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

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Complete List of Authors:	PEÑA CARRILLO, KENZY; Université Sorbonne Paris Nord, LEEC; Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Campo Experimental General Terán Lorenzi, Maria Cristina; Université Sorbonne Paris Nord, Laboratoire d'Ethologie Expérimentale et Comparée Brault, Maxence; Université Sorbonne Paris Nord, Laboratoire d'Ethologie Expérimentale et Comparée Devienne, Paul; Université Sorbonne Paris Nord, Laboratoire d'Ethologie Expérimentale et Comparée 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 Pavan, Gianni; Università degli Studi di Pavia Dipartimento di Scienze della Terra e dell'Ambiente, Dipartimento di Scienze della Terra e dell'Ambiente Poteaux, Chantal; Université Sorbonne Paris Nord, Laboratoire d'Ethologie Expérimentale et Comparée
Keywords:	ant acoustics, stridulations, stridulatory organ, species complex

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6 2 ***Ectatomminae*) produces a species-specific distress call**

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8 3 **Kenzy I. Peña-Carrillo^{1,2}, María Cristina Lorenzi¹, Maxence Brault¹, Paul Devienne¹,**
9
10 4 **Jean-Paul Lachaud^{3,4}, Gianni Pavan⁵, Chantal Poteaux¹**

11
12
13 5 ¹LEEC-Laboratoire d'Ethologie Expérimentale et Comparée, Université Sorbonne Paris Nord,
14
15 6 Sorbonne Paris Cité, 99 avenue J.-B. Clément, 93430 Villetaneuse, France.

16
17 7 ²INIFAP, Campo Experimental General Terán, km 31 carretera Montemorelos-China, 67400,
18
19 8 General Terán, N.L. México.

20
21
22 9 ³Depto. de Conservación de la Biodiversidad, El Colegio de la Frontera Sur, Avenida
23
24 10 Centenario Km. 5.5, 77074 Chetumal, Quintana Roo, México.

25
26
27 11 ⁴Centre de Recherches sur la Cognition Animale, Centre de Biologie Intégrative, Université
28
29 12 de Toulouse CNRS, UPS, 118 route de Narbonne, 31062 Toulouse Cedex 09, France.

30
31 13 ⁵Centro Interdisciplinare di Bioacustica e Ricerche Ambientali, Dipartimento di Scienze della
32
33 14 Terra e dell' Ambiente, Università degli Studi di Pavia, Via Taramelli 24, 27100, Pavia, Italy.

34
35
36 15 Corresponding author: kenzy.p@gmail.com, ORCID ID 0000-0003-0580-6700

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17 **Abstract**

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

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

32 **Introduction**

33 Communication is an important feature of animal life that allows individuals to share
34 information and its efficiency is essential for social life (Hölldobler 1999; d'Ettorre and Moore
35 2008). In social insects and particularly in ants, the efficient communication system and the
36 social organization are the primary bases for their evolutionary success; the efficient
37 transmission of information allows them to regulate the functional division of castes, cooperate
38 in rearing immature stages, gather food, defend the nest, explore new foraging grounds,
39 establish territorial borders and reject foreigners (Hölldobler 1995). Apart from chemical cues,
40 social insects communicate by using visual, tactile, and vibro-acoustic signals, among them
41 stridulations (Hölldobler and Wilson 1990; Hölldobler 1999; Leonhardt et al. 2016).

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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 45 plate of arrayed ridges (*pars stridens* or stridulatory file) located on the upper surface of the
10
11 46 first segment of the gaster (Hölldobler and Wilson 1990). The stridulation is produced when
12
13 47 the gaster is raised and lowered allowing the *plectrum* to rub on the ridges and produce a train
14
15 48 of pulses known as chirp (Grandi 1966, Golden and Hill 2016). The production of every pulse
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17 49 is due to the scraper's contact with one of the ridges (Tschuch and Brothers 1999; Polidori et
18
19 50 al. 2013).

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21
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23 51 For some taxa, the study of the stridulatory organs and/or the sounds they produce revealed
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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
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31 55 potential as taxonomic tools.

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

69 In *E. ruidum*, stridulations consist of sequences of pulse-trains organized in two subunits
70 (disyllabic chirps) composed by series of pulses with opposite phases (Pavan et al. 1997). In a
71 previous study (Pavan et al. 1997), the distress call of *E. ruidum* was analyzed in a limited
72 sample of workers from a single population in the south of Mexico (state of Chiapas), at a time
73 when the taxonomic complexity of the group and the existence of different species within the
74 complex were unacknowledged.

75 If communication systems are targets of selection (Endler 1992), we expected that, based on
76 the evidence that the *E. ruidum* species complex exhibits high variation in their chemical
77 communication system between and within species, divergence was also apparent in acoustic
78 signaling. Indeed, it was shown that bush-cricket populations characterized by distinctive song
79 types also diverge in their cuticular lipid proportions (Dutta et al. 2018). In addition, we
80 hypothesized that sound differences, if any, might be caused by morphometric differences in
81 the organ used to produce the sound. To test these hypotheses, we coupled the analysis of
82 stridulation recordings (distress call) with that of morphometry of the stridulatory file, to
83 investigate whether acoustic signals diverged between the species *E. ruidum* sp. 2, sp. 3-4 and
84 the newly reported *E. ruidum* sp. 5.

85 **Materials and Methods**

86 ***Collection of ants***

87 The ants of the *E. ruidum* complex were collected in the states of Oaxaca and Quintana Roo
88 (Mexico), and in Cali (Colombia) (Table 1); colonies from the same collection site were at least
89 1-2 m distant from each other. It is worth pointing that the different species of these ants cannot
90 be distinguished in the field and that collection sites are often difficult to reach. Therefore, only
91 after molecular or chemical investigations in the lab ants can be assigned to one of the species,

92 making it difficult to plan the number of colonies per species and per population to collect
93 during field trips. In particular, no replicate populations are possible for *E. ruidum* sp. 5, because
94 up to now only one population has been identified (Pena-Carrillo et al 2021).

95 ***Laboratory rearing and species identification***

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

99 The colonies from Coyula and Cali were identified as *E. ruidum* sp. 2, and those from Puerto
100 Escondido, Piedras Negras and Mazunte as *E. ruidum* sp. 3-4 by Meza-Lázaro et al. (2018).
101 The colonies from Punto 3 and Huaxpaltepec were identified as *E. ruidum* sp. 3-4 and sp. 5,
102 respectively by Peña-Carrillo et al. (2021), and those from Puerto Morelos as *E. ruidum* sp. 2
103 by Poteaux et al., based on barcoding sequences (COI gene) (unpublished data).

104 ***Stridulation recording and analysis***

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

107 We recorded the sounds produced by individual ants separated from their nestmates in a room
108 isolated from environmental sounds and electric interferences; experimental conditions were
109 the same in terms of temperature (room temperature 25 – 28 °C) and humidity (40 %). To
110 induce the distress call, we held each individual by the thorax with soft entomological forceps
111 and placed it at 1 cm from the ultrasonic microphone for at least one minute, following Pavan
112 et al. (1997) and Ferreira et al. (2010). Inducing the distress call and getting homogenous series
113 of chirps was time consuming, as often *E. ruidum* individuals exhibited a freezing behavior in
114 which the ants retracted their limbs and stayed immobile with their bodies hunching after
115 disturbance (Cupul-Magaña 2009). We used the ultrasonic USB microphone UltraMic 250 by
116 Dodotronic, and the Sea Pro 2.0 software package developed by Pavan G. at CIBRA for sound

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3 117 recording and real-time display. All recordings were performed at 250k sampling rate with 16
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5 118 bits dynamic range according to the AD converter of the microphone. After sound recording,
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7 119 ants were killed by freezing and kept in 95% ethanol for the analysis of the stridulatory file
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9 120 morphology.

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12 121 For each ant we analyzed five chirps which were selected only if they were included in
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14 122 continuous and homogenous series of chirps, and if they presented the disyllabic structure
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16 123 described by Pavan et al. (1997), that is, two pulse trains with opposite phase, separated by a
17
18 124 gap. Using the software Raven Lite (Bioacoustics Research Program, 2016), we measured the
19
20 125 following variables: chirp duration, duration of forward phase, inter-chip duration (GAP),
21
22 126 duration of backward phase and frequency range (intended as the upper limit of frequency range
23
24 127 of the pulses). Then, by using the “seewave” package (Sueur et al. 2008) in RStudio (Version
25
26 128 3.5.2), we counted the number of pulses in the forward and backward phase of the disyllabic
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28 129 chirps. We also calculated the total number of pulses (sum of pulses in the forward and
29
30 130 backward phase), the chirp rate (number of chirps / second) and the average pulse rate of the
31
32 131 forward and backward phase (number of pulses / second).

33 132 *Morphometry of the stridulatory file*

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36 133 After the sound recording, 42 out of the 85 workers used for the acoustical analyses were
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38 134 dissected under a stereoscope and we took pictures of their stridulatory file with a Leica S440
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40 135 Scanning Electron Microscope (SEM). For each ant, the fourth abdominal segment of the gaster
41
42 136 containing the stridulatory organ was cleaned by submersion in distilled water in an ultrasonic
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44 137 bath for approximately five minutes, and then air-dried and fixed in a drop of silver plate over
45
46 138 an aluminum stub. Once fixed, the samples were oven-dried for two to three hours at 50 °C,
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48 139 and coated with an 8-nm mixture of gold-palladium for being photographed with the SEM.
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50 140 Using the software Image J 1.51 (Schneider et al. 2012), we measured the following
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52 141 morphometric variables according to Ferreira et al. (2010): thorax length, total length of
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3 142 stridulatory file (TL), maximal width (MW), 1st, 2nd and 3rd quartile widths (Q1, Q2, Q3,
4
5 143 respectively), number of ridges and inter-ridge distance (ID) in the median portion of the
6
7 144 stridulatory file (for data analyses, inter-ridge distance was the average of five measures).
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9

10 145 ***Statistical analyses***

11
12 146 We reduced the number of acoustic and morphologic variables by performing separate Principal
13
14 147 Component Analyses (PCA) for acoustic and morphometric data. Then, we tested for
15
16 148 differences in PC values between species by running Generalized Linear Mixed Models
17
18 149 (GLMMs) for normally distributed data (identity link). In the GLMM, either the morphometric
19
20 150 or acoustic principal components (PCs) were the response variables, and species the fixed
21
22 151 factor; colony and population were used as random factors to account for the non-independence
23
24 152 of data. For the GLMM on acoustic data, individual identity was also used as a random factor
25
26 153 to account for non-independence of the measures from the same individual thus assuming a
27
28 154 different “baseline” measure for each subject. While the statistical comparison tests assess the
29
30 155 difference within and between individuals (or population or species), by entering a random
31
32 156 factor in the model we also controlled for individual differences. Frequency range values were
33
34 157 excluded from our statistical analyses due to variations mostly related to changes in the
35
36 158 microphone / ant distance and orientation, then, only robust temporal measures were analyzed.
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38 159 By combining the use of the acoustic and morphometric variables we calculated the percentage
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40 160 of rubbed ridges ((number of pulses x 100) / total number of ridges) and the speed of the rubbing
41
42 161 movement (length of rubbed surface / chirp duration of forward or backward phase) for both
43
44 162 phases. We used these data to test for variation in the sounds produced among species by
45
46 163 performing the Kruskal-Wallis test and then we checked for pairwise differences using Mann-
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48 164 Whitney test.
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51 165 All statistical analyses were conducted with the software IBM SPSS version 23.
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56 166 **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 (Kruskall-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: $U = 66$, $P =$

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3 192 0.002, N = 19; sp. 5 vs sp. 3-4: U = 89, P < 0.0001, N = 23; Backward phase rubbing - sp. 5 vs
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5 193 sp. 2: U = 63, P = 0.007, N = 19; sp. 5 vs sp. 3-4: U = 87, P < 0.0001, N = 23; these differences
6
7 194 were still significant after correcting for multiple comparisons) (Figure 3). In comparison to *E.*
8
9 195 *ruidum* sp. 2 and sp. 3-4, the ants *E. ruidum* sp. 5 produced chirps at the lowest rate because
10
11 196 they rubbed a higher percentage of ridges (approximately the double) thus producing at least
12
13 197 50% more pulses (Table 2). Ants *E. ruidum* sp. 2 and sp. 3-4 rubbed a similar percentage of
14
15 198 ridges (Forward phase rubbing – U = 113, P = 0.639, N = 32; Backward phase rubbing - U =
16
17 199 84, P = 0.116, N = 32) (Tables 2 and 3, Figure 3). The three species stridulated at similar speed
18
19 200 (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

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3 217 rubbed by the *plectrum* during its movement), due to the similarity in the morphological traits
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5 218 of the stridulatory files. However, although the difference in the acoustical traits is clear, the
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7 219 lack of morphological differences of the stridulatory file between the species may need further
8
9 220 investigations.

10
11 221 Ant stridulations are involved in different behaviors. *Solenopsis richteri* ants stridulate when
12
13 222 they are alarmed, when they attack or are stressed (e.g., stress induced by holding the body of
14
15 223 the ants) (Hickling and Brown, 2000). In *Atta cephalotes*, workers use a distress call to attract
16
17 224 nestmates in rescue behavior when they are trapped under the soil (Markl, 1965); to attract ants
18
19 225 of other castes to fight against parasites, and to recruit nestmates when cutting leaves to
20
21 226 provision the colony fungi (Roces et al. 1993; Hölldobler, 1999). In velvet ants, distress calls
22
23 227 have been interpreted as warning signals against predators (Wilson et al. 2012, 2018; Pan et al.
24
25 228 2017), and a possible role in intra-specific communication has been mentioned (Torricco-
26
27 229 Bazoberry and Muñoz 2019). In *E. ruidum*, stridulations may serve as a modulatory signal
28
29 230 enhancing short-range recruitment of nestmates during collective strategies of prey retrieval
30
31 231 (Schatz et al. 1997), although focused experiments are needed to confirm this hypothesis.
32
33 232 According to Tschuch and Brothers (1999), non-resonant structures as the stridulatory organ
34
35 233 serve for short-range intra and interspecific communication; however, their sound power level
36
37 234 might not be strong enough to be effective over a distance.

38
39 235 In the event that *E. ruidum* sp. 5 lived in sympatry with other species of the complex, its distress
40
41 236 call could reflect divergent selection faced by the species in the presence of another species,
42
43 237 although the benefit of producing species-specific distress calls might be argued. Up to now,
44
45 238 whether *E. ruidum* sp. 5 is sympatric with other species of the complex is unknown.

46
47 239 Acoustic signaling has been used as a means to distinguish species because calls have emerged
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49 240 as species-specific in different groups of insects, such as homopterans (Claridge, 1985),
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51 241 lacewings (Henry et al. 1993), flies (Ritchie and Gleason 1995), beetles (Kasper and
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3 242 Hirschberger 2005) and bush crickets (Chobanov et al. 2013; Zanthiev et al. 2017). For all these
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5 243 taxa, sound variation reflected selection on calls serving crucial communication functions, such
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7 244 as courtship calls. For instance, Kasper and Hirschberger (2005) studied the behavioral context
8
9 245 and acoustic structure of calls produced by different species of *Aphodius* beetles, as well as the
10
11 246 morphology of their stridulatory organs. They found that the beetles produced calls when they
12
13 247 were disturbed, and that males produced species-specific calls when they encountered females
14
15 248 in an experimental dung arena; the stridulatory organs of the beetles also presented species-
16
17 249 specific morphology.
18
19 250 In coevolutionary relationships, calls used for acoustic mimicry are also species-specific, as
20
21 251 reported in lycaenid butterflies among which some species spend their larval stages as parasites
22
23 252 in ant nests. Riva et al. (2016) studied the acoustic repertoire of parasitic and non-parasitic
24
25 253 lycaenid larvae and found that typically all species produced species-specific calls and that the
26
27 254 calls of parasitic species revealed their association with different species of host ants. That is,
28
29 255 the calls of parasitic species had a higher number of harmonics which overlapped their hosts'
30
31 256 frequency range, which in turn potentially increased their chances to stimulate host ant receptors
32
33 257 and get food from the ants.
34
35 258 The analysis of distress calls has also been useful to reveal inter-specific variation. For example,
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37 259 Polidori et al. (2013) compared the distress calls and morphology of the stridulatory file of
38
39 260 European velvet ants from different subfamilies and genera. They found acoustic differences
40
41 261 between the species, but statistics for all the studied species weakly supported the species-
42
43 262 specific association of sound variation with morphological variance. Carlos et al. (2014)
44
45 263 analyzed the stridulations and the stridulatory file of the workers from three species of Attini
46
47 264 ants and reported that the chirps' duration was species-specific. Their study demonstrated
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49 265 stridulations as an efficient taxonomic tool for species identification in the Attini tribe. Distress
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51 266 calls are useful taxonomic tools for cryptic species delimitation. By studying a set of cryptic
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3 267 species within the *Neoponera apicalis* complex, Ferreira et al. (2010) found that the calls of
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5 268 sympatric species differed from each other, probably because of their sympatric interactions. In
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7
8 269 addition, in combination with the distress calls, the analysis of the stridulatory file and
9
10 270 mitochondrial DNA sequences highlighted the cryptic species (Ferreira et al. 2010).

11
12 271 In comparison to the species of the *N. apicalis* complex, up to now there is no evidence whether
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14 272 the species of *E. ruidum* are sympatric, but populations of the different species live close
15
16
17 273 geographically. Yet, there seems to be no clear association between geographic distance and
18
19 274 sound differentiation. According to Aguilar-Velasco et al. (2016), *E. ruidum* sp. 2 and sp. 3-4
20
21 275 are located at fewer than 25 km of distance from each other, and the current results show that
22
23 276 their stridulations did not differ significantly. In contrast, *E. ruidum* sp. 2 and sp. 3-4
24
25 277 stridulations differed from that of *E. ruidum* sp. 5, whose colonies were located within the
26
27 278 distribution range of *E. ruidum* sp. 3-4 (with the closest population separated by ~ 60 km), and
28
29 279 close to a population of *E. ruidum* sp. 2 (located less than 20 km away, Peña-Carrillo et al.
30
31 280 2021).

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35 281 In some insects, morphological structures for sound production differ more than sounds
36
37 282 between species (Schmitt 1994; Schmitt and Traue 1990) while in other cases, the opposite was
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39
40 283 suggested. For example, like our results suggest, Grasso et al. (1998, 2000) found that the
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42 284 stridulations produced by the different castes of *Messor* ants varied not because of
43
44 285 morphometry, but because of the way the ants produce the sound, like the speed of the
45
46 286 movement and the duration of the gaps between phases in the chirps, as our results also suggest.

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49 287 Although the limited sampling used for morphological analyses did not allow us to detect any
50
51 288 significant variation in the structure of the stridulatory file of the workers, the combination of
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53 289 acoustic and morphological traits generated inter-specific variation in *E. ruidum* stridulation.
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56 290 Furthermore, the same acoustic traits that discriminated *E. ruidum* sp. 5 from the other species
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58 291 (this study) also discriminated between cryptic species of *Neoponera* ants (namely chirp
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3 292 duration, the number of pulses and velocity of the rubbing movement) (Ferreira et al. 2010),
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5 293 supporting the use of acoustic traits in integrative taxonomic studies. In fact, diverse taxa can
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7 294 be diagnosed through their acoustic production. For example, birds produce distinct songs
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9 295 which allow species identification, even when they are otherwise very similar morphologically
10
11 296 (Rheindt et al. 2008; Mahoney et al. 2020), and sound production is used to identify species in
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13 297 marine mammals (Oswald et al. 2007; Bauman-Pickering et al. 2013; Lin et al. 2014), and in
14
15 298 insects such as crickets and katydids (Lehmann et al. 2014; Brizio et al. 2020; Mc Neil and
16
17 299 Grozinger 2020). In ants the examples are less common (Ferreira et al. 2010), but do exist.
18
19 300 In the *E. ruidum*, a striking divergence was found in the olfactory cues that play a significant
20
21 301 role in nestmate discrimination (cuticular hydrocarbons) in most of the populations of the
22
23 302 species complex, including *E. ruidum* sp. 5 (Peña-Carrillo et al. 2021). Furthermore, in *E.*
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25 303 *ruidum* sp. 5, the phylogenetic tree based on mitochondrial sequences supported its
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27 304 differentiation from the species *E. ruidum* sp. 2 and 3-4 (Peña-Carrillo et al. 2021). Therefore,
28
29 305 differentiation in the acoustical characteristics of the distress call of *E. ruidum* sp. 5 could be
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31 306 expected in a scenario where the communication system of this species was under higher
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33 307 diverging selection pressure than those of *E. ruidum* sp. 2 and sp. 3-4. However, studies
34
35 308 including all the species of the complex are needed to know whether the variation of the distress
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37 309 call is exclusive to *E. ruidum* sp. 5.

38
39 310 Whatever the causes of the divergence of the distress call of *E. ruidum* sp. 5 from that of *E.*
40
41 311 *ruidum* sp. 2 and sp. 3-4, these results highlight that *E. ruidum* sp. 5 differs not only chemically
42
43 312 and genetically (Peña-Carrillo et al. 2021), but also acoustically. Adding evidence that this
44
45 313 taxon evolved separately from the other species of the complex, these results offer another
46
47 314 example of selection pressures acting on the communication system of ants.

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12
13 321 employed for seewave analyses.
14
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16 322 **Ethical statement**

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18
19 323 Our study followed the institutional and national ethical guidelines for scientific research in
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21 324 Mexico, Colombia and France. The species used in the experiments are not endangered or
22
23 325 protected in Mexico and Colombia and the collection of live ants complied with the current
24
25 326 laws in their country of origin.
26
27

28 327 **Disclosure statement**

29
30 328 The authors declared no potential conflict of interest.
31
32

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34
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36
37 331 M12A01 granted to CP.
38
39

40 332 **Data availability statement**

41
42 333 The datasets generated and/or analyzed during the current study are available from the
43
44 334 corresponding author on request.
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46

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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 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	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

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

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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 \pm 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 \pm 3 (17 – 56)	29 \pm 2 (15 – 42)	66 \pm 12 (41 – 107)
Percentage of rubbed ridges during the backward phase	39 \pm 3 (20 – 59)	34 \pm 2 (20 – 48)	64 \pm 9 (42 – 94)
Speed of the forward rubbing movement ($\mu\text{m} / \text{msec}$)	1.12 \pm 0.12 (0.526 – 1.81)	1.12 \pm 0.10 (0.513 – 1.83)	0.804 \pm 0.042 (0.729 – 0.968)
Speed of the backward rubbing movement ($\mu\text{m} / \text{msec}$)	1.50 \pm 0.17 (0.795 – 3.12)	1.34 \pm 0.07 (0.822 – 1.80)	0.989 \pm 0.075 (0.743 – 1.13)
Total number of ants	14	18	5

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3 490 **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
4 491 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

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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.

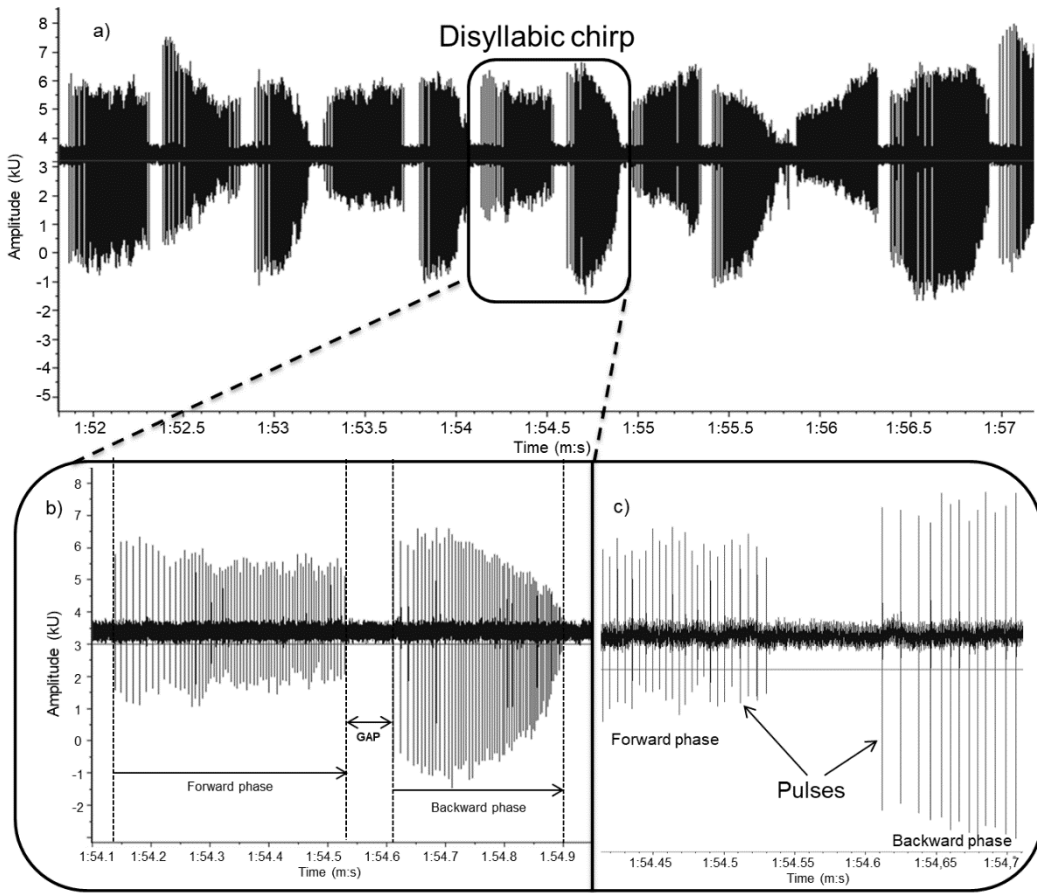
Workers	<i>E. ruidum</i> sp. 2	<i>E. ruidum</i> sp. 3-4	<i>E. ruidum</i> sp. 5
Thorax length (μm)	2891 \pm 86 (2192 - 3352)	3252 \pm 50 (2836 - 3607)	2912 \pm 63 (2640 - 3101)
Stridulatory file total length (μm)	352 \pm 9 (279 - 404)	390 \pm 6 (359 - 453)	376 \pm 13 (339 - 421)
Maximal width (μm)	270 \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

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3 506 **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
4 507 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 length			-	0.805

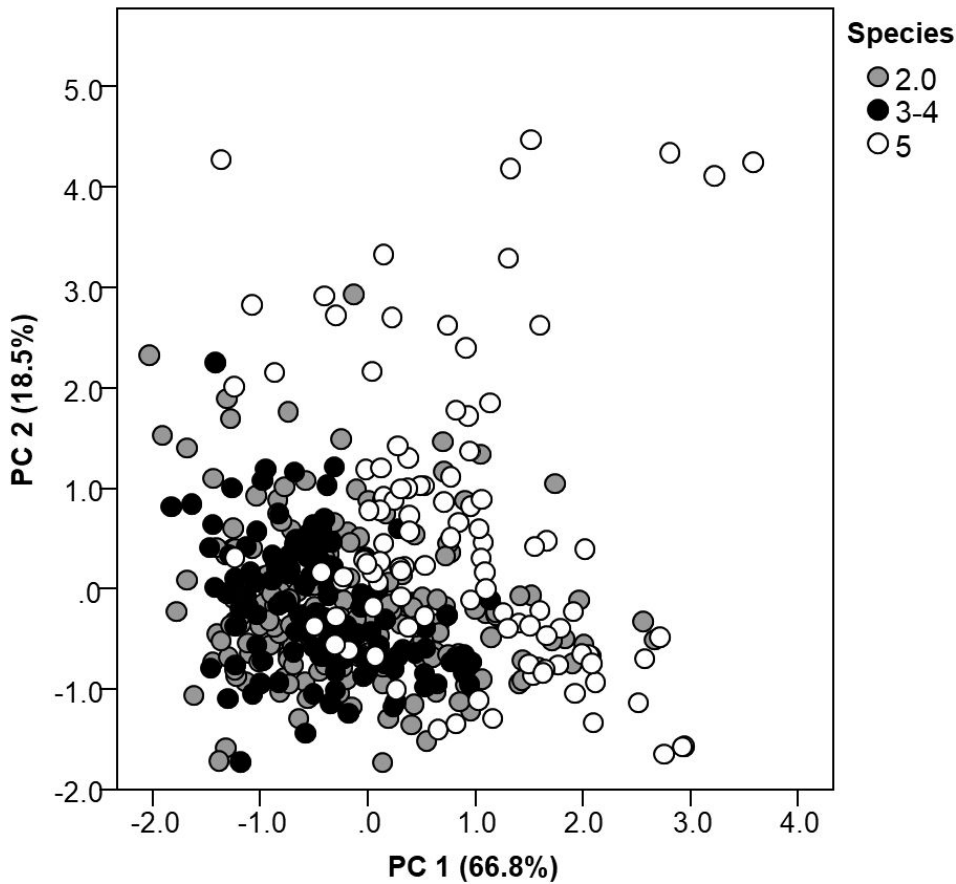
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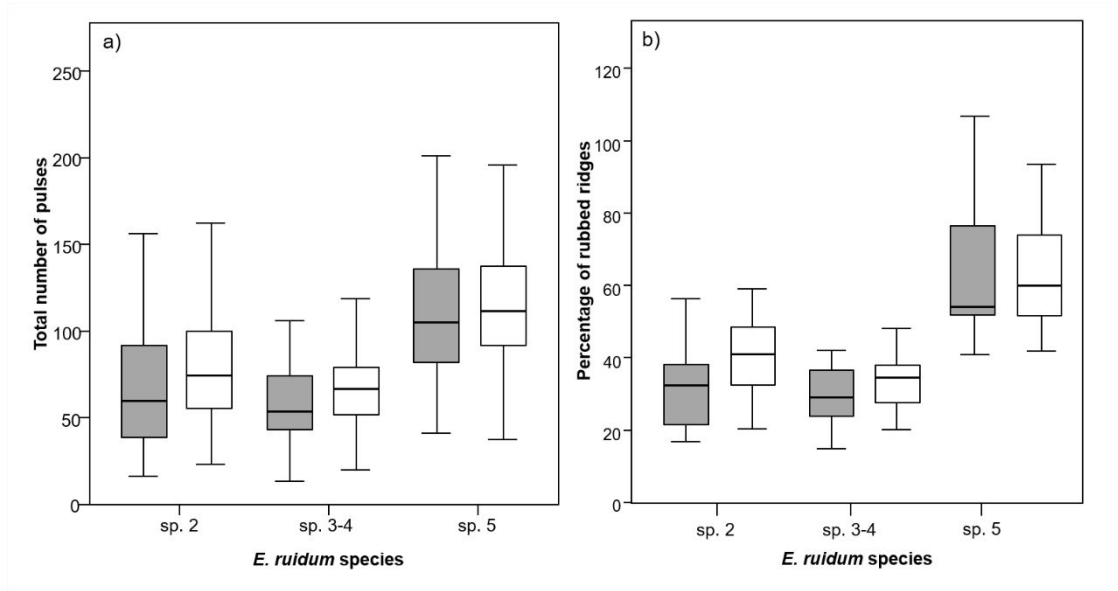
511 **Figure 1.**



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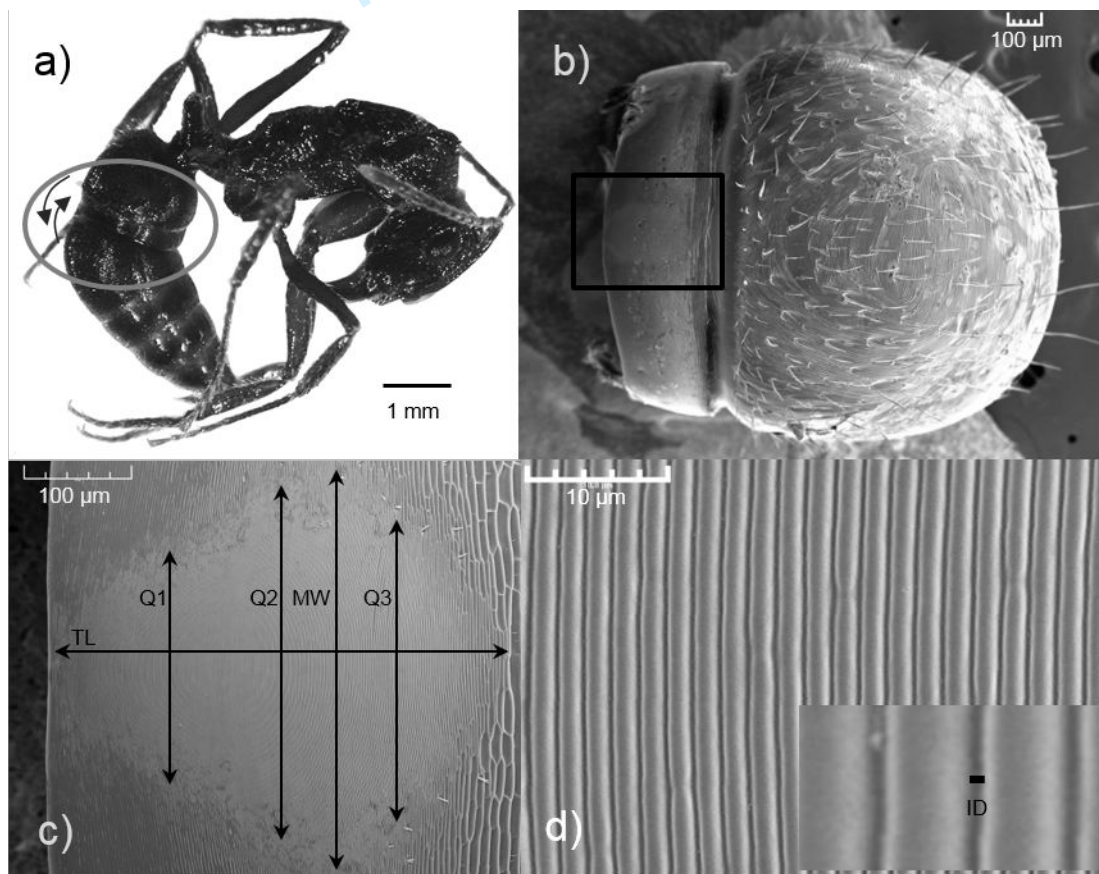
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514 **Figure 2.**



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516 **Figure 3.**



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518 **Figure 4.**

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3 520 **Figure 1.** (a) A sequence of disyllabic chirps in the *E. ruidum* stridulation. (b) Detailed view of a
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5 521 disyllabic chirp: forward phase, backward phase and the GAP (time space in between forward and
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7 522 backward phases). (c) Detailed view of pulses of the forward and backward phase around the gap
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9 523 from minute 1:54.45 to 1:54.7.

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12 524 **Figure 2.** PCA's plot of the acoustical traits in the three species of the *E. ruidum* complex.

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14 525 **Figure 3.** Acoustic variation of *E. ruidum*. (a) Total number of pulses per phase and species (b)
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16 526 Percentage of rubbed ridges per phase and species during the stridulation; bars colored with gray
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18 527 background represent the forward phase and bars with white background the backward phase of
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20 528 the chirps.

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23 529 **Figure 4.** The stridulatory file in *E. ruidum*. (a) Position of the file in the gaster; arrows indicate
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25 530 the backward and forward movement of the gaster when stridulating. (b) Postpetiolar segment of
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27 531 the gaster where the stridulatory file is located. (c) Morphometric variables taken from the
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29 532 stridulatory file: total length of stridulatory file (TL), maximal width (MW), 1st, 2nd and 3rd quartile
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31 533 widths (Q1, Q2, Q3, respectively). (d) Ridges in the stridulatory file and focus on the inter-ridge
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33 534 distance measure (ID).

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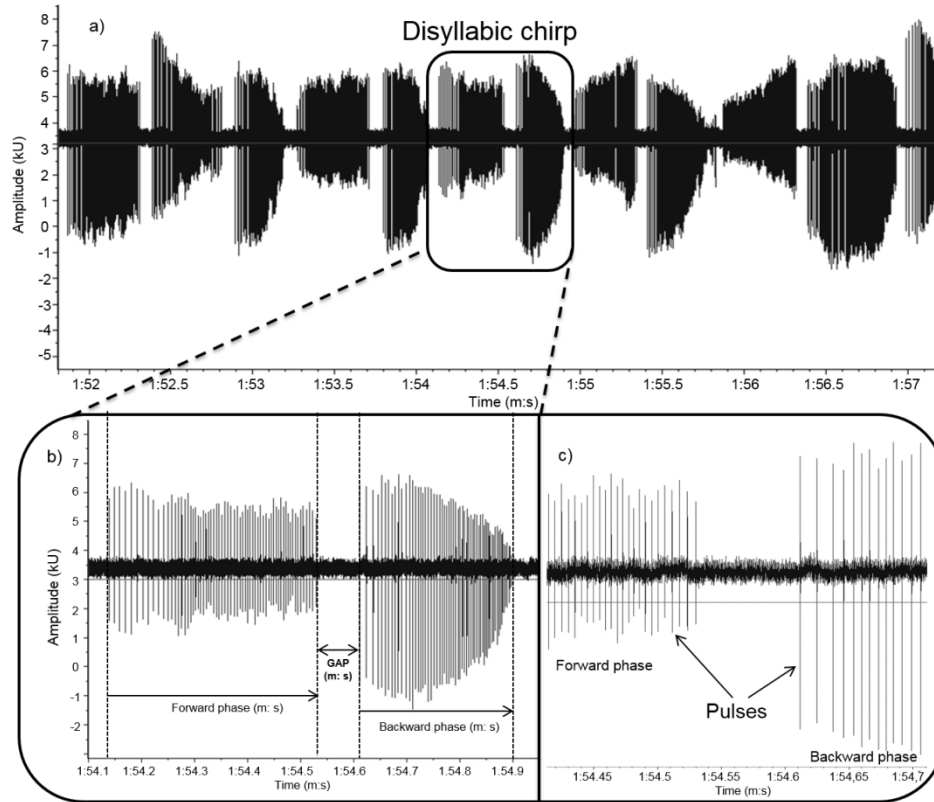


Figure 1. (a) A sequence of disyllabic chirps in the *E. ruidum* stridulation. (b) Detailed view of a disyllabic chirp: forward phase, backward phase and the GAP (time space in between forward and backward phases). (c) Detailed view of pulses of the forward and backward phase around the gap from minute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

241x195mm (150 x 150 DPI)

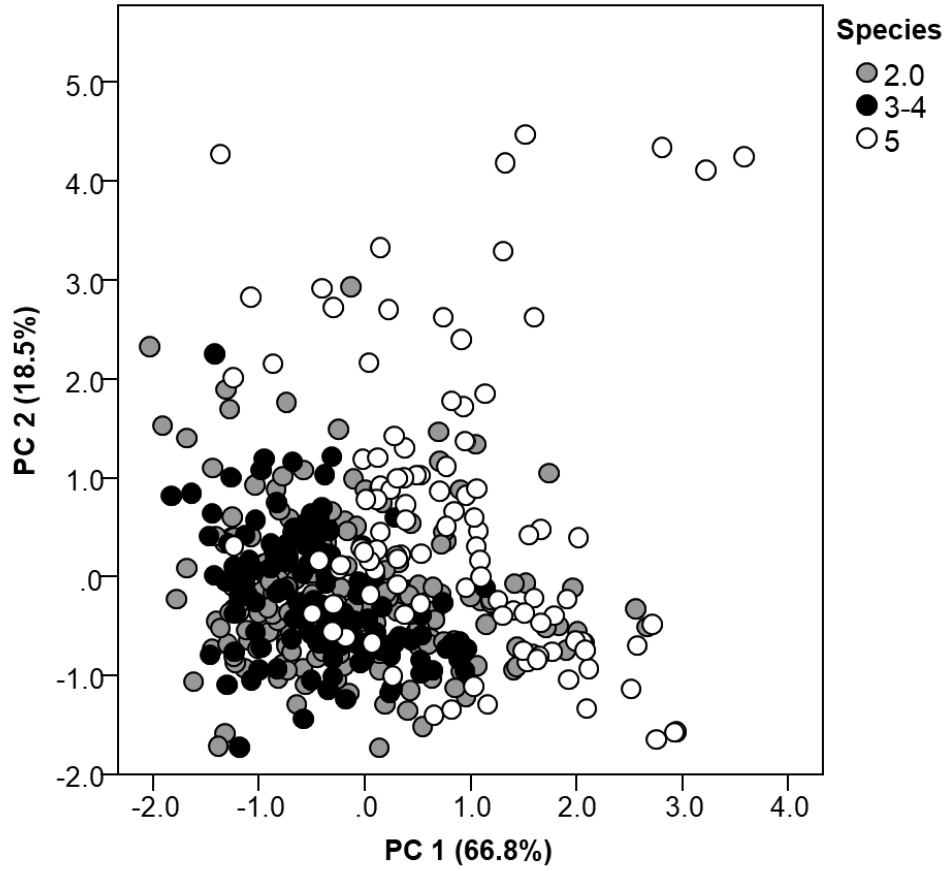


Figure 2. PCA's plot of the acoustical traits in the three species of the *E. ruidum* complex.

164x146mm (150 x 150 DPI)

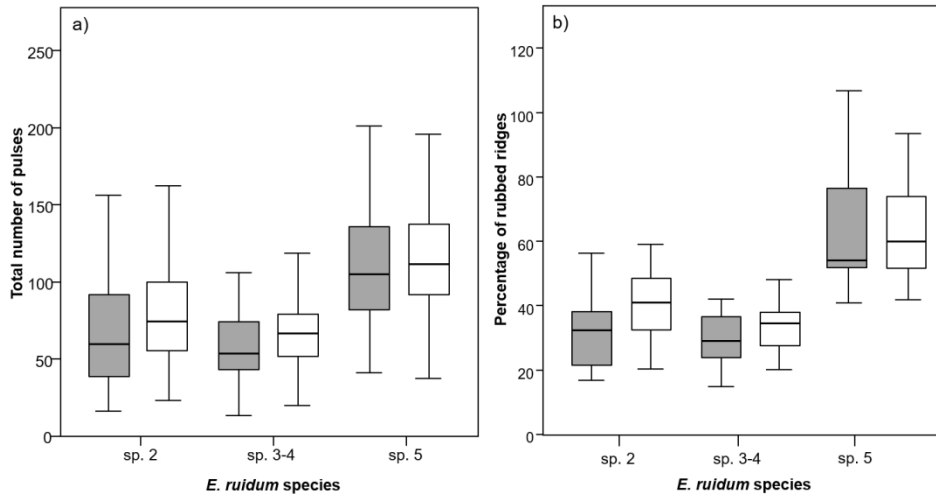


Figure 3. Acoustic variation of *E. ruidum*. (a) Total number of pulses per phase and species (b) Percentage of rubbed ridges per phase and species during the stridulation; bars colored with gray background represent the forward phase and bars with white background the backward phase of the chirps.

268x141mm (150 x 150 DPI)

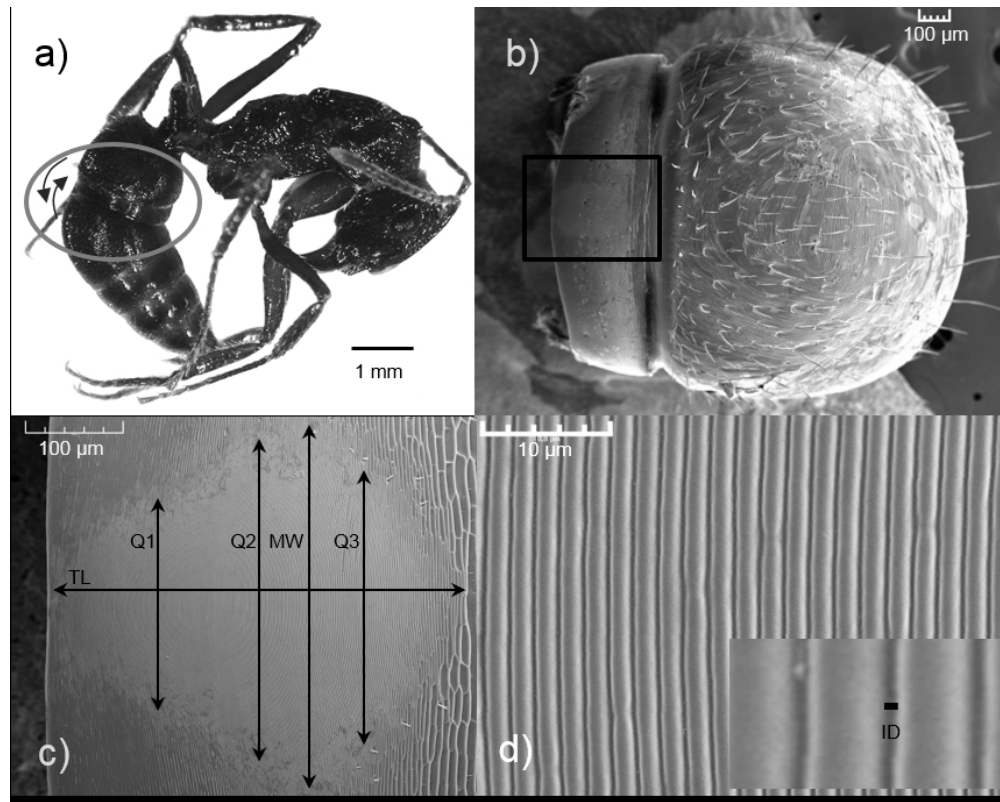


Figure 4. The stridulatory file in *E. ruidum*. (a) Position of the file in the gaster; arrows indicate the backward and forward movement of the gaster when stridulating. (b) Postpetiolar segment of the gaster where the stridulatory file is located. (c) Stridulatory file and its measures. (d) Ridges in the stridulatory file and focus on the inter-ridge distance measure.

145x115mm (150 x 150 DPI)