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

Kenzy Peña Carrillo, María Cristina Lorenzi, Maxence Brault, Paul Devienne, Jean-Paul Lachaud, Gianni Pavan, Chantal Poteaux

► To cite this version:

Kenzy Peña Carrillo, María Cristina Lorenzi, Maxence Brault, Paul Devienne, Jean-Paul Lachaud, et al.. A new putative species in the *Ectatomma ruidum* complex (Formicidae: Ectatomminae) produces a species-specific distress call. *Bioacoustics*, 2021, pp.1-16. 10.1080/09524622.2021.1938226 . hal-03357524

HAL Id: hal-03357524

<https://hal.science/hal-03357524>

Submitted on 28 Sep 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Journal:	<i>Bioacoustics</i>
Manuscript ID	TBIO-2021-0005.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	18-May-2021
Complete List of Authors:	<p>PEÑA CARRILLO, KENZY; Université Sorbonne Paris Nord, LEEC; Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Campo Experimental General Terán</p> <p>Lorenzi, Maria Cristina; Université Sorbonne Paris Nord, Laboratoire d'Ethologie Expérimentale et Comparée</p> <p>Brault, Maxence; Université Sorbonne Paris Nord, Laboratoire d'Ethologie Expérimentale et Comparée</p> <p>Devienne, Paul; Université Sorbonne Paris Nord, Laboratoire d'Ethologie Expérimentale et Comparée</p> <p>Lachaud, Jean-Paul; El Colegio De La Frontera Sur, Depto. de Conservación de la Biodiversidad; Université de Toulouse III, Centre de Recherches sur la Cognition Animale, Centre de Biologie Intégrative</p> <p>Pavan, Gianni; Università degli Studi di Pavia Dipartimento di Scienze della Terra e dell'Ambiente, Dipartimento di Scienze della Terra e dell'Ambiente</p> <p>Poteaux, Chantal; Université Sorbonne Paris Nord, Laboratoire d'Ethologie Expérimentale et Comparée</p>
Keywords:	ant acoustics, stridulations, stridulatory organ, species complex

SCHOLARONE™
Manuscripts

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

Kenzy I. Peña-Carrillo^{1,2}, María Cristina Lorenzi¹, Maxence Brault¹, Paul Devienne¹, Jean-Paul Lachaud^{3,4}, Gianni Pavan⁵, Chantal Poteaux¹

¹LEEC-Laboratoire d'Ethologie Expérimentale et Comparée, Université Sorbonne Paris Nord, Sorbonne Paris Cité, 99 avenue J.-B. Clément, 93430 Villetaneuse, France.

²INIFAP, Campo Experimental General Terán, km 31 carretera Montemorelos-China, 67400, General Terán, N.L. México.

³Depto. de Conservación de la Biodiversidad, El Colegio de la Frontera Sur, Avenida Centenario Km. 5.5, 77074 Chetumal, Quintana Roo, México.

⁴Centre de Recherches sur la Cognition Animale, Centre de Biologie Intégrative, Université de Toulouse CNRS, UPS, 118 route de Narbonne, 31062 Toulouse Cedex 09, France.

⁵Centro Interdisciplinare di Bioacustica e Ricerche Ambientali, Dipartimento di Scienze della Terra e dell'Ambiente, Università degli Studi di Pavia, Via Taramelli 24, 27100, Pavia, Italy.

Corresponding author: kenzy.p@gmail.com, ORCiD ID 0000-0003-0580-6700

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).

1
2
3 42 For stridulation production, insects use specialized devices or organs where opposite areas of
4
5 43 the exoskeleton serve as file and scraper (Masters et al. 1983). In ants, the stridulatory organ
6
7 44 typically consists of a ridge (*plectrum* or scraper) located on the edge of the post-petiole, and a
8
9
10 45 plate of arrayed ridges (*pars stridens* or stridulatory file) located on the upper surface of the
11
12 46 first segment of the gaster (Hölldobler and Wilson 1990). The stridulation is produced when
13
14 47 the gaster is raised and lowered allowing the *plectrum* to rub on the ridges and produce a train
15
16 48 of pulses known as chirp (Grandi 1966, Golden and Hill 2016). The production of every pulse
17
18 49 is due to the scraper's contact with one of the ridges (Tschuch and Brothers 1999; Polidori et
19
20 50 al. 2013).

21
22
23 51 For some taxa, the study of the stridulatory organs and/or the sounds they produce revealed
24
25 52 intra- or inter-species variation; for example, in mutillid wasps (Polidori et al. 2013), in ants
26
27 53 such as *Messor* (Grasso et al. 1998) and *Neoponera apicalis* (= *Pachycondyla*) (Ferreira et al.
28
29 54 2010), and in some species of the Myrmicinae subfamily (Castro et al. 2015), underlying their
30
31 55 potential as taxonomic tools.

32
33
34 56 *Ectatomma ruidum* Roger is a mainly Neotropical ant species that exhibits the peculiar
35
36 57 intraspecific behavior named cleptobiosis (food thievery between neighboring colonies), and
37
38 58 that was recently discovered to be a species complex (Aguilar-Velasco et al. 2016). Recent
39
40 59 molecular studies have shown that some species within the complex have faced selection in
41
42 60 molecular traits (mitochondrial DNA) (Meza-Lázaro et al. 2018). This marked genetic
43
44 61 diversification is paralleled by a very high level of variation in cuticular hydrocarbon profiles
45
46 62 between the species of the complex (Peña-Carrillo et al. 2021). Cuticular hydrocarbons serve
47
48 63 as a chemical signature that conveys information about castes, task specialization, reproductive
49
50 64 status and allow social insects to discriminate between nestmates and foreigners (Blomquist
51
52 65 and Bagnères 2010; van Zweden and d'Ettorre 2010). In contrast to the molecular and chemical
53
54 66 diversification of the *E. ruidum* species complex, morphology is relatively conserved across the
55
56
57
58
59
60

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. Indeed, it was shown that bush-cricket populations characterized by distinctive song types also diverge in their cuticular lipid proportions (Dutta et al. 2018). In addition, we hypothesized that sound differences, if any, might be caused by morphometric differences in the organ used to produce the sound. To test these hypotheses, we coupled the analysis of stridulation recordings (distress call) with that of morphometry of the stridulatory file, to investigate 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 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 2021).

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. (2021), 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

142 stridulatory file (TL), maximal width (MW), 1st, 2nd and 3rd quartile widths (Q1, Q2, Q3,
143 respectively), number of ridges and inter-ridge distance (ID) in the median portion of the
144 stridulatory file (for data analyses, inter-ridge distance was the average of five measures).

145 ***Statistical analyses***

146 We reduced the number of acoustic and morphologic variables by performing separate Principal
147 Component Analyses (PCA) for acoustic and morphometric data. Then, we tested for
148 differences in PC values between species by running Generalized Linear Mixed Models
149 (GLMMs) for normally distributed data (identity link). In the GLMM, either the morphometric
150 or acoustic principal components (PCs) were the response variables, and species the fixed
151 factor; colony and population were used as random factors to account for the non-independence
152 of data. For the GLMM on acoustic data, individual identity was also used as a random factor
153 to account for non-independence of the measures from the same individual thus assuming a
154 different “baseline” measure for each subject. While the statistical comparison tests assess the
155 difference within and between individuals (or population or species), by entering a random
156 factor in the model we also controlled for individual differences. Frequency range values were
157 excluded from our statistical analyses due to variations mostly related to changes in the
158 microphone / ant distance and orientation, then, only robust temporal measures were analyzed.
159 By combining the use of the acoustic and morphometric variables we calculated the percentage
160 of rubbed ridges ((number of pulses x 100) / total number of ridges) and the speed of the rubbing
161 movement (length of rubbed surface / chirp duration of forward or backward phase) for both
162 phases. We used these data to test for variation in the sounds produced among species by
163 performing the Kruskal-Wallis test and then we checked for pairwise differences using Mann-
164 Whitney test.

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

166 **Results**

167 *Acoustic traits*

168 *The characteristics of the stridulation and their variation*

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

175 The acoustical variation was summarized in two principal components that together explained
 176 85.3% of total variance. PC1, which explained 66.8% of variance and significantly
 177 discriminated among species (GLMM, $F_{2, 427} = 5.781$, $P = 0.003$), was mainly determined by
 178 the number of pulses that composed the chirps (total number of pulses, number of pulses in the
 179 forward and backward chirp) (Table 4) (Figure 2). Pairwise comparisons indicated that only *E.*
 180 *ruidum* sp. 5 differed significantly from *E. ruidum* sp. 2 and 3-4 in PC1 values (GLMM $F_{2, 427}$
 181 $= 5.781$: *E. ruidum* sp. 5 vs sp. 2 $P = 0.016$, *E. ruidum* sp. 3-4 vs sp. 2 $P = 0.228$; GLMM, $F_{2, 427}$
 182 $= 5.547$: *E. ruidum* sp. 5 vs sp. 3-4 $P = 0.001$).

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

186 *Variation of the sounds: the link between stridulations and morphology*

187 During the stridulation, the percentage of ridges rubbed was highly significantly different
 188 among species (Kruskal-Wallis test: forward phase - $K = 11.34$, $df = 2$, $P = 0.003$, $N = 37$,
 189 backward phase - $K = 11.83$, $df = 2$, $P = 0.003$, $N = 37$). Pairwise comparisons indicated that
 190 the differences in the percentage of ridges rubbed by the ants *E. ruidum* sp. 5 were significant
 191 compared to *E. ruidum* sp. 2 and sp. 3-4 (Forward phase rubbing - sp. 5 vs sp. 2: $U = 66$, $P =$

192 0.002, N = 19; sp. 5 vs sp. 3-4: U = 89, P < 0.0001, N = 23; Backward phase rubbing - sp. 5 vs
 193 sp. 2: U = 63, P = 0.007, N = 19; sp. 5 vs sp. 3-4: U = 87, P < 0.0001, N = 23; these differences
 194 were still significant after correcting for multiple comparisons) (Figure 3). In comparison to *E.*
 195 *ruidum* sp. 2 and sp. 3-4, the ants *E. ruidum* sp. 5 produced chirps at the lowest rate because
 196 they rubbed a higher percentage of ridges (approximately the double) thus producing at least
 197 50% more pulses (Table 2). Ants *E. ruidum* sp. 2 and sp. 3-4 rubbed a similar percentage of
 198 ridges (Forward phase rubbing – U = 113, P = 0.639, N = 32; Backward phase rubbing - U =
 199 84, P = 0.116, N = 32) (Tables 2 and 3, Figure 3). The three species stridulated at similar speed
 200 (K = 3.496, df = 2, P = 0.174, N = 37, backward phase - K = 5.557, df = 2, P = 0.062, N = 37).

201 ***Morphometric traits***

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

204 The PCA performed on the morphological traits of the stridulatory file (Table 5) summarized
 205 the overall variation in two principal components that together explained 67.4 % of total
 206 variance. PC1 (41 %) was mainly related to the variation in the width of the stridulatory file
 207 (width of Q1, Q2 and maximal width, Table 6). PC2 (26.3 % of the variance) was associated to
 208 the variation in the length of the stridulatory file, the number of ridges and the body size
 209 (represented by thorax length) (Table 6). However, neither PC1 nor PC2 scores differed
 210 significantly between species (GLMM, PC1: $F_{2,39} = 0.032$, P = 0.97, PC2; $F_{2,39} = 1.467$, P =
 211 0.24.

212 **Discussion**

213 Our results show that worker ants belonging to *E. ruidum* sp. 5, the new putative species
 214 recently reported by Peña-Carrillo et al. (2021), produce a distress call that differs from the
 215 closely related species *E. ruidum* sp. 2, and sp. 3-4. To our knowledge, the acoustic
 216 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 (Torricobazoberry 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.

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

242 Hirschberger 2005) and bush crickets (Chobanov et al. 2013; Zanthiev et al. 2017). For all these
243 taxa, sound variation reflected selection on calls serving crucial communication functions, such
244 as courtship calls. For instance, Kasper and Hirschberger (2005) studied the behavioral context
245 and acoustic structure of calls produced by different species of *Aphodius* beetles, as well as the
246 morphology of their stridulatory organs. They found that the beetles produced calls when they
247 were disturbed, and that males produced species-specific calls when they encountered females
248 in an experimental dung arena; the stridulatory organs of the beetles also presented species-
249 specific morphology.

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

258 The analysis of distress calls has also been useful to reveal inter-specific variation. For example,
259 Polidori et al. (2013) compared the distress calls and morphology of the stridulatory file of
260 European velvet ants from different subfamilies and genera. They found acoustic differences
261 between the species, but statistics for all the studied species weakly supported the species-
262 specific association of sound variation with morphological variance. Carlos et al. (2014)
263 analyzed the stridulations and the stridulatory file of the workers from three species of Attini
264 ants and reported that the chirps' duration was species-specific. Their study demonstrated
265 stridulations as an efficient taxonomic tool for species identification in the Attini tribe. Distress
266 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. 2021).

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, as our results also suggest. Although the limited sampling used for morphological analyses did not allow us to detect any significant variation in the structure of the stridulatory file of the workers, 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. 2021). 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. 2021). 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. 2021), 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.

Acknowledgments

This study is part of the PhD project of KIPC supported by a CONACyT-French government scholarship. Thanks to Miguel Quiroz, Franco Robles Guerrero, Rubí Meza-Lázaro, Gabriela Pérez-Lachaud, Alejandro Zaldivar Riverón and Carlos Santamaría for their help during the field trips in Mexico and Colombia. We thank Ovidiu Brinza from the LSPM-CNRS for his assistance while taking SEM photos and Christophe Féron for his valuable help with the scripts employed for seewave analyses.

Ethical statement

Our study followed the institutional and national ethical guidelines for scientific research in Mexico, Colombia and France. The species used in the experiments are not endangered or protected in Mexico and Colombia and the collection of live ants complied with the current laws in their country of origin.

Disclosure statement

The authors declared no potential conflict of interest.

Funding

This Project was partially funded by the Ecos-Nord-CONACYT Program, project number M12A01 granted to CP.

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on request.

References

- Aguilar-Velasco RG, Poteaux C, Meza-Lázaro R, Lachaud J-P, Dubovikoff D, Zaldivar-Riverón A. 2016. Uncovering species boundaries in the Neotropical ant complex *Ectatomma ruidum* (Ectatomminae) under the presence of nuclear mitochondrial paralogues. Zool J Linn Soc. 178:226–240.

- 340 Baumann-Pickering S, McDonald MA, Simonis AE, Solsona-Berga A, Merckens KPB, Oleson
341 EM, Roch MA, Wiggins SM, Rankin S, Yack TM, Hildebrand JA. 2013. Species-specific
342 beaked whale echolocation signals. *J Acoust Soc Am.* 134:2293–2301.
- 343 Blomquist GJ, Bagnères A-G. 2010. Insect hydrocarbons: biology, biochemistry and chemical
344 ecology. Cambridge (UK): Cambridge University Press; p. 79–81.
- 345 Brizio C, Buzzetti FM, Pavan G. 2020. Beyond the audible: wide band (0-125 kHz) field
346 investigation on Italian Orthoptera (Insecta) songs. *Biodivers J.* 11:443–496.
- 347 Carlos AA, Barbero F, Casacci LP, Bonelli S. 2014. Bioacoustics of *Trachymyrmex fuscus*,
348 *Trachymyrmex tucumanus* and *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). *J*
349 *Acoust Soc Am.* 136:2074.
- 350 Castro S, Alvarez M, Mungira ML. 2015. Morphology of the stridulatory organs of Iberian
351 myrmicinae ants (Hymenoptera: Formicidae). *Ital J Zool.* 82:387–397.
- 352 Chobanov DP, Grzywacz B, Iorgu IŞ, Ciplak B, Ilieva MB, Warchałowska-Śliwa E. 2013.
353 Review of the Balkan Isophya (Orthoptera: Phaneropteridae) with particular emphasis on
354 the *Isophya modesta* group and remarks on the systematics of the genus based on
355 morphological and acoustic data. *Zootaxa.* 3658:1–81.
- 356 CIBRA: Centro Interdisciplinare di Bioacustica e Ricerche Ambientali. 2017. Pavia, Italy:
357 Università degli Studi di Pavia; [accessed 2021, October 19th].
358 (<http://www.unipv.it/cibra/seapro.html>)
- 359 Claridge MF. 1985. Acoustic signals in the Homoptera: behavior, taxonomy and evolution.
360 *Annu Rev Entomol.* 30:297–317.
- 361 Cocroft RB, Rodríguez RL. 2005. The behavioral ecology of insect vibrational communication.
362 *BioScience.* 55:323–334.

- 363 Cupul Magaña FG. 2009. Primera observación del comportamiento defensivo por muerte
364 simulada de la hormiga *Ectatomma ruidum* (Roger, 1861) (Formicidae, Ponerinae). Acta
365 Zool Mex. 25: 199–201.
- 366 d’Ettorre P, Moore AJ. 2008. Chemical communication and the coordination of social
367 interactions in insects. In: d’Ettorre P, Hughes DP, editors. Sociobiology of communication:
368 an interdisciplinary perspective. New York: Oxford University Press; p.81–96.
- 369 Dutta R, Balakrishnan R, Tregenza T. 2018. Divergence in potential contact pheromones and
370 genital morphology among sympatric song types of the bush cricket *Mecopoda elongata*.
371 Front Ecol Evol. 6:158.
- 372 Endler JA. 1992. Signals, signal conditions, and the direction of evolution. Am Nat. 139:S125–
373 S153.
- 374 Ferreira RS, Poteaux C, Delabie JHC, Fresneau D, Rybak F. 2010. Stridulations reveal cryptic
375 speciation in neotropical sympatric ants. PLoS ONE. 5:e15363.
- 376 Golden TMJ, Hill PSM. 2016. The evolution of stridulatory communication in ants, revisited.
377 Insect Soc. 63:309–319.
- 378 Grandi G. 1966. Istituzioni di Entomologia Generale. Bologna: Calderini; p.655.
- 379 Grasso DA, Mori A, Le Moli F, Giovannotti M, Fanfani A. 1998. The stridulatory organ of four
380 *Messor* ant species (Hymenoptera, Formicidae). Ital J Zool. 65:167–174.
- 381 Grasso DA, Priano M, Pavan G, Mori A, Le Moli F. 2000. Stridulation in four species of *Messor*
382 ants (Hymenoptera: Formicidae). Ital J Zool. 67:281–285.
- 383 Henry CS, Martínez WM, Pupedis RJ. 1993. Hidden taxonomic diversity within *Chrysoperla*
384 *plorabunda* (Neuroptera: Chrysopidae): two new species based on courtship songs. Ann
385 Entomol Soc Am. 86:1–13.
- 386 Hickling R, Brown RL. 2000. Analysis of acoustic communication by ants. J Acoust Soc Am.
387 108:1920–1929.

- 388 Hölldobler B. 1995. The chemistry of social regulation: Multicomponent signals in ant
389 societies. *Proc Natl Acad Sci.* 92:19–22.
- 390 Hölldobler B. 1999. Multimodal signals in ant communication. *J Comp Physiol A.* 184:129–
391 141.
- 392 Hölldobler B, Wilson EO. 1990. *The Ants*. Cambridge (MA): Harvard University Press; p. 227–
393 259.
- 394 Kasper J, Hirschberger P. 2005. Stridulation in *Aphodius* dung beetles: Songs and morphology
395 of stridulatory organs in North American *Aphodius* species (Scarabaeidae). *J Nat Hist.*
396 39:91–99.
- 397 Lehmann GUC, Frommolt K-H, Lehmann AW, Riede K. 2014. Baseline data for automated
398 acoustic monitoring of Orthoptera in a Mediterranean landscape, the Hymettos, Greece. *J*
399 *Insect Conserv.* 18:909–925.
- 400 Leonhardt SD, Menzel F, Nehring V, Schmitt T. 2016. Ecology and evolution of
401 communication in social insects. *Cell.* 164:1277–1287.
- 402 Lin T-H, Yu H-Y, Chou L-S, Chen C-F. 2014. Passive acoustic monitoring on the seasonal
403 species composition of cetaceans from marine cable hosted observatory. *Oceans. IEEE.* 1-6.
- 404 Mahoney SM, Reudink MW, Pasch B, Theimer TC. 2020. Song but not plumage varies
405 geographically among willow flycatcher *Empidonax traillii* subspecies. *J Avian Biol.* 51
406 [Accessed 2020 Dec 27] <https://doi.org/10.1111/jav.02621>
- 407 Markl H. 1965. Stridulation in leaf cutting ants. *Science.* 149:1392–1393.
- 408 Masters WM, Tautz J, Fletcher NH, Markl H. 1983. Body vibration and sound production in an
409 insect (*Atta sexdens*) without specialized radiating structures. *J Comp Physiol A.* 150:239–
410 249.

- McNeil DJ, Grozinger CM. 2020. Singing in the suburbs: point count surveys efficiently reveal habitat associations for nocturnal Orthoptera across an urban-to-rural gradient. *J Insect Conserv.* 24:1031–1043.
- Meza-Lázaro RN, Poteaux C, Bayona-Vásquez NJ, Branstetter MG, Zaldívar-Riverón A. 2018. Extensive mitochondrial heteroplasmy in the neotropical ants of the *Ectatomma ruidum* complex (Formicidae: Ectatomminae). *Mitochondrial DNA A.* 29:1203–1214.
- Oswald JN, Rankin S, Barlow J, Lammers MO. 2007. A tool for real-time acoustic species identification of delphinid whistles. *J Acoust Soc Am.* 122:587–595.
- Pan AD, Williams KA, Wilson JS. 2017. Are diurnal iguanian lizards the evolutionary drivers of New World female velvet ant (Hymenoptera: Mutillidae) Müllerian mimicry rings? *Biol J Linn Soc.* 120:436–447.
- Pavan G, Priano M, De Carli P, Fanfani A, Giovannotti M. 1997. Stridulatory organ and ultrasonic emission in certain species of Ponerinae ants (Genus: *Ectatomma* and *Pachycondyla*, Hymenoptera, Formicidae). *Bioacoustics.* 8:209–221.
- Peña-Carrillo KI, Poteaux C, Leroy C, Meza-Lázaro RN, Lachaud J-P, Zaldívar-Riverón A, Lorenzi MC. 2021. Highly divergent cuticular hydrocarbon profiles in the cleptobiotic ants of the *Ectatomma ruidum* species complex. *Chemoecology.* 31:125–135.
- Polidori C, Pavan G, Ruffato G, Asís JD, Tormos J. 2013. Common features and species-specific differences in stridulatory organs and stridulation patterns of velvet ants (Hymenoptera: Mutillidae) *Zool Anz.* 252:457–468.
- Raven Lite: Interactive Sound Analysis Software. Version 2.0.0. Ithaca, NY: The Cornell Lab of Ornithology. URL <http://www.birds.cornell.edu/raven>.
- Rheindt FE, Norman JA, Christidis L. 2008. DNA evidence shows vocalizations to be a better indicator of taxonomic limits than plumage patterns in *Zimmerius* tyrant-flycatchers. *Mol Phylogenetics Evol.* 48:150–156.

- 1
2
3 436 Ritchie MG, Gleason JM. 1995. Rapid evolution of courtship song pattern in *Drosophila*
4
5 437 *willistoni* sibling species. J Evol Biol. 8:463–479.
6
7 438 Riva F, Barbero F, Bonelli S, Balleto E, Casacci LP. 2016. The acoustic repertoire of lycaenid
8
9 439 butterfly larvae. Bioacoustics. 26:77–90.
10
11
12 440 Roces F, Tautz J, Hölldobler B. 1993. Stridulation in leaf cutting ants. Naturwissenschaften 80:
13
14 441 521–524.
15
16
17 442 RStudio Team. 2016. RStudio: Integrated Development for R. Version 3.5.2. Boston, MA:
18
19 443 RStudio, Inc. URL <http://www.rstudio.com/>.
20
21 444 Schatz B, Lachaud J-P, Beugnon G. 1997. Graded recruitment and hunting strategies linked to
22
23 445 prey weight and size in the ponerine ant *Ectatomma ruidum*. Behav Ecol Sociobiol. 40:337–
24
25 446 349.
26
27
28 447 Schmitt M. 1994. Stridulation in leaf beetles (Coleoptera, Chrysomelidae). In: Jolivet PH, Cox
29
30 448 ML, Petitpierre E, editors. Novel aspects of the biology of Chrysomelidae. Dordrecht:
31
32 449 Kluwer Academia Publishers; p.319–325.
33
34
35 450 Schmitt M, Traue D. 1990. Morphological and bioacoustic aspects of stridulation in Criocerinae
36
37 451 (Coleoptera Chrysomelidae). Zool Anz. 225:225–240.
38
39
40 452 Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image
41
42 453 analysis, Nat methods 9: 671–675.
43
44
45 454 Sueur J, Aubin T, Simonis C. 2008. Seewave: a free modular tool for sound analysis and
46
47 455 synthesis. Bioacoustics. 18: 213–226.
48
49 456 Tan MK, Yong CYH, Ingrisch S, Ahmad Sah HH, Wahab RBHA, Johns PM. 2018. Inferring
50
51 457 species boundaries using acoustic and morphological data in the ground cricket genus
52
53 458 *Gymnogryllus* (Orthoptera: Grylloidea: Gryllinae). Syst Biodivers. 16:731–742.
54
55
56 459 Torrico-Bazoberry D, Muñoz MI. 2019. High frequency component in the distress stridulation
57
58 460 of Chilean endemic velvet ants (Hymenoptera: Mutillidae). Rev Chil Entomol. 45:5–13.
59
60

- 1
2
3 461 Tschuch G, Brothers DJ. 1999. Modeling vibration and sound production in insects with
4
5 462 nonresonant stridulatory organs. *J Acoust Soc Am*. 106:3706–3710.
6
7
8 463 van Zweden JS, d’Ettorre P. 2010. Nestmate recognition in social insects and the role of
9
10 464 hydrocarbons. In: Blomquist GJ, Bagnères A-G, editors. *Insect hydrocarbons: biology,*
11
12 465 *biochemistry and chemical ecology*. Cambridge (UK): Cambridge University Press; p.
13
14 466 222–243.
15
16
17 467 Wilson JS, Williams KA, Forister ML, von Dohlen CD, Pitts JP. 2012. Repeated evolution in
18
19 468 overlapping mimicry rings among North American velvet ants. *Nat Commun*. 3:1272.
20
21 469 Wilson JS, Pan AD, Limb ES, Williams KA. 2018. Comparison of African and North American
22
23 470 velvet ant mimicry complexes: another example of Africa as the ‘odd man out’. *PLoS ONE*.
24
25 471 13:e0189482.
26
27
28 472 Zhantiev RD, Korsunovskaya OS, Benediktov A. 2017. Acoustic signals of the bush-crickets
29
30 473 *Isophya* (Orthoptera: Phaneropteridae) from Eastern Europe, Caucasus and adjacent
31
32 474 territories. *Eur J Entomol*. 114:301–3011.
33
34
35 475
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

476 **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	7	5
				10Z3	9	-
	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	10	6
				92	7	-
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	5	6
				Huax1-1	5	-
				Huax2-8	5	-
				Huax2-14	5	-
Total number of individuals					85	42

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)

	<i>E. ruidum</i> sp. 2	<i>E. ruidum</i> sp. 3-4	<i>E. ruidum</i> 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

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)).

	<i>E. ruidum</i> sp. 2	<i>E. ruidum</i> sp. 3-4	<i>E. ruidum</i> 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

1
2
3
494
4
495
5
6
496
7
8
497
9
10
11
498
12
13
499
14
15
500
16
17
501
18
19
502
20
21
503
22
23
24
504
25
26
27
505
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

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

Figure 1.

Figure 2.

Figure 3.

Figure 4.

1
2
3 520 **Figure 1.** (a) A sequence of disyllabic chirps in the *E. ruidum* stridulation. (b) Detailed view of a
4
5 521 disyllabic chirp: forward phase, backward phase and the GAP (time space in between forward and
6
7 522 backward phases). (c) Detailed view of pulses of the forward and backward phase around the gap
8
9 523 from minute 1:54.45 to 1:54.7.
10
11
12 524 **Figure 2.** PCA's plot of the acoustical traits in the three species of the *E. ruidum* complex.
13
14 525 **Figure 3.** Acoustic variation of *E. ruidum*. (a) Total number of pulses per phase and species (b)
15
16 526 Percentage of rubbed ridges per phase and species during the stridulation; bars colored with gray
17
18 527 background represent the forward phase and bars with white background the backward phase of
19
20 528 the chirps.
21
22
23 529 **Figure 4.** The stridulatory file in *E. ruidum*. (a) Position of the file in the gaster; arrows indicate
24
25 530 the backward and forward movement of the gaster when stridulating. (b) Postpetiolar segment of
26
27 531 the gaster where the stridulatory file is located. (c) Morphometric variables taken from the
28
29 532 stridulatory file: total length of stridulatory file (TL), maximal width (MW), 1st, 2nd and 3rd quartile
30
31 533 widths (Q1, Q2, Q3, respectively). (d) Ridges in the stridulatory file and focus on the inter-ridge
32
33 534 distance measure (ID).
34
35
36
37 535
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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.

241x195mm (150 x 150 DPI)

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

164x146mm (150 x 150 DPI)

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.

268x141mm (150 x 150 DPI)

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) Stridulatory file and its measures. (d) Ridges in the stridulatory file and focus on the inter-ridge distance measure.

145x115mm (150 x 150 DPI)