Org Divers Evol (2015) 15:543–553
DOI 10.1007/s13127-015-0215-9

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

Biogeography, cryptic diversity, and queen dimorphism evolution of the Neotropical ant genus *Ectatomma* Smith, 1958 (Formicidae, Ectatomminae)

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Received: 27 October 2014 / Accepted: 15 April 2015 / Published online: 3 May 2015

Abstract Due to its high biodiversity and its complex climatic and geological history, the Neotropical region has caught the attention of evolutionary and conservation biologists. The Neotropics have an understudied and probably extensive cryptic diversity, stemming from old lineages that have persisted through time with highly similar morphology or from new morphologically undifferentiated sibling species. The wide-ranging Neotropical ant genus *Ectatomma* currently has only 15 described species, some of which present limited distribution. These ants provide an excellent system for the study of diversification and cryptic diversity in the Neotropics. *Ectatomma* also displays queen-size dimorphism in some northern populations of its two most common species: a case of true microgyny and a recently described parasitic species. We performed a phylogenetic and biogeographic analysis of *Ectatomma* species using two mitochondrial genes and one nuclear gene. We also explored the relationship between the history of the genus and the appearance of miniaturized queens. Our analysis recovered a monophyletic *Ectatomma* that originated in the Parana region of South America. We recorded three likely events of colonization of the Caribbean–Mesoamerican region. We also detected ample evidence of cryptic divergence that deserves a full taxonomic revision of the genus. Miniature queens—microgynes and parasites—represent two independent evolutionary events that appeared in the recent history of the genus.

Keywords Cryptic species · Neotropical biogeography · Speciation events · Ant queens · Miniaturized queens · Social parasitism

Electronic supplementary material The online version of this article (doi:10.1007/s13127-015-0215-9) contains supplementary material, which is available to authorized users.

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Introduction

The Neotropical region is an area of special interest to evolutionary biologists due to its high biodiversity. The complex geological and climatic events that have occurred in the continental Neotropics since the Paleocene have played a crucial role in determining its extant biodiversity. These events include several episodes of tectonic uplift which concluded around 10 million years ago (MYA), promoting vicariance by rivers and mountain range formations (Rull 2011), Miocene–Pliocene marine incursions from 15 to 4.2 MYA (Hubert and Renno 2006), the uplift of the Central American Isthmus during the late Pliocene (5–3 MYA), climatic shifts from the Eocene to the Miocene in southern South America (Morrone 2006), and Quaternary glaciation and deglaciation cycles (Rull 2011).

Recently, Moreau and Bell (2013) concluded that the Neotropics acted as a museum (where old lineages persist thorough time) and as a cradle (where recent speciation occurs and new species are being created) for ant evolution. Their

conclusion corresponds to the historical complexity of the Neotropics and the extent of Neotropical arthropod biodiversity. Even though most studies where cryptic species are reported correspond to temperate regions, the Neotropics are more likely to present cryptic species (Bickford et al. 2007; Ferreira et al. 2010). Cryptic species complexes are organisms that are described as a single nominal species due to their highly similar morphology, whether diverging for several million years or representing recent speciation events (Lucas et al. 2002; Bickford et al. 2007; Scheffers et al. 2012). Molecular phylogenetic studies of wide-range Neotropical ant taxa coupled with biogeographic methods can be a powerful tool to determine the presence of cryptic species and to improve our understanding of the processes behind their diversification and our ability to conserve arthropod biodiversity (Schlick-Steiner et al. 2006; Steiner et al. 2010; Wilson et al. 2012).

Ectatomma is a small genus of ants belonging to Ectatomminae, currently located in the Formicoid group (Moreau et al. 2006). The genus *Ectatomma*, with currently 15 recognized species, includes relatively large ants, endemic of the Neotropics (Kugler and Brown 1982). Most of these species are relatively common in diverse habitats (wet forest, dry forest, and savannah) below an altitude of 1500 min the Neotropics (Brown 1958). Some of them are commonly found in plantations where they are considered ecologically dominant (Majer et al. 1994; Schatz and Lachaud 2008). Most of them are generalist predators (Brown 1958), and the two most common species *Ectatomma tuberculatum* and *Ectatomma ruidum* were extensively studied as they are regarded as important natural biological control agents against cotton, coffee, cocoa and maize pests in Guatemala, Panama, Nicaragua and Mexico (Perfecto 1990; Ibarra-Núñez et al. 2001). By contrast, some *Ectatomma* species present a restricted distribution, and their taxonomy is surprisingly poorly known. Brown (1958) was the first author to recognize the difficulties in delimitating some *Ectatomma* species. Some of these problems were partly solved by Kugler and Brown in 1982, who suggested some synonymies and recognized 12 species. Recently, three new species were described or redescribed (Almeida 1986, 1987; Arias-Penna 2006; Feitosa et al. 2008), but nothing is known on their relationships.

Although the geographical distribution of most *Ectatomma* species is in South America, the two most common species, *E. ruidum* and *E. tuberculatum*, display variation in their colony structures, both regarding the number of queens (strictly monogynous vs polygynous populations in *E. tuberculatum*) and their phenotypes, presenting miniaturized queens (Hora et al. 2005; Lenoir et al. 2011) in the northern part of their range. The nature of the miniaturized queens is highly dissimilar between both species. In *E. ruidum*, microgynes—an isometric reduction of the large morph—are related to polygyny and dependent colony founding (Lachaud et al. 1999a, b; Lenoir et al. 2011). Microgynes are present in species of the ant subfamilies Pseudomyrmecinae (Janzen 1973), Formicinae (Heinze and Hölldobler 1993), Myrmicinae (Elmes 1991; McInnes and Tschinkel 1995; Hamaguchi and Kinomura 1996; Schlick-Steiner et al. 2005; Lenoir et al. 2010), Amblyoponinae (Molet et al. 2007) and Ectatomminae (Lachaud et al. 1999b). On the other hand, small queens in *E. tuberculatum* colonies are inquiline parasites that have been described as a different species, *Ectatomma parasiticum* (Hora et al. 2005, 2009; Feitosa et al. 2008). Inquiline parasite queens lack worker progeny but coexist in the nest with the host queen(s), a type of permanent parasitism without slavery (Buschinger 2009). Buschinger (1990) proposed that inquilines could have emerged from microgynes in a genetic isolation event from the polygynous host species, by way of sympatric speciation. Therefore, we expect that these social parasites are closely related to their hosts (Emery 1909) corresponding to the strict form of Emery's rule (LeMasne 1956). In this paper, we first present a molecular phylogenetic study of the Neotropical genus *Ectatomma* that aims to improve our understanding of the evolutionary and biogeographic relationships between its species. Second, we use phylogenetic inferences to answer the following questions: (1) How many times has queen miniaturization arisen in *Ectatomma* and (2) where is *E. parasiticum* positioned in the phylogeny in relation with its host species *E. tuberculatum*? We discuss our results and their implications for the current state of knowledge on the diversity of this Neotropical genus.

Materials and methods

Sampling

The analysis includes a total of 55 specimens representing 12 of the 15 species of *Ectatomma* from Central and South America. Three genera outside of the *Ectatomma* genus but belonging to the Ectatomminae subfamily were included as outgroups (*Gnamptogenys*, *Rhytidoponera*, and *Typhlomyrmex*). Table S1 contains a full list of specimens, their taxonomic status (Bolton 2003), biogeographic origin, and GenBank accession numbers. Voucher specimens were deposited in the collection of the National Museum of Natural History, in Paris, or came from established research collections for most of the colonies sampled in this study (Table S1)

DNA isolation and polymerase chain reaction (PCR) amplification

Field-collected individuals were stored in 70–95 % EtOH and kept in the laboratory until DNA extraction. Total genomic DNA was extracted from thorax, legs, and head of worker ants (except for *E. parasiticum*, where a parasitic queen was used), using the DNAeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's protocol.

For the phylogenetic analyses, we obtained data from two mitochondrial genes and one nuclear gene. We amplified a portion of the cytochrome oxidase subunit 1 region (*COI*, ~850 bp) of the mitochondrial DNA (mtDNA) with the primers Jerry (5′-CAACATTTATTTTGATTTTTTGG-3′) and Pat (5′-TCCAATGCACTAATCTGCCATATTA-3′) (Simon et al. 1994). A fragment of mtDNA cytochrome b (*Cyt B*, ~800 bp) was amplified with primers CB1 (5′-TATG TACTACCATGAGGACAAATATC-3′) and tRS (X) (Jermiin and Crozier 1994). The nuclear gene *wingless* was assessed with primers Wg578F (5′-TGCACNGTGAARACYTGCTG GATGCG-3′) (Ward and Downie 2005) and Wg1032R (5′-ACYTCGCAGCACCARTGGAA-3′) (Abouheif and Wray 2002). Each gene was sequenced from two–three individuals of each species or two individuals per site when available. Details of sampling and accession numbers are in Table S1.

The PCR cocktail contained 5-μL 10× buffer, 1.5–2.5 μL 25 mM $MgCl_2$, 0.25 μL 100 mM dNTP, 1.2 μL of each primer (10 μM), 0.25 μL of a Taq DNA Polymerase (Promega), and ultrapure water in a 50-μL reaction. All reactions were initially denatured at 94 °C for 3 min in a T1 Thermal Cycler (Biometra) and then subjected to 35 cycles of 3 min at 94 °C for denaturation, 1 min at 45–50 °C (annealing temperature depending on gene amplified) for annealing, and 1 min at 72 °C extension, plus a final extension of 5 min at 72 °C.

Amplified products obtained were sequenced by Genoscreen (http://www.genoscreen.fr), using dye terminator cycle sequencing following the protocol specified by the ABI PRISMTM Dye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer, Norwalk, CT, USA). Primers used for amplification served as forward and reverse sequencing primers in separate reactions.

Phylogenetic analyses

Sequences were edited and aligned using the programs CHROMAS (Technelysium, 2005) and GENEDOC (Nicholas et al. 1997) and adjusted by eye. A 9-bp-long fragment of *Cyt B* presented excessive variability between samples and was thus eliminated from the analysis. To examine gene congruence, we performed the partition homogeneity test in PAUP 4.0b (Swofford 2003) using 100 replicates with five random addition sequences. This test resulted in non-significant differences among partitions, and further analysis was performed on the combined data set. Phylogenetic analysis was computed using maximum likelihood (ML) and Bayesian approaches. We estimated the best model of evolution for the two mitochondrial and the nuclear loci separately using JMODELTEST (Darriba et al. 2012). Model selection was based on Akaike information criterion (AIC) scores provided by the software (*COI* and *Cyt B*: TIM2 + I + G [GTR + I + G], freqA=0.314, freqC=0.125, freqG=0.085, freqT=0.446, R[AC]=2.748, R[AG]=8.864, R[AT]=2.749, R[CG]=1.00, R[CT]=25.268, R[GT]=1.00, p-inv=0.528, gamma shape=1.464; *wingless*: HKY + I freqA=0.2343, freqC=0.2894, freqG=0.2841, freqT=0.1922, kappa=14.03[ti/tv=6.874], p-inv=0.7140). ML analyses were performed on the combined data set in GARLI 2.01 (Zwickl 2006) in the CIPRES Science Gateway portal (Miller et al. 2010). The best-likelihood-score tree was determined from eight search replicates using a five million generation termination threshold; the maximum score change and topological change before termination were set at default values (0.01 and 0.05, respectively). Nodal support was obtained with 100 bootstrap replicates using 2.5 million generations as termination threshold.

Bayesian analyses were carried out with MR. BAYES 3.2 (Ronquist et al. 2012) using selected model parameters as uniform priors. We assumed a Dirichlet distribution for base frequencies and substitution rates. A fixed prior was set for the gamma parameter, transition–transversion ratio, and the proportion of invariable sites. We also assumed an uninformative prior topology. Two independent analyses with three heated chains and one cold chain were run for 3,000,000 generations. Samples were taken every 500 generations with a burnin of 30 % of samples. Convergence was checked by visualizing the mixing of chains for all parameters with TRACER v1.4 (Rambaut and Drummond 2007) and examining the average standard deviation of split frequencies (final value after 3,000,000 generations=0.0035).

We estimated divergence times from the complete data set using the relaxed-clock Bayesian approach as implemented in BEAST 2.1.2 (Drummond et al. 2012). The molecular clock was tested using PAUP 4.0b (Swofford 2003) and was rejected. For divergence time estimation, we used the evolutionary models presented above. We used the uncorrelated lognormal clock rate and a Yule birth process tree prior. Based on previously estimated mutation rates for mitochondrial DNA in arthropods (Brower 1994) and *wingless* (Swallow et al. 2005), we assumed normal priors for the clock rate with means of 0.023 and 0.004 sequence divergence per million years for each partition, respectively. The root of the tree prior (including outgroups) was uniform from 43.0 to 53.0 million years considering the most recent age estimation for the Ectatommini based on phylogenetic and fossil reconstruction (Moreau and Bell 2013). We obtained the highest clade probability tree and 95 % highest posterior density (HPD) divergence time intervals for each node using BEAST 2.1 (Drummond et al. 2012) companion software. The first 15,000 trees were eliminated from the analysis in order to ensure that trees were selected once convergence was attained.

We reconstructed *Ectatomma* ancestral areas using the Bayesian binary method implemented in RASP ver.2.1 (Yu et al. 2011). For this analysis, terminal taxa geographic distributions were coded into five areas according to the biogeographic regions described in Morrone (2006) (Table S1). Ten chains of Markov chain Monte Carlo (MCMC) runs were performed on

the Mr. Bayes Bayesian consensus tree for 1,000,000 cycles; the first 300 samples were discarded. Coded areas for *Ectatomma* samples were as follows: (A) Parana, (B) Amazonian, (C) Caribbean–Mesoamerican, (D) Chacoan, and (E) Caribbean–north-western South American (Morrone 2006). We recorded ancestral area posterior probability for the main clades detected. Finally, in order to test if queen dimorphism arose once or twice in the evolution of the genus, we used the same Bayesian approach to reconstruct microgyny evolution.

Results

Phylogenetic analyses

The two mitochondrial (*COI* and *Cyt B*) and the nuclear (*wingless*) sequenced genes rendered a 1830 character matrix (1446 mitochondrial and 384 nuclear). A total of 648 characters were variable and 479 were parsimony informative. The Bayesian and ML analyses produced nearly identical trees (Fig. 1). *Ectatomma* monophyly is strongly supported (BPP=1.0; 100 % bootstrap support). We identify four main *Ectatomma* clades in our tree (Fig. 1). Clade 1 comprises three described species with a predominant distribution in South America: *Ectatomma edentatum*, *E. muticum* and *Ectatomma suzanae*. Clade 2 contains the wide-range *E. tuberculatum*, its recently described inquiline parasite, *E. parasiticum* (Feitosa et al. 2008), and *Ectatomma vizottoi* from Brazil. One of the remaining principal clades, clade 3, encompasses the other wide-range species *E. ruidum*, which is a sister to the Central American endemic *Ectatomma gibbum.* The fourth clade contains four South American species (*Ectatomma permagnum*, *Ectatomma lugens*, *Ectatomma brunneum*, and *Ectatomma opaciventre*).

Within described species, monophyly support varies across the genus. *E. ruidum*, *E. lugens*, *E. brunneum*, and *E. opaciventre* present the highest support as monophyletic clades (BPP=1.0; 100 % bootstrap support). *E. tuberculatum* is paraphyletic because *E. parasiticum* is nested within it; furthermore, a single *E. tuberculatum* sample from Brazil forms a clade with *E. vizottoi* (BPP=0.98; 65 % bootstrap support) that is a sister to the rest of *E. tuberculatum* and *E. parasiticum*. The *E. tuberculatum–E. parasiticum–E. vizottoi* clade is strongly supported (BPP=1; 100 % bootstrap support). *E. suzanae* and *E. muticum* are both nested within *E. edentatum*, which is also paraphyletic. *E. permagnum* and *E. gibbum* could not be evaluated as monophyletic because they were represented by a single collection event. However, they are a sister to well-defined clades and species: *E. lugens* and *E. ruidum*, respectively.

Deep relationships within the *Ectatomma* genus are not clearly defined in our results. Some deep-level nodes present low support, including clade 2 as a sister to clade 3 and clade 4 (BPP=0.57; <50 % bootstrap support). Within clade 1 and clade 3, some species relationships are weakly supported. In clade 1, *E. muticum* has low support as sister to part of the paraphyletic *E. edentatum* and *E. suzanae*. There is weak support for both *E. gibbum* being a sister to *E. ruidum* in clade 3 and clade 4 being monophyletic.

Divergence time estimation

Our results support that the genus *Ectatomma* originated in the early or middle Miocene, between 15.8 and 22.8 MYA (Fig. 2). The most basal group is represented by clade 1, which diversified between the early and late Miocene. It is highly probable that the other three clades originated between the second half of the middle Miocene and the late Miocene. Time of origin varied widely across species. *E. lugens*, *E. brunneum*, and *E. opaciventre* are "recent" species, which originated between the second half of the Pliocene and the Pleistocene. *E. tuberculatum* and *E. ruidum* appeared earlier, the former during the middle and late Miocene and the latter during the late Miocene–Pliocene. *E. edentatum*'s origin can be traced back to the origin of clade 1: the end of the early Miocene, more than 15 MYA.

Historical biogeography

RASP ancestral area reconstructions are shown in Fig. 3. The ancestral area reconstructed for the genus *Ectatomma* is the Parana (A) of South America. This area is also most likely to be the ancestral area for clades 1, 2, and 4. Most species also had a higher posterior probability ancestral area within South America. However, clade 3 presents a higher probability of originating in the Caribbean–Mesoamerican region (C; 0.93 posterior probability) principally within Central America. Within clade 3, *E. ruidum* presents two separate lineages represented both in Central America and South America. Its sister species, *E. gibbum*, is represented in this study by a Central American sample; however, *E. gibbum* is also present in neighbouring northern South America (Arias-Penna 2008).

In our reconstruction, *E. tuberculatum*—the species with the most northernmost range of the genus—originated in South America in the Parana (A) regions and subsequently dispersed to the Caribbean–Mesoamerican area (C). Finally, the fourth dispersal event to the Caribbean–Mesoamerican area (C) was detected within clade 1 for *E. edentatum*. All Central American or Mexican *Ectatomma* clades are more closely related to northern South American clades from Ecuador, Venezuela (Caribbean–north-western South American, E), or French Guiana (Amazonian), than to central or southern South American clades (Parana, A; or Chacoan, D), which can be interpreted as a pattern of northward dispersal. Accordingly, sampled Brazilian lineages of *E. tuberculatum* and *E. edentatum* tend to be more basal in the species clade.

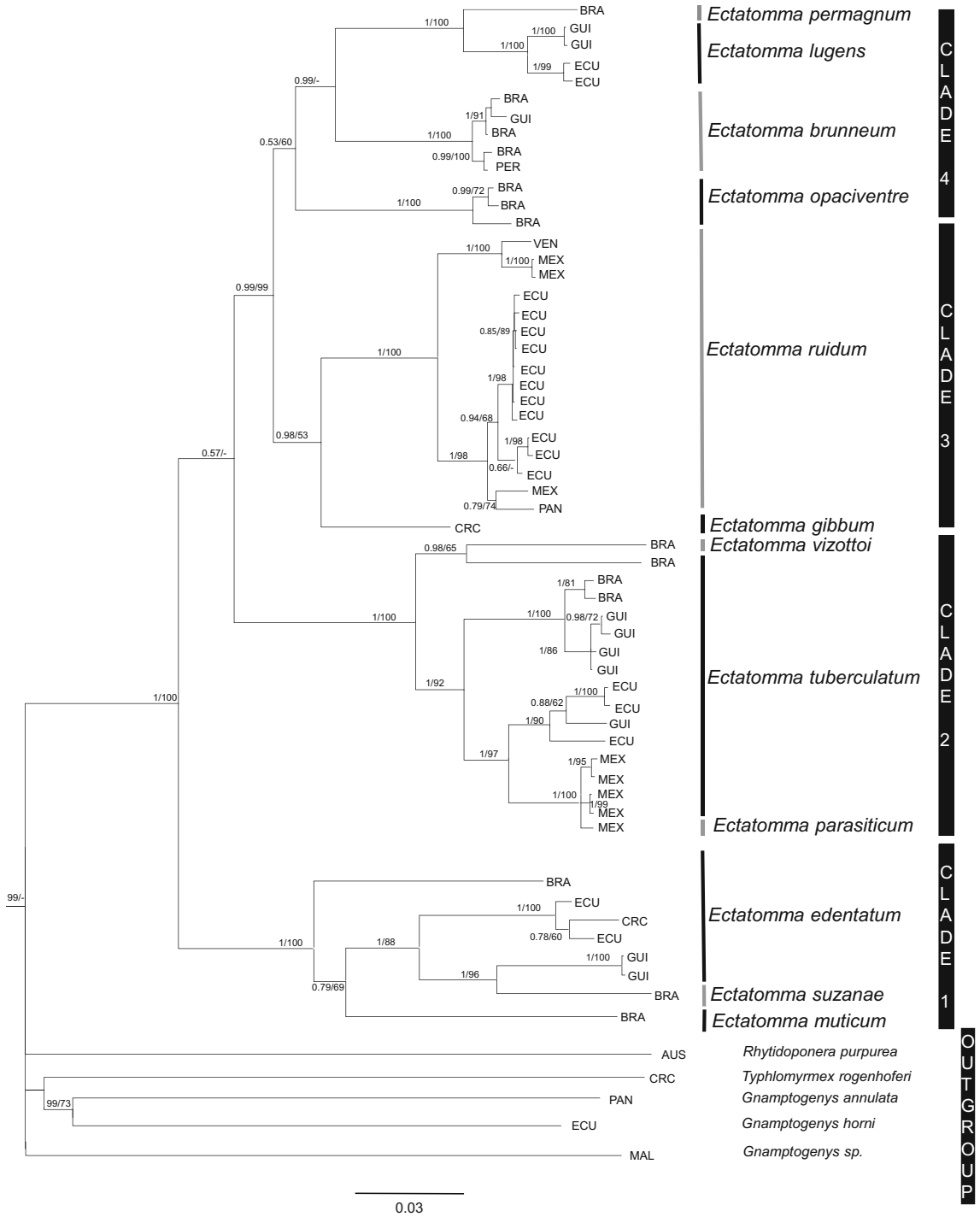

Fig. 1 *Ectatomma* ant species Bayesian phylogram obtained by Mr. Bayes analyses of two mitochondrial and one nuclear gene sequences. Node support is shown by two numbers, the first one corresponding to Bayesian posterior probabilities and the second one to bootstrap support from maximum likelihood analysis. *BRA* Brazil, *GUI* French Guiana, *ECU* Ecuador, *PER* Peru, *VEN* Venezuela, *MEX* Mexico, *PAN* Panama, *CRC* Costa Rica, *AUS* Australia, *MAL* Malaysia

Evolution of microgyny and parasitism

For the sampled species, small queen occurrence within *Ectatomma* represents two evolutionarily independent events: the first one because of the presence of microgyny in a small clade within *E. ruidum* (clade 3) and the second as an inquiline parasite evolution within the northernmost clade of *E. tuberculatum* (clade 2). Correspondingly, reconstructed ancestral states show a high posterior probability of non-small-queen ancestors for all nodes within the two species (results not shown). From our results, we are unable to confirm Emery's rule between *E. parasiticum* and its host population

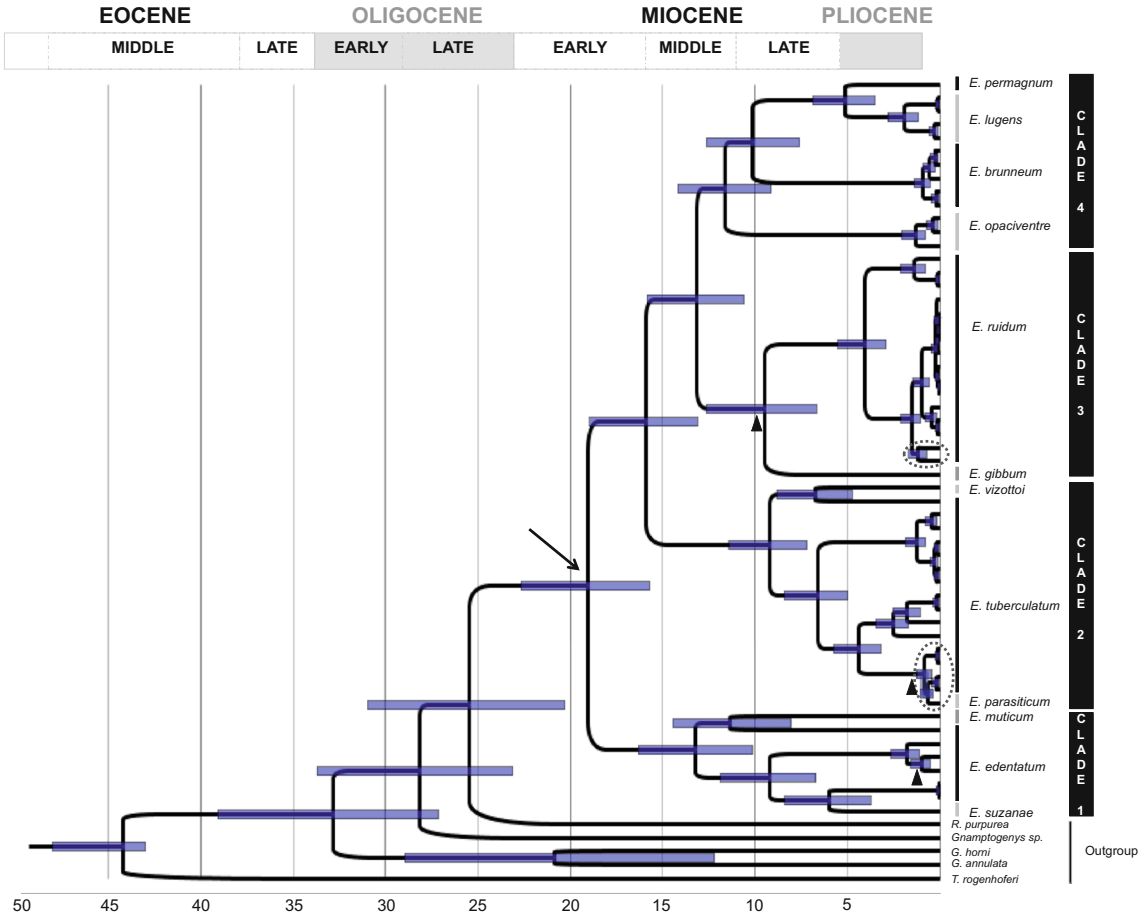

Fig. 2 Ultrametric tree of *Ectatomma* ants obtained from BEAST Bayesian relaxed molecular clock analysis of two mitochondrial genes and one nuclear gene. Ninety-five per cent highest posterior density divergence time estimates are presented as *bars*. *Numbers on the scale at the foot of the figure* represent millions of years. Corresponding geological epochs and their subdivisions are represented with *differential background shadings and dotted lines*. *Circled terminal clades* represent the smallest monophyletic groups from our phylogenetic analysis that present small queens. *Bold triangles* indicate nodes that represent dispersal events to Central America according to our ancestral area reconstruction. *Arrows* represent the most recent common ancestor of *Ectatomma*

because the clade is unresolved and also contains other Mexican *E. tuberculatum* populations. Regarding the divergence time estimation, clades with the presence of small queens are of recent origin; both the smallest clade that contains *E. parasiticum* and *E. tuberculatum* and the smallest *E. ruidum* clade that presents microgynes are dated between the end of the Pliocene and the beginning of the Pleistocene (Fig. 2).

Discussion

Ectatomma species relationship and diversification

Until recently, Ectatomminae were considered as poneromorphs due to their ancestral ant morphology and behaviour (Bolton 2003); today, they are nested within the Formicoid clade (Moreau and Bell 2013; Ouellette et al. 2006). The taxonomy of *Ectatomma* has been revised several times, mainly by Brown (1958) and Kugler and Brown (1982) who recognized 12 species of *Ectatomma* but synonymized some species. Three species have been described or redescribed after these revisions (Almeida 1987; Arias-Penna 2006; Feitosa et al. 2008). However, the identity of several species remains uncertain. Our phylogenetic analysis unveils cases of unrecognized or cryptic diversity in *Ectatomma* with the exception of clade 4, where all species appear as relatively compact, monophyletic groups. The rest of the tree shows relatively deep lineage diversification within species going as far back as the early Miocene (the earliest of their 95 % highest posterior density divergence time estimates) in *E. edentatum* or 5.5 and 13 MYA, in *E. ruidum* and *E. tuberculatum*, respectively. This deep lineage diversification is consistent with the above species-level divergence as shown for other Hymenoptera (Jansen et al. 2010; Lucky and Sarnat 2010; Lucky 2011; Blaimer 2012).

Within clade 1, *E. edentatum* presents paraphyletic lineages scattered within *E. suzanae* and *E. muticum* (clade 1, Fig. 1).

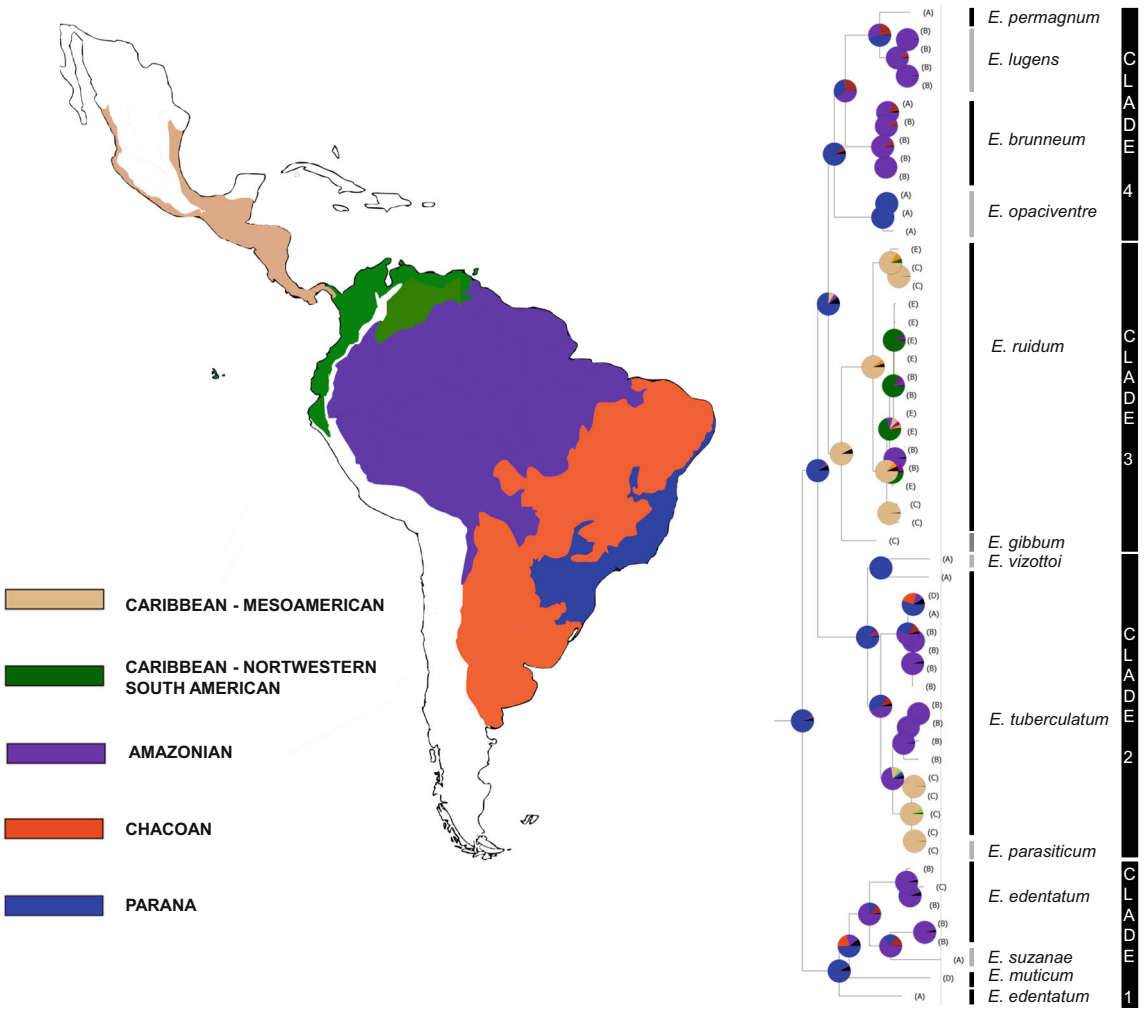

Fig. 3 Ancestral area reconstruction results from RASP Bayesian analyses of *Ectatomma* ants based on biogeographic regions of Morrone (2006). *Pie chart colours* correspond to the posterior probability frequencies for each node. *Letters in parenthesis* correspond to biogeographic regional codes for each sample: (A) Parana, (B) Amazonian, (C) Caribbean–Mesoamerican, (D) Chacoan, and (E) Caribbean–north-west South American

Ectatomma edentatum was first considered as two geographically separated species (Brown 1958): *E. edentatum*, restricted to southern South America, and *Ectatomma morgani*, present in the Amazon and Orinoco basins. The species *E. morgani* was later synonymized under *E. edentatum* by Kugler and Brown (1982), with an extension of its range to include Panama and Costa Rica. *E. suzanae*, on the other hand, is a recently described species (Almeida 1986). Most lineage splits within clade 1 represent relatively deep divergences; a new revision of all these taxa is required. Brown (1958), in an early examination of the genus, considered the morphology of *E. edentatum* as very similar to *E. ruidum*; however, in our analyses, they prove to be unrelated.

In the case of the two most common species, *E. ruidum* and *E. tuberculatum*, our results show that both represent independent evolutionary lineages. *E. ruidum* presents two differentiated clades that colonized Central America independently. These two clades differentiated between 5.5 and 2.5 MYA. The first clade included samples from a Mexican population of *E. ruidum* without microgynes together with samples from Venezuela, while samples from a Mexican population with microgynes grouped with samples from Ecuador and Panama. However, to date, no microgynes have been reported in *E. ruidum* from Ecuador or Panama (Breed et al. 1990, 1999; Guénard and McGlynn 2013). *E. ruidum* microgynes have only been found in samples from Mexico (Lachaud et al. 1999a, b). The two lineages of Mexican *E. ruidum* live in sympatry on a coastal plain in southern Mexico, with less than 15 km and no noticeable geographical barriers separating the sampled populations. Although some life traits are common in both *E. ruidum* lineages (e.g. feign of death when workers are disturbed, Cupul-Magaña 2009), in addition to the presence/absence of microgynes, they are differentiated by other biological characters: date of sexuals production (Lachaud, unpublished data) and chemical compounds in their hydrocarbon profiles (Poteaux, unpublished data). At the beginning of the

20th century, another species was described in central–southwestern Mexico (Michoacan State) by Emery (1901): *Ectatomma aztecum*. It was revised by Kugler and Brown (1982) and is currently considered to be a junior synonym of *E. ruidum*. However, the morphology of both *E. ruidum* forms analyzed here is distinct from the described *E. aztecum*'s morphology (F. Fernandez, personal communication). Therefore, the two *E. ruidum* lineages may represent two different species: one corresponding to the 'true' *E. ruidum* and the other to a new, undescribed species.

Furthermore, Kugler and Brown (1982) considered in their revision that *Ectatomma confine* is a very close relative of *E. ruidum* (only differing in the processes of the mesosoma) and could eventually represent an extreme variant of this species (locality series). We can thus hypothesize that the diversity in *E. ruidum*—as in other *Ectatomma* lineages—is underestimated over its distribution range and that a new taxonomic revision based on a holistic approach, combining different fields such as morphology, ecology, and molecular biology, is required.

E. gibbum was first described as a new species by Kugler and Brown (1982), with morphology very similar to *E. ruidum*. Our results show that *E. gibbum* represents a well-differentiated lineage that might share a common ancestor with the *E. ruidum* lineage. Our divergence time estimation and ancestral area reconstruction results place the origin of clade 3 (*E. ruidum* + *E. gibbum*) in Central America during the Middle or Late Miocene. The closure of the Central American Seaway and consequent formation of the Central American Isthmus occurred during the end of the Pliocene, implying that the ancestor of these species was able to migrate northwards either to an island or to the southernmost part of North America before the isthmus closed. This hypothesis would have to be confirmed with a finer evolutionary reconstruction that includes samples of *E. gibbum* from northern South America.

Within *E. tuberculatum*, most samples formed two clearly differentiated clades that diverged between 8 and 5 MYA. One of those clades includes samples from Brazil and French Guiana; the other—where *E. parasiticum* is nested—includes samples from Mexico, Ecuador, and French Guiana. *E. tuberculatum* is reported to be a facultative polygynous ant in Brazil with a functional polygyny and a mean relatedness of 0.3 among nestmate workers (Hora et al. 2005; Zinck et al. 2007). However, in Mexico, *E. tuberculatum* is known to be monogynous in different places (e.g. only 1 % of 253 colonies collected in the south of the country were polygynous, including only two queens, and the parasite was never found, see Pérez-Lachaud et al. 2011) except in Apazapan (Veracruz) where *E. parasiticum* was discovered (Fénéron et al. 2013). Furthermore, in this population, polygyny was functional but with oophagy events and unequal sharing of reproduction between queens (Hora et al. 2007).

A single *E. tuberculatum* sample forms a clade along with the recently described *E. vizottoi* (Almeida, 1987) both from the Parana Region in Brazil; the level of divergence within these two specimens is greater than the differentiation found within *E. ruidum*, and therefore, a thorough taxonomic revision is also recommended.

We acknowledge that our divergence estimates would be greatly improved with the addition of more nuclear markers that are inherited independently and have different coalescence times and historical migration patterns to our predominantly mitochondrial data set. Divergence time estimates from fast-evolving mitochondrial genes could result in overestimation of species divergence times because of their high substitution rate (Dornburg et al. 2014; Zheng et al. 2011). Furthermore, mitochondrial genomes in some groups tend to introgress between related species at a high rate, which would yield inaccurate phylogenetic reconstructions and would impact species divergence time estimations under total evidence analysis (Near and Keck 2013). As stated above, we propose a thorough revision of some specific relationships between *Ectatomma* species and within *E. ruidum* and *E. tuberculatum*, which should include a higher number of markers, as well as other biosystematic studies.

Evolution of miniaturized queens in Ectatomma

Small queens in *Ectatomma* originated twice independently but around the same time frames in small clades within *E. ruidum* and *E. tuberculatum*. In *E. ruidum* the appearance of microgynes occurred between 0.60 and 1.6 MYA while *E. parasiticum* originated between 0.4 and 1.3 MYA in *E. tuberculatum*. Consistent with our results, studies on both small queens—microgynes for *E. ruidum* and inquiline miniaturized queens for *E. parasiticum*—indicate that both display quite dissimilar relationships with the nests that they dwell upon (Feitosa et al. 2008; Lenoir et al. 2011). In *E. ruidum*, Lenoir et al. (2011) showed that microgynes constitute an alternative reproductive strategy unrelated to any kind of parasitism: Young mated microgynes are readopted by a conspecific colony, most often their natal nest, and can contribute to colony growth by egg laying. In certain ecological conditions, polygyny can be advantageous since large and stable habitats can be rapidly colonized by polygynous ant species (Hölldobler and Wilson 1990; Savolainen and Vepsäläinen 2003). Conversely, small queens of *E. parasiticum*—the only parasitic species described in the Ectatomminae—are obligatory inquiline parasites of their host ant, *E. tuberculatum* at a single location in eastern Mexico.

Queen size reduction, whether true microgyny or adaptation to inquilinism, is linked to polygyny (Bourke and Franks 1991; Rüppell and Heinze 1999). Polygynous colonies have been detected in several species within *Ectatomma* besides *E. ruidum*, *E. tuberculatum*, and *E. parasiticum*:

E. brunneum (Lachaud and Pérez-Lachaud, unpublished data), *E. permagnum* (Paiva and Brandão 1989), *Ectatomma planidens* (referred to as *E. edentatum*, Antonialli-Junior and Giannotti 2003), and *E. vizottoi* (Vieira et al. 2007). Of the four main clades detected in our study, only species from clade 1 lack a description of polygynous colonies. Dealate miniature queens can be easily mistaken with worker ants and may not be evident to the untrained eye. We hypothesize that other *Ectatomma* species present cases of queen miniaturization but that they have not been detected or reported yet.

Emery (1909) proposed that social parasitic insects are closely related to their hosts. Emery's rule (LeMasne 1956) was generalized in two forms: (1) the loose form of the rule where social parasites belong to the same genus as their host, or a closely related genus, and (2) the strict form of Emery's rule (noted SFER in Jansen et al. 2010) where social parasites are the closest relatives of their respective hosts. The SFER hypothesis is evolutionarily attractive since it is an argument in favour of sympatric speciation (Bourke and Franks 1991; Savolainen and Vepsäläinen 2003; Vepsäläinen et al. 2009), a particularly intriguing mode of evolution (Buschinger 1990; Bolnick and Fitzpatrick 2007). Due to the lack of resolution of the smallest clade containing *E. parasiticum*, we are unable to determine if *E. parasiticum* follows the strict Emery's rule pattern of differentiation with its host. Although evidence for SFER is highly debated in the evolutionary community (see Steiner et al. 2006; Smith et al. 2007), it has been repeatedly suggested that ant inquilines may diverge from their sister host species (or from a common ancestor) through intraspecific parasitism (Buschinger 1990, 2009; Bourke and Franks 1991). In that case, reproductive isolation in sympatry would be probably facilitated by the social biology and ecology of the host ant species. In particular, polygyny and later miniaturization of polygynous queens are considered as prerequisites for this scenario, as it is assumed for *Myrmica* (Savolainen and Vepsäläinen 2003; Jansen et al. 2010) and *Acromyrmex* (Sumner et al. 2004). This could also be the case for *E. tuberculatum* and *E. parasiticum* (Hora et al. 2005) where differentiation is weak (see difference of cuticular hydrocarbon profiles in Savarit and Fénéron 2014). All these arguments support a recent divergence between both species, corroborated by our results on divergence time within the Pleistocene. Testing this hypothesis would require markers with higher resolution to establish the fine-scale evolutionary relationships between *E. parasiticum*, its host population in eastern Mexico and other *E. tuberculatum* populations in Mexico.

Acknowledgments We thank J. Delabie, T. Delsinne, D. Donoso, R. Rodrigues, S. Lacau, J. Longino, A. López, C. Moreau, C. Schmidt, and C. Villemant for sending samples for analyses. We are grateful to L.R. Pérez-Marcelín for help with manuscript preparation, J. Flawell for correcting the English and two anonymous reviewers for their comments. The Fyssen Foundation granted postdoctoral funds to support A. Nettel-Hernanz in conducting this study.

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