density and pressure of a medium and it is propagated as waves. Graphically, waves are represented as wave-forms when in pressure-versus-time plots. The frequency of the sound measures how many times complete cycles of sound waves occur/repeat per second and its fundamental unit is expressed in Hertz (Hz). The most common way to characterize animal sound signals is with spectrograms (also named sonograms). Acoustic signals spread in air or water and sounds are attenuated or amplified by vegetation, physical barriers, acoustic impedance, but also by the morphology or posture of the animal that generates the sound (Greenfield 2016). Insects produce acoustic signals in different ways: a) by rubbing one body part against another, as in stridulations; b) by hitting some body part; c) by vibrating some body part; d) by vibrating drum-like membranes called tymbals; and e) by forcibly ejecting air or fluids.

In general, insects produce sounds according to daily periods (likely determined by light intensity, temperature or humidity) or when the presence or activities of other organisms promote it, i.e., alarm sounds or distress calls (Alexander 1957). Particularly, ants produce sounds in situations of alarm, recruitment, after the end of mating by females (Hölldobler and Wilson 1990) and when they dig soils (Pielström and Roces 2012). The most common sound produced by ants is the stridulation, which is produced by specialized structures consisting of a scraper and a plate of arrayed ridges (stridulatory file) typically located in the post-petiole and the first gastral segment (Stuart and Bell 1980; Hölldobler and Wilson 1990). Stridulations are produced by the dorso-ventral movement of the gaster of ants, which telescope the scrapper beneath the stridulatory file (Stuart and Bell 1980). According to Hikling and Brown (2000), ant stridulations are transmitted by the air, and their reception is thought to occur through their hairlike sensilla located on the antennae. Nonetheless, according to Roces and Tautz (2001) ants perceive stridulations as substrate-borne vibrational signals because they are unable to perceive airborne sounds.

Acoustic signals have the advantages that they can be detected at a distance; hence, costs associated with direct encounters are minor with respect to those that are direct. Also, because they encode information about the identity of the signaler, acoustic signals are suited to mediate discrimination between species (Wilkins et al. 2013). One of the best examples of species-specific sounds is represented by the *Laupala* crickets (Jacob and Hedwig 2019). In these crickets, sister species exist that often live in sympatric communities; they are morphologically

similar but differ in the temporal patterns of their songs (Shaw 1996, 2002). In solitary hymenopterans as velvet ants (Mutilidae), stridulations can vary at both the inter- and intra-specific level (Polidori et al. 2013). Indeed, Polidori and coworkers (2013) documented that different taxonomic groups of velvet ants exhibit species-specific patterns in both temporal parameters of their stridulation, and in the morphology of their stridulatory organ. Also, in closely related ant species of the *Neoponera apicalis* complex, species-specific variation was reflected in the stridulation produced by sympatric ants and in the morphology of their stridulatory organ (Ferreira et al. 2010). In such cases, stridulations represent potential taxonomic tools. In fact, differences in acoustic patterns of communication have been found to drive speciation in closey related species of insects (Henry 1994; Hoikkala et al. 1994; Mendelson and Shaw 2005). As an illustration, in the previously cited genus of crickets *Laupala*, the variation of temporal patterns of songs is closely linked to female signal preferences (i.e., sexual selection) (Shaw 2000; Jacob and Hedwig 2019).

I.4. The model system *Ectatomma ruidum* species complex

The genus Ectatomma *Smith*. The ants of the genus *Ectatomma* are one of the most common groups of foraging ants in the neotropics. This genus comprises 15 species whose habitats include wet forest, dry forest and savannah (Nettel et al. 2015). These ants are typically generalist predators of small arthropods and earthworms; additionally, they also collect honeydew from homopters and nectar from plant sources (Kugler and Brown 1982).

Biological aspects of *Ectatomma ruidum* (Roger)

Geographic distribution. *E. ruidum* is one of the most common species of its genus. It is distributed from the state of Tamaulipas in the Northeast of Mexico to at least the northern border of Brazil in the region of the "Tepuis", but it is not reported far inside the Amazonas Basin (Krugler and Brown 1982; Schatz et al. 1997; Meza-Lázaro et al. 2018). *E. ruidum* represents a complex of different putative species (Aguilar-Velasco et al. 2016), hence, along its large distribution, different putative species could be found (see below).

Habitat. These ants occur in damp rain-forests and in disturbed habitats (Schatz 1997) such as plantations of cocoa, coffee and maize (Lachaud 1990), as well as dry and semi-desertic zones, secondary forests and urban areas (Fernandez 1991) from sea level to altitudes between 1500-1600 m (Lachaud 1999).

Nest architecture and nest density. Nests of *E. ruidum* are hypogeal with one circular entrance with a diameter of about 3 mm; entrances are frequently masked by soil vegetation. From the entrance, a sloping tunnel connects the chambers situated at different levels, up to 2 m deep below the surface; sometimes nests can have two or three close entrances (Lachaud 1990; de Carli 1997) (**Figure 2**). The size of a mature colony varies between 50 and 200 ants and nest density varies from 360 to 11,200 nests/ hectare (Lachaud 1990; Lachaud et al. 1996). In cultivated habitats, they can reach really high nest density, up to 11500 nests/ha (Schatz and Lachaud 2008) leading to overlap of foraging areas and local competition. Because of the high nest density the species is regarded as a dominant one and has an important role of natural biological control agent in Neotropical countries (Perfecto and Sediles 1992; Lachaud et al. 1996).

Diet. E. ruidum has an omnivore diet that also includes plant remains (Breed 1990; Arias-Penna 2008) and during dry seasons *E. ruidum* have mainly predator and necrophagic behaviors, as it was reported in the region of the south of Mexico known as the Soconusco in Chiapas, Mexico (Lachaud 1996, **Figure 2**)

Foraging behavior. These ants are diurnal earth-dwelling (Lachaud et al. 1999); they forage individually during daylight but nocturnal foraging has also been reported during dry seasons (Lachaud 1990). *E. ruidum* foragers hunt solitary but, depending on prey size and weight, they can recruit other foragers in numbers which match the weight of the prey (Schatz et al. 1997). Also, when live prey are especially abundant, hunters are subdivided into two categories: stingers (specialized in killing prey) and transporters (specialized in carrying dead prey one by one to the nest) (Schatz et al. 1999).

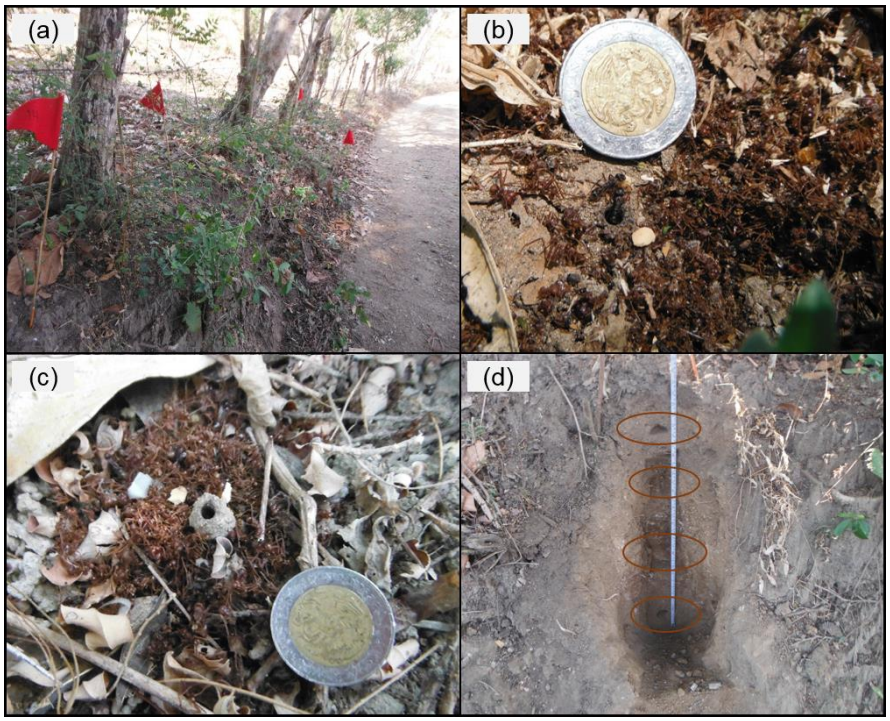


Figure 2. Nests of *E. ruidum* in Oaxaca, Mexico. (a) Nests located on the border of a dirt road in the municipality of Huaxpaltepec. (b) and (c) Typical nest entrance which allows ants to pass only one by one; nest entrances are notably surrounded by corpses of other species of ants and other insects. These photos were taken in the municipality of Pinotepa Nacional. (d) A nest with four chambers, each highlighted by a brown-circle; only one queen was found in the deepest chamber, approximately 80 cm deep from the soil surface. All photos were taken during the dry season in May 2019. The diameter of the coin used as a size reference is 2.5 cm.

Social structure (number of queens). Colonies of *E. ruidum* have a single queen (monogyny), but facultative polygyny (presence of more than one reproductive female per colony), has been found in two populations of *E. ruidum* Chiapas, México (Lachaud et al. 1999). Polygynous colonies present macrogynes or exhibit two morphological queen types: microgynes (isometric reduction of macrogynes) and macrogynes (Schatz et al. 1996; Lachaud et al. 1999). Microgynes and macrogynes can produce eggs of both queen morphs; however, the nest foundation capacity of microgynes is lower than that of macrogynes. Therefore, microgynes are thought to represent an alternative and less costly reproductive strategy (Lachaud et al. 1999). Indeed, once mated, microgynes are usually re-adopted into their natal nest (Lenoir et al. 2011). Up to know, the presence of microgynes has been reported in populations from the Yucatan Peninsule and south-east Mexico, as well as in western Colombia (Lachaud et al. 1999; Nettel-Hernanz et al. 2015). In two populations from Chiapas, Mexico, mother-daughter genetic relationships showed that functional monogyny occurs in the presence of several dealate and mated females. This result confirmed that queens are able to inhibit the laying capacities of other dealate females. Populations without microgynes were originally thought to represent an evolutionary lineage distinct from the populations were the presence of microgynes occurs (Nettel et al. 2015). Finally, with respect to males, polyandry (females mating with more than one male) has been reported in polygynous colonies but seems unfrequent (Passera et al. 1994; Lenoir et al. 2011). In addition, according to the lack of inbreeding and to field observations in

Chiapas, Mexico, gene dispersion was suggested to be done mainly by flying males in populations of *E. ruidum* Lenoir et al. (2011).

Cleptiobiosis and parasitism. *E. ruidum* is one of the few ant species where cleptobiosis has been reported. Cleptobiotic or thief ants steal food, nest materials or other items of value from other colonies (Breed et al. 2012). Concerning *E. ruidum*, intraspecific cleptobiosis has commonly been reported in populations from Costa Rica and Mexico (Breed et al. 1990; de Carli et al. 1998), while in a population from Nicaragua it was suggested they steal from colonies of other ant species (Perfecto and Vandermeer 1993). The behavior of thief ants differs from that of foragers: when thieves steal food from other colonies, they walk slowly, pause frequently and avoid encountering conspecifics in route (Mc Glynn et al. 2015). They also hide quickly when disturbed (Jandt et al. 2015). Their behavior is so peculiar that Mc Glynn and co-workers (2015) proposed they represent a distinct caste of foragers. For *E. ruidum*, cleptobiosis was found to have low fitness cost in populations with high nest-density, because the experimental removal of thiefs did not caused fitness gains in the affected colonies. Also, the permissive acceptance threshold exhibited by these ants when they regularly come into contact with conspecifics from neigbouring nests was proposed as an explanation for the persitance of cleptobiosis in the species (Jandt et al. 2015).

Besides thievery, colonies of *E. ruidum* can be affected by the presence of parasites as the *Mermis* worms (Wheeler 1910; Weber 1946 in Arias-Penna 2008) and eucharitid parasitic wasps (Lachaud and Pérez-Lachaud 2009). According to Lachaud and Perez-Lachaud (2009) despite the presence of the eucharitid wasps affected the growth of colonies, the high nest density reached by these ants decrease the impact of wasp parasitism in their populations.

Taxonomy of *E. ruidum*

E. ruidum was firstly described as *Ponera ruida* by Roger J. in 1861 from specimens collected in Colombia, but other localities included Brazil and Cayenne in the French Guiana. Later, it was renamed as *Ectatomma ruidum* by Kugler and Brown (1982), but in their study the authors synonymized this species with *Ectatomma aztecum* Emery 1901 by analyzing specimens collected in Acapulco, Veracruz and other localities of the lowland southern of Mexico (Figure 3). Kugler and Brown (1982) mentioned that *E. aztecum* appeared to be a very hispid variant of *E. ruidum*. Their arguments relied on slight differences in morphological features: they mentioned that some of the Mexican specimens of *E. aztecum* tend to have the first gastric (postpetiolar) tergum with more nearly transverse costulation (less strongly arched than usual) over the anterior half or more of the disc. The type specimen of *E. aztecum* was collected in Michoacan, Mexico and is located in the Museum of natural history "Giacomo Doria" in Genoa, Italy (Figure 3).

The diagnostic traits to identify *E. ruidum* are as follows: head in the perfect full-face view with posterior outline transverse, nearly straight over most of the distance between the eyes or indented mesad; median eminence of pronotum low, angular in front as seen from the side; lateral teeth prominent, acute or rectangular (Krugler and Brown 1982). In lateral view the petional node is high and thin, at least the upper half with the anterior and posterior surfaces

vertical and subparallel (Arias Penna 2008). The size of the ants is approximately 7-9 mm in length and they weight around 5-12 mg (Brown 1958 in Schatz et al. 1997).

Figure 3. Pictures of the type specimen of *E. ruidum* (worker) previously described as *E. aztecum* by Krugler and Brown (1982), specimen No. CASENT0903841. Photographs (a)-(c) by Will Ericson and (d) by Alexandra Westrich; all photos were dowloaded from: AntWeb. Version 8.54.9. California Academy of Science, online at https://www.antweb.org. Accessed 12 March 2021.

In a previous article focused on the evolution, biogeography and phylogenetic relationships of ants of the genus *Ectatomma* in relation with the presence of small queens, Nettel et al. (2015) reported first evidence about the existence of two different sister clades of *E. ruidum*, one consisting of populations where microgynes were present and another where they were not. Later, based on the previous findings of Nettel et al. (2015), Aguilar-Velasco and co-workers (2016) performed a study focusing on populations of *E. ruidum* from several localities along its geographic distribution (Figure 4). They analyzed the morphology of the specimens and performed phylogenetic molecular analyses for which two mitochondrial (COI and Cytb genes) and one nuclear genetic marker (H3 gen) were sequenced. Aguilar-Velasco et al. (2016) reported the presence of at least four morphospecies within *E. ruidum* and one putative hybrid population, with specimens sharing characters between the *E. ruidum* sp. 2 and *E. ruidum* sp. 3; for this reason they assigned those individuals as *E. ruidum* sp. 2x3. The morphologic descriptions of the morphotypes and their pictures are described in the Table 2 and Figure 6.

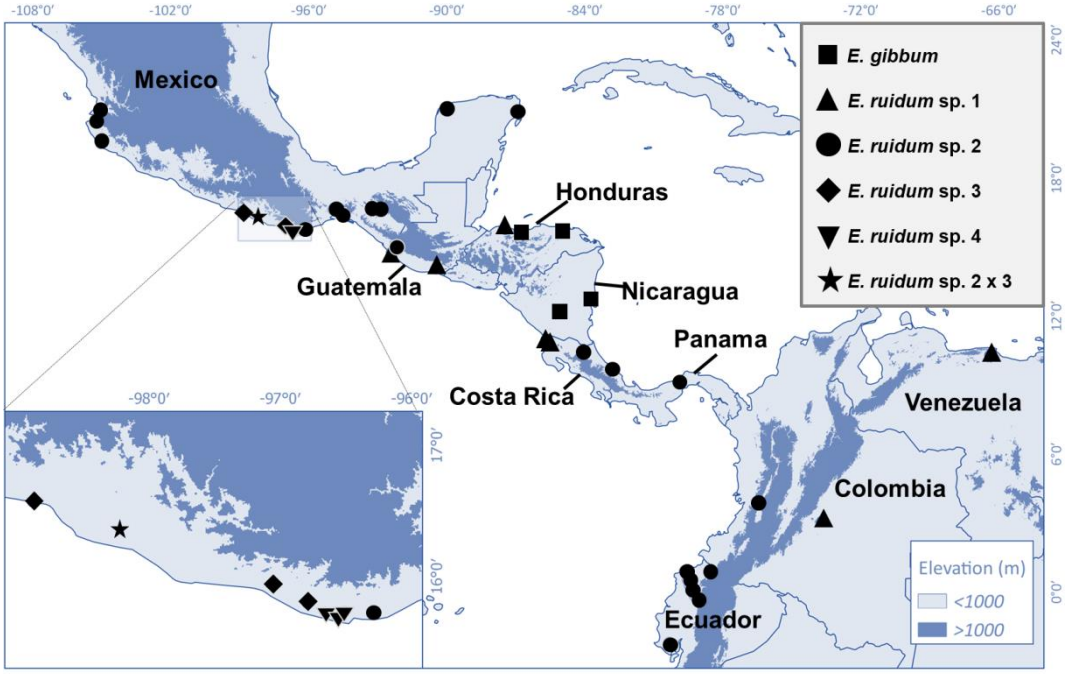


Figure 4. The geographic distribution of the different morphotypes proposed by Aguilar-Velasco et al. (2016). Adapted from Aguilar-Velasco et al. (2016).

With respect to genetic analyses, the phylogenetic tree inferred with sequences of the COI gen presented two main clades, one containing specimens from the morphotypes *E. ruidum* sp. 1 at a basal position and another one divided in two, one containing *E. ruidum* sp.2 on one side, and another one grouping *E. ruidum* spp. 3 and 4 (Figure 5). The analysis of nuclear sequences confirmed the putative hybrid origin of the samples of the morphotype *E. ruidum* sp. 2x3, reason why those samples were not included in the phylogenetic analyses of mitochondrial sequences. Additionally, a number of sequences of the different morphotypes presented highly polymorphic sites that were considered as *numts* (also called pseudogenes; non-functional fragments of mitochondrial DNA integrated into the nuclear genome). These sites were excluded from the further analyses. This finding showed that identifying the presence of pseudogenes is essential given that they can interfere with phylogenetic inferences, for example because of their different mutation rates. Therefore, the researchers highlighted the necessity to detect them, in order to avoid lineage overestimation due to *numts*.

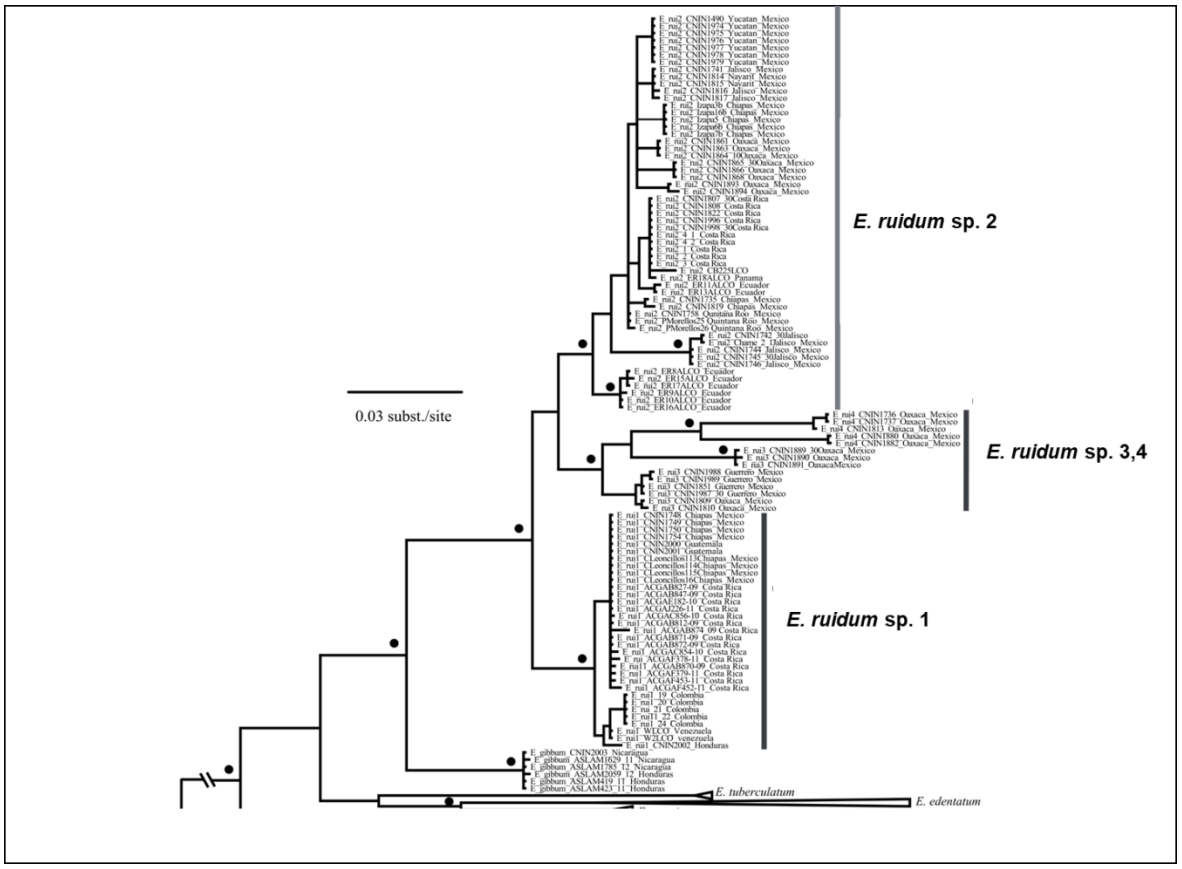


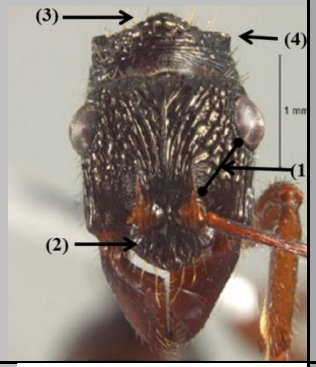
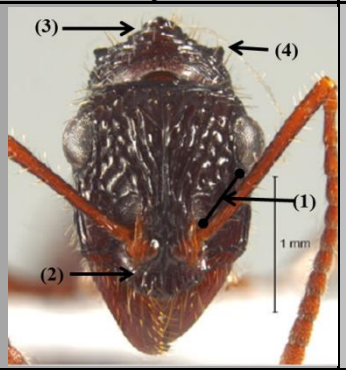
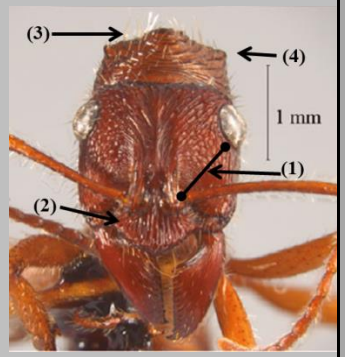
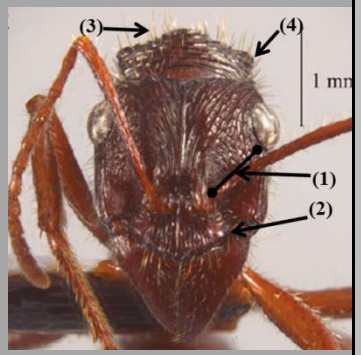
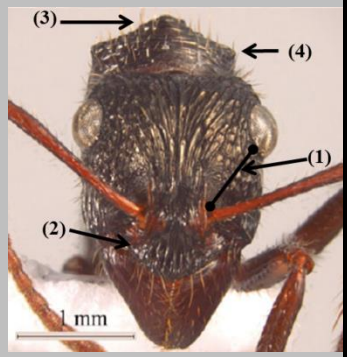
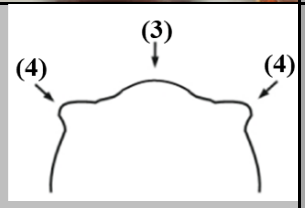
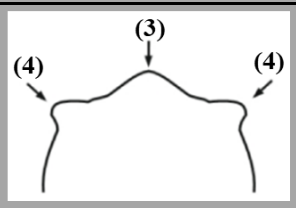
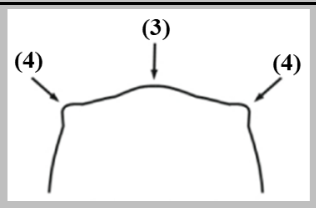
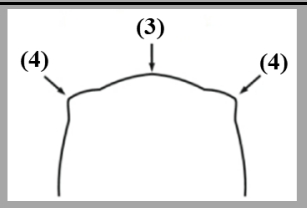
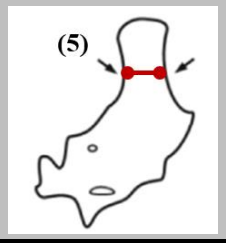
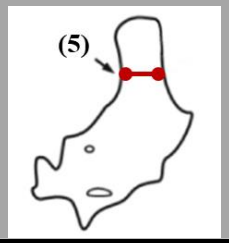
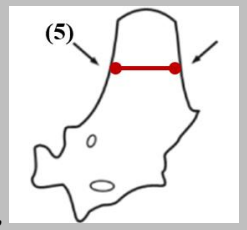
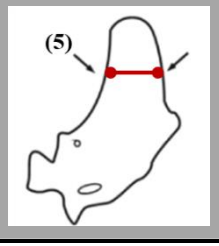
Figure 5. Phylogenetic tree inferred with the sequences of COI gen from the morphotypes *E. ruidum* spp. 1-4, branches with black dots indicate support values > 0.95; this figure was adapted from Aguilar-Velasco et al. (2016).

In their study, Aguilar-Velasco and co-workers (2016) showed that the putative *E. ruidum* sp. 1 and *E. ruidum* sp. 2 has the widest distribution. *E. ruidum* sp. 1 occurs from the extreme South of Mexico (Chiapas, at the border with Guatemala), across all Central America and from northern and north-eastern South America, while *E. ruidum* sp. 2 is distributed along the Atlantic and Pacific coasts. Additionally, these putative species differed by the presence or absence of microgynes: *E. ruidum* sp. 1 does not present microgynes while *E. ruidum* sp. 2 does. About *E. ruidum* sp. 3 and 4 and sp. 2x3, the authors highlighted that they presented a limited distribution restricted to a region of the Pacific coast of Mexico. Moreover, the finding of putative hybrids between *E. ruidum* sp. 2 and sp. 3 suggested the existence of a hybrid zone situated between the distribution ranges of both species, near the pacific coast of Oaxaca in Mexico. Furthermore, the possibility that *E. ruidum* sp. 3 belonged to the ancient type specimen of *E. aztecum* was proposed, because they had a high morphological similarity and their field collection sites overlapped.

Table 2. Morphological features of the different putative species of the *E. ruidum* complex; adapted from Aguilar-Velasco et al. (2016). Numbers between parenthesis indicate the characters in Figure 6.

		Morphospecies of *E. ruidum*				
	Character	**sp. 1**	**sp. 2**	**sp. 3**	**sp. 4**	**sp. 2x3**
Head	Space between eye and frontal carina (1)	Eight or fewer longitudinal striae; space between striae rugulose	Eight or fewer longitudinal striae; space between striae slightly rugulose	10-16 longitudinal striae running from base of antennae to top level of eye	10-16 longitudinal striae running from base of antennae to top level of eye	10-16 longitudinal striae running from base of antennae to top level of eye; space between striae slightly rugulose
	Clypeus (2)	With fewer irregular longitudinal striae; posterior outline of head straight	With few irregular longitudinal striae; posterior outline of head straight	With well-defined longitudinal striae; posterior outline of head straight	With well-defined longitudinal striae; posterior outline of head straight	With well-defined longitudinal striae; posterior outline of head straight
Thorax	Pronotal hump (3)	Low, moderately acute, slightly higher than lateral teeth	Low, strongly acute, distinctly higher than lateral teeth,	Low, rounded, same level as lateral teeth,	Pronotal hump low, rounded, same level as lateral teeth,	Low, rounded, slightly higher than lateral teeth,
	Lateral pronotal teeth (4)	Prominent, pointed,	Prominent, pointed,	Reduced, rectangular,	Rectangular	Reduced, rectangular;
	Propodeum	With transverse striae, oblique rugae anteriorly and/or medially	With transverse striae, oblique rugae anteriorly and/or medially	With well-defined transverse striae	Propodeum with well-defined transverse striae.	Propodeum with transverse, irregular striae
	Propodeal teeth	Distinct and sharp	Distinct/reduced, sharp	Distinct/reduced, sharp	Absent/reduced, sharp	Reduced, sharp
Abdomen	Petiolar node (5)	High, narrow, medially constrained, subparallel dorsally	Moderately high, narrow, slightly arched anteriorly, almost straight posteriorly	Low wide, distinctly arched anteriorly, straight posteriorly.	High, moderately wide, straight, slightly arched anteriorly	Low, wide, distinctly arched anteriorly, straight posteriorly.

Figure 6. Photographs and drawings showing the morphological characters (indicated in Table 2) and differences between individuals of the different morphospecies proposed by Aguilar-Velasco et al. (2016); all photos were adapted from their article and their available supplementary files.

		Morphospecies of *E. ruidum*				
		sp. 1	**sp. 2**	**sp. 3**	**sp. 4**	**sp. 2x3**
HEAD						
THORAX	Pronotal hump					
	Petiolar node					

In a more recent study performed by Meza et al. (2018), the mitogenomes of the different morphospecies proposed by Aguilar-Velasco et al. (2016) were assembled and used to infer their phylogenetic relationships. One of the questions the researchers tried to answer was if the origin of the mitochondrial DNA polymorphism in *E. ruidum* was due to heteroplasmy (i.e. the presence of multiple mitochondrial DNA haplotypes in a single organism, Magnacca and Brown 2010).

As previously showed by Nettel et al. (2015) and Aguilar-Velasco et al. (2016), the phylogenetic analyses supported the separation of the morphospecies *E. ruidum* sp. 1 and sp. 2 as two different putative species; in contrast, the mitogenomes of the species *E. ruidum* spp. 3, 4 and 2x3 were grouped together, suggesting they represented the same lineage (Figure 7). Also, the presence of heteroplasmy was confirmed in the morpho species *E. ruidum* spp. 3, 4 and 2x3. In addition, the different haplotypes contained in each heteroplasmic individual were analyzed separately for COI and Cytb genes to reconstruct additional phylogenetic trees. These analyses documented that the heteroplasmic haplotypes were functional. Moreover, their phylogenetic analyses indicated that the heteroplasmic haplotypes were highly divergent, characterized by long branches (Figure 7, 8), that their presence did not allow the separation of the different putative species and would be considered as a putative single species (Figure 7, 8). Instead, the clustering of heteroplasmic individuals suggested their populations share heteroplasmy. The authors suggested that the origin of the heteroplasmy in *E. ruidum* cannot be explained by occasional paternal leakage because it was limited among individuals, but might be due to a prevalent source of a second mitochondrial lineage.

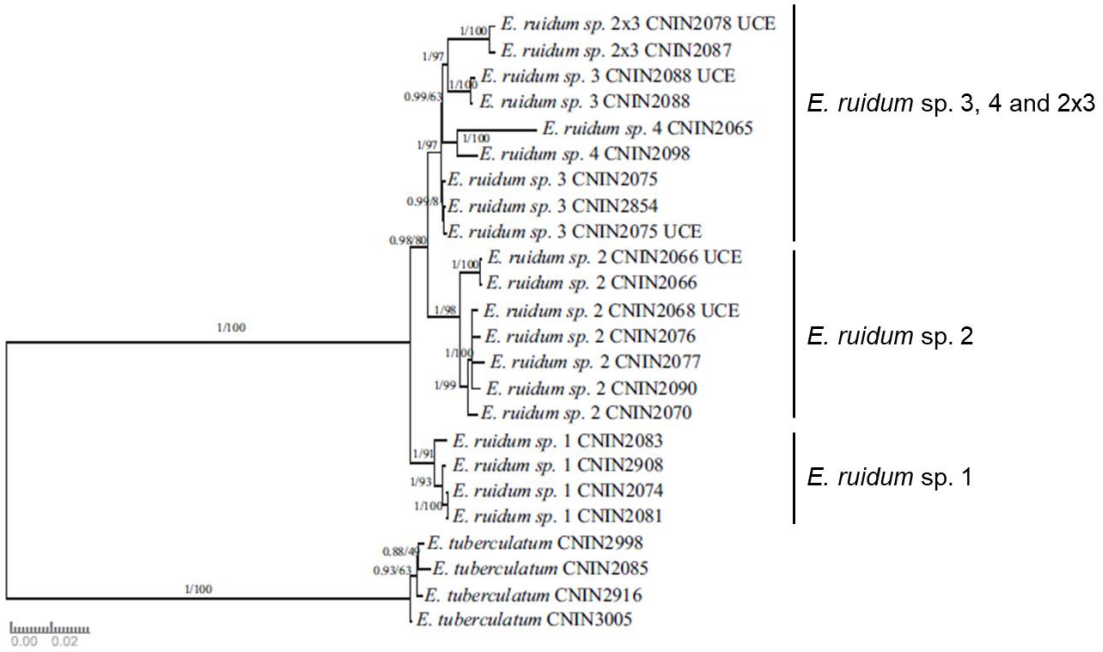


Figure 7. Bayesian phylogenetic tree showing the phylogenetic relations between the different morphotypes of the *E. ruidum* species complex; the tree was constructed with the protein-coding gene data set derived from their mitogenome. Adapted from Meza et al. (2018).

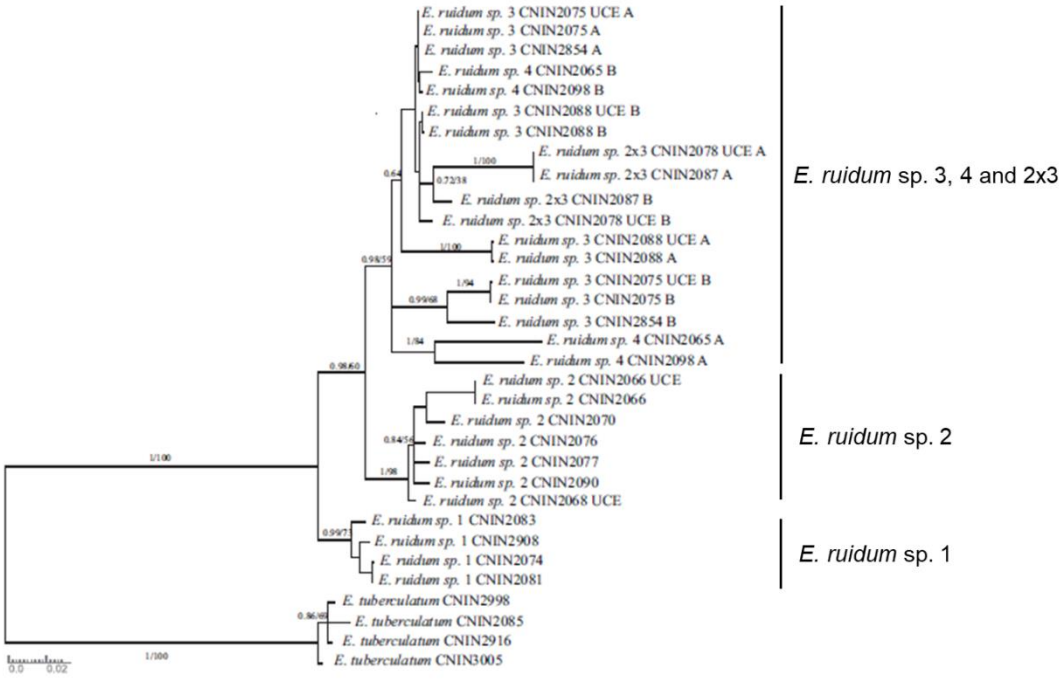


Figure 8. Bayesian phylogenetic tree constructed with the different COI (gen) haplotypes contained in heteroplasmic individuals; the longest branches correspond to secondary heteroplasmic haplotypes. Adapted from Meza et al. (2018).

I.5. Aim of the study

The ants of the *Ectatomma ruidum* species complex represent an excellent model to study evolution: they exhibit a fascinating biology that includes cleptobiotic behavior, social polymorphism and queen dimorphism. Also, the high genetic variability and the presence of heteroplasmy restricted to a limited geographic zone, suggest that the south of Mexico (and particularly the region of the southern Sierra Madre) could represent a hot spot for the diversification of the species. Speciation may be promoted by divergence in communication signals (West-Eberhard 1983) and for social insects such as ants, diversification in major life-history traits such as recognition cues may promote speciation (Brodetzki et al. 2019). Also, in complex and heterogenous environments, population structure may be affected by factors different from geographical distance (de Queiroz et al. 2017). Spatial differences in environmental features may promote different local selective pressures that could drive evolution (Wang et al. 2014). Indeed, the geographic region that up to now concentrates the highest variability in *E. ruidum* (i.e., different putative species) and where a putative hybrid zone was suggested, is considered an environmentally heterogenous area, which might favor speciation events in different taxa (Santiago-Alvarado et al. 2016; Salas-Morales et al. 2018 in Oaxaca).

The aim of this project was to study phenotypic and genotypic traits to understand the possible mechanisms involved in the population divergence that lead to the species diversification of the *E. ruidum* species complex. This study could represent a channel for the identification of potential local selection pressures along a possible geographic hot spot for the diversification of this group of ants. Furthermore, these results will provide evidence to support the separation of the different putative species of the *E. ruidum* species complex. In the **Chapter 1** of this thesis, I studied the cuticular hydrocarbon profiles of the different putative species of the complex to investigate their variation and test their potential as taxonomic tools. I applied these analyses to one new putative species we discovered during a sampling performed in 2019 and to the different morphospecies of the *E. ruidum* complex. I discussed the possible causes of variation in chemical cues with respect to thievery and other biotic and abiotic factors. In the **Chapter 2**, I compared acoustic and morphologic data to test for inter-specific variation among the different putative species of the complex. These analyses tested if acoustic and morphologic traits could represent potential taxonomic tools to discriminate among these species. Finally, in the **Chapter 3** of this thesis, the species boundaries of the different putative species of the complex and the putative hybrid population were studied. The application of cutting-edge sequence techniques, combined with the study of variation in phenotypic characters (cuticular hydrocarbons) would help to clarify the phylogenetic relationships of the different putative species of the complex. These analyses also highlighted that the study of heteroplasmic individuals could be problematic while performing phylogenetic inferences only based on mtDNA. At the end of the thesis, I included an **Appendix** section with a brief description of an ongoing project (Characterization of the genetic structure of populations from the *E. ruidum* species complex along a transect in Oaxaca, with microsatellite loci) started in the last year of my PhD studies.

2.

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).

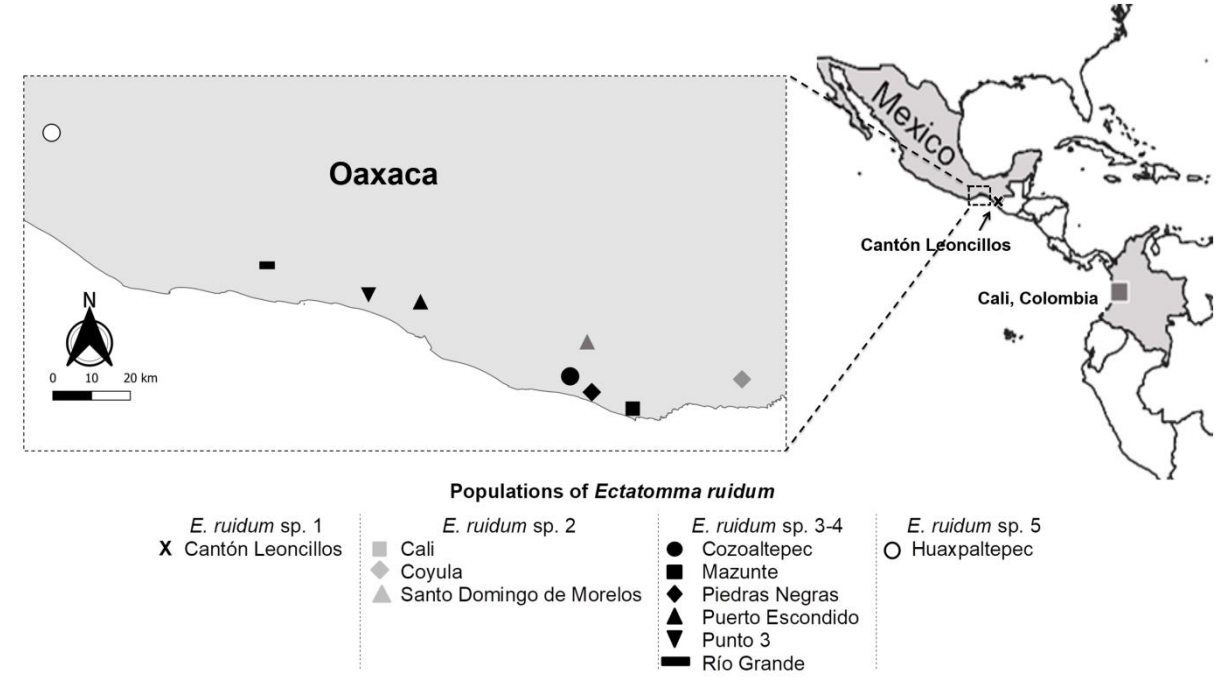

Fig. 1 Map showing the examined populations of the *E. ruidum* species complex

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.

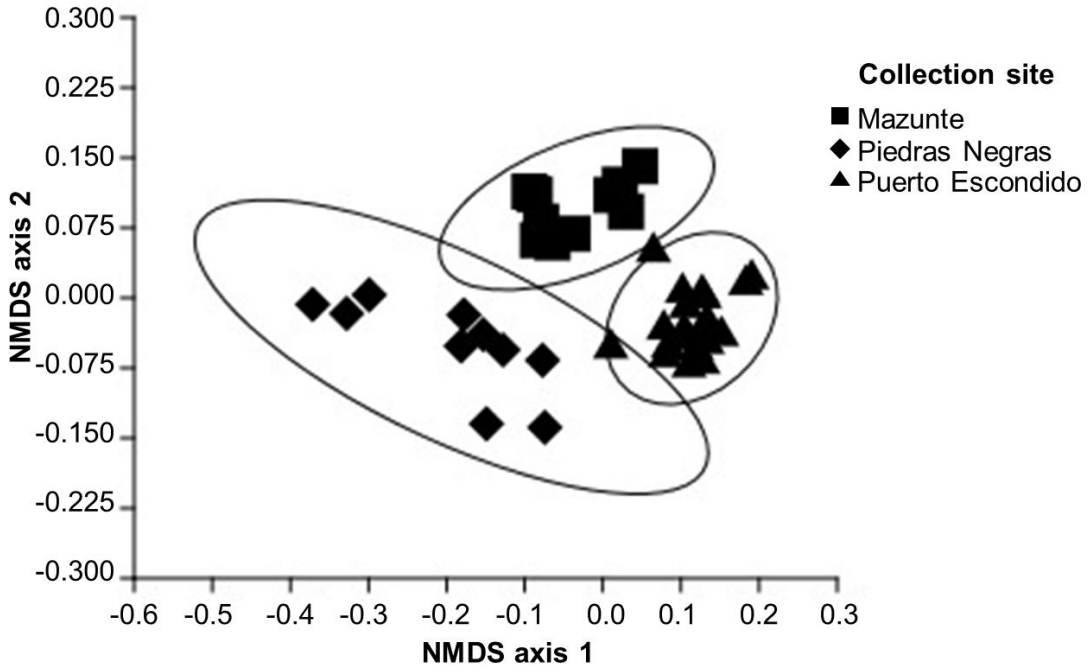

Fig. 2 Two-dimensional Nonmetric Multidimensional Scaling ordination of the chemical profiles of *E. ruidum* sp. 3-4

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 < 0.0001), alkenes ($F_{2,37}$ = 21.628, P < 0.0001), 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).

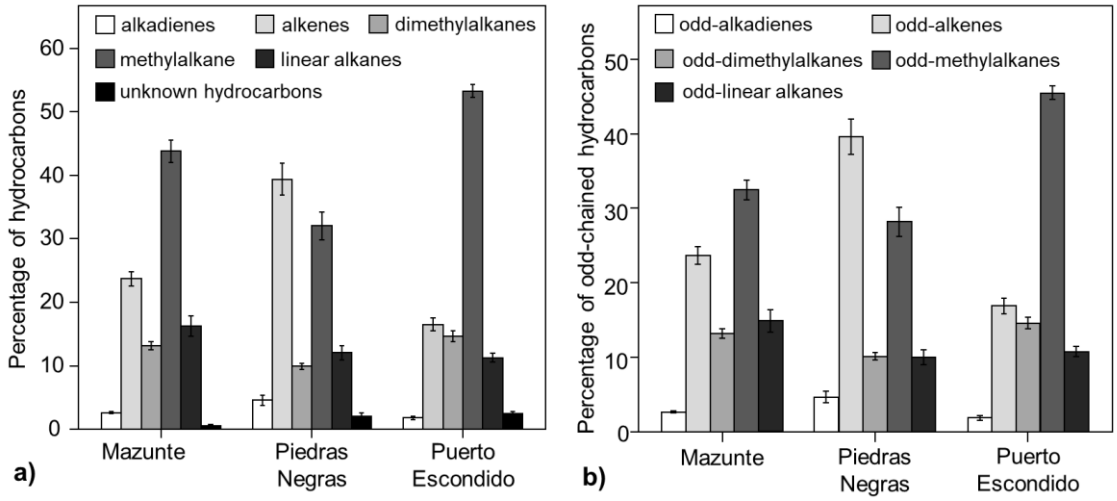

Fig. 3 Mean proportions of: a) cuticular hydrocarbons and b) odd-chain hydrocarbons of ants *E. ruidum* sp. 3-4 by class

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

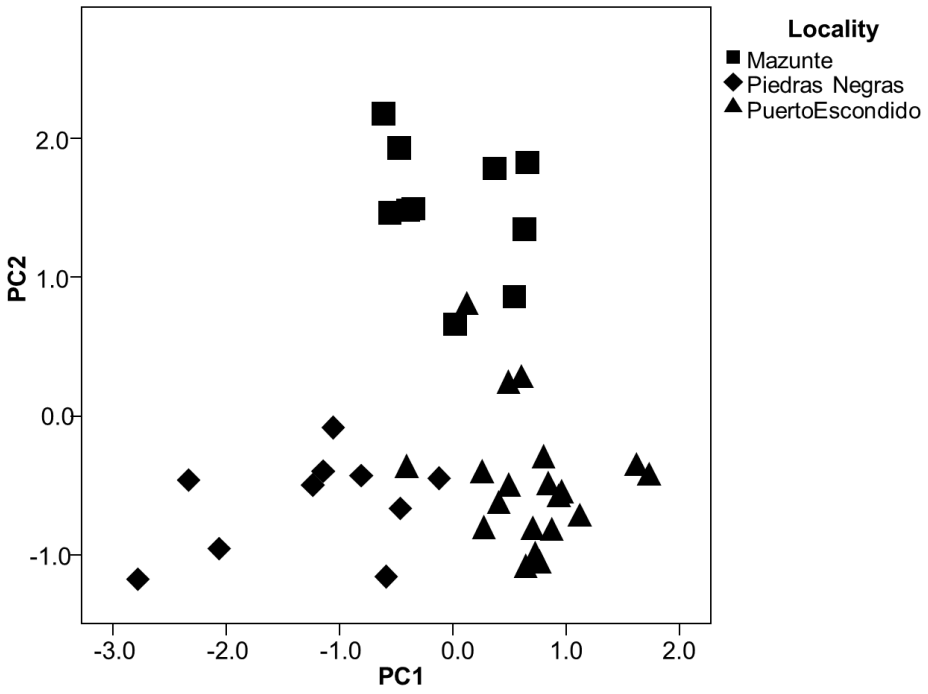

Fig. 4 The PCA's plot of the nine traits describing the cuticular hydrocarbon profiles of ants of *E. ruidum* sp. 3-4

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.

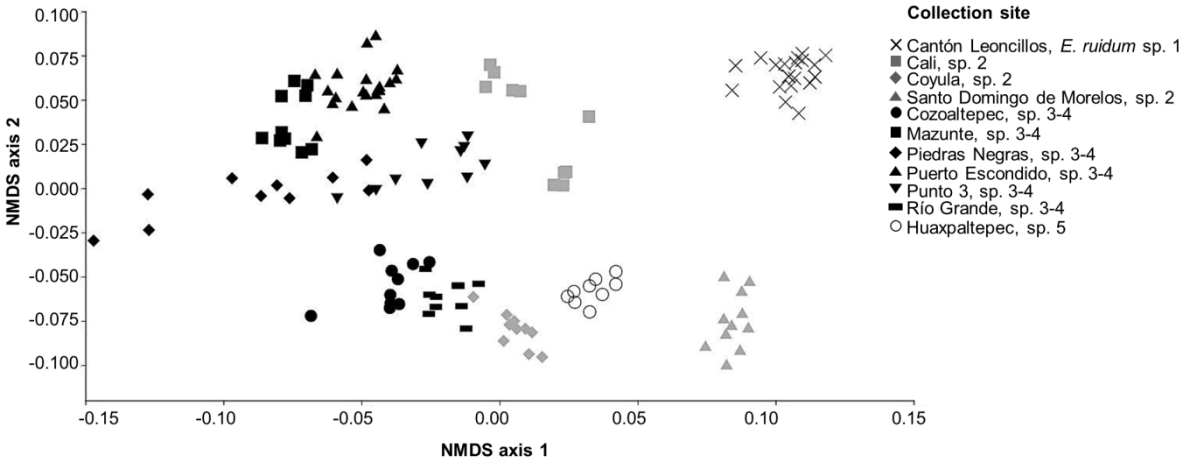

Fig. 5 Two-dimensional Nonmetric Multidimensional Scaling ordination of the chemical profiles of the *E. ruidum* species complex

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.

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3.

A new putative species in the *Ectatomma ruidum* complex (Formicidae: Ectatomminae) produces a species-specific distress call

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Under revision in *Bioacoustics*

Abstract

Social insects communicate by using chemical, visual, tactile, and acoustic signals, including stridulations. *Ectatomma ruidum* is a mainly Neotropical ant species complex that has faced strong divergence at the genetic level; the species have a highly variable blend of cuticular hydrocarbons and a relatively conserved morphology. Based on evidence for genetic and chemical differentiation, we tested for variation in acoustic traits. We compared the stridulations produced by the species *E. ruidum* sp. 2, sp. 3-4 and the new putative species *E. ruidum* sp. 5, as well as the morphology of the stridulatory file. We found that the stridulations produced by *E. ruidum* sp. 5 were statistically different from those of the other species in a number of traits. The differences in stridulatory traits might rely more on the way the ants produce the sound (rubbed area percentages) than on the morphology of the stridulatory file, for which we did not find variation. Our results highlight the use of acoustic traits as potential taxonomic tools for integrative taxonomic studies and suggest that the acoustic traits of *E. ruidum* species complex have been subjected to selection.

Keywords: ants, acoustics, stridulations, stridulatory organ, species complex.

Introduction

Communication is an important feature of animal life that allows individuals to share information and its efficiency is essential for social life (Hölldobler 1999; d'Ettorre and Moore 2008). In social insects and particularly in ants, the efficient communication system and the social organization are the primary bases for their evolutionary success; the efficient transmission of information allows them to regulate the functional division of castes, cooperate in rearing immature stages, gather food, defend the nest, explore new foraging grounds, establish territorial borders and reject foreigners (Hölldobler 1995). Apart from chemical cues, social insects communicate by using visual, tactile, and vibro-acoustic signals, among them stridulations (Hölldobler and Wilson 1990; Hölldobler 1999; Leonhardt et al. 2016).

For stridulation production, insects use specialized devices or organs where opposite areas of the exoskeleton serve as file and scraper (Masters et al. 1983). In ants, the stridulatory organ typically consists of a ridge (plectrum or scraper) located on the edge of the post-petiole, and a plate of arrayed ridges (pars stridens or stridulatory file) located on the upper surface of the first segment of the gaster (Hölldobler and Wilson 1990). The stridulation is produced when the gaster is raised and lowered allowing the plectrum to rub on the ridges and produce a train of pulses known as chirp (Grandi 1966, Golden and Hill 2016). The production of every pulse is due to the scraper's contact with one of the ridges (Tschuch and Brothers 1999; Polidori et al. 2013).

For some taxa, the study of the stridulatory organs and/or the sounds they produce revealed intra- or inter-species variation; for example, in mutillid wasps (Polidori et al. 2013), in ants such as *Messor* (Grasso et al. 1998) and *Neoponera apicalis* (=*Pachycondyla*) (Ferreira et al. 2010), and in some species of the Myrmicinae subfamily (Castro et al. 2015), underlying their potential as taxonomic tools.

Ectatomma ruidum (Roger) is a mainly Neotropical ant species that exhibits the peculiar intraspecific behavior named cleptobiosis (food thievery between neighboring colonies), and that was recently discovered to be a species complex (Aguilar-Velasco et al. 2016). Recent molecular studies have shown that some species within the complex have faced selection in molecular traits (mitochondrial DNA) (Meza-Lázaro et al. 2018). This marked genetic diversification is paralleled by a very high level of variation in cuticular hydrocarbon profiles between the species of the complex (Peña-Carrillo et al. 2020). Cuticular hydrocarbons serve as a chemical signature that conveys information about castes, task specialization, reproductive status and allow social insects to discriminate between nestmates and foreigners (Blomquist and Bagnères 2010; van Zweden and d'Ettorre 2010). In contrast to the molecular and chemical diversification of the *E. ruidum* species complex, morphology is relatively conserved across the species (Aguilar-Velasco et al. 2016), while knowledge about the acoustic organ morphology and characteristics of the sounds produced are scarce.

In *E. ruidum*, stridulations consist of sequences of pulse-trains organized in two subunits (disyllabic chirps) composed by series of pulses with opposite phases (Pavan et al. 1997). In a previous study (Pavan et al. 1997), the distress call of *E. ruidum* was analyzed in a limited sample of workers from a single population in the south of Mexico (state of Chiapas), at a time

when the taxonomic complexity of the group and the existence of different species within the complex were unacknowledged.

If communication systems are targets of selection (Endler 1992), we expected that, based on the evidence that the *E. ruidum* species complex exhibits high variation in their chemical communication system between and within species, divergence was also apparent in acoustic signaling. Sound differences, if any, could be supported by morphometric differences in the organ used to produce the sound. To test this hypothesis, we coupled the analysis of stridulation recordings (distress call) and morphometric analyses of the stridulatory file, to test whether acoustic signals diverged between the species *E. ruidum* sp. 2, sp. 3-4 and the newly reported *E. ruidum* sp. 5.

Materials and Methods

Collection of ants

The ants of the *E. ruidum* complex were collected in the states of Chiapas, Oaxaca and Quintana Roo (Mexico), and in Cali (Colombia) (Table 1); colonies from the same collection site were at least 1-2 m distant from each other. It is worth pointing that the different species of these ants cannot be distinguished in the field and that collection sites are often difficult to reach. Therefore, only after molecular or chemical investigations in the lab ants can be assigned to one of the species, making it difficult to plan the number of colonies per species and per population to collect during field trips. In particular, no replicate populations are possible for *E. ruidum* sp. 5, because up to now only one population has been identified (Pena-Carrillo et al 2020).

Laboratory rearing and species identification

In laboratory, all colonies were kept in artificial nests at 25 ± 1 °C, with a photoperiod of 12 h L: 12 h D and a relative humidity of 40 %, they were fed three times per week with a mixture of honey, apple and crickets, and were given with water *ad libitum*.

The colonies from Coyula and Cali were identified as *E. ruidum* sp. 2, and those from Puerto Escondido, Piedras Negras and Mazunte as *E. ruidum* sp. 3-4 by Meza-Lázaro et al. (2018). The colonies from Punto 3 and Huaxpaltepec were identified as *E. ruidum* sp. 3-4 and sp. 5, respectively by Peña-Carrillo et al. (2020), and those from Puerto Morelos as *E. ruidum* sp. 2 by Poteaux et al., based on barcoding sequences (COI gene) (unpublished data).

Stridulation recording and analysis

We randomly choose 85 workers from 13 colonies of three putative species of the *E. ruidum* complex for the acoustic analyses (4-5 colonies per species, see Table 1).

We recorded the sounds produced by individual ants separated from their nestmates in a room isolated from environmental sounds and electric interferences; experimental conditions were the same in terms of temperature (room temperature 25 – 28 °C) and humidity (40 %). To induce the distress call, we held each individual by the thorax with soft entomological forceps and placed it at 1 cm from the ultrasonic microphone for at least one minute, following Pavan et al. (1997) and Ferreira et al. (2010). Inducing the distress call and getting homogenous series of chirps was time consuming, as often *E. ruidum* individuals exhibited a freezing behavior in which the ants retracted their limbs and stayed immobile with their bodies hunching after disturbance (Cupul-Magaña 2009). We used the ultrasonic USB microphone UltraMic 250 by Dodotronic, and the Sea Pro 2.0 software package developed by Pavan G. at CIBRA for sound recording and real-time display. All recordings were performed at 250k sampling rate with 16 bits dynamic range according to the AD converter of the microphone. After sound recording, ants were killed by freezing and kept in 95% ethanol for the analysis of the stridulatory file morphology.

For each ant we analyzed five chirps which were selected only if they were included in continuous and homogenous series of chirps, and if they presented the disyllabic structure

described by Pavan et al. (1997), that is, two pulse trains with opposite phase, separated by a gap. Using the software Raven Lite (Bioacoustics Research Program, 2016), we measured the following variables: chirp duration, duration of forward phase, inter-chip duration (GAP), duration of backward phase and frequency range (intended as the upper limit of frequency range of the pulses). Then, by using the "seewave" package (Sueur et al. 2008) in RStudio (Version 3.5.2), we counted the number of pulses in the forward and backward phase of the disyllabic chirps. We also calculated the total number of pulses (sum of pulses in the forward and backward phase), the chirp rate (number of chirps / second) and the average pulse rate of the forward and backward phase (number of pulses / second).

Morphometry of the stridulatory file

After the sound recording, 42 out of the 85 workers used for the acoustical analyses were dissected under a stereoscope and we took pictures of their stridulatory file with a Leica S440 Scanning Electron Microscope (SEM). For each ant, the fourth abdominal segment of the gaster containing the stridulatory organ was cleaned by submersion in distilled water in an ultrasonic bath for approximately five minutes, and then air-dried and fixed in a drop of silver plate over an aluminum stub. Once fixed, the samples were oven-dried for two to three hours at 50 °C, and coated with an 8-nm mixture of gold-palladium for being photographed with the SEM. Using the software Image J 1.51 (Schneider et al. 2012), we measured the following morphometric variables according to Ferreira et al. (2010): thorax length, total length of stridulatory file (TL), maximal width (MW), 1^{st}, 2^{nd} and 3^{rd} quartile widths (Q1, Q2, Q3, respectively), number of ridges and inter-ridge distance (ID) in the median portion of the stridulatory file (for data analyses, inter-ridge distance was the average of five measures).

Statistical analyses

We reduced the number of acoustic and morphologic variables by performing separate Principal Component Analyses (PCA) for acoustic and morphometric data. Then, we tested for differences in PC values between species by running Generalized Linear Mixed Models (GLMMs) for normally distributed data (identity link). In the GLMM, either the morphometric or acoustic principal components (PCs) were the response variables, and species the fixed factor; colony and population were used as random factors to account for the non-independence of data. For the GLMM on acoustic data, individual identity was also used as a random factor to account for non-independence of the measures from the same individual thus assuming a different "baseline" measure for each subject. While the statistical comparison tests assess the difference within and between individuals (or population or species), by entering a random factor in the model we also controlled for individual differences. Frequency range values were excluded from our statistical analyses due to variations mostly related to changes in the microphone / ant distance and orientation, then, only robust temporal measures were analyzed.

By combining the use of the acoustic and morphometric variables we calculated the percentage of rubbed ridges ((number of pulses x 100) / total number of ridges) and the speed of the rubbing movement (length of rubbed surface / chirp duration of forward or backward phase) for both phases. We used these data to test for variation in the sounds produced among species by

performing the Kruskal-Wallis test and then we checked for pairwise differences using Mann-Whitney test.

All statistical analyses were conducted with the software IBM SPSS version 23.

Table 1. Origin and number of workers of the *Ectatomma ruidum* species complex used for the acoustic and morphological analyses.

Species	Locality	State, country	Geographic coordinates	Colony	Number of individuals for acoustic analyses	Number of individuals for morphometric analyses
sp. 2	Puerto Morelos	Quintana Roo, Mexico	20°50'38.82"N, 86° 54'11.46W	7Z5 10Z3	7 9	5 -
	Coyula	Oaxaca, Mexico	15°45'2.85"N, 96°17'51.05"W	Coyula 19	5	5
	Cali	Cali, Colombia	3°22’39"N, 76°31’52”W	118 92	10 7	6 -
sp. 3-4	Puerto Escondido (Yerba Santa)	Oaxaca, Mexico	15°56'24"N, 97°4'15.60"W	YS1	7	5
	Piedras Negras	Oaxaca, Mexico	15°43'30"N, 96°39'36.00"W	17	5	5
	Mazunte (Puente Zapotal)	Oaxaca, Mexico	15°41’6”N, 96°33’54”W	6	6	5
	Punto3	Oaxaca, Mexico	15°57'24.84"N, 97°11'46.32"W	Punto3	9	5
sp. 5	Huaxpaltepec	Oaxaca, Mexico	16°20'26.00"N, 97°57'34.00"W	Huax13 Huax1-1 Huax2-8 Huax2-14	5 5 5 5	6 - - -
Total number of individuals					85	42

Results

Acoustic traits

The characteristics of the stridulation and their variation

The stridulations produced by the ants of *E. ruidum* sp. 2, 3-4 and 5 were composed of sequences of chirps, each with a disyllabic structure (two opposite phases separated by a gap); each syllable consisted of a pulse train (Figure 1). Among the species, the upper frequency limit of stridulation ranged around 60-70 kHz. The acoustic traits of *E. ruidum* sp. 5 were distinctly variable with respect to the other species; in general *E. ruidum* sp. 5 had the highest mean values for all acoustic traits, while *E. ruidum* sp. 3-4 had the lowest values (Table 2, 3).

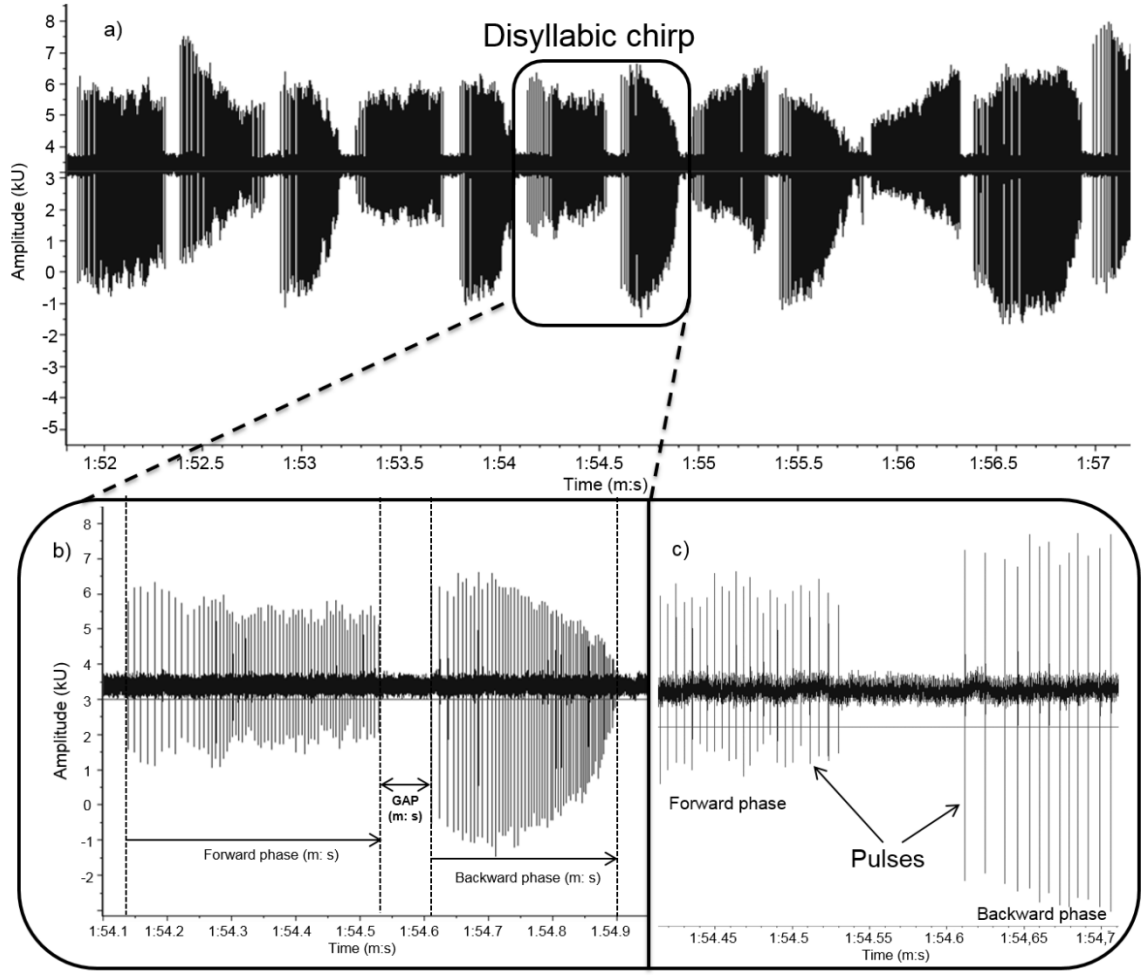

Figure 1. (a) A sequence of disyllabic chirps in the *E. ruidum* stridulation. (b) Detailed view of a disyllabic chirp: forward phase, backward phase and the GAP (time space in between forward and backward phases). (c) Detailed view of pulses of the forward and backward phase around the gap from minute 1:54.45 to 1:54.7.

The acoustical variation was summarized in two principal components that together explained 85.3% of total variance. PC1, which explained 66.8% of variance and significantly discriminated among species (GLMM, $F_{2, 427} = 5.781$, $P = 0.003$), was mainly determined by the number of pulses that composed the chirps (total number of pulses, number of pulses in the

forward and backward chirp) (Table 4) (Figure 2). Pairwise comparisons indicated that only *E. ruidum* sp. 5 differed significantly from *E. ruidum* sp. 2 and 3-4 in PC1 values (GLMM $F_{2, 427}$ = 5.781: *E. ruidum* sp. 5 vs sp. 2 P = 0.016, *E. ruidum* sp. 3-4 vs sp. 2 P = 0.228; GLMM, $F_{2, 427}$ = 5.547: *E. ruidum* sp. 5 vs sp. 3-4 P = 0.001).

PC2, which explained 18.5% of variance, summarized the duration of chirps (total chirp duration, duration of the forward phase and of the GAP duration) (Table 4) and did not discriminate among species (GLMM, $F_{2, 426}$ = 0.831; P = 0.436) (Figure 2).

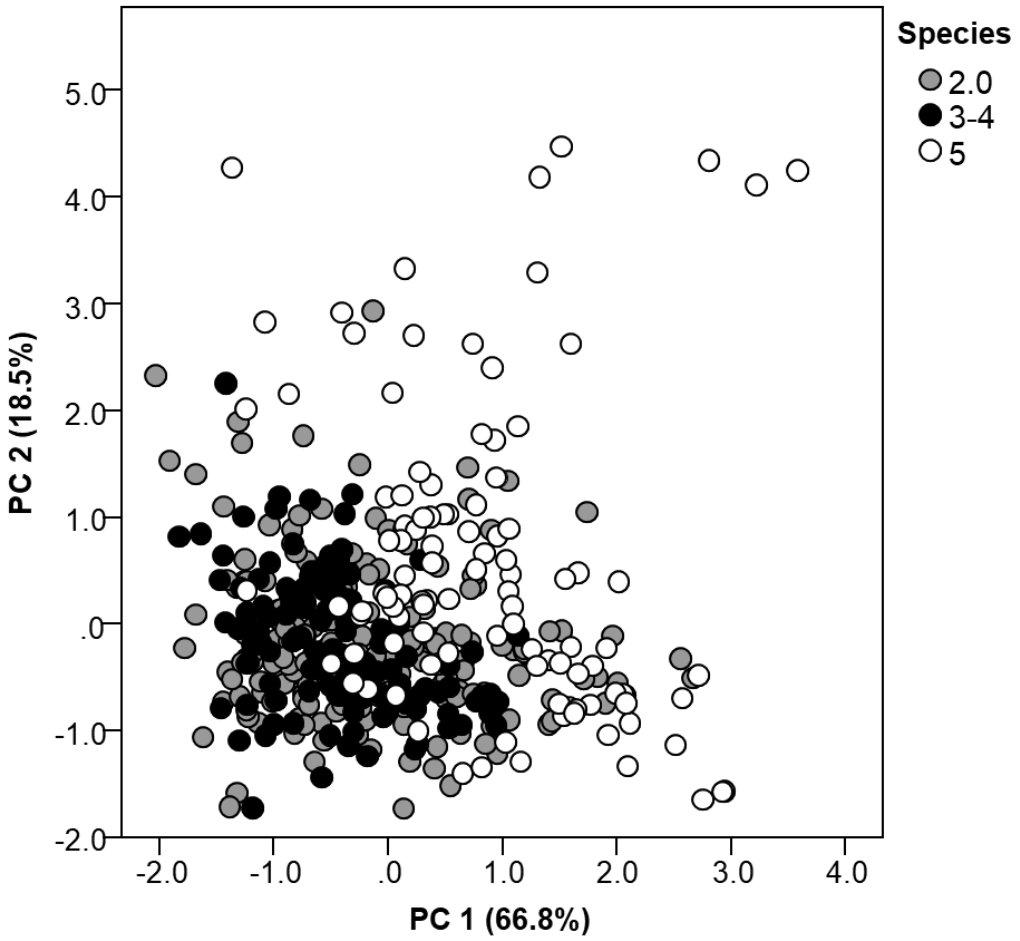

Figure 2. PCA's plot of the acoustical traits in the three species of the *E. ruidum* complex.

Table 2. Mean values ± standard error and min-max range (between parentheses) of the stridulatory sound variables of the *E. ruidum* species 2, 3-4 and 5. Measures are given in seconds (duration) or per second (number or rate).

	E. ruidum sp. 2	*E. ruidum* sp. 3-4	*E. ruidum* sp. 5
Chirp duration	0.248 ± 0.006 (0.069 - 0.602)	0.229 ± 0.005 (0.094 - 0.389)	0.390 ± 0.018 (0.173 – 1.132)
Duration of forward phase	0.112 ± 0.003 (0.032 - 0.321)	0.105 ± 0.003 (0.036 - 0.231)	0.190 ±0.011 (0.082 - 0.707)
Duration of backward phase	0.112 ± 0.003 (0.024 - 0.347)	0.101 ± 0.003 (0.034 - 0.208)	0.169 ± 0.006 (0.068 - 0.405)
Inter chirp duration (GAP)	0.024 ± 0.001 (0.010 - 0.066)	0.023 ± 0.001 (0.009 - 0.079)	0.032 ± 0.002 (0.010 - 0.114)
Number of forward pulses	66 ± 2 (16 – 176)	59 ± 2 (13 – 135)	112 ± 4 (41 – 248)
Number of backward pulses	81 ± 3 (22 - 196)	68 ± 2 (19 - 142)	115 ± 3 (37 – 224)
Total number of pulses	147 ± 4 (40 – 338)	127 ± 3 (50 - 232)	226 ± 7 (99 – 442)
Chirp rate	4.51 ± 0.12 (1.66 – 14.57)	4.62 ± 0.10 (2.57 – 10.67)	2.96 ± 0.01 (0.88 – 5.78)
Average pulse rate of forward phase	609 ± 18 (183 – 1497)	591 ± 19 (194 – 1231)	672 ± 25 (257 – 1491)
Average pulse rate of backward phase	620 ± 20 (107 – 1669)	641 ± 30 (120 – 2027)	730 ± 30 (208 – 1642)
Total number of ants	38	27	20

Table 3. The percentage of rubbed ridges and the speed of the rubbing movement of the *E. ruidum* species 2, 3-4 and 5 (Mean ± standard error (min-max range).

	E. ruidum sp. 2	*E. ruidum* sp. 3-4	*E. ruidum* sp. 5
Percentage of rubbed ridges during the forward phase	32 ± 3 (17 – 56)	29 ± 2 (15 – 42)	66 ± 12 (41 – 107)
Percentage of rubbed ridges during the backward phase	39 ± 3 (20 – 59)	34 ± 2 (20 – 48)	64 ± 9 (42 – 94)
Speed of the forward rubbing movement (µm /msec)	1.12 ± 0.12 (0.526 – 1.81)	1.12 ± 0.10 (0.513 – 1.83)	0.804 ± 0.042 (0.729 – 0.968)
Speed of the backward rubbing movement (µm / msec)	1.50 ± 0.17 (0.795 – 3.12)	1.34 ± 0.07 (0.822 – 1.80)	0.989 ± 0.075 (0.743 – 1.13)
Total number of ants	14	18	5

Table 4. Scores of the PCA factor loadings on the acoustical traits of worker ants (only r > 0.700). The variables are sorted by loading size. High loadings indicated that the trait was highly correlated with the PC.

Rotated Component Matrix	**Principal Component**	
	1	2
Total number of pulses	0.965	-
Number of forward pulses	0.886	-
Number of backward pulses	0.869	-
GAP duration	-	0.891
Total chirp duration	-	0.824
Forward chirp duration	-	0.760

Variation of the sounds: the link between stridulations and morphology

During the stridulation, the percentage of ridges rubbed was highly significantly different among species (Kruskall-Wallis test: forward phase - K = 11.34, df = 2, P = 0.003, N = 37, backward phase - K = 11.83, df = 2, P = 0.003, N = 37). Pairwise comparisons indicated that the differences in the percentage of ridges rubbed by the ants *E. ruidum* sp.5 were significant compared to *E. ruidum* sp. 2 and sp. 3-4 (Forward phase rubbing - sp. 5 vs sp. 2: U = 66, P = 0.002, N = 19; sp. 5 vs sp. 3-4: U = 89, P < 0.0001, N = 23; Backward phase rubbing - sp. 5 vs sp. 2: U = 63, P = 0.007, N = 19; sp. 5 vs sp. 3-4: U = 87, P < 0.0001, N = 23; these differences were still significant after correcting for multiple comparisons) (Figure 3). In comparison to *E. ruidum* sp. 2 and sp. 3-4, the ants *E. ruidum* sp. 5 produced chirps at the lowest rate because they rubbed a higher percentage of ridges (approximately the double) thus producing at least 50% more pulses (Table 2). Ants *E. ruidum* sp. 2 and sp. 3-4 rubbed a similar percentage of ridges (Forward phase rubbing – U = 113, P = 0.639, N = 32; Backward phase rubbing - U = 84, P = 0.116, N= 32) (Tables 2 and 3, Figure 3). The three species stridulated at similar speed (K = 3.496, df = 2, P = 0.174, N = 37, backward phase - K = 5.557, df = 2, P = 0.062, N = 37).

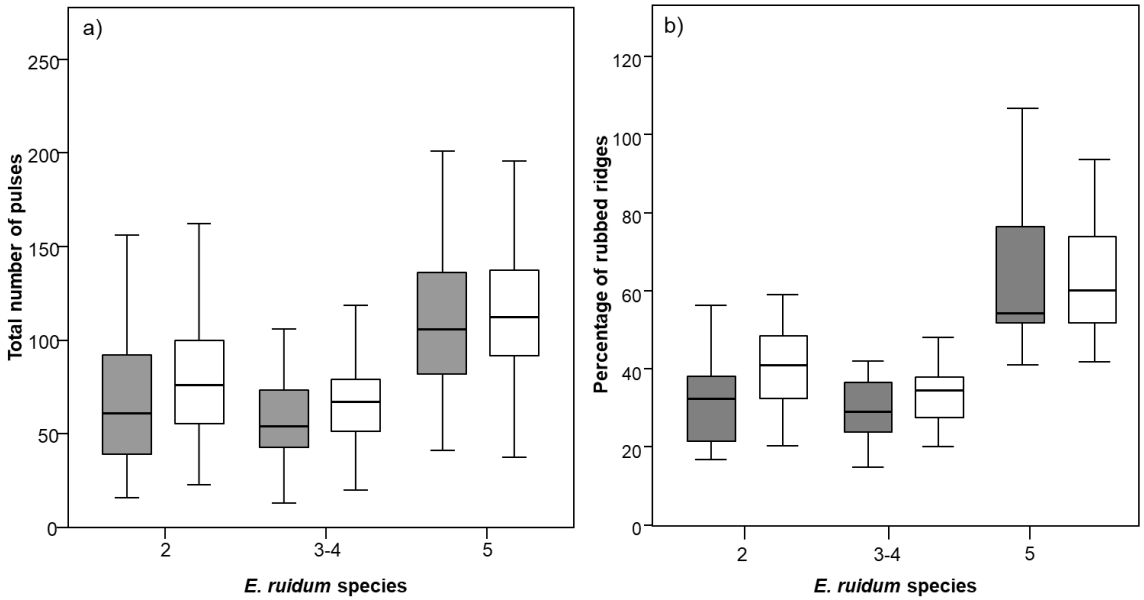

Figure 3. Acoustic variation of *E. ruidum*. (a) Total number of pulses per phase and species (b) Percentage of rubbed ridges per phase and species during the stridulation; bars colored with gray background represent the forward phase and bars with white background the backward phase of the chirps.

Morphometric traits

In all three species of the *E. ruidum* complex the stridulatory file was located at the anterior edge of the first gastral segment of the abdomen (Figure 4).

The PCA performed on the morphological traits of the stridulatory file (Table 5) summarized the overall variation in two principal components that together explained 67.4 % of total variance. PC1 (41 %) was mainly related to the variation in the width of the stridulatory file

(width of Q1, Q2 and maximal width, Table 6). PC2 (26.3 % of the variance) was associated to the variation in the length of the stridulatory file, the number of ridges and the body size (represented by thorax length) (Table 6). However, neither PC1 nor PC2 scores differed significantly between species (GLMM, PC1: $F_{2,39} = 0.032$, P = 0.97, PC2; $F_{2,39} = 1.467$, P = 0.24.

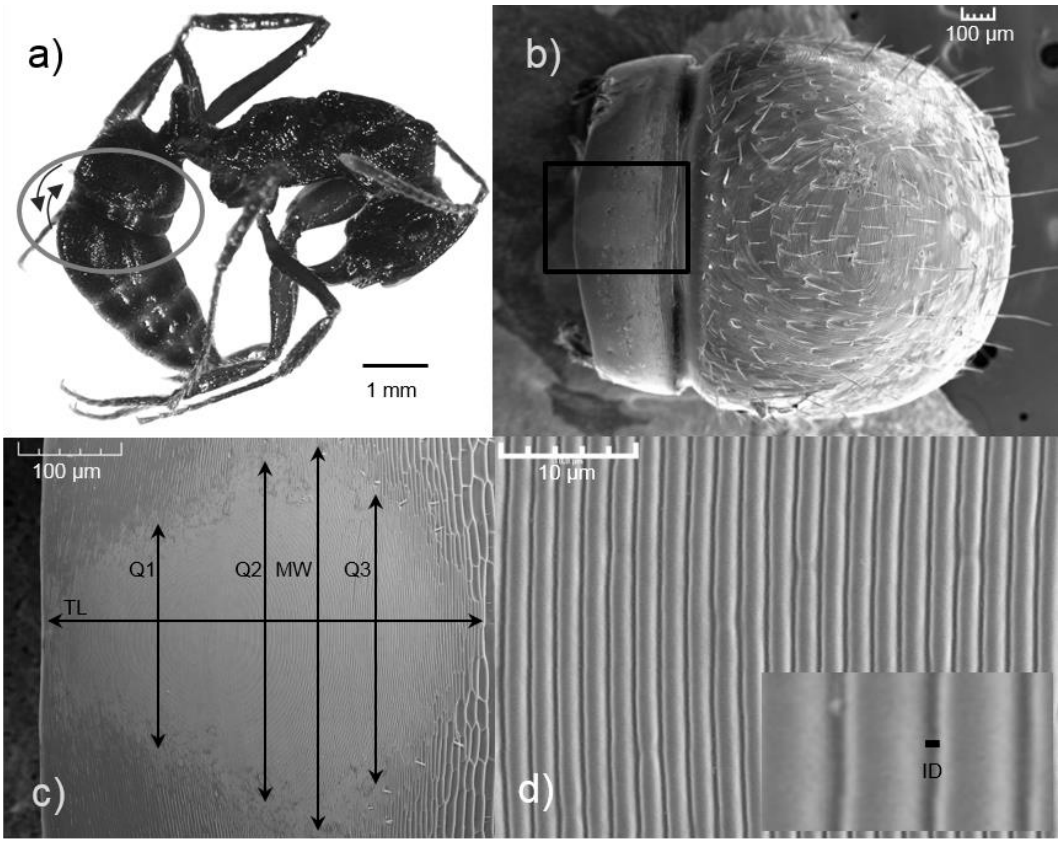

Figure 4. The stridulatory file in *E. ruidum*. (a) Position of the file in the gaster; arrows indicate the backward and forward movement of the gaster when stridulating. (b) Postpetiolar segment of the gaster where the stridulatory file is located. (c) Morphometric variables taken from the stridulatory file: total length of stridulatory file (TL), maximal width (MW), 1st, 2nd and 3rd quartile widths (Q1, Q2, Q3, respectively). (d) Ridges in the stridulatory file and focus on the inter-ridge distance measure (ID).

Table 5. Mean values ± standard error (and min-max range in parentheses) of the morphometric variables of the stridulatory file of the *E. ruidum* species 2, 3-4 and 5.

Workers	*E. ruidum* sp. 2	*E. ruidum* sp. 3-4	*E. ruidum* sp. 5
Thorax lenght (µm)	2891 ± 86 (2192 - 3352)	3252 ± 50 (2836 - 3607)	2912 ± 63 (2640 - 3101)
Stridulatory file total length (µm)	352 ± 9 (279 - 404)	390 ± 6 (359 - 453)	376 ± 13 (339 - 421)
Maximal width (µm)	270 ± 16 (75.5 - 336)	266 ± 8 (191 - 317)	293 ± 6 (274 - 316)
Q1 width (µm)	233 ± 10 (158 - 306)	225 ± 8 (165 - 291)	202 ± 6 (185 - 219)
Q2 width (µm)	275 ± 10 (200 - 356)	260 ± 7 (183 - 304)	286 ± 6 (270 - 310)
Q3 width (µm)	220 ± 10 (145 - 295)	231 ± 7 (146 - 285)	256 ± 8 (236 - 280)
Total number of ridges	192 ± 5 (160 – 238)	199 ± 3 (176 – 219)	193 ± 4 (177 – 202)
Mean of distance between ridges (µm)	0.298 ± 0.018 (0.223 - 0.515)	0.349 ± 0.019 (0.218 - 0.557)	0.298 ± 0.011 (0.250 - 0.326)
Total	16	20	6

Table 6. Scores of the PCA factor loadings on the morphometric traits (only r > 0.700). The variables are sorted by loading size. High loadings indicated that the trait was highly correlated with the PC.

Rotated Component Matrix	**Principal Component**	
	1	2
Q2 width	0.891	-
Q1 width	0.801	-
Maximal width of stridulatory file	0.761	-
Total length of stridulatory file	-	0.887
Total number of ridges	-	0.845
Thorax lenght	-	0.805

Discussion

Our results show that *E. ruidum* sp. 5, the new putative species recently reported by Peña-Carrillo et al. (2020), produces a distress call that differs from the closely related species *E. ruidum* sp. 2, and sp. 3-4. To our knowledge, the acoustic differentiation might rely mainly in in the way ants produce the sound (percentage of ridges rubbed by the plectrum during its movement), due to the similarity in the morphological traits of the stridulatory files. However, although the difference in the acoustical traits is clear, the lack of morphological differences of the stridulatory file between the species may need further investigations.

Ant stridulations are involved in different behaviors. *Solenopsis richteri* ants stridulate when they are alarmed, when they attack or are stressed (e.g. stress induced by holding the body of the ants) (Hickling and Brown, 2000). In *Atta cephalotes*, workers use a distress call to attract nestmates in rescue behavior when they are trapped under the soil (Markl, 1965); to attract ants of other castes to fight against parasites, and to recruit nestmates when cutting leaves to provision the colony fungi (Roces et al. 1993; Hölldobler, 1999). In velvet ants, distress calls have been interpreted as warning signals against predators (Wilson et al. 2012, 2018; Pan et al. 2017), and a possible role in intra-specific communication has been mentioned (Torrico-Bazoberry and Muñoz 2019). In *E. ruidum*, stridulations may serve as a modulatory signal enhancing short-range recruitment of nestmates during collective strategies of prey retrieval (Schatz et al. 1997), although focused experiments are needed to confirm this hypothesis. According to Tschuch and Brothers (1999), non-resonant structures as the stridulatory organ serve for short-range intra and interspecific communication; however, their sound power level might not be strong enough to be effective over a distance.

In the event that *E. ruidum* sp. 5 lived in sympatry with other species of the complex, its distress call could reflect divergent selection faced by the species in the presence of another species, although the benefit of producing species-specific distress calls might be argued. Up to now, whether *E. ruidum* sp. 5 is sympatric with other species of the complex is unknown. Additionally, we do not know whether the different species use soils with different characteristics to build their nests. Indeed, stridulations produce vibrations that can diverge because of the characteristics of the microhabitat were insects live (Crocoft and Rodriguez 2005).

Acoustic signaling has been used as a means to distinguish species because calls have emerged as species-specific in different groups of insects, such as homopterans (Claridge, 1985), lacewings (Henry et al. 1993), flies (Ritchie and Gleason 1995), beetles (Kasper and Hirschberger 2005) and ground crickets (Tan et al. 2018). For all these taxa, sound variation reflected selection on calls serving crucial communication functions, such as courtship calls. For instance, Kasper and Hirschberger (2005) studied the behavioral context and acoustic structure of calls produced by different species of *Aphodius* bettles, as well as the morphology of their stridulatory organs. They found that the beetles produced calls when they were disturbed, and that males produced species-specific calls when they encountered females in an experimental dung arena; the stridulatory organs of the beetles also presented species-specific morphology.

In coevolutionary relationships, calls used for acoustic mimicry are also species-specific, as reported in lycaenid butterflies among which some species spend their larval stages as parasites in ant nests. Riva et al. (2016) studied the acoustic repertoire of parasitic and non-parasitic lycaenid larvae and found that typically all species produced species-specific calls and that the calls of parasitic species revealed their association with different species of host ants. That is, the calls of parasitic species had a higher number of harmonics which overlapped their hosts' frequency range, which in turn potentially increased their chances to stimulate host ant receptors and get food from the ants.

The analysis of distress calls has also been useful to reveal inter-specific variation. For example, Polidori et al. (2013) compared the distress calls and morphology of the stridulatory file of European velvet ants from different subfamilies and genera. They found acoustic differences between the species, but statistics for all the studied species weakly supported the species-specific association of sound variation with morphological variance. Carlos et al. (2014) analyzed the stridulations and the stridulatory file of the workers from three species of Attini ants and reported that the chirps' duration was species-specific. Their study demonstrated stridulations as an efficient taxonomic tool for species identification in the Attini tribe. Distress calls are useful taxonomic tools for cryptic species delimitation. By studying a set of cryptic species within the *Neoponera apicalis* complex, Ferreira et al. (2010) found that the calls of sympatric species differed from each other, probably because of their sympatric interactions. In addition, in combination with the distress calls, the analysis of the stridulatory file and mitochondrial DNA sequences highlighted the cryptic species (Ferreira et al. 2010).

In comparison to the species of the *N. apicalis* complex, up to now there is no evidence whether the species of *E. ruidum* are sympatric, but populations of the different species live close geographically. Yet, there seems to be no clear association between geographic distance and sound differentiation. According to Aguilar-Velasco et al. (2016), *E. ruidum* sp. 2 and sp. 3-4 are located at fewer than 25 km of distance from each other, and the current results show that their stridulations did not differ significantly. In contrast, *E. ruidum* sp. 2 and sp. 3-4 stridulations differed from that of *E. ruidum* sp. 5, whose colonies were located within the distribution range of *E. ruidum* sp. 3-4 (with the closest population separated by ~ 60 km), and close to a population of *E. ruidum* sp. 2 (located less than 20 km away, Peña-Carrillo et al. 2020).

In some insects, morphological structures for sound production differ more than sounds between species (Schmitt 1994; Schmitt and Traue 1990) while in other cases, the opposite was suggested. For example, like our results suggest, Grasso et al. (1998, 2000) found that the stridulations produced by the different castes of *Messor* ants varied not because of morphometry, but because of the way the ants produce the sound, like the speed of the movement and the duration of the gaps between phases in the chirps. The stridulation of the ant *Neoponera apicalis* (previously referred as *Pachycondyla apicalis*) is produced by the rubbing of stridulatory file only in one direction of the movement, while the contact is avoided during the return movement (Pavan et al. 1997). While in the study by Grasso et al. (2000) the different sounds were produced by different castes, in *E. ruidum* they were all produced by workers, but

these workers belonged to different putative species. Nevertheless, in both *Messor* and *E. ruidum* ants, morphology appears to be less variable than the acoustic output.

Although the limited sampling used for morphological analyses did not allow us to detect any significant variation in the structure of the stridulatory file, the combination of acoustic and morphological traits generated inter-specific variation in *E. ruidum* stridulation. Furthermore, the same acoustic traits that discriminated *E. ruidum* sp. 5 from the other species (this study) also discriminated between cryptic species of *Neoponera* ants (namely chirp duration, the number of pulses and velocity of the rubbing movement) (Ferreira et al. 2010), supporting the use of acoustic traits in integrative taxonomic studies. In fact, diverse taxa can be diagnosed through their acoustic production. For example, birds produce distinct songs which allow species identification, even when they are otherwise very similar morphologically (Rheindt et al. 2008; Mahoney et al. 2020), and sound production is used to identify species in marine mammals (Oswald et al. 2007; Bauman-Pickering et al. 2013; Lin et al. 2014), and in insects such as crickets and katydids (Lehmann et al. 2014; Brizio et al. 2020; Mc Neil and Grozinger 2020). In ants the examples are less common (Ferreira et al. 2010), but do exist.

In the *E. ruidum*, a striking divergence was found in the olfactory cues that play a significant role in nestmate discrimination (cuticular hydrocarbons) in most of the populations of the species complex, including *E. ruidum* sp. 5 (Peña-Carrillo et al. 2020). Furthermore, in *E. ruidum* sp. 5, the phylogenetic tree based on mitochondrial sequences supported its differentiation from the species *E. ruidum* sp. 2 and 3-4 (Peña-Carrillo et al. 2020). Therefore, differentiation in the acoustical characteristics of the distress call of *E. ruidum* sp. 5 could be expected in a scenario where the communication system of this species was under higher diverging selection pressure than those of *E. ruidum* sp. 2 and sp. 3-4. However, studies including all the species of the complex are needed to know whether the variation of the distress call is exclusive to *E. ruidum* sp. 5.

Whatever the causes of the divergence of the distress call of *E. ruidum* sp. 5 from that of *E. ruidum* sp. 2 and sp. 3-4, these results highlight that *E. ruidum* sp. 5 differs not only chemically and genetically (Peña-Carrillo et al. 2020), but also acoustically. Adding evidence that this taxon evolved separately from the other species of the complex, these results offer another example of selection pressures acting on the communication system of ants.

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4.

Species diversification of the Neotropical ant species complex *Ectatomma ruidum* (Ectatomminae) based on genome-wide evidence and cuticular chemical cues

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In preparation

Abstract

Ectatomma ruidum is perhaps the most widely distributed ant taxon in the *Ectatomma* genus, as it occurs in a wide range of habitats in the Neotropics. Its taxonomic complexity, peculiar behavior and the presence of heteroplasmy in some of its populations make this group interesting from an evolutionary point of view. The aim of our study was to integrate genome-wide screening, barcoding sequences and cuticular hydrocarbon analyses to untangle the phylogenetic relations between the multiple species identified within this species complex and to infer their taxonomic boundaries. We focused on populations that inhabit the southern Sierra Madre of Mexico and also referred to the influence of heteroplasmy on its speciation process. Specifically, we coupled DNA sequence information gathered via two reduced genome representation techniques (3RAD and Ultra Conserved Elements - UCEs), data obtained both from a large data set of a mitochondrial DNA (COI gene) and cuticular hydrocarbon analyses. Our results allow to delimitate at least five different species within the complex and to separate species that in past studies merged because of the presence of a secondary fast evolving lineage (heteroplasmy). Also, the strong correlation between genetic and chemical distances suggests that cuticular hydrocarbons may have had a role in the diversification of the species complex. These results add to the complexity and variety of mechanisms that promote speciation and biodiversity.

Introduction

The species is considered as the fundamental unit in evolutionary biology but this concept has been strongly debated since decades (de Quieroz 2007). One of the many definitions considers species as groups of organisms that are diagnosable distinct, cohesive or exclusive, and reproductively isolated (Harrison and Larson, 2014). Then, speciation can be defined as the origin of reproductive barriers among populations that permit the maintenance of genetic and phenotypic distinctiveness of these populations in geographical proximity (Seehausen et al., 2014). A long maintained idea about reproductive isolation is that it could only happen between populations that were spatially isolated (Boomsma and Nash, 2014) and therefore, allopatric speciation has been considered the most common and plausible mode (Coyne & Orr 2004, p. 142). However, sometimes adaptive radiations do not coincide with phylogeographic splits associated with hard dispersal barriers (Wollenberg-Valero et al 2019). In such cases, the consideration of intrinsic factors may help to explain the patterns of distribution and species divergence (Seehausen et al., 2014).

Speciation driven by intrinsic barriers often result from epistatic incompatibilities (Presgraves, 2010; Seehausen et al., 2014). Intrinsic reproductive barriers can be initiated either by the evolution of genetic incompatibilities, through genetic drift, as an indirect consequence of selection, or through genomic conflict (Seehausen et al., 2014). One proposed mechanism at the origin of reproductive barriers initiated by genomic conflict is the evolution of incompatibilities in co-functioning mitochondrial (mt) and nuclear genes (Gershoni et al. 2009; Lane 2009; Chou and Leu 2010; Burton and Barreto 2012; Crespi and Nosil 2013; Hill 2016).

In eukaryotes, mitochondria are predominantly inherited from the maternal gamete, even in unicellular organisms (Birky, 1995; Sato and Sato, 2013; Radzvilavicius et al., 2017). Combination of a nuclear genome with an alien mitochondrial can result in inter-specific hybrids that display so-called cytoplasmic incompatibilities (Grun, 1976). Such incompatibilities can create hybridization barriers and contribute to speciation (Hill 2015, 2016, 2019). This hypothesis predicts that the mt genotype will be functionally distinct between species and that introgression of mt genomes will be prevented by mitonuclear incompatibilities that arise when heterospecific mt and nuclear genes attempt to cofunction to enable aerobic respiration (Hill 2017, 2018, 2019; Sloan 2018).

At the phenotypic level, communication signals play an important role in species identification and may contribute to or even drive reproductive isolation (Bradbury and Vehrencamp 2011). In insects, chemical communication plays a major communicative role, and among the different compounds, cuticular compounds have been the focus of a huge amount of studies, especially in social insects (Blomquist and Bagnères 2010; Sprenger and Menzel 2020). Cuticular hydrocarbons are expressed on the insect's cuticle and are essential to prevent desiccation; secondarily, they have evolved communication roles such as nestmate/non-nestmate discrimination, information about caste, task specialization, etc. (Blomquist and Bagnères 2010; van Zweden, d'Ettore 2010). Cuticular hydrocarbons are also involved in discrimination between species (Blomquist & Bagnères 2010; Adams & Tsutsui 2020; Chung & Carrol 2015) and have been used as taxonomic tools, for instance, to detect morphologically similar but

chemically different lineages, as in some *Tetramorum* species (Schlick-Steiner et al. 2006), *Crematogaster levior* (Hartke et al. 2019) and to differentiate cryptic species in *Neoponera villosa* (Lucas et al. 2002).

The ant genus *Ectatomma* Smith, 1858 (Ectatomminae) currently includes 15 valid species with mainly Neotropical distribution (https://www.antweb.org/notLoggedIn.jsp; 1st of April 2020). Of all the described species, *Ectatomma ruidum* (Roger) is perhaps the most frequent and widely distributed. This species was originally described from different sites in Brazil, Cayenne (French Guiana) and Colombia (Roger, 1861), though its type locality was subsequently restricted to Colombia (Kugler and Brown, 1982). Currently, *E. ruidum* is known to be distributed from northern Mexico in the state of Tamaulipas to central Brazil, as well as in some Caribbean islands (Aguilar-Velasco, et al., 2016; https://www.antweb.org). *E. ruidum* occurs in a wide range of habitats from the sea level to up to 1600 m of elevation (Kugler and Brown 1982; Santamaría et al., 2009).

From an evolutionary point of view, *E. ruidum* is a particular and an interesting species because of its taxonomic complexity and because of the presence of heteroplasmy up to now known to occur only in certain populations. By analyzing external morphological traits and DNA sequences (nuclear and mitochondrial DNA), Aguilar-Velasco et al. (2016) identified four evolutionary lineages and a presumably hybrid population and assigned them to separate species. By performing next generation sequencing techniques, Meza et al. (2018) documented that the mitochondrial genome of the species *E. ruidum* spp. 3, 4 and the putative hybrids presented extensive heteroplasmy (i.e., the presence of a secondary mitochondrial lineage in a single individual, Magnacca and Brown 2010), which suggested these three lineages might correspond to only a single species with a complex evolutionary history.

At the phenotypic level, investigations highlighted large divergence between lineages in the *E. ruidum* species complex. Peña-Carrillo et al. (2021) documented broad divergence in cuticular hydrocarbons, the chemical cues that are used in nestmate discrimination. Additionally, acoustic traits of the the distress call differ significantly among certain lineages of this species (Chapter 3; Peña-Carillo et al. submitted).

The high phenotypic variation documented in the species complex unveiled traits that might have been the target of selection, and raises the question about the drivers of divergence involved in the species diversification of this ant complex, for which species boundaries remain unclear.

Integrating molecular and phenotypic data and using different methodologies is widely accepted as a valid and effective approach for delimiting species (Dayrat, 2005; Will et al. 2005; Schlick-Steiner et al., 2010; Pante et al., 2014). Integrative taxonomy has provided statistical rigor for species delimitation, validation of candidate species, and assignment of individual specimens to a given species group, and has improved the detection of cryptic diversity and the inference among species relationships (Leaché and Fujita, 2010; Schlick-Steiner et al., 2010; Edwards and Knowles, 2014; Leavitt et al., 2015).

Here, we have integrated genome-wide screening, barcoding sequences and cuticular hydrocarbon analyses to provide a robust test of species diversification within the *E. ruidum* species complex. We focus on populations that inhabit the southeast of Mexico and also referred to the influence of heteroplasmy on the speciation process. Specifically, we combine DNA sequence information generated with two reduced genome representation techniques, 3RAD (Bayona-Vásquez et al., 2019) and Ultra Conserved Elements (UCEs; Faircloth et al., 2012; Faircloth, 2017), together with data obtained both from a large data set of a sequence fragment of the Cytochrome oxidase I (cox1) mitochondrial DNA gene (Barcoding locus; Hebert et al., 2003) and cuticular hydrocarbon analyses.

Material and methods

Taxon sampling

Our taxon sampling varied according to the data source examined, though in all cases we included representative specimens of the four putative species that were proposed by Aguilar-Velasco et al. (2016) for the *E. ruidum* complex. For COI, we analysed a 626 bp fragment for 251 specimens assigned to *E. ruidum* and five of *Ectatomma gibbum*, with the latter being employed as outgroup. Of these sequences, 110 came from Aguilar-Velasco et al. (2016) and six from Meza Lázaro et al. (2018), whereas 135 sequences were newly generated. We excluded from the COI data set all potential nuclear mt paralogs that were detected based on the presence of internal stop codons or incorrect relationships (Song et al. 2014). We also excluded all the fast-evolving secondary mitochondrial copies detected by Meza-Lázaro et al. (2018) which were present in the populations assigned to E. *ruidum* sp. 3, *E. ruidum* sp. 4 and *E. ruidum* sp. 2 x 3.

For the 3RAD and UCE data, we generated sequences for 35 (34 of *E. ruidum*, one of *E. tuberculatum* as outgroup) and 14 (13 of *E. ruidum*, one of *E. gibbum* as outgroup) specimens, respectively.

For the cuticular hydrocarbon (CHC) analyses, we collected workers from several colonies per locality, and analyzed the chemical profiles of 5-10 individuals per colony (n = 211). A list with the specimens examined for the different data sources, their taxon assignment, geographic origin and DNA voucher and Genbank accession numbers are reported in the supplementary material (Table S1).

DNA sequencing protocols and assembly procedures

All specimens were preserved in 96% ethanol until they were processed in the laboratory for DNA sequencing. We extracted total genomic DNA from the whole specimens using the EZ-10 Spin Column Genomic DNA Minipreps kit (BIO BASIC®, Toronto, Canada) and quantified it using the Qubit fluorometer system (High Sensitivity DNA kit, Life Technologies Inc., Carlsbad, CA, USA). For COI amplification, we followed the procedure described in Aguilar-Velasco et al. (2016), and used 1:10 and 1:30 dilutions of DNA template.

We generated wide-genome data with the 3RAD method, which uses three restriction enzymes, two for the construction of dual-digest RADseq libraries and a third that cuts apart adapter-dimers formed by the phosphorylated adapter, thus increasing the efficiency of adapter ligation (Glenn et al., 2017). We digested 250 ng of the extracted genomic DNA for each sample using the restriction enzymes XbaI and EcoRI-HF (New England Biolabs; Beverly, MA, USA), which leave different sticky ends, and NheI (New England Biolabs; Beverly, MA, USA), used to digest iTru adapter dimers. We ligated double-stranded iTru R1 and iTru R2.1 adapters onto each fragment of DNA, and then ran a short PCR cycle (13 -15 cycles) with the iTru5 and iTru7 primers obtained from Adapterama (Glenn et al. 2016). The resulting libraries were size-selected in a broad window (200-800 bp) and sequenced at the GSL facilities at Berkeley

University, CA, USA. Libraries were sequenced using the 150 SRR HiSeq2500 Rapid, 10 pM, INDEX (124M Reads, 72% PhiX Aligned).

We used the process_radtags program implemented in the software pipeline Stacks 2.0 (Catchen et al., 2011; 2013) to demultiplex, clean and trim the sequence data. We discarded any read with an uncalled base (-c) or with low quality scores (-q). We processed demultiplexed reads using the software pipeline ipyrad Ethos v 0.6.19 (Eaton, 2014) on the Miztli supercomputer owned by the Dirección General de Cómputo y de Tecnologías de Información y Comunicación, National Autonomous University of Mexico (DGTIC, UNAM). Reads from each sample were clustered using the program VSEARCH v 2.0.3 (https://github.com/torognes/vsearch) and aligned with MUSCLE v 3.8.31 (Edgar, 2004). We used a range of clustering thresholds (80–98% in 2-3% increments) to assess the level of sequence similarity at which two fragments are considered homologous and avoid the potential of false heterozygous calls due to clustering of paralog (optimum clustering threshold; Eaton, 2014).

To assess the level of sequence similarity at which two fragments are considered homologous and avoid the potential for false heterozygous calls due to clustering of paralog (optimum clustering threshold; Eaton, 2014), we implemented the analysis described by Ilut et al. (2014). This approach minimizes the number of false homozygous (the splitting of a single locus into two) and false heterozygous (clustering of paralogs) in a clustering threshold series. We analyzed each reads library in a clustering threshold series ranging from 0.80 to 0.98%. The sequence rate of similarity converged at a value of 0.94% and used this value to produce matrices. However, preliminary phylogenetic analyses of the matrices yielded odd topologies. Therefore, we tested clustering threshold values from 94 to 99 in combination with different mean sample locus (15, 18, 20, 23, 25, 28, 30 and 33) and ran ML and Bayesian analyses using the matrices produced. We obtained the same odd topologies using low mean sample locus 15, 18, 20 and 23 regardless of the clustering threshold. We obtained, the most stable, coherent and well supported results using 98 as clustering threshold value. We therefore built our 3RAD matrices for subsequent analyses using 98 as clustering threshold value in combination with the following min_samples_locus values= 25, 28, 30, 33. Hereinafter, we name the 3RAD matrices according to the min-sample-locus (from 25 to 33) and the inclusion (io) or exclusion of the outgroup (eo).

For the UCE data, we obtained sequencing libraries following Branstetter et al.'s (2017) protocol. We fragmented up to 50 ng of input DNA to an average fragment distribution of 400-600 bp using a Qsonica Q800R (Qsonica LLC, Newton, CT) or a BioRuptor® Pico sonicator (Diagenode). Following DNA fragmentation, we constructed sequencing libraries using Kapa library preparation kits (Kapa Biosystems Inc., Wilmington, MA) and custom dual-indexing barcodes (Glenn et al., 2016). We evaluated amplification measuring DNA concentration. We purified PCR reactions 0.8 to 1.0X using Sera-Mag™ SpeedBeads (Thermo-Scientific, Waltham, MA, USA) (Rohland and Reich, 2012).

For UCE enrichment, we pooled 10-12 libraries at equimolar concentrations and adjusted pool concentrations to 147 ng/µl. For each enrichment, we used a total of 500 ng of DNA (3.4 µl

each pool). We enriched each pool using the bait set ('ant-specific hym-v2') (Branstetter et al., 2017), which has 9,446 custom-designed probes (MYcroarray, Inc.) targeting 2,524 UCE loci plus 452 baits targeting 16 commonly sequencedexons. The enriched library quality was verified using an Agilent TapeStation 2200 (Agilent Tech). We sent pools to either the University of Utah genomics core facility on an Illumina HiSeq 2500 instrument (PE150) or the Georgia Genomics Facility at the University of Georgia, Athens, USA, on an Illumina NextSeq v2300 (PE150). The sequencing facilities demultiplexed and converted raw data from BCl to FASTQ.

We used the program PHYLUCE version 1.5.0 and its subprograms (Faircloth, 2016) for assembly and alignment of the UCE data. We cleaned and trimmed raw reads using ILLUMIPROCESSOR (Faircloth, 2013). The cleaned and trimmed reads were assembled *de novo* using the program ABySS version 1.3.6 (Simpson et al., 2009). We mapped the assembled contigs to the hym-v2 bait database to identify individual UCE loci, remove paralogs and generate a list of shared UCE loci. We sorted out data by locus and aligned each one with the program MAFFT version 7.130b (Katoh and Standley, 2013). The resulting alignments were filtered and trimmed with the program Gblocks version 0.91b (Castresana, 2000; Talavera and Castresana, 2007), and removed loci that had data for less than 100% taxa. We used the 100% filtered matrix for most analyses, but evaluated the number of loci that were present in matrices filtered to have 75, 80, 90, 95 % taxon occupancy (Table X).

We also followed the Tutorial II: Phasing UCE data (https://phyluce.readthedocs.io/en/latest/tutorial-two.html) to call for SNPs. This tutorial derives from the procedure described by Andermann et al. (2018) (https://doi.org/10.1093/sysbio/syy039). The above workflow requires of individual-specific "reference" that can be aligned against raw reads. We used edge-trimmed exploded alignments as reference contigs, aligned raw reads to them, and used the exploded alignments and raw reads to phase the data. We exploded the edge trimmed alignments to create separate FASTA files for each sample using phyluce_align_explode_alignments. We used bwa mem to map the fastq read files against the contig reference database for each sample. We sorted the reads within each bam file into two separates bam files using phyluce_snp_phase_uces. We built three final matrices based on filtering UCE loci for three different levels of taxon occupancy (% of taxa required to be present in each locus): 85%, 90%, and 100%. UCE matrices included four males, since hymenopteran males are generally haploid. Male samples were phased in the same way than worker (female) samples, from which we only expected homozygous loci.

Phylogenetic analyses and haplotype network reconstruction

3RAD. We conducted a Bayesian phylogenetic analysis with the program ExaBayes v.1.5 (Aberer et al., 2014). Analyses were run using the generalized time-reversible model (GTR+G) and five independent MCMC chains of 1,000,000 generations each. The first 100,000 trees (10%) were discarded as burn-in for each MCMC run prior convergence (i.e. when maximum discrepancies across chains <0.1). We assessed burn-in, convergence among runs and run performance examining the resulting parameter files with the program TRACER v1.6.0 [S16] and, we computed consensus trees using the consense utility implemented in ExaBayes.

UCEs. We performed a ML three for which we conducted concatenated matrix analyses with the program RAxML v8 (Stamatakis, 2014), and performed the best tree plus rapid bootstrap search ('-f a' option) and 200 bootstrap replicates. We used the GTR+Γ model of sequence evolution (best tree and bootstrap searches) and analyzed all the concatenated matrices partitioned by locus. We selected the best scheme partition with the program PartitionFinder version 2 (Lanfear et al., 2017) based on the Bayesian Information Criterion (BIC) and the rcluster option, which is more appropriate for larger data sets.

For species tree estimation, we generated gene trees for the matrix with 100% phased loci alignments with the program RaxML with 200 bootstrap replicates. We calculated species tree support with 200 multi-locus bootstrap replicates (Seo, 2008). All 3RAD and UCEs phylogenetic analyses were run on the CIPRES Web Portal (Miller et al., 2010).

COI. With the mtDNA sequences we constructed a parsimony network using the TCS method (Clement et al. 2002). This methodology allowed us to visualize genelogical information at shallow divergence levels; additionally TCS recovers accurate population-scale phylogeographic patterns even when genetic differentitation is low (Perez-Moreno et al. 2017). The haplotype network was generated with the software PopArt (Leigh and Bryant 2015).

Demographic and species delineation analyses

3RAD. To test if some individuals might be of hybrid origin and assigned to different genetic clusters, we used the program STRUCTURE v.2.3.4 (Pritchard et al., 2000) for the 3RAD data, which is implemented in the ipyrad.analysis toolkit (https://ipyrad.readthedocs.io/analysis.html). We used the 98_33 matrix (clustering threshold=98 and min_sample_locus = 33, without outgroup) to perform an individual-based Bayesian clustering analysis. We used an admixture model with correlated frequencies and assessed values of population differentiation (K) in 15 independent runs for K between 2 and 8. All runs were conducted for 1 million MCMC iterations with a burn-in of 250,000. Figures were generated based on the iterations with the highest posterior probability. We selected the K value based on the highest average value of the likelihood [LnP(D)] obtained (Evanno et al. 2005).

Genetic distances

We calculated genetic distances for the four data sets: two for the reduced genome representation techniques (3RAD and UCEs) and two for the COI sequences (primary and secondary haplotype matrices, see Meza-Lázaro et al. 2018). COI matrices were computed using population average distance per species, and in the case of the 3RAD sequences genetic distances were only calculated using all variable sites per locus (matrix 98: 33). All genetic distances were calculated using the software Mega X (Kumar et al. 2018).

Chemical distances based on cuticular hydrocarbon analyses

A thoroughly analysis of the cuticular hydrocarbons of *E. ruidum* ants was recently published (Peña-Carrillo et al. 2020) of which we extracted the chemical data (Table S1). In the current analysis, we expanded population samples and included two new populations, Puerto Morelos (*E. ruidum* sp. 2) and Cuatode (*E. ruidum* sp. 4) which were not included in the previous analyses. Cuticular extractions were performed as in Peña-Carrillo et al. (2020) (see Table S1).

We calculated the chemical distances between individuals by using Bray-Curtis distances as the dissimilarity measures based on the relative abundances of the simplified chemical profile of the ants (i.e., obtained by merging the percentages of hydrocarbons with the same carbon-chain length by class, see Peña-Carrillo et al. 2020). Then, we calculated the chemical distances between populations (centroids) and used them to obtain a hierarchical cluster dendrogram using the Primer 6 & Permanova + software (Primer – E Ltd).

We performed Mantel tests based on Pearson's product-moment correlation with a maximum of 999 permutations to correlate chemical, genetic and geographic distances of populations of the different species of the *E. ruidum* complex. Mantel tests were performed with the Vegan package (Oksanen 2013) in R studio (Version 3.5.2).

Results

Genome-wide data

The samples included in the 3RAD data set had from 75,193 to 100,7069 reads, and the generated matrices from 896 to 8,731 loci. The outgroup, *E. tuberculatum*, shared with the ingroup from 1,764 to 5,496 loci (98-15-io and 94-15-io matrices, respectively). The generated matrices derived from the unphased UCE data, on the other hand, had from 642 to 2,196 loci and from 508,859 to 1,817,455 characters in the matrices with 100 and 75% taxon occupancy, respectively. The phased UCE matrices had considerably less loci than the matrices with unphased data (85%= 737 loci, 100%= 390 loci). However, phased matrices contained more parsimoniously informative sites. SNP codification as a binary matrix for the UCE data showed the presence of heterozygous loci in the male samples. Those heterozygous sites actually exist in the nucleotide alignments.

Phylogenetic analyses and haplotype network reconstruction

In general, our 3RAD data phylogenetic analysis yielded highly supported topologies (Figure 1). The Exabayes analyses recovered with significant support the following six clades: 1) *E. ruidum* sp. 1, two separate clades with samples assigned to *E. ruidum* sp. 2 (hereafter referred as, sp. 2A and sp. 2B), one for *E. ruidum* sp. 3, *E. ruidum* sp. 4, and a clade of *E. ruidum* sp. 6, formed by a sample from Pinotepa Lagunilla, Oaxaca and another one from Fogos, Guerrero, Mexico (Figure 1). One of the clades formed by samples assigned to *E. ruidum* sp. 2A was exclusively composed by specimens from Oaxaca, Mexico, ranging from the states of Michoacan, Jalisco, Nayarit and the northeast in the state of Tamaulipas. The second clade (*E. ruidum* sp. 2B) was composed by specimens from Puerto Morelos, Quintana Roo, Mexico to Central and South America. The clades of *E. ruidum* sp.3, sp. 4 and sp. 6 clades were also strongly supported (Figure 1).

Similarly, the ML analysis from UCE data recovered four putative species (*E. ruidum* sp. 1, 2, 3, 4) as monophyletic, as well as a clade formed by samples from Pinotepa Lagunilla, Oaxaca and Guerrero (*E. ruidum* sp. 6) (Figure 2).

With respect to the haplotype network (Figure 3), it displayed evident genetic structuring at species level, that is, all samples from *E. ruidum* sp. 1 were separated from the rest of species by a number of mutations. Samples of *E. ruidum* sp. 2 had a high number of haplotype diversity; samples belonging to the clade 2A were mainly sharing the same hypothetic haplotype; in general, most of samples from this groups proceeded from Mexico and for some groups evident genetic substructuring was observed (e.g. samples from circles 11 and 12). Similarly, samples from the clade 2B shared the same hypothetic haplotype and were grouped together. Some samples also identified as *E. ruidum* sp. 2 were separated from the groups 2A and 2B (e.g. circles 5 and 20-22), but in any case they remained separated from the rest of species by several mutations. Samples of *E. ruidum* spp. 3, 4 and 6 were separated from the rest by several mutations, but, they seemed to be very close to each other. *E. ruidum* sp. 5 was separated from the rest by a number of mutations as *E. gibbum* the sister species used as a reference (Figure 3).

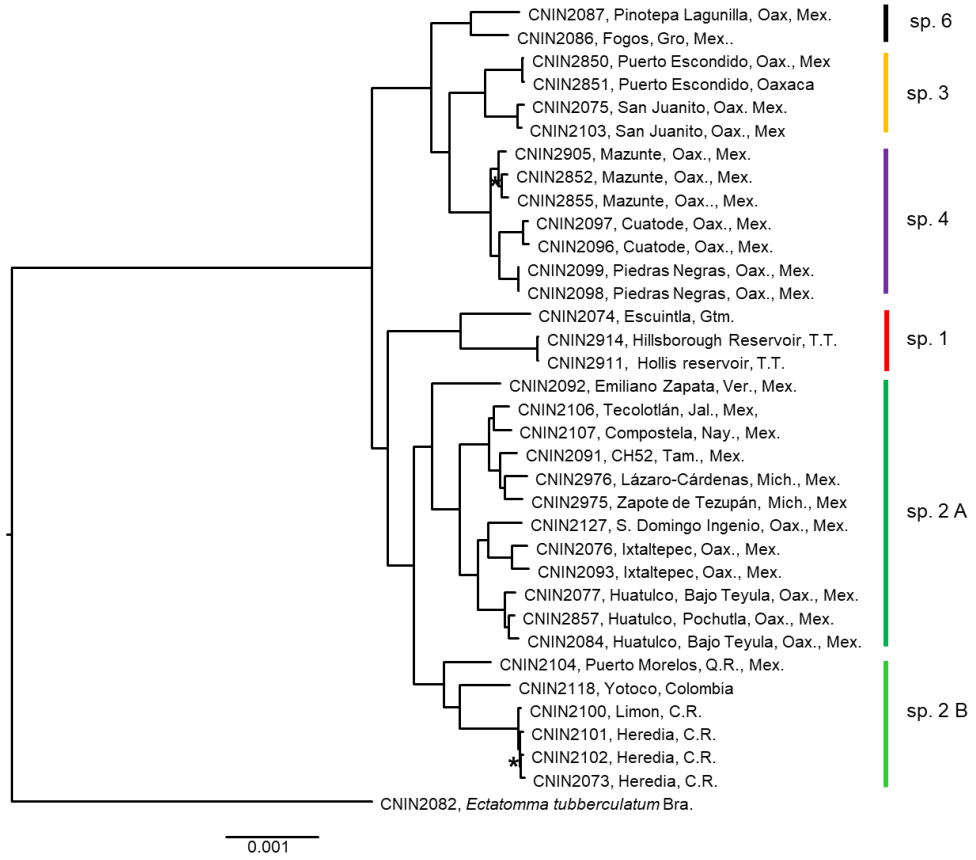

Figure 1. Bayesian concatenated tree constructed with 3RADseq data. Asterisks next to branches indicate support of ± 0.8 and for the rest of branches support values were equal to 1. On the right side of branches the different species of *E. ruidum* are indicated; the sister species *Ectatomma tuberculatum* was included as external group.

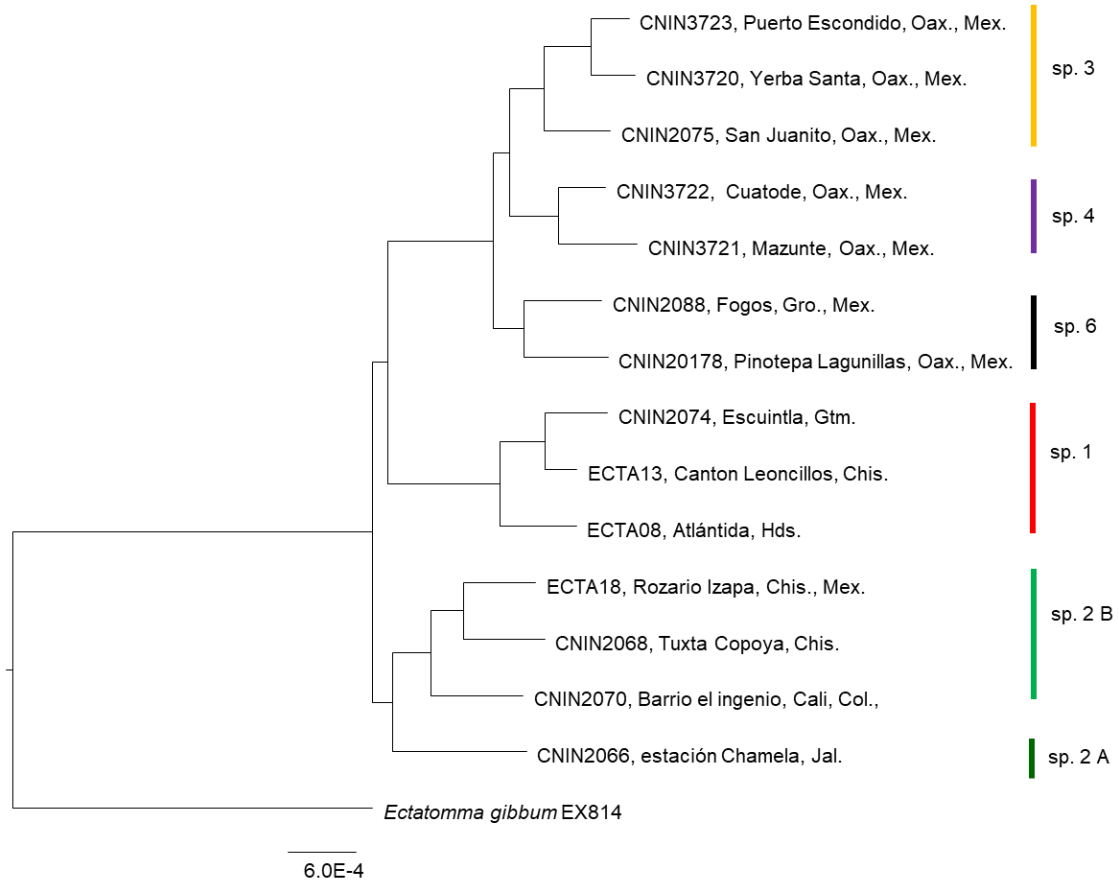

Figure 2. Maximum likelihood tree constructed with UCE data. On the right side of branches the different species of *E. ruidum* are signaled; all branches had a 100% of support and the sister species *E. gibbum* was included as external group.

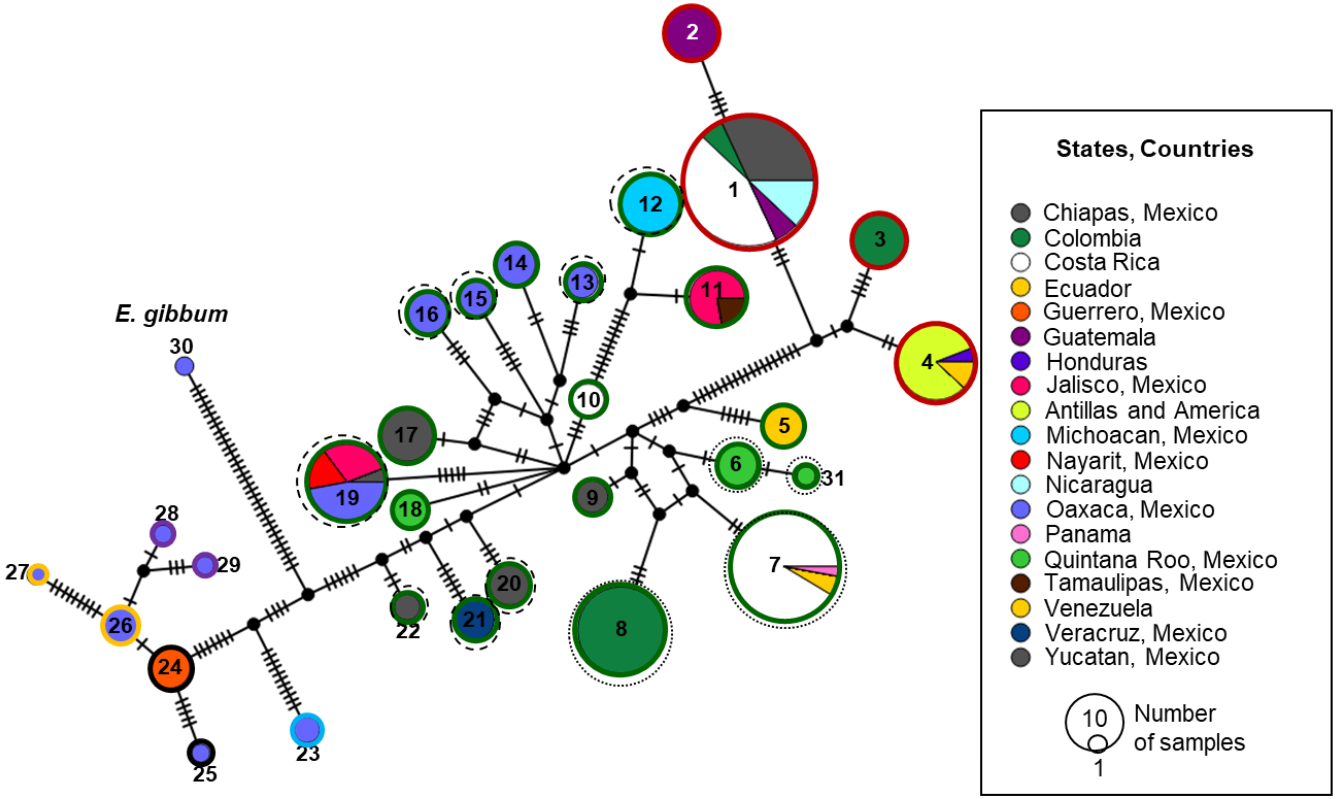

Figure 3. TCS parsimony mtDNA haplotype network. Legend denotes the different countries were samples were collected, each circle represents a group samples with the same haplotype and the number them inside indicates its ID (see Table xx. Samples information). Relative sizes of circles indicate the number samples included in each one and outlines of circles indicate to which species of *E. ruidum* they belong: sp. 1 red outline, sp. 2 green, sp. 3 yellow, sp. 4 purple, sp. 5 sky blue, sp. 6 black. Secondary outlines among samples of the *E. ruidum* sp. 2 indicate samples from the two clades: secondary dashed lines represent samples from the clade 2A, while pointed secondary outlines samples from the clade 2B; samples lacking of secondary outlines were not represented in wide genomic analyses. Black lines connecting haplotypes represent the number of mutations that occurred among the groups and black dots represent hypothetical haplotypes (missing intermediate sequences). The sister species *E. gibbum* was included as a reference.

Demographic and species delineation analyses

The STRUCTURE analyses displayed the highest value of Ln P (D) at K = 7 and the Evanno method peaked at K = 3, indicating strong support for three clusters (Figure 4). In general, the *E. ruidum* sp. 4 was mostly supported at different K. At k = 2 a cluster containing the members of *E. ruidum* sp. 1 and *E. ruidum* sp. 2 and a second with those of *E. ruidum* sp. 3, *E. ruidum* sp. 4 and *E. ruidum* sp. 2 x 3 were recovered. At K = 3, we recovered the cluster of *E. ruidum* sp. 2 only with samples from localities situated along the Mexican Pacific coast, a second exclusively composed of samples of *E. ruidum* sp. 4 and a third one with an admixture of genes of samples assigned to *E. ruidum* sp. 1, *E. ruidum* sp. 2 from the Mexican Caribbean and Colombia, *E. ruidum* sp. 3 and *E. ruidum* sp. 2 x 3. At K = 4, the latter cluster was further divided into two, one having admixing genes between samples of the former two taxa and the second one of the latter two. At subsequent K values, *E. ruidum* sp. 2 x 3 and the samples of *E. ruidum* sp. 1 from Trinidad and Tobago in the Caribbean were each recovered as exclusive clusters, whereas at K = 7 the samples of *E. ruidum* sp. 3 and the single one of *E. ruidm* sp. 1 from Guatemala also were recovered separately (Figure 4).

Chemical distances based on cuticular hydrocarbon analyses

The cluster analysis on the cuticular hydrocarbons yielded two main branches (Figure 5). One belonged to the hydrocarbon profiles of *E. ruidum* sp. 1 and was largely separated from the second one, which contained two clusters, one corresponding to *E. ruidum sp.* 2 and the second to *E. ruidum* sp. 3 and 4. The branch of *E. ruidum* sp. 2 showed that the cuticular hydrocarbon proportions of the populations of Cali (Colombia) and Puerto Morelos (Mexico) were more similar to each other than those of the population of Coyula (Mexico) (Figure 5). The cluster corresponding to *E. ruidum* sp. 3 and sp. 4, highlighted a large differentiation between populations of *E. ruidum* sp. 4 (Figure 5).

Chemical distances were strongly and significantly, positively correlated with the genetic distances obtained with distances calculated with both the 3RAD and UCEs techniques (Mantel test, for 3RAD: r = 0.855, P = 0.001; for UCEs: r = 0.876, P = 0.016). Similarly, there was a strong and significant, positive correlation between chemical and genetic distances when the latter were calculated with the primary haplotypes of COI sequences (r = 0.847, P = 0.004). On the contrary, the genetic distances from the secondary COI haplotypes were not correlated with chemical distances (r = 0.343, P = 0.073) (Supplementary figure 1).

There was no significant correlation between geographic and chemical distances (r = -0.022, P = 0.354), nor between geographic and genetic distances calculated from 3RAD (r = 0.322, P = 0.141), UCEs (r = 0.052, P = 0.225), COI primary haplotypes (r = 0.217, P = 0.205) and COI secondary haplotypes (r = -0.258, P = 0.826).

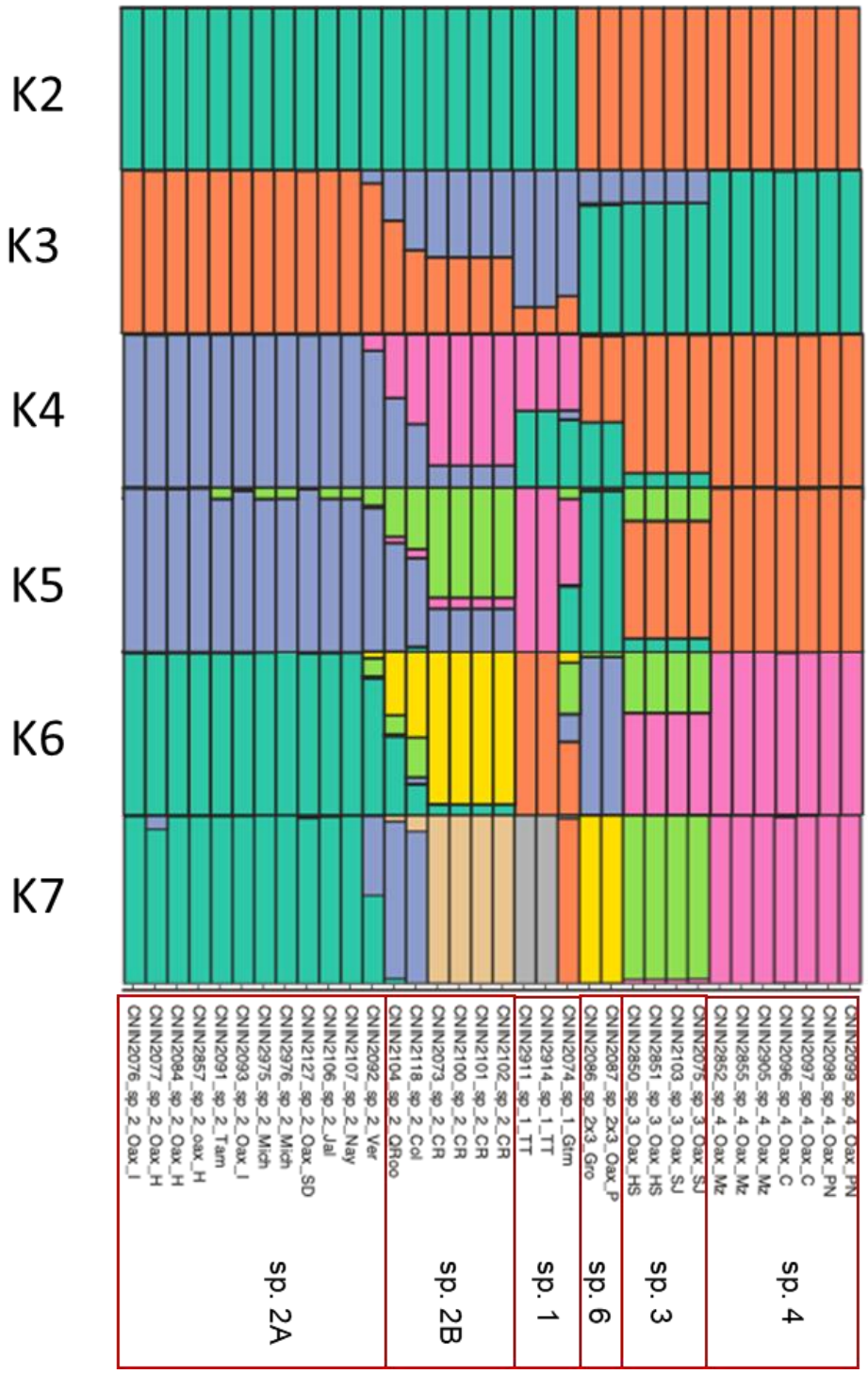

Figure 4. Structure analysis. including haplotypes of the species *E. ruidum* sp. 1-4 and *E. ruidum* sp. 6. According to the Evanno method K = 3, indicates the strongest support.

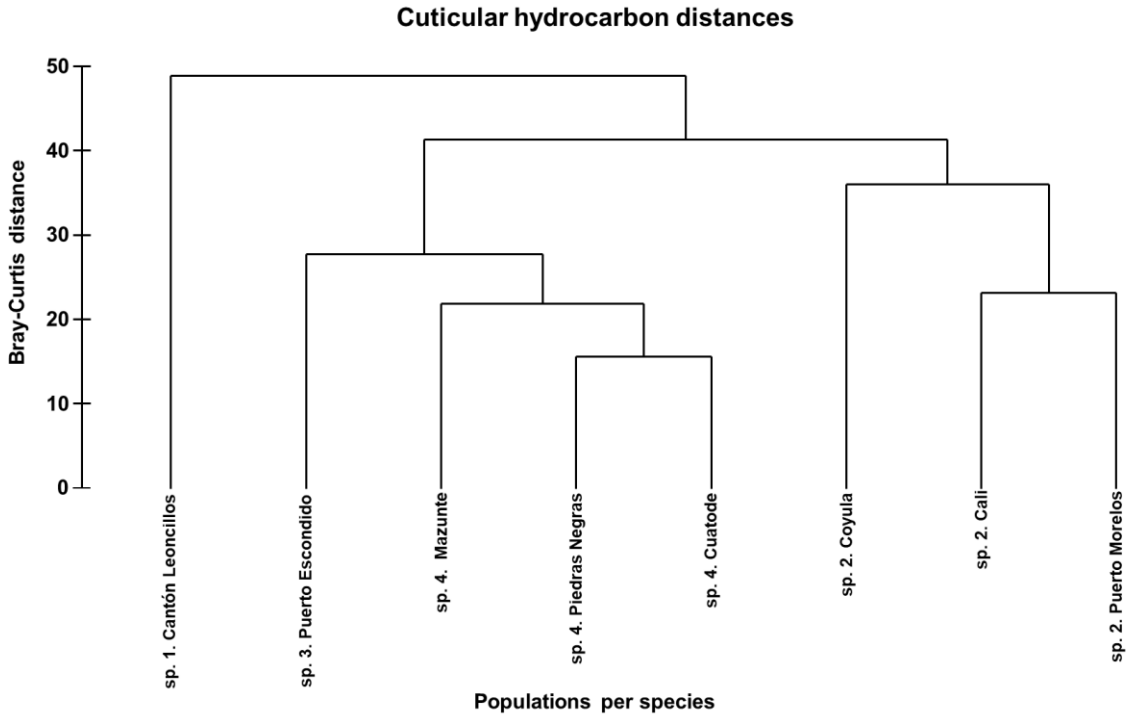

Figure 5. Hierarchical cluster dendrogram constructed from the dataset of cuticular hydrocarbons (simplified chemical profile) of some populations of the *E. ruidum* species complex.

Discussion

Species delimitation

The current molecular-based analyses (COI, 3RAD and UCEs data) and cuticular hydrocarbon variation strongly support the separation of the species *E. ruidum* spp. 1 and 2 and 3RAD, UCE and hydrocarbon profiles strongly suggested the separation of E. ruidum spp. 3 and 4 previously proposed by Aguilar-Velasco et al. (2016). Additionally, our results suggest that the complex is composed of at least five distinct species, co-occurring in the lowlands of the Southern Sierra Madre in Mexico (a mountain range in southern Mexico which extends from southern Michoacan state to the Isthmus of Tehuantepec in Easter Oaxaca state, Prebus, 2020). Moreover, all analyses suggest that diversity within the complex might be even higher, hence, offering evidence that the lowlands of the Southern Sierra Madre have been a hotspot for the diversification of the *E. ruidum* species complex, probably in the absence of physical barriers.

We clearly recovered *E. ruidum* sp. 1 as a well differentiated species regardless the data set used (genetic or chemical). 3RAD and UCE analyses consistently recovered *E. ruidum* sp 1 as a strongly supported clade, although *E. ruidum* sp. 1 was poorly represented in both datasets. COI-based network also suggested that *E. ruidum* sp. 1 has genetic structure, although haplogroups are not highly divergent between each other. Mirroring molecular results, cuticular hydrocarbon profiles supported the differentiation of *E. ruidum* sp. 1 with respect to the other species.

About *E. ruidum* sp. 2, genetic analyses identified it as a well-supported monophyletic group composed by two geographically differentiated clades, which might represent additional lineages. RAD and UCE analyses yielded the clade 2B as monophyletic with respect to the clade 2A, however, the limited sampling employed for UCEs suggests this separation should be corroborated. Similarly, COI-based network also suggested the existence of two different groups of *E. ruidum* sp. 2. But, given that the network included additional samples not present in the previous genome wide analyses (3RAD and UCEs), the haplotype network weakened the exclusivity of the clade 2A suggested as exclusive to Mexico in 3RAD analyses (i.e., samples from Costa Rica close to samples from Jalisco, Mex.), and suggesteds the existence of additional population structure. The cuticular hydrocarbon profiles of *E. ruidum* sp. 2 were also clearly differentiated from all other species and the differentiation of chemical profiles of populations of Cali and Puerto Morelos with respect to those of Coyula supported the existence of two different groups in *E. ruidum* sp. 2. Therefore genetic and chemical data offer evidence for two different clades within *E. ruidum* sp2 (named spp. 2A and 2B in the present work).

The species *E. ruidum* sp. 3 and *E. ruidum* sp. 4 that were previously considered as genetically the same species (Aguilar-Velasco et al., 2016; Meza-Lázaro et al. 2018), in this study were also supported as two different but closely related species by the current genetic and chemical data. In the previous analyses by Aguilar-Velasco et al. (2016) and Meza-Lázaro et al. (2018), haplotype genealogies failed to identify them as reciprocally monophyletic, reason why they were reported as a single evolutionary lineage. In the current study, we did not rely only on monophyly to delimit species, since species can be non-monophyletic because of several causes

such as introgression, incomplete lineage sorting or low phylogenetic signal (Funk and Omland, 2003). However, our low sampling precluded a robust monophyly evaluation.

Our coalescent-based species delimitation analyses (phylogenies) allowed us to identify independent evolutionary lineages with statistical rigor, even when the sampling was low. We found consistent support for *E. ruidum* sp. 3 and *E. ruidum* sp. 4 as different species. In addition we found evidence for the presence of a sixth species that includes the putative hybrid population proposed by Aguilar et al. (2016) and one population from Guerrero, Mexico that was previously identified as *E. ruidum* sp. 3 due do the presence of heteroplasmy (Meza-Lázaro et al., 2018). Recently, species delimitations methods based on the multiple species coalescent were criticized for delimiting populations instead of species in cases where metapopulations exhibited high geographical structure (Sukumaran and Knowles, 2017; Chambers and Hills, 2020). However, in the current study the analysis of chemical profiles allowed us to confirm the genetic delimitation at phenotypic level.

Species diversification

Species diversification and morphological evolution are shaped by different processes, for example, niche adaptation (Evans et al., 2009; Ricklefs, 2010), developmental constraints (Porto et al., 2015), interspecific competition (Rabosky, 2013; Price et al., 2014) and predation (Langerhans et al., 2004 and Arbuckle and Speed, 2015). In some groups of ants speciation has been linked to divergence in traits linked to communication systems and social behavior (Brodetzki et al., 2019). For the *E. ruidum* species complex, our results suggest that cuticular hydrocarbons have been involved in the diversification of species.

Our results showed that cuticular hydrocarbon distances mirrored the phylogenetic relations of the *E. ruidum* species complex. That is, cuticular hydrocarbon variation supported the genetic differentiation of *E. ruidum* sp. 1 with respect to the other species, as well as the intraspecific variation between different populations of *E. ruidum* sp. 2. Also, the (relatively small) variation in the cuticular hydrocarbon profiles of *E. ruidum* sp. 3 and sp. 4 matched the close phylogenetic relationships between these two species. On the other side, we did not find support for the hypothesis of isolation by distance (Wright, 1943); geographic and genetic distances (3RAD, UCEs, COI) were not correlated. Similarly, cuticular chemical distances did not correlate with geographic distances. Indeed, Peña-Carrillo et al. (2021) showed that the cuticular hydrocarbon profiles of the geographically close populations of *E. ruidum* sp. 4 (Mazunte and Piedras Negras, separated by less than 20 km) differed significantly.

The chemical components of the cuticle of insects have been proved to be correlated with genetic distances in termites (Dronnet et al., 2006), stingless bees (Leonhardt et al., 2013) and ants (Hartke et al., 2019). For instance, Hartke et al. (2019) studied the species status of the neotropical ant *Camponotus femoratus* and *Crematogaster levior*, two species which live in mutualistic (parabiotic) association. Although these two ant species share the nest and exhibit tolerant interactions, they exhibit species-specific cuticular chemical profiles; in addition, two different chemotypes were discovered in both species (Emery and Tsutsui, 2013; Menzel et al., 2014). Genetic analyses confirmed the existence of two lineages, i.e., cryptic species (Hartke et al 2019). That is, the existence of intraspecific lineages within both species was supported

by both chemical and genetic data. Similarly, in the *E. ruidum* species complex, the fact that for all species chemical and genetic distances (3RAD, UCEs and COI) were highly correlated, indicates that cuticular hydrocarbons are effective taxonomic tools and suggests that they might have directly contributed to the phylogenetic divergence.

According to Menzel et al. (2017), the abundance of hydrocarbons should be under stronger selection than the simple presence/absence of hydrocarbons and their quantitative variation may quickly respond to selective pressures. By analyzing the quantitative chemical profile of the ants of the *E. ruidum* species complex (i.e., analyses were based on cuticular hydrocarbon proportions rather than on their presence/absence), Peña-Carrillo et al. (2021) documented that the hydrocarbon profiles of ants might have largely diverged both between and within the species complex. Peña-Carrillo et al (2021) suggested that the divergence might be associated to role of cuticular hydrocarbons in nestmate/non-nestmate communication, and to evidence for competitive interactions between colonies within the complex (intraspecific thievery). Besides their function in nestmate/non-nestmate discrimination (Van Zweden and d'Ettorre, 2010), cuticular hydrocarbons mediate mating interactions and may play a role in pre-mating isolation (Savarit et al., 1999; Smadja et al., 2009; Snellings et al., 2018). For different insect taxa, a role of cuticular hydrocarbons in speciation has been suggested (Grillet et al., 2012; Schwander et al., 2013; Chung and Carrol, 2015).

Finally, the heteroplasmy found in *E. ruidum* sp. 3, 4, and 6 might reflect other factors involved in the diversification of the complex. For example, it may represent the existence of incomplete reproductive isolation between heteroplasmic populations and consequently, reflect their recent speciation. In *Cataglyphis* ants, Eyer and Hefetz (2018) found heteroplasmic individuals in populations from geographic areas where several species co-occur. They also found genetic flow among those sympatric populations of different *Cataglyphis* species. Therefore, they proposed that hybridization could be a factor linked to the origin of the heteroplasmic individuals. In our case, the demographic analysis performed with 3RAD and UCE did not offer conclusive evidence for the existence of hybrid populations, however, the demographic analysis was based on a limited sampling, where putative hybrids would have not been represented enough in our data. Further studies including population genetics and behavioral tests based on a larger sampling would help to clarify if the presence of heteroplasmy in these three species may be linked to hybridization or if a source of a secondary mitochondrial genome could exist.

Further directions

Our study showed strong support for the existence of at least five species of the *E. ruidum* species complex. However, studies based on an extensive sampling and aimed to test for the existence of gene flow among populations of the different putative species are encouraged. Furthermore, population genetic studies will contribute to know if the most supported species, *E. ruidum* sp. 1 and sp. 2 (up to now supported by morphological, chemical and genetic characters) are reproductively isolated. For the other species putative (*E. ruidum* spp. 3-6), a more extensive sampling combined with the study of different phenotypic traits will help to obtain conclusive evidence to support their separation and to understand the selection pressures linked to their divergence.

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Supplementary table 1. Sampling ID and information about the *E. ruidum* species complex.

Species	Country	Sate	Locality	Coordinates		Type of analysis				Voucher name for DNA extraction	Number in haplotype network
1	Colombia	San Andrés Island	San Andrés	12.58	-81.695			COI		POTEAUX1,6,8	1
1	Colombia	Meta	El Caduceo, San Martin.	3.665364	-73.66001			COI		Erui_Colomb_19-22, 24, CNIN3019-3021, CNIN-2759	3
1	Costa Rica	Guanacaste	Costa Rica, Guanacaste, Santa Rosa Nancite	10.8	-85.65			COI			1
1	Costa Rica	Guanacaste	Costa Rica, Guanacaste, Santa Rosa	10.838	-85.619			COI			1
1	Costa Rica	Guanacaste	Costa Rica, Cacao-1044					COI			1
1	Costa Rica	Guanacaste	Costa Rica, Nancite	10.807	-85.699			COI			1
1	Costa Rica	Guanacaste	Costa Rica, Santa Rosa-Nancite,	10.8	-85.65			COI			1
1	Costa Rica	Puntarenas	Aprox 3 Km Curré	8.9754	-83.3036			COI			1
1	Costa Rica	Guanacaste	Costa Rica, Guanacaste, Junquillal	10.969	-85.685			COI			1
1	France	Guadeloupe	Basse-Terre	16.218	-61.599			COI		EruiPOTEAUX9	4
1	France	Guadeloupe	Grande-Terre	16.208	-61.507			COI		POTEAUX10-12	4
1	France	Guadeloupe	Grande-Terre	16.235	-61.536			COI		POTEAUX13-15, CNIN-3022	4
1	France	Marie Galante	Grand Bourg	15.883	-61.311			COI		CNIN2949-50	4
1	France	Martinique	Fort-de-France	14.601	-61.067			COI		POTEAUX2,3,4	4
1	Guatemala	Esquintla	Carretera Puerto Quetzal en cruce con Obrero	14.52050	-90.47180	RAD	UCE			CNIN2074_sp_1_Gtm	
1	Guatemala	Escuintla	Carr. Puerto Quetzal, cruce con Obero,	14.00868	90.78649			COI		CNIN-2000-01,2081, 2156-63	2
1	Honduras	Atlántida	7km SSW Tela				UCE			ECTA08_sp_1_Hds	-

1	Honduras	Atlántida	2km SSW Tela.	15.764282	-87.45171			COI		CNIN-2002, 2947	4
1	Mexico	Chiapas	Cantón Leoncillos	14.768888	-91.59722		UCE			ECTA13_sp_1_Chis	-
1	Mexico	Chiapas	Ecosur Instalaciones	14.88728	-92.2867			COI		CNIN-3016-18	1
1	Mexico	Chiapas	México, Chiapas, Tapachula, Cantón Leoncillos	14.7689	-92.403				CHC		
1	Mexico	Chiapas	México, Chiapas, Tapachula, Cantón Leoncillos	14.7689	-92.403			COI		CNIN-1748-50, 52-54, 56-57, CLeoncillos113-116	1
1	Nicaragua	Managua						COI		CNIN-2941-46	1
1	Trinidad y Tobago	Trinidad	Holllis Res			RAD				CNIN2911_sp_1_TT	-
1	Trinidad y Tobago	Tobago	Hillsborough			RAD				CNIN2914_sp_1_TT	-
1	Trinidad y Tobago	Trinidad	Union	10.307	-61.108			COI		POTEAUX5	4
1	Trinidad y Tobago	Trinidad	Victoria Mayaro Reserve	10.198	-61.061			COI		CNIN2950Tri	4
1	Trinidad y Tobago	Trinidad	Victoria Mayaro Reserve	10.137	-61.081			COI			4
1	Venezuela	Acevedo	Caucagua	10.296667	-66.38527			COI		AGI56835, W1LCO, W2LCO	4
2	Colombia	Cali	Nido 1, Barrio El ingeniero	3.38674	-76.53150		UCE			CNIN2070_sp_2_Col	-
2	Colombia	Valle del Cauca	Yotoco			RAD				CNIN2118_sp_2_Col	-
2	Colombia	Valle del Cauca	Cali, CIAT	3.503955	-76.35933			COI		CNIN-2716-20	8
2	Colombia	Valle del Cauca	Cali, Finca Palmira	3.553471	-76.199			COI		CNIN-2721-25	8
2	Colombia	Valle del Cauca	Cali, Universidad del Valle	3.377500	-76.53111				CHC		
2	Colombia	Valle del Cauca	Cali, Barrio Ingenio	3.388403	-76.52896			COI		CNIN-2726-30	8
2	Colombia	Valle del Cauca	Yotoco	3.881472	-76.437			COI		CNIN-2769-71, Erui2CB225LCOCol	8
2	Colombia	Valle del Cauca	Buena Ventura	3.899	-77.04063			COI		CNIN-2753-55	8
2	Costa Rica	Heredia	Estación La Selva	104.29000	-84.00900	RAD				CNIN2101_sp_2_CR	-
2	Costa Rica	Heredia	Puerto Viejo de Sarapiquí, Muelle			RAD				CNIN2102_sp_2_CR	-

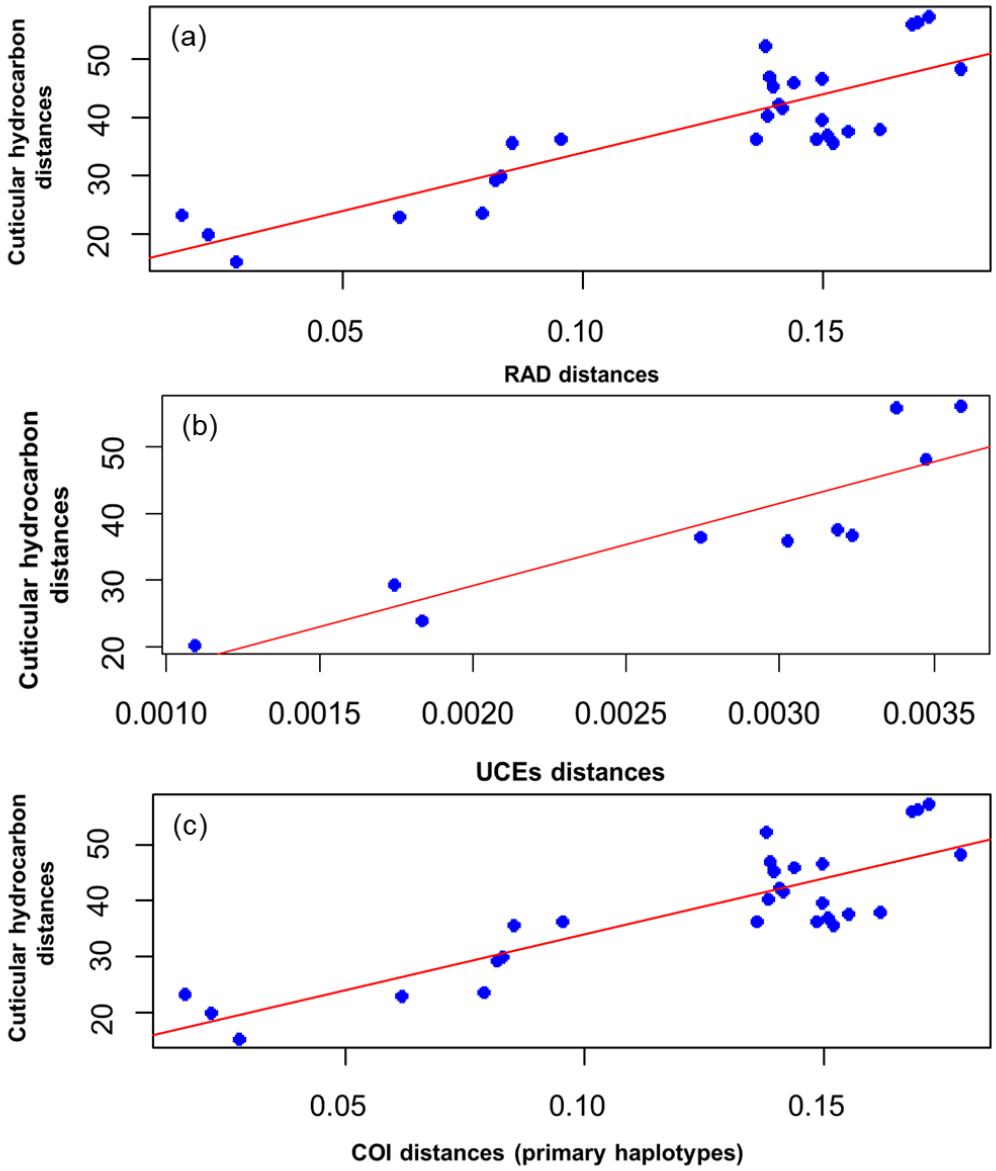
2	Costa Rica	Heredia	Puerto Viejo, Saquiquí, orilla de carretera			RAD				CNIN2073_sp_2_CR	-
2	Costa Rica	Heredia	Heredia, Est. Biol. La Selva	10.43	-84.007			COI		CNIN-1822, CR4-1,4-2,1, 2, 3	7
2	Costa Rica	Heredia	Puerto Viejo de Sarapiquí					COI		CNIN-2692-2700	7
2	Costa Rica	Heredia	Heredia, Est. Biol. La Selva,	10.429	-84.009			COI		CNIN-1996-98	7
2	Costa Rica	Limón	Puerto Viejo de Talamanca			RAD				CNIN2100_sp_2_CR	-
2	Costa Rica	Limón	Parque Nal. Cahuita	9.734	-82.828			COI		CNIN-1807, 08	7
2	Costa Rica	Limón	Puerto Viejo de Talamanca					COI		CNIN-2706-2710	7
2	Costa Rica	Limón	Manzanillo					COI		CNIN-2701-05	7
2	Costa Rica	Puntarenas	Parque nacional Carara					COI		CNIN-2731,32,35	10
2	Ecuador	Imbabura		0.8685	-78.46361			COI		ER8ALCO	5
2	Ecuador	Cantón Esmeraldas	Ecuador, Esmeraldas	0.86825	-79.65402			COI		ER9ALCO	5
2	Ecuador	Cantón Esmeraldas	Ecuador, Esmeraldas,	0.530528	-79.53836			COI		ER10ALCO	5
2	Ecuador	Cantón Esmeraldas	Ecuador, Esmeraldas	0.303	-79.45716			COI		ER15ALCO	5
2	Ecuador	Cantón Esmeraldas	Ecuador, Esmeraldas	0.919306	-79.66513			COI		ER16ALCO	5
2	Ecuador	Pichincha	Ecuador, Pichincha	0.124556	-79.23802			COI		ER17ALCO	7
2	Ecuador	Santa Elena	Ecuador, Santa Elena					COI		ER13ALCO	7
2	Mexico	Tamaulipas	CH52			RAD				CNIN2091_sp_2_Tam	-
2	Mexico	Tamaulipas	Gómez Farías	23.0359	-99.101			COI		CNIN-2090-91	11
2	Mexico	Veracruz	Emiliano Zapata, SSE			RAD				CNIN2092_sp_2_Ver	-
2	Mexico	Oaxaca	Ixtaltepec, Mazahua	16.63300	-94.95000	RAD				CNIN2093_sp_2_Oax_I	-
2	Mexico	Oaxaca	Ixtaltepec, Mazahua	16.63300	-94.95000	RAD				CNIN2076_sp_2_Oax_I	-
2	Mexico	Oaxaca	Coyula	15.75079	-96.29751				CHC		
2	Mexico	Oaxaca	Santa María Huatulco, Bajo Teyula	15.73300	-96.29000	RAD				CNIN2077_sp_2_Oax_H	-
2	Mexico	Oaxaca	Santa María Huatulco, Bajo Teyula	15.73300	-96.29000	RAD				CNIN2084_sp_2_Oax_H	-
2	Mexico	Quintana Roo	Puerto Morelos						CHC		
2	Mexico	Quintana Roo	Puerto Morelos			RAD				CNIN2104_sp_2_QRoo	-

2	Mexico	Quintana Roo	Puerto Morelos.	20.844117	-86.90318			COI		CNIN-1758-,59, 61,62, PMorelos25-26	6, 31
2	Mexico	Quintana Roo	Santa Gertrudis	19.802076	-88.77541			COI		CNIN-2749-52	18
2	Mexico	Jalisco	Tecolotlán, Sierra de Quila			RAD				CNIN2106_sp_2_Jal	-
2	Mexico	Jalisco	Estación Biológica Chamela, camino Chachalaca	19.49930	-105.0383		UCE			SSCNIN2066_sp_2_Jal	-
2	Mexico	Jalisco	Pto. Vallarta, Palo María.					COI		CNIN-1739-40, 1816-17	19
2	Mexico	Jalisco	Est. Biol. Chamela, camino Chachalaca	19.4993	-105.0383			COI		Chame2_1Jal1, CNIN-2066,2673-74, 1741-42, 44-47	11
2	Mexico	Nayarit	Compostela, Fortuna de Vallejo	20.99480	-105.1192	RAD				CNIN2107_sp_2_Nay	-
2	Mexico	Nayarit	Compostela, Fortuna de Vallejo	20.9948	-105.1192			COI		CNIN-1738, 1814-15	19
2	Mexico	Yucatán	Camino a Celestún	20.91209	-89.96168			COI		CNIN-1489-90, 1974-79	17
2	Mexico	Oaxaca	Santa María Huatulco, La Unión, Pochutla	15.73850	-96.29460	RAD				CNIN2857_sp_2_oax_H	-
2	Mexico	Oaxaca	Asunción Ixtaltepec, Mazahua	16.633	-94.95			COI		1893-94	16
2	Mexico	Oaxaca	Asunción Ixtaltepec, Santiago Ixtaltepec	16.694	-94.904			COI		CNIN-1861, 63-65, 84-85	19
2	Mexico	Oaxaca	Santo Domingo Ingenio, La Blanca	16.588	-94.692			COI		CNIN-1866, 68	15
2	Mexico	Oaxaca	Santa María Huatulco, Bajo Teyula	15.733	-96.297			COI		CNIN-1886-87, 95, 97, 1967, 69	13
2	Mex	Chiapas	Tuxtla, Copoya	16.71300	-93.11700		UCE			CNIN2068_sp_2_Chis	-
2	Mex	Chiapas	Rosario Izapa				UCE			ECTA18_sp_2_Chis	-
2	Mexico	Chiapas	Rosario Izapa					COI		E_rui2_Izapa3b, 5, 6b,7b,16b_Chis	20
2	Mexico	Chiapas	Tuxtla Gutiérrez, Copoya	16.713	-93.117			COI		CNIN-1898	22
2	Mexico	Chiapas	Chiapa de Corzo, Emiliano Zapata	16.665	-92.952			COI		CNIN-1735, 1819	9
2	Mexico	Michoacan	2Km SE Zapote de Tezupán	18.19694	-103.0219	RAD				CNIN2975_sp_2_Mich	-
2	Mexico	Michoacan	Carretera Lázaro Cárdenas- Manzanillo	18.03972	-102.5294	RAD				CNIN2976_sp_2_Mich	-
2	Mexico	Michoacán	2km SEZapote de Tezupán	18.196944	-103.1052			COI		CNIN-3030-3034	12

2	Mexico	Michoacán	Lázaro Cárdenas, Carretera Federal 200 Km22	18.039722	-102.5294			COI		CNIN-3025-3029	12
2	Mexico	Veracruz	Emiliano Zapata	19.32795	-96.63279			COI		CNIN2151-2155	21
2	Panama		Barro Colorado					COI		ER18ALCO	7
3	México	Oaxaca	Mpio. San Pedro Mixtepec, 8km N Pto. Escondido, Rancho Hierba Santa	15.94	-97.071			COI		CNIN-1809, 10	26
3	México	Oaxaca	Santa María Tonameca, San Juanito o La Botija	15.796	-96.796			COI		CNIN-1889-91, 1970, 71	-
3	México	Oaxaca	Santa María Tonameca, San Juanito, La Botija	15.79600	-96.79600			COI		CNIN-2075	26
3	Mexico	Oaxaca	Santa María Tonameca, San Juanito, La Botija	15.79600	-96.79600	RAD				CNIN2075_sp_3_Oax_SJ	-
3	Mexico	Oaxaca	Santa María Tonameca, San Juanito, La Botija	15.79600	-96.79600	RAD				CNIN2103_sp_3_Oax_SJ	-
3	Mexico	Oaxaca	San Pedro Mixtepec, Hierba Santa, 8Km N Pto. Escondido	15.94000	-97.07000	RAD				CNIN2850_sp_3_Oax_HS	-
3	Mexico	Oaxaca	San Pedro Mixtepec, Hierba Santa, 8Km N Pto. Escondido	15.94000	-97.07000	RAD				CNIN2851_sp_3_Oax_HS	-
3	Mexico	Oaxaca	San Pedro Mixtepec, 8km N Pto. Escondido, Rancho Hierba Santa	15.94000	-97.07100				CHC		
3	Mexico	Oaxaca	San Pedro Mixtepec, 8km N Pto. Escondido, Rancho Hierba Santa	15.94000	-97.07100		UCE			CNIN3720_sp_3_Oax_HS	-
3	Mexico	Oaxaca	San Pedro Mixtepec, 8km N Pto. Escondido, Rancho Hierba Santa	15.94000	-97.07100		UCE			CNIN3723_sp_3_Oax_HS	-
3	Mexico	Oaxaca	Santa María Tonameca, San Juanito, La Botija	15.79600	-96.79600		UCE				-

3	Mexico	Oaxaca	San Pedro Mixtepec, 8km N Pto. Escondido, Rancho Hierba Santa	15.94000	-97.07100		UCE			ECTA02_sp_3_Oax_PE	-
4	Mexico	Oaxaca	Mpio. Tonameca, 3km N Mazunte	15.6854	-96.5618				CHC		
4	México	Oaxaca	Mpio. Tonameca, 3km N Mazunte	15.6854	-96.5618			COI		CNIN-1736-37, 1813	-
4	México	Oaxaca	Santa María Tonameca, Cuatode	15.732	-96.509			COI		CNIN-1880, 82, 1972-73	-
4	México	Oaxaca	Santa María Tonameca, entrada a Piedras Negras,	15.725	-96.66			COI		CNIN-1991-92	-
4	Mexico	Oaxaca	Santa María Tonameca, 3Km N Mazunte	15.685073	-96.56504			COI		CNIN-2065	29
4	Mexico	Oaxaca	Santa María Tonameca, Cuatode	15.73200	-96.50900	RAD				CNIN2096_sp_4_Oax_C	-
4	Mexico	Oaxaca	Santa María Tonameca, Cuatode	15.73200	-96.50900	RAD				CNIN2097_sp_4_Oax_C	-
4	Mexico	Oaxaca	Mao Santa María Tonameca, Piedras Negras	15.72500	-96.66000				CHC		
4	Mexico	Oaxaca	Mao Santa María Tonameca, Piedras Negras	15.72500	-96.66000	RAD		COI		CNIN2098_sp_4_Oax_PN	28
4	Mexico	Oaxaca	Mao Santa María Tonameca, Piedras Negras	15.72500	-96.66000	RAD				CNIN2099_sp_4_Oax_PN	-
4	Mexico	Oaxaca	Santa María Tonameca, 3Km W Mazunte	15.68540	-96.56180	RAD				CNIN2852_sp_4_Oax_Mz	-
4	Mexico	Oaxaca	Santa María Tonameca, 3Km W Mazunte	15.68540	-96.56180	RAD				CNIN2855_sp_4_Oax_Mz	-
4	Mexico	Oaxaca	Santa María Tonameca, 3Km W Mazunte	15.68540	-96.56180	RAD				CNIN2905_sp_4_Oax_Mz	-
4	Mexico	Oaxaca	Santa María Tonameca, 3Km W Mazunte	15.68540	-96.56180		UCE			CNIN3721_sp_4_Oax_Mz	-

4	Mexico	Oaxaca	Santa María Tonameca, Puente Quatode	15.73200	-96.50900				CHC		
4	Mexico	Oaxaca	Santa María Tonameca, Puente Quatode	15.73200	-96.50900		UCE			CNIN3722_sp_4_Oax_Q	-
5	Mexico	Oaxaca	Huaxpaltepec					COI	-		23
6	Mexico	Oaxaca	Pinotepa Nacional	16.35300	-98.21800	RAD				CNIN2087_sp_2x3_Oax_P	-
6	Mexico	Oaxaca	Pinotepa Nacional, Lagunillas	16.35300	-98.21800		UCE				-
6	México	Oaxaca	Pinotepa Nacional, Lagunilla	16.353	-98.218			COI		CNIN-1993-95	-
6	Mexico	Guerrero	Copala	16.56750	-98.88806	RAD				CNIN2086_sp_2x3_Gro	-
6	Mexico	Guerrero	Copala, Fogos, Microondas	16.56750	-98.88806		UCE			CNIN2088_sp_5_Gro	-
6	México	Guerrero	Copala, Est. Microondas Fogos	16.5675	-98.8881					CNIN-1851, 1987-89	-
E. gibbum	Nicaragua	Región Autónoma del Atlántico Sur	Reserva Natural Kahka Creek	12.657	-83.743			COI		CNIN-2003	30
E. gibbum	Nicaragua		Nicaragua				UCE			EX814_E_gibbum	-
E. tuberculatum	Brazil	Minas Gerais	Passos, Parque Emilio			RAD				CNIN2082_E_tub_Bra	-



Supplementary figure 1. Correlation between cuticular hydrocarbon distances and (a) 3RAD genetic distances, (b) UCE genetic distances and (c) mtDNA (COI) genetic distances distances.

5.

Discussion

The central theme of my thesis was to provide evidence to support the separation of the different putative species within the *Ectatomma ruidum* species complex and to identify potential local selection pressures at phenotypic and genotypic levels. Up to know evidence supporting the separation of the putative species was based only on two mitochondrial genes. Also, along the wide neotropical distribution of *E. ruidum*, the distribution patterns of the putative species raised questions about which could be the mechanisms that separated them; in some geographic regions certain putative species exist in quasi sympatry, while in other cases, species appear to have a very large distribution. To analyze phenotypic traits, I studied variation in recognition cues among different species of the complex (Peña-Carrillo et al. 2021; **Chapter 2**); and studied acoustic variation in the distress call they produced (Peña-Carillo et al. submitted; **Chapter 3)**. Finally, I integrated the analysis of genetic sequences and cuticular hydrocarbon profiles to infer species boundaries (**Chapter 4**). Overall, the results showed that the mechanisms and driving forces involved in the diversification of the *E. ruidum* species complex are not only reflected at genetic level, but are also expressed at phenotypic level. This study supports that *E. ruidum* is fragmented in multiple species which are relatively conserved morphologically but relatively well separated in their nuclear and mitochondrial genomes as well as in phenotypic traits other than morphology. These results provide strong evidence for the separation of some species of the complex, and raise questions about what pressures resulted in such a high diversification level within a relatively limited geographical range, i.e. which mechanisms separated populations and, later on, species along their distribution range.

Phenotypic variation in *Ectatomma ruidum*

For social groups, communication is essential to delineate group membership, coordinate activity and establish ranks of individuals (Richard and Hunt 2013). Communication is subject to social selection which acts on interacting phenotypes (d'Ettorre and Hughes 2008), and it can occur in different modalities as, for example, via chemical cues and acoustic signals.

Chemical cues

In social insects, chemicals are important vectors of necessary messages for the functioning of the activities in the colonies, and in particular, cuticular hydrocarbons play a major role in communication (Blomquist and Bagnères 2010). Cuticular hydrocarbons have been proposed as potential dual traits (Chung and Caroll 2015). That is, they have physiological roles (Blomquist and Bagnères 2010) but also serve as communication cues, i.e., nestmate recognition cues (van Zweden and d'Ettorre 2010). In the *E. ruidum* species complex, the results reported in this thesis show that cuticular hydrocarbons are highly variable between species at quantitative level. The data I obtained show that the between-species variation does not result from geographic distance (Pena-Carillo et al 2021; Chapter 1). Typically, the cuticular hydrocarbon profiles of conspecific social insects include the same set of substances, and nestmates are recognized by variation in their quantities (Kleeber et al. 2017; Sprenger and Menzel 2020). High quantitative variation of cuticular hydrocarbons has been found among closely related species of social insects as *Euglossa* bees (Pokorny et al. 2014), the ants *Crematogaster levior* and *Camponotus femoratus* (Hartke et al. 2019), *Cataglyphis niger* species complex (Brodetzki et al. 2019). In the latter species, quantitative differences of

cuticular hydrocarbons differentiated the different species of the complex from sympatric populations (Brodetzki et al. 2019).

In contrast to quantitative differences, qualitative variation is mainly found between species, hence, different species usually present a highly different cuticular hydrocarbon set up (Sprenger and Menzel 2020); inclusively it can be specific enough to be used as a taxonomic tool (Kather and Martin 2012). In the current study, the striking variation of ant hydrocarbons reveals a higher level of divergence within the species complex than that highlighted by previous genetic studies (Aguilar-Velasco et al. 2016; Meza-Lázaro et al. 2018, Chapter 2). For example, different populations of the putative *E. ruidum* sp. 3-4 studied in the Chapter 2, were differentiated only by their highly divergent hydrocarbons while mitochondrial DNA was not able to do it (Peña-Carrillo et al. 2021). But, what could be the reasons for this high variation? Our results revealed that the most variable cuticular traits were related to alkanes, alkenes and alkadienes, which are likely to play a major role in nestmate discrimination (van Zweden and d'Ettorre 2010). Cuticular hydrocarbons are evolutionary labile (Sprenger and Menzel 2020) and the quantity of a given hydrocarbon may quickly respond to selection pressures (Menzel et al 2017). For example, the impact of individual hydrocarbons on the waterproofing capacity of an insect's cuticle depends on their relative abundance, while the presence/ absence of a given hydrocarbon may be relatively conserved among phylogenetically close taxa (Menzel et al. 2017). Among the examples of extrinsic factors influencing hydrocarbon plasticity, even in the short term, temperature and humidity are often mentioned (Menzel et al. 2017; Sprenger and Menzel 2020). Moreover, one of the major drivers of diversity in recognition cues over evolutionary times may be the selective pressure imposed by intra and inter specific social parasites (Crozier 1986; Kleeberg et al. 2017; Lorenzi et al. 2011). Indeed, a form of intra-specific social parasitism has been reported in *E. ruidum* ants. Thievery (close to a form of parasitic interaction, Breed et al. 2012) has been reported in different populations of *E. ruidum* (Breed 1990; de Carli et al. 1998; Perfecto and Vandemeer 1993). Although we do not know which, among the multiple species of the species complex, is affected by thievery (e.g., thievery was reported before the description of *E. ruidum* as a species complex by Aguilar-Velasco and co-workers in 2016), it could have promoted the variation of ant cuticular hydrocarbon profiles. The ability to discriminate intruders can help ants to prevent the entry of cleptobionts and other territorial interactions (Breed et al. 2012) and divergence in recognition cues between populations has been associated both to interspecific parasitism (Martin et al 2010) and to intraspecific parasitism (Lorenzi et al. 2017). Additional factors as nest materials, diet, microorganisms and pathogens are also known to affect cuticular hydrocarbon profiles (Sprenger and Menzel 2020) and their effects in the *E. ruidum* species should be tested in the future.

Acoustic signals

In contrast to the high variation in chemical cues, the analyses of the acoustic traits of the species of *E. ruidum* complex unveiled a very different scenario. The stridulations emitted by *E. ruidum* spp. 2 and 3-4 do not differ significantly, and only the new putative species *E. ruidum* sp. 5 produced its own, different stridulation. Sound differences in the latter were linked to the way ants produced their sound. As shown in Chapter 3, the number of pulses was one of the

most variable traits where every pulse represented a rubbed ridge, but the total number of ridges contained in the stridulatory file was similar among the different species. This means that despite all species had a similar number of ridges in the stridulatory file, the ants of the *E. ruidum* sp. 5 rubbed a larger area of the stridulatory plate and this consequently modified their stridulation. Stridulating insects such as ants produce stridulations in different situations such as alarm, mating, recruitment and as warming signals (Markl, 1965; Hölldobler, 1999; Hickling and Brown, 2000; Wilson et al. 2012). Similarly to other acoustic signals, stridulations depend on their clear reception for a successful communication. The stridulations produced by *E. ruidum* are ultrasounds (~ 75 kHz, Pavan et al. 1997), and for this kind of sounds, the environmental and social conditions in which they are produced could be restricted by the intrinsic properties of their transmission (Arch and Narins 2008). For example, ultrasonic frequencies could be limited by atmospheric attenuation, directionality and susceptibility to scattering, but these limitations for transmission can also be beneficial because they restrict the transmission of acoustic information only to receivers in proximity (Pye and Langbauer 1998; Arch and Narins 2008). In this respect; the power level of sound produced in non-resonant structures like the stridulatory file is supposed not to be effective over a distance (Tschuch and Brothers 1999). But in the case of ants this will depend on the way stridulations are perceived by ants, which up to now is a controversial issue (Hickling and Brown 2000; Roces and Tautz 2001; Golden and Hill 2016).

The variation in acoustic communication (i.e. in mating signals) has been well studied among certain groups of insects such as crickets, treehoppers and lacewings because it is involved in mating processes and consequently in sexual selection (Wilkins et al. 2013). As an example, the two sibling species of crickets *Gryllus rubens* and *G. texensis* diverge whereas they share gene flow; differences in males' mating calls and strong female preferences for certain sound traits were promoters of such divergence (Blankers et al. 2015, 2019). Despite sexual selection has been proposed as the primary driver of acoustic divergence in this group of crickets, the mechanisms for the divergence are not well understood (Wilkins et al. 2013). However, recent demographic analyses demonstrated the existence of physical linkage between mating calls produced by males and preference loci in females (Blankers et al. 2019). Up to now stridulations involved in courtship behavior of ants have been studied in few groups like *Pogonomyrmex, Cardiocondyla* and *Pachycondyla* (Markl et al. 1977; Mercier et al. 2007; Ferreira et al. 2014). In the analysis of courtship behavior of different *Cardiocondyla* species, males of *C. elegans* had antennal contact with females and produced stridulations that were considered necessary for the pre-copulatory behavior (Mercier et al. 2007). Unfortunately, in the case of *E. ruidum* there is no information about mating behavior, hence, we do not know whether acoustic signals are involved on it. For some species acoustic communication is costly in terms of fitness. For instance, the differences between the stridulation of queens and workers of *Myrmica* ants are exploited by parasitic butterflies *Maculinea* to integrate into *Myrmica* colonies with the highest rank (Barbero et al. 2009). *Maculinea* caterpillars produce stridulations which match those of the ant queen (acoustical mimicry), thus probably getting rescued as first if the colony is disturbed and larvae need to be got to safety, or getting food even if the colony is starving; these responses occurring usually only towards queens (Barbero et al. 2009).

The examples above highlight the role of acoustical signals in ant societies. As a further point, stridulations can be used as taxonomic tools. For instance, Ferreira et al. (2010) investigated the distress calls that ants produced when their nests were disturbed or they were targeted by predators (Ferreira et al. 2014). They found that the calls produced by different cryptic species of the *Neoponera apicalis* (= ex *Pachycondyla*) ant species complex differed (Ferreira et al. 2010). The highly divergent stridulations reinforced the divergence found in morphological traits of the stridulatory file/ organ of these ants.

The results of this thesis (Chapter 3) suggest that similarly to *Neoponera* ants, the analysis of acoustic traits of the *E. ruidum* sp. 5 coupled with the analysis of other characters could be useful for its taxonomical identification. In any case, more thoroughly studies including a higher number of colonies per population are needed to confirm the lack of morphological variation among the other species of the complex. Additionally, further studies are needed to untangle the function(s) of the stridulations in the *E. ruidum* species and to know if they have a primary role in communication. It is known that, in sound producing animals, some acoustic signals evolved from non-primary functions (exapted, *sensu* Gould and Vrba 1982) that consistently influenced the behavior of conspecifics. For example, the primary function of bats echolocation is prey capture and navigation, but it exapted for use in intraspecific communication (Arch and Narins 2008). Then, the lack of acoustic variation might suggest that distress calls are not under divergent selection pressures in most *E. ruidum* species, whereas they have been in *E. ruidum* sp. 5. However, which selection pressures are involved in the divergence of their stridulations are currently unknown.

Multitrait divergence in *E. ruidum*

Throughout the different chapters of this thesis, I have been discussing how phenotypic and genetic characters differ among species of *E. ruidum* complex and I present evidence supporting that variation is consistent between chemical and genetic traits between most of the species. In other words, the results I am presenting suggest that variation in different phenotypic traits has played different roles along the evolutionary history of these species complex. For example, acoustic traits seem not to have been involved in the diversification of the different species, while cuticular hydrocarbons, whose distances were highly correlated to genetic ones, might have been (Chapter 4). The results of this work offer evidence for the existence of a total of six species: *E. ruidum* sp. 1, *E. ruidum* sp. 3, *E. ruidum* sp. 4, *E. ruidum* sp. 5, *E. ruidum* sp. 6, and the group of *E. ruidum* sp. 2 which contains two distinct clades (*E. ruidum* spp. 2A and 2B, see Chapter 4). The separation between the putative species *E. ruidum* spp. 1-4 was highly supported by a strong correlation between differences in their nuclear genome (3RADseq and UCEs) and variation in their cuticular hydrocarbon profiles. *E. ruidum* sp. 5 was clearly separated from the putative *E. ruidum* sp. 2-4 by sequences of the mitochondrial DNA marker COI, by a high variation of its chemical profile and by its divergent stridulation. *E. ruidum* sp. 6 was strongly separated from *E. ruidum* sp. 1-4 by the differences in its nuclear genome (3RAD and UCEs). Phylogenetically, *E. ruidum* sp. 6 was previously referred as the putative hybrid population *E. ruidum* sp. 2x3 (from Pinotepa Nacional Lagunilla, Oaxaca, Mexico, see Chapter 4 for details) and one population *E. ruidum* sp. 3 from the state of Guerrero in Mexico (see

Chapter 4 for details). In the next section, I will discuss potential evolutionary processes that underlie speciation in *E. ruidum* species complex using an integrated perspective.

Among all the species of the complex, *E. ruidum* sp. 1 is the most differentiated lineage supported by different types of characters. The results show this species was always separated from the other species by analyzing genetic (Chapter 4) and chemical data (Chapter 2 and 4). These results confirm previous studies (Nettel et al. 2015; Aguilar-Velasco et al. 2016). Aguilar-Velasco et al. (2016) showed that *E. ruidum* sp. 1 is one of the two putative species with the widest geographic distribution. Also they differ slightly morphologically from the other morphotypes, by the form of the pronotal hump and the petiolar node (pronotal hump pronotal is located in the first dorsal segment of the thorax and the petiolar node is located in the metasomal, see Table 2a). Actually, the researchers indicated that species 1 matched the original morphological description of the type specimen as reported by Roger (1861). Moreover, in the study of Aguilar et al. (2016) the separation of *E. ruidum* sp. 1 based on COI was also observed with mitochondrial sequences of Cytb, as well as by the phylogenetic analyses generated via mitogenome sequences (Meza-Lazáro et al. 2018). Populations of *E. ruidum* sp. 1 were also characterized by the absence of microgynes, which confirmed the hypothesis of Nettel et al. (2015) who originally proposed that these ants could be divided into two lineages, one characterized by the absence of microgynes (now referred as *E. ruidum* sp. 1) and another with. Accordingly, up to now this species has been well supported by different phenotypic and genetic traits; therefore, in agreement with previous studies, the separation of *E. ruidum* sp. 1 is strongly suggested. Nevertheless, additional population genetic studies should be performed to know if *E. ruidum* sp. 1 is reproductively isolated from other putative species, and follows the biological species concept adopted for this study. Additionally, the study of acoustic traits in this species could serve to determine if they represent potential taxonomic characters and what their role is in the communication system.

Similarly to the former species, *E. ruidum* sp. 2 is likely a group whose identity is supported by the results based on the different types of characters investigated. In the current work, its identity is supported by genetic variations both in mitochondrial and in nuclear DNA (3RAD and UCEs) and by differences in their CHCs profiles. Previously, Aguilar-Velasco et al. (2016) mentioned that *E. ruidum* sp. 2 could also be morphologically differentiated from the rest of morphotypes of *E. ruidum*, and their genetic analyses based on mitochondrial DNA Cytb also support its separation. The identity of *E. ruidum* sp. 2 was further supported by the mitogenome sequences assembled by Meza-Lazáro et al. (2018). With respect to Meza-Lazáro et al. (2018), the current results, based on a combination of genetic analyses (mtDNA, 3RAD and UCEs) and hydrocarbon profiles, suggest that *E. ruidum* sp. 2 should be further subdivided in two different clades: *E. ruidum* sp. 2A and sp. 2B. However regarding acoustic traits, the ants belonging to *E. ruidum* sp. 2 did not show any variation with respect to the other species.

According to the 3RAD analyses, the clade *E. ruidum* sp. 2A includes mostly samples collected in the Southern Sierra Madre of Mexico (extending from the states of Jalisco, Michoacan, Guerrero, Oaxaca and part of Puebla), a few collected in the Oriental and Occidental Sierra Madre of Mexico. But in the haplotype network, the structure of the clade 2A changes and is not restricted only to samples from Mexico. It includes samples from Costa Rica and others

from the eastern side of the Yucatan Peninsula (such as the locality Santa Gertudis) that were not included in the 3RAD analyses but share the same hypothetical haplotype where most of samples from the clade 2A were located. In general, the high diversity of haplogroups within this clade might suggest a higher intraspecific diversity, which up to now is corroborated at the mitochondrial and nuclear levels. Despite chemical analyses also suggested a striking variation in hydrocarbon profile, the sampling was less extensive than the one used for genetic analyses. Hence, at this moment it is questionable whether the high genetic diversity within *E. ruidum* sp. 2A is also revealed as chemical divergence because of the lower number of populations used for chemical analyses. According to the geographic distribution of the samples, the geographic area of the Southern Sierra Madre seems to have had an important role in the diversification within this clade. This mountain chain possesses a remarkably high biological diversity and is home to a high number of endemisms (Blancas-Calva et al. 2010; Santiago-Alvarado et al. 2016). Moreover, the Southern Sierra Madre presents a complex geological composition that creates a remarkable heterogeneity of habitats that promotes its high biological diversity (Santiago-Alvarado et al. 2016). In other groups of ants such as *Temnothorax,* this mountain chain has been a scene for speciation. For instance, Prebus (2020) performed a multi-approach study addressing the species delimitation of the *Temnothorax salvini* group and proposed that the species *T. aztecus* reached its most recent expansion phase in this region due to a taxon cycle, a process to explain species distribution across island archipelagos. Although the sampling of *E. ruidum* sp. 2 used in the present study was relatively small to claim conclusive evidence; its geographic representation was diverse and large enough to support the divergence of the clade *E. ruidum* sp. 2A from sp. 2B and its potential diversity at the mitochondrial and the nuclear levels (See Chapter 4). Therefore, further studies including data from multiple sources (e.g., cuticular hydrocarbons, behavior, biogeography, population genetics), and a more extensive sampling of the populations included in the clade *E. ruidum* sp. 2A will help to uncover its taxonomic status.

With respect to the clade of *E. ruidum* sp. 2B, 3RAD and UCEs data suggest that this clade is composed mainly of populations geographically distributed from South America to the south of Mexico in the state of Chiapas (very close to the border with Guatemala) and on the east side of the Yucatan Peninsula. The same distribution was supported by the haplotype network.

With respect to *E. ruidum* sp. 3 and sp. 4, the current research revealed that they are separated genetically using 3RAD and UCEs data and phenotypically by differences in their cuticular chemical profiles (Chapter 4). In the study of Meza et al. (2018) both species were clustered together with the species *E. ruidum* sp. 6 (before named *E. ruidum* sp. 2x3) originated from a putatively hybrid population between *E. ruidum* sp. 2 and sp. 3. Heteroplasmy (the presence of multiple mitochondrial DNA haplotypes in a single organism), observed here in *E. ruidum* spp. 3, 4 and 6, is not widely known in ants, and has been reported only in ants of the *Cataglyphis albicans* species complex. In a study about the phylogeographic relationships of this species complex, Eyer and Hefetz (2018) analyzed sequences from nuclear and mitochondrial genes, as well as microsatellites markers to test for the occurrence of gene flow through hybridization between sympatric but genetically distinct lineages of the complex. Their results revealed that mitochondrial DNA did not recover any of the nuclear species, due to the presence of several

strongly divergent sequences that were predominantly found in a restricted area where several species co-occur. Furthermore, one species of the complex exhibited heterozygous sequences in the mitochondrial DNA revealing the presence of heteroplasmy; the heteroplasmic individuals did not carry diagnostic nuclear sequences or microsatellites alleles suggesting their hybrid origin. Eyer and Hefetz (2018) suggested that the heteroplasmy may have originated through hybridization or mitochondrial DNA recombination. Indeed, gene flow was found between the species of the complex. When the researchers performed phylogenetic analysis without the highly divergent and heteroplasmic sequences, the mitochondrial phylogenetic trees supported the genetic clusters obtained from nuclear sequences and microsatellites analyses. For this reason, they highlighted that the identification of the *Cataglyphis albicans* species complex should not rely only on mitochondrial barcoding genes.

Similarly to the observation of Eyer and Hefetz (2018), populations from other *E. ruidum* species are close to the previously proposed hybrid population of *E. ruidum* sp. 6 (Aguilar-Velasco et al. 2016). For example, the closest population of *E. ruidum* sp. 2 (see annex) is located approximately less than 15 km from *E. ruidum* sp. 6, and there is another one of *E. ruidum* sp. 5 approximately 30 km away from it. Sequences of *E. ruidum* sp. 2 and sp. 5 did not reveal signals of heteroplasmy or pseudogenes (non-functional fragments of mtDNA integrated into the nuclear genome, Bensasson et al. 2001) like incorrect phylogenetic placement in a phylogenetic tree (Chapter 3). Furthermore, the demographic analysis performed with 3RAD and UCE data were not conclusive about the delimitation of all the putative species, except for *E. ruidum* sp. 4 that was the only one delimited, and did not show conclusive evidence that *E. ruidum* sp. 6 could be linked to putative hybrids. Consequently, future studies based on a more representative sampling per population and with the aim to test for the existence of gene flow between populations should shed light on the mechanisms that maintain or restrict heteroplasmy in the populations of *E. ruidum* spp. 3, 4 and 6, given that, up to now, the separation of the species is based on few individuals.

About our discovery of *E. ruidum* sp. 5, this species has been consistently separated by using mitochondrial DNA, chemical data and acoustics traits (Chapter 2, 3). Therefore, in this thesis results from multiple disciplines converged on its delimitation as different species. An important next step for the delimitation of this putative species should be to demonstrate its reproductive isolation from other *E. ruidum* species. Up to now, the only known population of *E. ruidum* sp. 5 (16 nests have been collected, see annex) is geographically close to another of *E. ruidum* sp. 2 (see annex) separated by less than 15 km away. So, the geographic proximity of both populations raises questions about the mechanisms that promoted its divergence in a geographic area that seems to be very similar. A more extensive sampling would help to know about the geographic distribution of this new putative species, and to identify the contact zone between *E. ruidum* sp. 5 and other species.

With respect to the social structure of *E. ruidum*, the presence of multiple mated queens (polygyny) has been reported only in a population from Rosario Izapa, Chiapas which corresponds to the clade *E. ruidum* sp. 2B. In this population, polygyny was considered as facultative because polygynous colonies were found in a lower proportion than monogynous ones (Lachaud et al. 1999). In contrast, in the population of Cali, Colombia which also

corresponds to the clade 2B, colonies only contain a single queen (Poteaux C., unpublished data) as in two other populations of *E. ruidum* sp. 1 investigated extensively in Chiapas (>500 colonies collected, J-P Lachaud, pers. comm).

Polymorphism in social structure has been associated to speciation in ants. For example, the sympatric populations of ants of the *Cataglyphis niger* species complex (including *C. niger, C. drusus* and *C. savigyi*), are differentiated by different mitochondrial haplotypes (mitotypes) and in their cuticular hydrocarbon profiles. Also, in behavioral tests ants show aggressive responses when they were confronted to ants of a different species, but not when confronting individuals of the same lineage. But because the three species share gene flow and apparently assortative mating does not occurs, there is no conclusive evidence so far to separate the three species (Brodetzki et al. 2019). However, the fact that the three *Cataglyphis* species maintain their mitochondrial DNA differentiation indicates a strong structuration and low female dispersal. In addition, social structure is present in the species complex (polygyny in *C. niger*, monogyny in the others). Hence, Brodetzki et al. (2019) proposed that if the three species were considered as a single one, their sympatric speciation based on differences in social structure might be possible. In *E. ruidum* polygyny is considered as facultative, but up to now it has been reported only in *E. ruidum* sp. 2, then, in a scenario where polygynous populations were common in the past, their existence might have been involved in the high diversity contained in this putative group of species (*E. ruidum* sp. 2A and 2B).

Polygyny has been linked to differences in genotypes (Keller and Parker 2002), and in fire ants (*Solenopsis invicta*) and alpine silver ants (*Formica selysi*), a social chromosome (which means that a supergene region contained in a pair of chromosomes will determine if a colony will have a single or multiple queens) was responsible of social polymorphism (Wang et al. 2013; Purcell et al. 2014). Also it is likely to be influenced by environmental factors like competition for food and nest limitation (Hölldobler and Wilson 1977; Bourke and Francks 1995; McGlynn 2010). Polygynous populations of *E. ruidum* have been found in places where high nest densities have been reached (Lachaud et al. 1999). Then, additional studies with the aim of investigate the occurrence of polygyny in other populations of *E. ruidum* sp. 2, and their relation with possible environmental pressures or the presence of the social chromosome are encouraged.

Besides polygyny, in *E. ruidum* queen size dimorphism has been reported, particularly in the population from Rosario Izapa, Chiapas (Lachaud et al. 1999). Queen size dimorphism refers to the presence of queens with different body sizes in the same colony; miniaturized queens are named microgynes and are an isometric reduction of the normal large queen morph, referred as macrogynes (Lachaud et al. 1999). Microgynes have been found in additional populations of *E. ruidum* sp. 2 such as: Puerto Morelos, Quintana Roo (*E. ruidum* sp. 2B) and Coyula (*E. ruidum* sp. 2A), as well as in the putative species *E. ruidum* sp. 3 (Yerba Santa, Oaxaca, Mexico) and *E. ruidum* sp. 4 (Mazunte, Oaxaca, Mexico) (Poteaux C, unpublished data).

According to Seifert (2010), microgyny is involved in the speciation process of ants and can be accompanied by polygyny or having it as a preliminary step. If the dispersal abilities of microgynes are reduced and they mate inside their nests because they have weaker wing muscles to fly away, while macrogynes (which have big wing muscles) still continue to fly and

disperse away, mating place separation could be favored and facilitate genetic divergence. The model of Seifert (2010) was based on a parasitic ant species (*Myrmica rubra*); however, he highlighted the fact that the combination of polygyny and intranidal mating trigger speciation. In the case of *E. ruidum*, previous studies focused in the population Rosario Izapa, Chiapas, Mexico (*E. ruidum* sp. 2B), showed that in monogynous and polygynous colonies, macrogynes and microgynes can be re-adopted by their mother colony, which represents a low dispersal strategy. However, in the same studies no viscosity was found within populations (Lenoir et al. 2011). In agreement with the study of Aguilar-Velasco et al. (2016), up to now *E. ruidum* sp. 1 seems to be the only species where microgynes are absent, suggesting that queen polymorphism may also be involved in the diversification of this highly divergent species. Future research focused on the analysis of colonies of the putative *E. ruidum* spp. 5 and 6 will help to determine if the absence of microgynes may also be a characteristic of the putative *E. ruidum* sp. 1.

As stated in the introduction, the purpose of this thesis was to provide evidence to support the separation of the different putative species of the *E. ruidum* species complex and identify potential selection pressures. One of the most remarkable result of this study is the highly correlation found between cuticular hydrocarbons and genetic distances. This high correlation strongly suggests that selection pressures acting on recognition cues have been involved along the diversification of the species complex. These results raise questions about the role of recognition cues in these ants. According to Chung and Caroll (2015), changes in particular cuticular hydrocarbons would have an indirect effect on another. By a way of example, the synthesis of cuticular hydrocarbons follows a common pathway (Howard and Blomquist 2005). Then, in the biosynthetic pathways, changes in individual or in a class of cuticular hydrocarbons that regulate waterproofing properties or mating success may alter the production of other cuticular hydrocarbons (Chung and Caroll 2015).

Additionally, the presence of important diverging traits, like polygyny, the presence of queen polymorphism and heteroplasmy could serve to understand the mechanisms that shape the separation of the species of this complex.

6.
Conclusion

With the results included in this PhD thesis, I provided phenotypic and genotypic evidence that suggests the separation of the species of the complex as follows: 1) *E. ruidum* sp. 1 was supported by mitochondrial, nuclear and chemical information; 2) *E. ruidum* sp. 2 was supported as different by mitochondrial, nuclear and chemical information; in addition, beyond its simple separation from the other species, the three kinds of data supported that *E. ruidum* sp. 2 might be divided into two different clades here proposed as 2A and 2B. Furthermore, the clade 2A might also include more diversity, reason why future work would determine if it could be considered as a group of species; 3) similarly to the former species, *E. ruidum* sp. 3 and *E. ruidum* sp. 4 are supported as separate species but only by the analysis of highly polymorphic nuclear markers and cuticular hydrocarbons. This suggest that population genetic studies are needed to untangle if both species are reproductively isolated or if they represent incipient species that share genetic flow and have in common the presence of heteroplasmy; 4) *E. ruidum* sp. 5 was recently discovered and up to now its separation is supported by mitochondrial DNA, cuticular hydrocarbon analysis and acoustic traits. Because the results are based on a single population, more studies on the geographic distribution and the life traits of this species are needed; 5) The delimitation of *E. ruidum* sp. 6 was only possible by using the most highly polymorphic molecular markers and relied on only very few individuals. Nonetheless, a higher sampling is needed to corroborate its separation by studing their recognition signals, acoustic traits and population genetics.

Globally, this study agrees with the idea that using mitochondrial DNA alone for species delimitation of the *E. ruidum* species complex is problematic, could lead to misidentifications or is not useful when analyzing heteroplasmic organisms. The obtained results allowed identifying important mechanisms that might be involved in the diversification of the species complex at a large scale. In addition, they allowed to confirm that the Southern Sierra Madre of Mexico represents a hotspot for the diversification of *E. ruidum*.

7.

Appendix

7. Appendix (ongoing project)

In this section I present a brief theoretical background and a short description of the materials and methods used for one ongoing project started in the last year of my PhD studies.

Genetic structure of *Ectatomma ruidum* populations along a putative diversification transect in Oaxaca, based on microsatellite loci variation

Background

Ectatomma ruidum is a widely distributed neotropical ant and is one of the few species that present a cleptobiotic behavior (Breed, 1990). Also, in some populations social polymorphism and queen dimorphism (Lachaud et al. 1999a, b) is present. *E. ruidum* was previously proposed to be a complex of different species (*E. ruidum* spp. 1-4) with a putative hybrid population (*E. ruidum* 2x3 (Aguilar-Velasco et al. 2016). Some species as the putative *E. ruidum* spp. 3, 4 and 2x3, up to now have been only found in restricted geographic area along the lowlands of the state of Oaxaca, in a region where a putative hybrid zone was proposed (Aguilar-Velasco et al. 2016). In addition, the study of the mitochondrial genome of the species of this complex (Meza-Lázaro et al. 2018) revealed that *E. ruidum* spp. 3, 4 and 2x3 are heteroplasmic, which means that individuals present more than one type of mitochondrial genome (Magnacca and Brown 2010). Heteroplasmy was suggested to originate from a prevalent source of a second mitochondrial lineage (Meza-Lázaro et al. 2018). Recently, the study of the recognition cues of the species complex supported the differentiation of the putative species, by showing that populations of *E. ruidum* have highly divergent cuticular hydrocarbon profiles. Indeed, for some species high differences between populations was found (i.e., *E. ruidum* sp. 4). Moreover, in the same study a new putative species was reported (*E. ruidum* sp. 5). Thievery was proposed to be a selection pressure linked to the high variation in recognitions cues, but the possible influence of biotic and abiotic factors was not discarded (Peña-Carrillo et al. 2021; Chapter 2). In another study (Chapter 3), the putative *E. ruidum* sp. 5 was shown also to diverge from the others because it produces a different distress call (stridulation), while the morphology of the organ used to produce the sound was similar to that of the other species (Peña-Carrillo et al. *submitted;* Chapter 3). Because the presence of heteroplasmy makes it more difficult to investigate the genetic delimitation of the species in this complex, their nuclear genome (obtained by 3RAD and UCEs sequences) was analyzed together with data from recognition cues. The genetic distances obtained from the genetic and chemical data where highly correlated, suggesting that recognition cues haven been involved along the divergence of the species complex (Meza-Lázaro et al. *in preparation;* Chapter 4). Finally, the genomic data supported the separation of the *E. ruidum* sp. 2 in two different evolutionary lineages, as well as the identification of *E. ruidum* spp. 3, 4 and 6 (previously named sp. 2x3)as true species. The evidence of genetic (mitochondrial and nuclear genomes) and phenotypic (recognition cues and acoustic signals) variation supported the divergence of the different species and raised questions about the mechanism that allow their differentiation. The region of Oaxaca, Mexico, was previously proposed as an hybridization zone because different species inhabit close areas without clear geographic barriers between them. Additionally, the only species (*E. ruidum* spp. 3, 4 and 6) where heteroplasmy was found inhabit this putative hybridization zone.

Studies focused on populations variations may allow understanding the first steps that have promoted population divergence and eventually the speciation (Mallet 2009), as here for the *E. ruidum* species.

The aim of this study is to investigate if reproductive isolation exists among the different species of the complex. These results would be an important step for supporting the species status of the different species of the complex. Furthermore, the presence of possible hybridization and/or introgression between the populations of heteroplasmic species will be tested as a putative explanation for its origin.

This objective will be pursuit by analyzing polymorphic nuclear markers (microsatellites). Microsatellites are evolutionary relevant because of their instability, they have fast mutation rates (Gemayel et al. 2012), are widely distributed throughout the genome and are useful for the study of evolution, diversity and gene flow estimations at intraspecific level in non-model taxa (Vieira et al. 2016). Also, these markers are widely used in studies of population structure and genetic mapping (Vieira et al. 2016) and could be used to untangle species relationship when they have recently diverged (Noble et al. 2010; Cordonnier et al. 2019). For instance, they have served for species discrimination in *Tetramorium* ant species, even in hybridizing populations (Cordonnier et al. 2019). Here we expect they detect putative hybridization between species in Oaxaca.

Material and methods

Sampling. Individuals of the different putative species (*E. ruidum* spp. 1-5) from previously studied populations (Aguilar-Velasco et al. 2016, Peña-Carrillo et al. 2021) were collected along a transect of approximately 120 km along the lowlands of the state of Oaxaca (Figure 1). 15-20 colonies (1 individual/colony, Table 1) were analyzed.

DNA extraction. The DNA of each workers was extracted from ethanol-preserved tissues (mainly head, or head and thorax) using a standard Chelex 10 % (Biorad) procedure.

Microsatellite loci. We used two sets of microsatellite loci designed in *E. ruidum*: 7 loci from Lenoir et al. (2011) and 12 from an unpublished set obtained during a collaboration with a Colombian laboratory (Herrera J, pers. comm, Ecos Nord C12A02). These 12 loci come from a set of 30 loci that were chosen according to their polymorphism, previously tested in 10 Colombian populations (Herrera J. unpublished); so the group of loci used here was different from the ones initially designed for multiplexing.

PCR reaction. PCR were performed according to procedures in Lenoir et al. (2011) and Herrera (pers. comm).

Genotyping. Due to the large range of alleles observed for some loci in some species, we merged the different PCR products in 5 different mixes (see Table 1). The multilocus products were mixed with the internal size marker GeneScan™ Liz500 (Applied Biosystems, Foster City, CA, USA) and run on an automated ABi 3100 Sequencer (Applied Biosystems) in the UMR 9191-EGCE laboratory in Gif-sur-Yvette, as service provider. Fragment length was scored using the freeware application Peak Scanner™ v1.0 (Applied Biosystems).

Statistical analyses. We will calculate number of alleles per locus and average over loci, observed heterozygosity (H_o) and Nei's unbiaised expected heterozygosity (H_e) using Genetix 4.05.2 (Belkhir et al. 2004). Exact tests for departures from the Hardy–Weinberg equilibrium for each locus and over loci and linkage disequilibrium using will be performed using Genepop on the web (Raymond & Rousset, 1995; Rousset 2008). The presence and frequency of null alleles will be estimated with Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004).

To determine the number of genetically homogeneous groups and to test for the presence of putative hybrids, we'll use Bayesian clustering algorithm and assignment methods implemented in STRUCTURE v. 2.3.1 (Pritchard et al., 2000), among others.

Results

Allelic diversity per locus and the range of allelic size are given for each population in Table 2. However, some data were still missing and it was not possible to test for the different parameters yet.

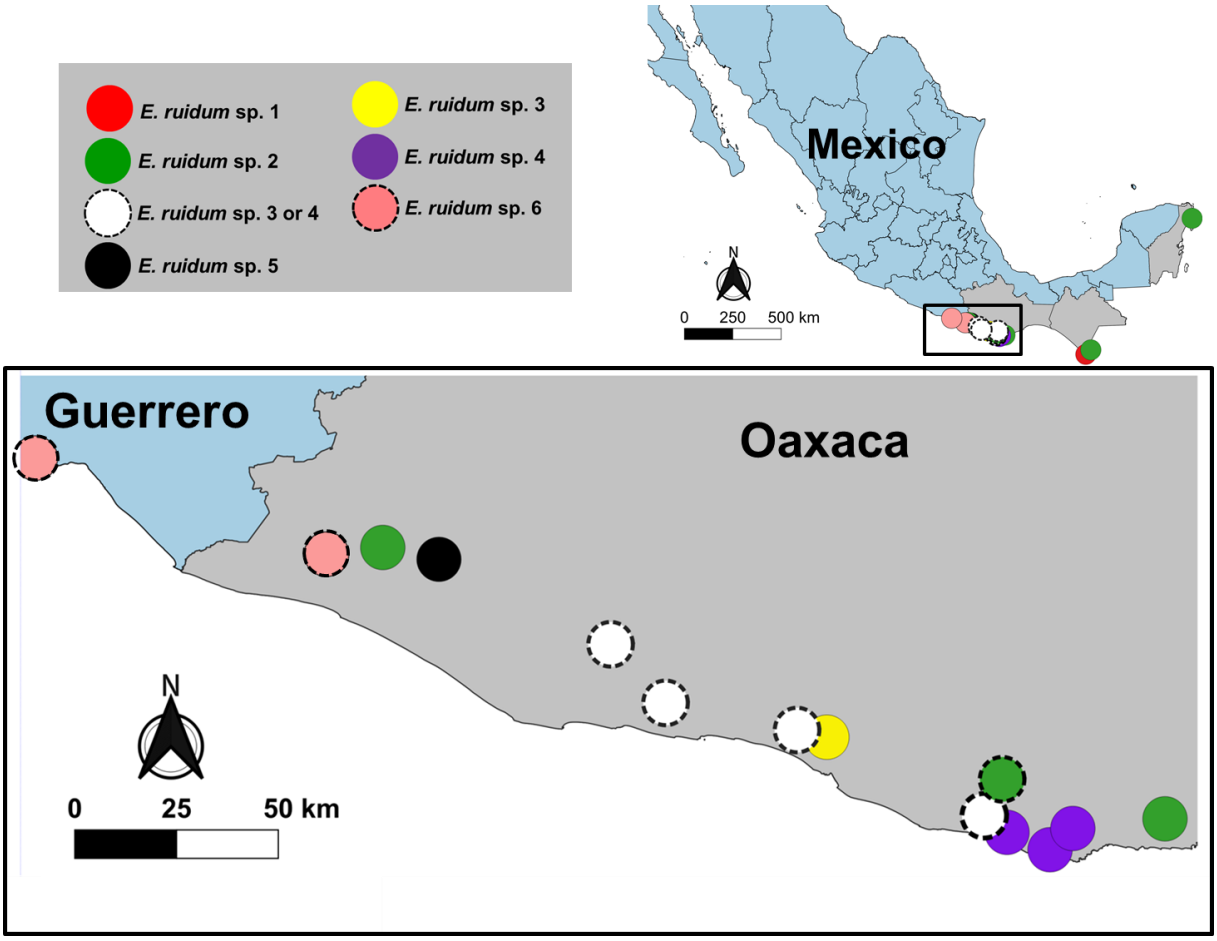


Figure 1. Transect of study. Circles with dotted outline are not included in the study.

Table 1. Sampling sites.

E. ruiudm species	Locality	State, Country	Coordinates	Number of individuals analysed (1 per colony)	GenBank accession number (previously published sequences)
sp. 1	Cantón Leoncillos	Chiapas, Mexico	14°45'59.00"N/ 92°24'12.00"W	12	KU570636-39
	San Martin, el Caduceo	Colombia	N 3°40'5/ W 73°39'28	14	-
sp. 2	Cali	Cali, Colombia	3° 22' 39"N/ 76°31'52"W	15	MG870224
	Coyula	Oaxaca, Mexico	15°45'2.85"N/ 96°17'51.05"W	20	MN957982-85
	Pinotepa Nacional	Oaxaca, Mexico	15°50'34.00"N/ 96°40'11.00"W	16	MN848418-19
	Puerto Morelos	Quintana Roo, Mexico	20°50'38.82"N/ 86°54'11.46"W	15	KU570581, 611-12
	Rosario Izapa	Chiapas, Mexico	14°58'08"N/ 92°09'18"W	20	KU570606-10-
sp. 3	Puerto Escondido (Yerba Santa)	Oaxaca, Mexico	15°56'24.00"N / 97° 4'15.60"W	19	KU570668
sp. 4	Mazunte	Oaxaca, Mexico	15°41'7.44"N/ 96°33'42.48"W	20	KU570662-64
	Piedras Negras (P. Zapotal)	Oaxaca, Mexico	15°43'30.00"N/ 96°39'36.00"W	21	MG870246
	Puente Cuatode	Oaxaca, Mexico	15°43'55.20"N/ 96°30'32.40"W	17	MN848420-21
sp. 5	Huaxpaltepec	Oaxaca, Mexico	16°20'26.00"N/ 97°57'34.00"W	16	MN848413-14

Table 2. Number of alleles and range of allelic size (in base pairs) for each microsatellite locus per population of the *E. ruidum* species complex.

E. ruidum species	Population		Mix 1				Mix 2				Mix 3				Mix 4				Mix 5		
			Er 2050	Er 3157	Er 2	Er 6	Er 2038	Er 30	L 92	Er 4160	Er 9	Er 17	Er 11	Er 4	Er 2035	Er 24	Er 10	Er 5042	Er 7	Er 3	Er 8
sp.1	San Martin	# of alleles	8	3	2	7	1	4	14	2	3	3	1	4	2	2	8	4		4	6
		range size	132-152	198-206	384-386	446-462	127	214-218	206-268	309-311	147-151	207-213	261	348-355	86-104	186-190	234-261	289-305		260-268	380-463
	Cantón Leoncillos	# of alleles	4	2	1	3	3	-	8	2	5	3	1	2	1	1	4	4		1	6
		range size	112-122	214-241	389	429-458	149-156		253-270	314-316	140-159	212-222	264	334-350	86	186	227-251	289-328		260	395-425
sp. 2	Pinotepa Nacional	# of alleles	6	10	3	3	1	1	7	5	7	10	5	1	8	6	-	3		5	12
		range size	100-118	211-250	384-388	422-429	132	228	229-257	284-314	145-161	212-238	264-285	334	100-126	160-184		289-320		250-270	403-478
	Rosario Izapa	# of alleles	2	8	1	1	3	1	8	3	2	3	2	1	4	3	1	3		5	7
		range size	112-122	198-270	389	427	164-168	236	247-271	314-321	149-150	212-218	264-267	334	106-124	177-185	232	293-314		248-309	416-432
	Coyula	# of alleles	2	20	4	2	8	4	10	9	15	8	2	1	13	2	7?	3		6	12
		range size	101-103	210-272	378-386	424-430	130-162	213-228	233-251	298-316	149-184	207-230	264-267	334	91-132	176-180	244-260	288-290		250-260	402-436
	Puerto Morelos	# of alleles	7?	12	3	6	9	1	10 ?	4	5	4	2	2	9	5	7	5		13	13
		range size	111-132	211-251	386-390	423-435	158-171	224	248-279	313-319	140-151	213-221	264-267	349-351	86-130	174-185	219-246	300-310		270-297	397-445
	Cali	# of alleles	7?	3	3	4	5	3	3	4	5	5?	4	1	1	7?	6?	4		4	8
		range size	109-117	192-215	392-396	429-437	162-170	218-222	242-252	284-317	155-170	212-225	273-285	336	86	180-192	236-243	294-314		258-287	386-404
sp. 3	Puerto Escondido (Yerba Santa)	# of alleles	1	10	ON	9	9	2	10?	3	3	8	9	1	1	5	6	3		11	9
		range size	99	199-229		437-471	147-168	226-228	225-252	294-298	144-149	209-238	301-339	328	89	181-197	226-240	294-302		277-313	389-434
sp. 4	Puente Cuatode	# of alleles	4	6	8	11	5	3	6	8	5	9	5	2	3	3	7	5?		8	12
		range size	99-104	221-246	377-496	433-475	134-145	220-230	217-229	296-323	160-170	224-270	279-297	332-334	87-91	185-193	219-238	286-294		242-287	383-430
	Mazunte (P. Zapotal)	# of alleles	4	16	5	9	7	4	8	6	11	8	2	2	6	4	7	5		9	11
		range size	95-103	216-274	377-475	434-471	130-160	225-234	221-233	284-302	155-182	230-252	279-291	332-334	87-92	177-193	229-242	286-291		248-297	374-424
	Piedras Negras	# of alleles	2	6	5	3	6	4	7	4	5	6	3	1	4	2	3	2		6	6
		range size	99-103	229-270	413-436	450-454	134-161	222-234	200-240	294-302	159-169	232-269	279-285	332	87-90	185-189	238-242	286-287		242-264	408-442
sp. 5	Huaxpaltepec	# of alleles	6	15	3	2	4	1	6	5	9	7	6	7	10	3		4		2	21
		range size	100-122	221-272	382-386	424-426	132-142	209	241-260	308-325	136-169	207-227	255-273	335-355	110-130	172-184	a refaire	290-323		254-256	401-493

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ABSTRACT

To describe and understand biodiversity, the identification of species is essential. Because some species diversify without revealing any morphologic change, the use of different taxonomic tools is highly recommended. Among the advantages of employing different traits for species classification, one of the most remarkable is that at the same time we obtain information about which traits have been involved in the diversification of species. In this study I investigated the variation observed in the ant species *Ectatomma ruidum* as an evidence of different taxa. *E. ruidum* is a widely distributed ant from the Neotropics and in previous studies based on mitochondrial sequences the species was proposed to include at least four different taxa. The geographic distribution patterns of the putative species shows that some of them are restricted to small areas, without any apparent geographic barrier separating populations, which raised the question about which mechanisms separated them. By analyzing recognition cues, acoustic signals, morphological acoustic traits and DNA sequences (mitochondrial DNA COI gene, 3RAD and UCE) I provide evidence supporting the separation for most of the previously proposed species. Additionally, the combination of phenotypic and genetic information unveiled that recognition cues may have had a very important role in the diversification of the species complex. Overall, this study adds evidence in favor of the use of a multi trait approach for the delimitation of closely related species.

Keywords: Ants, *Ectatomma ruidum*, cuticular hydrocarbons, acoustic signals, speciation.