

1 **Original Article**

2
3 **Title**

4 The first fossil replete ant worker establishes living food storage in the Eocene

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32 **Abstract**

33 Worker specialization extends the behavioral and ecological repertoire of ant colonies.
34 Specialization may relate to colony defense, brood care, foraging, and, in some taxa, storage.
35 Replete workers swell the crop and gaster to store liquid food, which can be accessed by other
36 colony members through trophallaxis. This storage ability, known as repletism, has
37 independently evolved across several ant lineages, but the temporal history of this trait has not
38 yet been investigated. Here, we describe the first fossil replete in the extinct species
39 *Leptomyrmex neotropicus* Baroni Urbani, 1980 preserved in Miocene-age Dominican amber.
40 Together with new evidence of repletism in *L. neotropicus'* extant sister species, *L. relictus*
41 Boudinot et al., 2016, we reconstruct the pattern of acquisition and descent in this storage-
42 linked trait. Our ancestral state reconstruction suggests that *Leptomyrmex* acquired replete
43 workers in the Eocene and may therefore represent the earliest instance of so-called
44 “honeypot” ants among all known ants, both living and extinct.

45
46

47 **Introduction**

48 Eusociality is a profound phenotypic phenomenon that shapes morphology as well as
49 behavior. Division of labor is central to advanced sociality in insects and workers may exhibit
50 a range of behavioral or morphological specializations related to task performance (WILSON &
51 HÖLLDOBLER 2005). A striking example of caste specialization is repletism. Replete workers
52 serve as living food storage within the colony by retaining liquid food within their gaster
53 (terminal abdominal segments). Food storage takes place in the crop, a region of the
54 alimentary canal in the foregut between the esophagus and the proventriculus. The crop swells
55 to accommodate large amounts of food, distending the gaster to large proportions via elastic
56 intersegmental membranes located between each tergite (CARNEY 1969, WILSON 1974). This
57 elasticity enables repletes to distend their gaster dramatically in some species, which renders
58 replete individuals visibly distinct from other workers (CONWAY 1994) and may limit mobility
59 (CHARBONNEAU & al. 2017). During times of scarcity, the stored contents of the replete crop
60 are redistributed to colony members. Food is regurgitated from one ant to another, a process
61 known as trophallaxis. Prior to trophallaxis, ants concentrate the stored material by reducing
62 its water content and they add components of their internal fluids in the crop (MEURVILLE &
63 LEBOEUF 2021). This creates a network of fluid and nutrient exchange in the colony. Because
64 trophallaxis is a common feature across ant lineages, many taxa have the capacity to distend
65 the crop and gaster as part of a colony-wide “social stomach” (MEURVILLE & LEBOEUF 2021).
66 Taxa with a replete caste are ostensibly less vulnerable to fluctuating resource availability,
67 particularly during seasons when food sources are limited (VAN ELST & al. 2021). Trophallaxis
68 may regulate the flow of nutrients among the colony with repletes, in particular, providing a
69 reliable source of a “higher quality” of food (BORGESSEN 2000).

70

71 Repletism is a convergently evolved trait that has been observed in several ant lineages. While
72 a precise definition of repletism is lacking, well-documented replete castes are reported from
73 20 genera (ANDERSEN 2002, BORGESSEN 2000, CASADEI-FERREIRA & al. 2020, COSENS &
74 TOUSSAINT 1985, GLANCEY & al. 1973, EYER & al. 2012, CONWAY 1992, KHALIFE & PEETERS
75 2020, LORINCZI 2016, MOFFETT 1986, 1988, RUANO & TINAUT 1999). Repletes are typically
76 classified as either crop repletes, storing liquid carbohydrates in the social stomach (i.e., crop),
77 or fat body repletes, containing lipids in hypertrophied fat bodies (CHARBONNEAU & al. 2017,
78 TSCHINKEL 1987). Both types of repletes play a role in regulating nutrient storage in the colony
79 and can provide sustenance to the colony during times of scarcity (BORGESSEN 2000,
80 CHARBONNEAU & al. 2017, KHALIFE & PEETERS 2020). Crop repletes are often referred to as
81 honeypot ants or honey ants, while fat body repletes are referred to as corpulents or “false
82 honeypot ants” (BORGESSEN 2000, LORINCZI 2016). Nearly all origins of repletism occur in the
83 subfamilies Formicinae and Myrmicinae, while a single dolichoderine genus – *Leptomyrmex*
84 MAYR, 1862 – exhibits honeypot workers (Fig. 1).

85

86 While most of the 29 described *Leptomyrmex* species are endemic to Australia, New Guinea,
87 and New Caledonia, two species are known from the neotropics: a single fossil species from

88 the Dominican Republic and a recently discovered extant species in Brazil (BARDEN & al. 2017,
89 BOUDINOT & al. 2016, LUCKY & WARD 2010). Numerous Australasian *Leptomyrmex* species
90 exhibit replete workers, which are frequently found outside of the nest and apparently use their
91 distended crops for liquid food transport as well as storage (WHEELER 1915). Until now, the
92 replete status of neotropical *Leptomyrmex* species has remained unknown, obscuring the
93 temporal and biogeographic origin of this trait. Following the recent discovery of *L. relictus* in
94 the Brazilian cerrado (BOUDINOT & al. 2016), *Leptomyrmex* is hypothesized to have originated
95 in the Neotropics during the Eocene before dispersing to Australasia prior to the glaciation of
96 Antarctica (BARDEN & al. 2017), a route that has been documented in other lineages (DLUSSKY
97 & RADCHENKO 2013, SANMARTIN & RONQUIST 2004). Were replete workers gained recently in
98 Australasia or did repletes evolve in the Neotropics prior to their long-distance migration and
99 diversification? Are there multiple origins of repletism in *Leptomyrmex*?

100
101 Here, we report new fossil and extant evidence of repletism in the Neotropics. Through
102 microCT imaging we confirm the replete status of the now extinct Caribbean species *L.*
103 *neotropicus* Baroni Urbani, 1980 and report replete workers in the extant sister species *L.*
104 *relictus* Boudinot et al., 2016 for the first time. With these natural history data, we estimate the
105 approximate age and retention of repletism in the genus *Leptomyrmex* through ancestral state
106 reconstruction. Our approach illuminates the evolutionary history of extreme morphology-
107 assisted food storage in ants.

108 109 110 **Materials and methods**

111 **Fossil imaging:**

112 Photomicrographs were taken using a Nikon SMZ25 stereomicroscope equipped with a DS-
113 Ri2 digital camera. Individual images were digitally stacked using Nikon NIS Elements to
114 generate a high-resolution extended focus montage image. X-ray computed tomography data
115 were generated at the New Jersey Institute of Technology Otto H. York Center for
116 Environmental Engineering and Science using a Bruker SkyScan 1275 micro-CT scanner. The
117 specimen was scanned at a voltage of 38kV and current of 190 μ A for 65ms exposure times
118 averaged over four frames per rotation with a voxel size of 8.00 μ m. Z-stacks were generated
119 using NRecon (Micro Photonics, Allentown, PA), segmented using 3D Slicer v4.9 (FEDOROV &
120 al. 2012), and rendered in Blender v.3.2.1.

121 122 **Ancestral state reconstruction:**

123 We reconstructed ancestral states of repletism across *Leptomyrmex* workers using the
124 phylogeny of BARDEN & al. (2017) and a survey of natural history observations. Replete codings
125 were derived from a literature survey as well as published iNaturalist accounts of reliably
126 identified *Leptomyrmex* species (Supplemental Table 1). Given the uncertainty associated with
127 some species regarding the presence of repletes, species were coded in a probability matrix:
128 terminals were assigned a 1/0 replete/non-replete status if known to have repletes, a 0/1
129 replete/non-replete status if known to not have repletes, and assigned 0.5/0.5 if the presence
130 of repletes was uncertain. We assumed a flat uninformative prior probability distribution for
131 uncertain states rather than attempting to assess the probability of repletes vs non-repletes in
132 uncertain species, given the lack of natural history information for many species. We conducted
133 ancestral state reconstruction using stochastic character state mapping implemented with the
134 prior probability matrix for character states. We inferred the reconstruction under the equal
135 rates (ER) model, based on prior comparisons of the Akaike information criterion (AIC) using
136 different character state evolution models (symmetrical (SYM) and all rates different (ARD),
137 and ran the simulation for 200 trees (nsim=200)). State changes were summarized across all
138 200 trees. Ancestral state construction was conducted in R version 4.2.0 using the package
139 phytools (REVELL 2012).

140 141 **Results**

142 **A fossil replete:**

143 Specimen BALDR-0155 is a *Leptomyrmex neotropicus* worker preserved as an inclusion within
144 amber dated to the Upper Miocene (~16 Ma; ITURRALDE-VINCENT & MACPHEE 1996) from the
145 Northern mines of near La Cumbre, Dominican Republic. The gastral elastic intersegmental
146 membrane is significantly distended (Fig. 2) while there are no signs of taphonomic distortion
147 across the cuticle. X-ray computed tomography recovers a sharp difference in inclusion density
148 in the region of the gaster and head (Fig. 3), consistent with air. This heterogeneous density is
149 the result of void space within the cuticle, a common feature recovered through X-ray imaging
150 as internal features degrade after an insect is entombed in resin (DIERICK & al. 2007).

151

152 **Extant repletes in the Neotropics:**

153 We (LC, HMM) observed replete workers of *Leptomyrmex relictus* entering and exiting a
154 disturbed nest entrance, with some repletes carrying brood or unidentified objects in their
155 mandibles (Supplemental Video 1 and 2). Replete gasters are conspicuously enlarged and
156 distended relative to nearby non-repletes. This documentation in *L. relictus* confirms mobility
157 and multiple task performance of repletes, as described in other *Leptomyrmex* species
158 (PLOWMAN 1981). Observations took place across the months of July, August, and September
159 2020 in Parque Cesamar (-10.209838, -48.322934) city of Palmas, state of Tocantins, Brazil.
160 Two videos were recorded from the same nest within the park (Supplemental Video 1, 2).

161

162 **The evolution of repletism in *Leptomyrmex*:**

163 We found strong support for repletism as the ancestral condition of *Leptomyrmex* (Fig. 4;
164 posterior probability 0.89 repletism). State changes were relatively infrequent; across all trees,
165 we estimated the average number of gains and losses as 2.5. Our reconstruction suggests
166 that once repletism evolved in *Leptomyrmex*, it infrequently or perhaps never reverted. The
167 preponderance of ancestral nodes estimated as replete suggests that many *Leptomyrmex*
168 species are likely to have a replete caste upon further study, though because our probability
169 matrix used a flat uninformative prior due to lack of ecological data, this may have biased some
170 more recent ancestral nodes towards repletism.

171

172 In extreme cases of repletism (e.g., *Myrmecocystus* Wesmael, 1838), replete workers tend to
173 be immobile and confined to the nest, solely serving as subterranean food storage (CONWAY
174 1977). In fat repletes, once workers have depleted the resources in their fat bodies, usually
175 during the season after storage, they also become foragers (WILLIAMS AND LUCKY 2020). In
176 other taxa, repletes are mobile, performing other tasks, such as carrying brood or foraging
177 (COSENS & TOUSSAINT 1985, CONWAY 1992, PLOWMAN 1981, SKINNER 1980). Several species
178 of *Leptomyrmex* are documented as mobile repletes, foraging on plants and transporting liquid
179 food to the nest (PLOWMAN 1981, DAVIDSON & al. 2004). Our report of mobile repletes in *L.*
180 *relictus*, and the presence of a *L. neotropicus* replete worker in fossil amber suggests that
181 mobility and replete foraging was ancestral in this lineage.

182

183 **Discussion**

184 We recover a single origin of replete workers in the last common ancestor of all extant and
185 extinct *Leptomyrmex* species in the Eocene ~45 Ma (Fig. 4). Our results suggest that living
186 food storage was present in a Neotropical ancestor and that this trait was retained as the genus
187 expanded into Australasia. The expansion of grasslands and increases in global temperatures
188 during the Eocene-Miocene transition may have contributed to the retention of repletism even
189 across continents and tens of millions of years (AZEVEDO & al. 2020, DLUSSKY & RADCHENKO
190 2013). The retention of this trait in *Leptomyrmex* is unexpected because repletism is frequently
191 ascribed to species that inhabit dry climates or are winter active (HÖLLDOBLER & WILSON 1990,
192 KRONAUER & al. 2004), while some replete species in *Leptomyrmex* are found in wet forests.
193 Several other genera, including *Pheidole* WESTWOOD, 1839 (TSUJI 1990), exhibit repletes
194 within species that are endemic to wet habitats; even as climate is strongly linked to living food
195 storage in some lineages, it does not appear to be a requirement for repletism.

196 It is notable that in our ancestral state reconstruction (Figure 4), and throughout ant lineages
197 that contain repletism (Table 1), there remain many species with an unknown status.

198 Repletism is often difficult to demonstrate if the replete workers are immobile and confined to

199 the nest. These ants can be difficult to extract from underground, and nestmates often move
200 repletes to deeper chambers to avoid exposure. There is therefore a bias toward
201 underreporting replete castes, where they may exist, and it is more likely that fossilized
202 lineages will exhibit evidence of mobile repletes since taxa with immobile repletes are
203 unlikely to be aboveground and therefore caught in resin- or sediment-based preservation
204 modes.

205
206 This study marks the first ancestral state reconstruction of repletes in any genus of ants, and
207 the estimated ages of lineages that contain replete species provide an opportunity to assess
208 the temporal distribution of living food storage (Tab. 1). Molecular-based divergence estimates
209 suggest that crown-group *Carebara* WESTWOOD, 1840, *Leptothorax* MAYR, 1855, and
210 *Monomorium* MAYR, 1855 each originated in the early Eocene, prior to *Leptomyrmex*.
211 Repletism is present but not pervasive in these older taxa, which prevents a clear
212 reconstruction of replete origins – it is not yet known whether repletes evolved once early in
213 the history of these lineages and were subsequently lost in several descendants, or if repletism
214 was recently acquired across multiple distantly related species, for example. Although
215 *Leptomyrmex* is not the oldest lineage to contain repletes, the definitive reconstruction of
216 ancestral repletism here establishes the first clear indication that ants with “honeypot” repletes
217 were present in the Eocene. Future ancestral reconstructions of replete workers across ant
218 lineages will further reveal the tempo of replete evolution as a striking case of morphology-
219 enabled division of labor.

220

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226

227 **References**

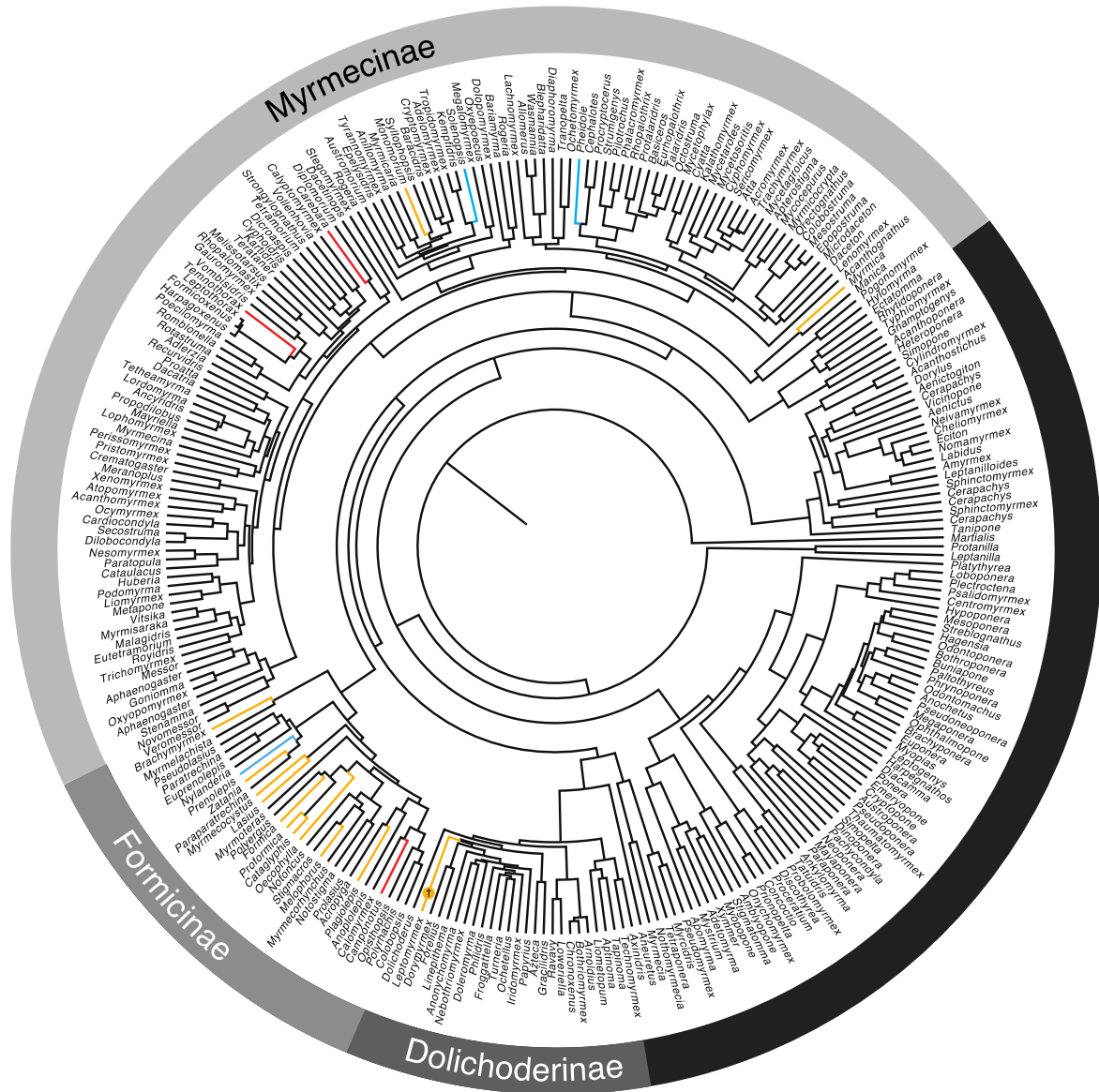
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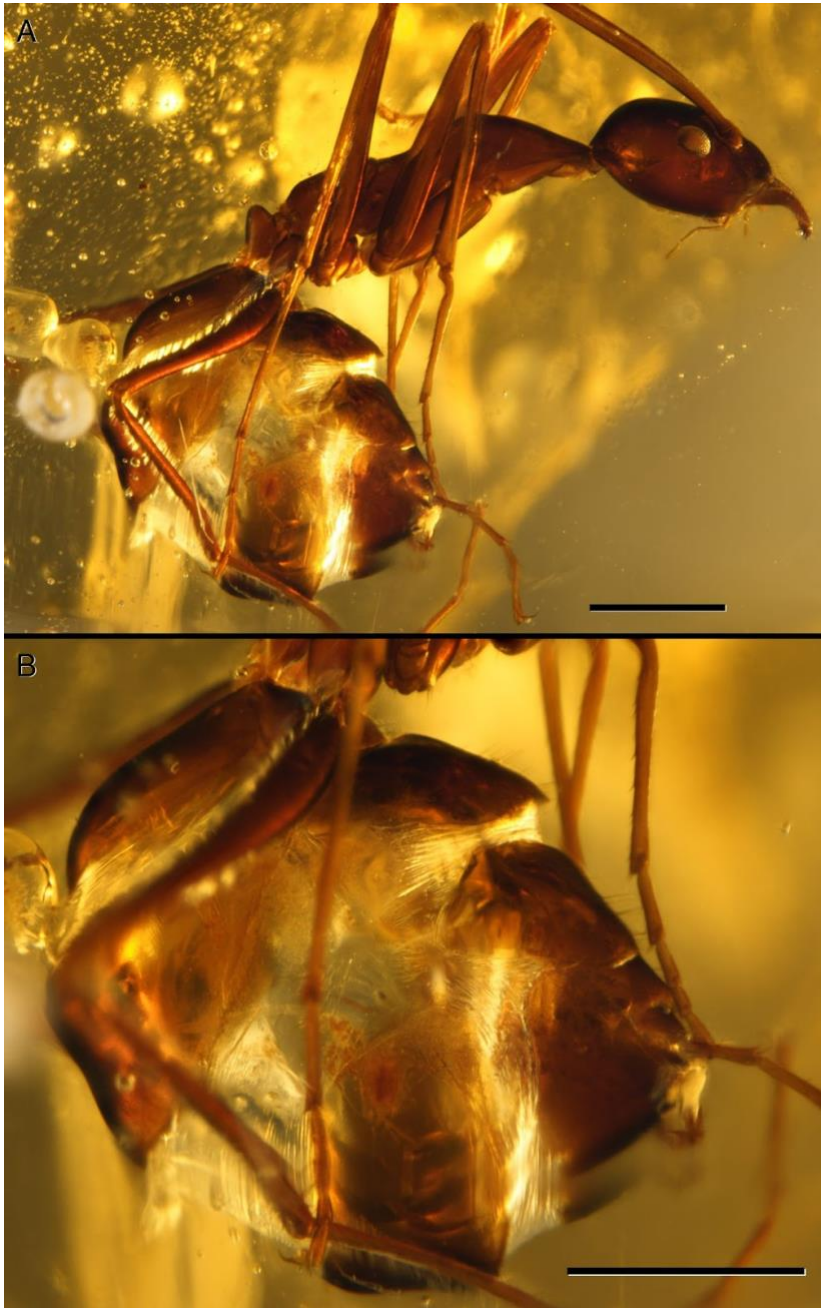
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370 **Table and figure captions**
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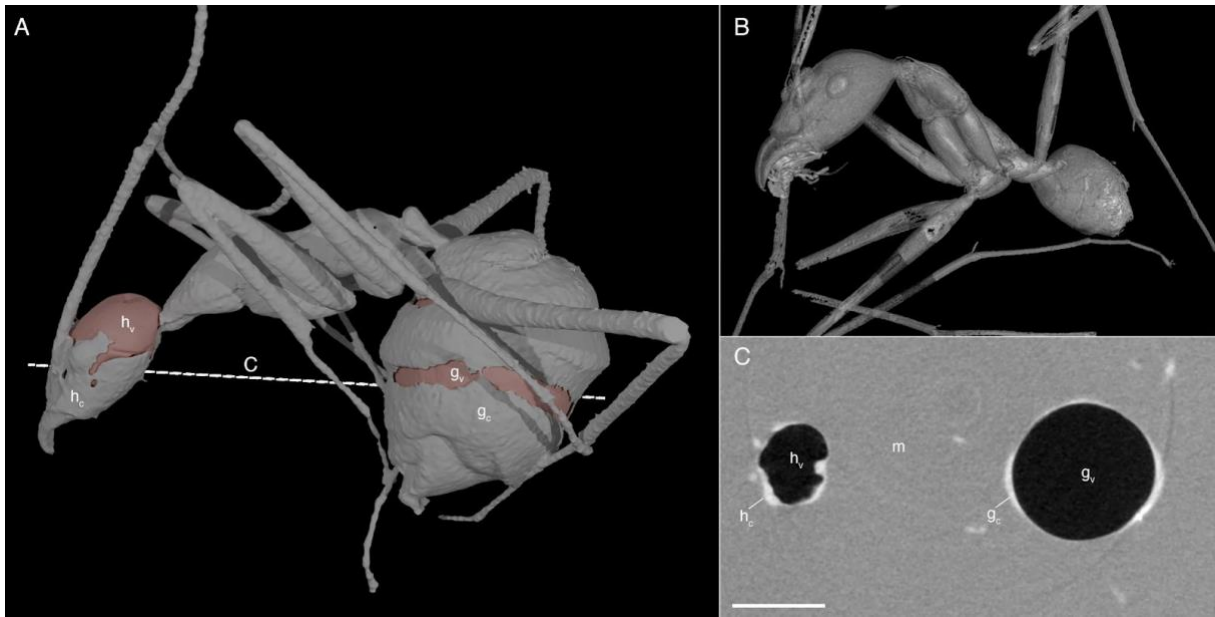
372 **Figure 1. Phylogenetic distribution of known repletes.** Topology adapted from Moreau
373 and Bell, 2017. Lineage color reflects presence of repletes. Yellow: crop repletes; blue: fat
374 body repletes; red: crop/fat body repletes. The fossil species *Leptomyrmex neotropicus* is
375 denoted by the dagger † symbol.
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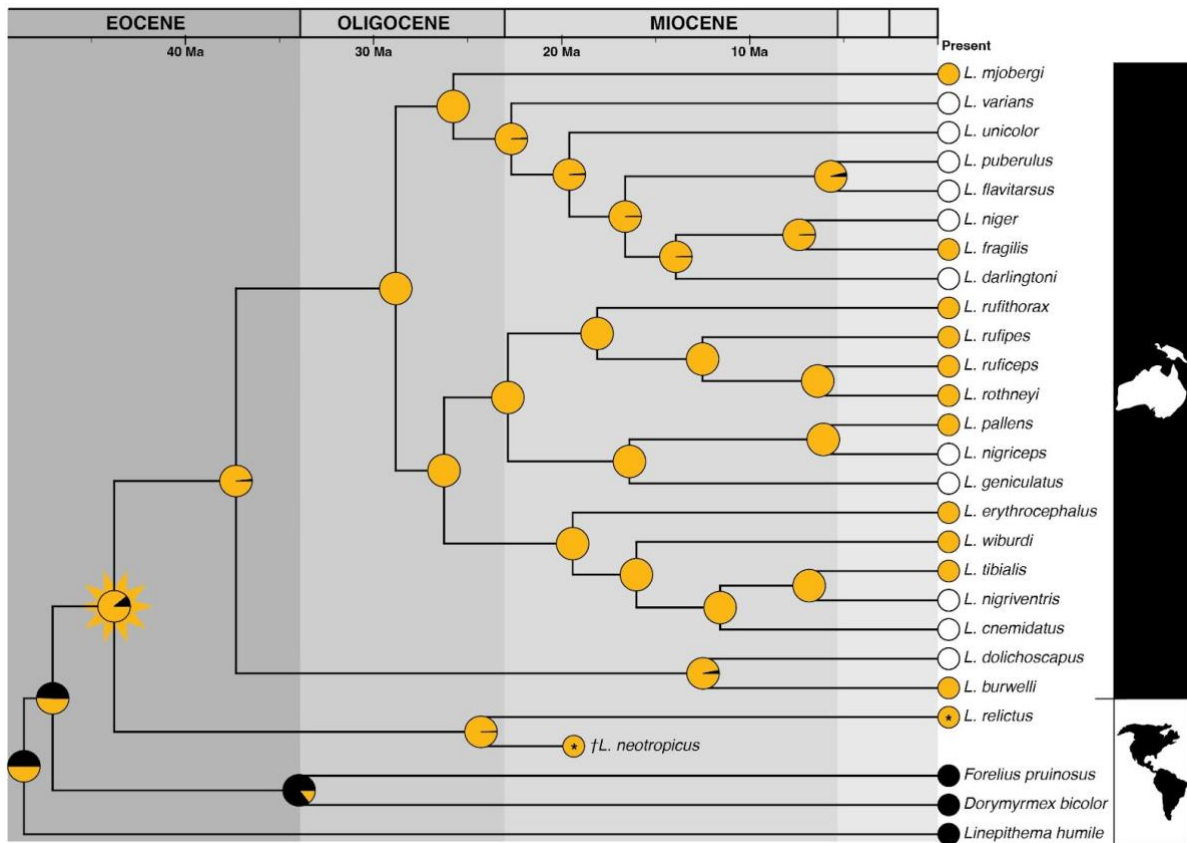
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Figure 2. Photomicrograph of *L. neotropicus* replete specimen BALDR-0155 preserved in Miocene-age Dominican amber. (A) Lateral view. (B) Enlarged, lateral view of distended gaster. Scale = 1mm.



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Figure 3. X-ray computed tomography images of *L. neotropicus* (A) Lateral view of replete *L. neotropicus* worker specimen BALDR 0155. (B) Lateral view of a non-replete *L. neotropicus* worker (AMNHDR-13-85 modified from Barden et al., 2017). (C) Z-stack cross section of specimen BALDR 0155 head and gaster denoted by dotted line in sub-panel A. hc = head cuticle; hv = voidspace of head; m = amber matrix; gc = gaster cuticle; gv = voidspace of gaster. Scale = 1mm



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Figure 4. Ancestral state reconstruction of replete workers across *Leptomyrmex*. Summary of 200 simulated stochastic character histories under an equal rates (ER) model. Node pie charts represent posterior probabilities of states at each node. Yellow = replete; black = no repletes; white = unknown, these were coded as ambiguous in ancestral state reconstruction. Most recent common ancestor of all *Leptomyrmex* is indicated with star icon. Topology and mean node ages from Barden et al. (2017).

Table 1. Summary of major replete lineages and their estimated crown ages.

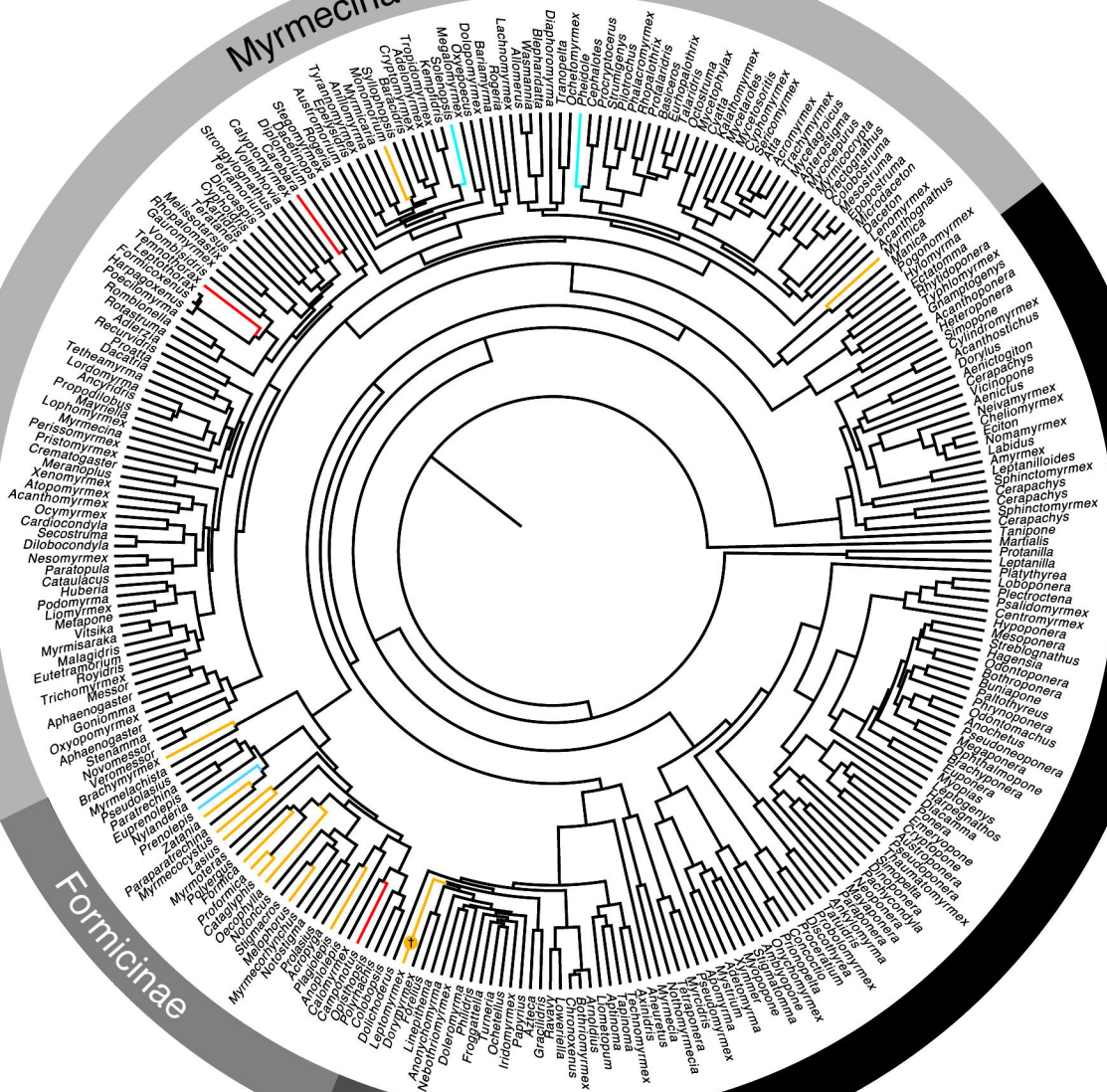
Replete genera	Type of repletism	Mean lineage age (Ma)	Age reference	Replete reference
<i>Agraulomyrmex</i>	Fat body	~23	Blaimer et al. (2016)	Prins (1983)
<i>Brachymyrmex</i>	Crop	~17 [27.5 – 7.5]	Boudinot et al. (2022)	Zolessi et al. (1978)
<i>Camponotus</i>	Crop	~24 [30 – 13]	Boudinot et al. (2022)	Lubbock (1880); Froggatt (1896) Heterick (2022)
<i>Cataglyphis</i>	Crop	~15 (divergence)	Tinaut and Ruano (2021)	Eyer et al. (2013)
<i>Colobopsis</i>	Crop	~24.5 [35 – 15]	Boudinot et al. (2022)	Wilson (1974); Hasegawa (1993)
<i>Lasius</i>	Crop	~21.9 [28.6 – 15.3]	Boudinot et al. (2022)	Cammaerts (1996)
<i>Melophorus</i>	Crop	~44.2 [51 – 20] (divergence)	Blaimer et al. (2015)	Conway (1992); Heterick (2017)
<i>Myrmecocystus</i>	Crop	~14.1 [19.9 – 10.2]	Van Elst (2021)	Froggatt (1896) Snelling (1976)
<i>Plagiolepis</i>	Crop	~11.2 [24 – 3] (low sample size)	Blaimer et al. (2015)	Heterick (2022)
<i>Prenolepis</i>	Fat body	~15 [19 – 9]	Boudinot et al. (2022)	Tschinkel (1983)
<i>Proformica</i>	Crop	~20 (divergence)	Tinaut and Ruano (2021)	Galkowski (2017)
<i>Zatania</i>	Crop	~15 [18.5 – 9]	Boudinot et al. (2022)	Wheeler (1936)
<i>Formica</i>	Crop	~17 [21 – 4]	Boudinot et al. (2022)	Cosens & Toussaint (1985)
<i>Leptomyrmex</i>	Crop	~43.8 [54 – 35.2]	Barden et al. (2017)	
<i>Solenopsis</i>	Fat body	~39.1 [31.4 – 47.1]	Ward et al. (2015)	Glancey (1973)
<i>Leptothorax</i>	Crop	[~48 – 25]	Ward et al. (2015)	Børgesen (2000)
<i>Myrmica</i>	Crop	~34	Jansen et al. (2010)	Børgesen (2000)
<i>Monomorium</i>	Crop	[~50 – 30]	Ward et al. (2015)	Børgesen (2000)
<i>Carebara</i>	Crop	~43 [50 – 30]	Ward et al. (2015)	Azorsa & Fisher (2018)
<i>Pheidole</i>	Fat body	35.2 [46.6 – 24.9]	Ward et al. (2015)	Tsuji (1990)

476 Lineage dates are derived from published molecular-based divergence estimates and indicate the age of the last common
477 ancestor for each genus, except where otherwise noted. Lineage dates denoted with (divergence) correspond with the last
478 common ancestor of the focal genus and its closest living relative sampled in the corresponding phylogeny – such instances
479 reflect inadequate sampling to confidently estimate crown-ages and are therefore overestimates. Instances of single species
480 repletes are excluded; it is not possible to estimate the age of these lineages with current available data. Color legend:
481 Formicinae (orange), Dolichoderinae (green), Myrmicinae (blue).
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483 **Supplemental Video 1. Replete workers of *Leptomyrmex relictus*.** Video
484 evidencing mobile replete workers emerging from disturbed nest. Filming took place
485 on the morning of August 20, 2020 (~9:00), at an external temperature of 29.3°C and
486 27.8°C inside the nest (nest humidity ranging from 30% to 60%).
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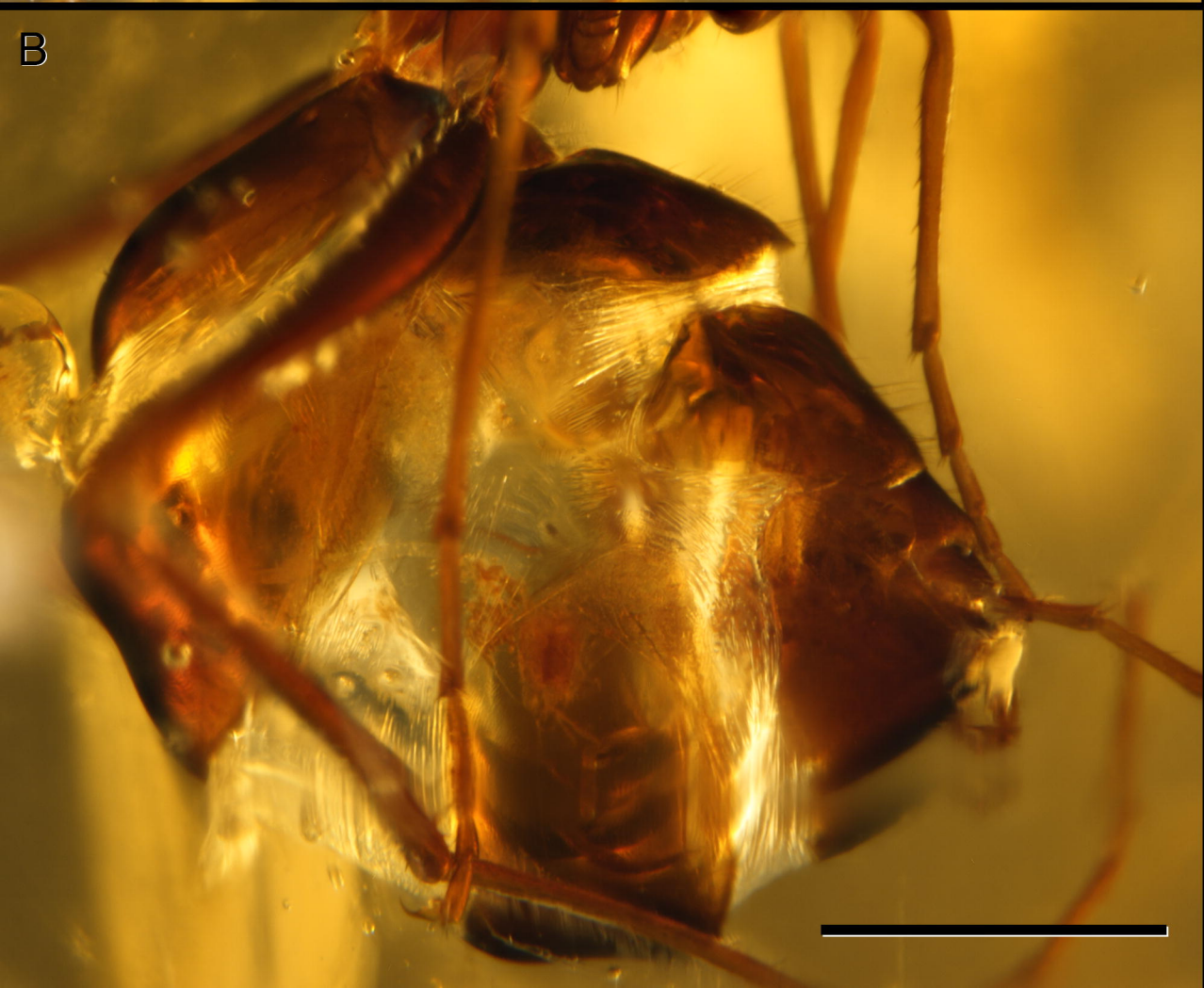
488 **Supplemental Video 2. Replete workers of *Leptomyrmex relictus*.** Video
489 evidencing mobile replete workers emerging from disturbed nest. Filming took place
490 on the afternoon of August 20, 2020 (~14:00), external temperature of 34.5°C and
491 internal temperature of 29.4° (nest humidity ranging from 30% to 60%).

Myrmecinae

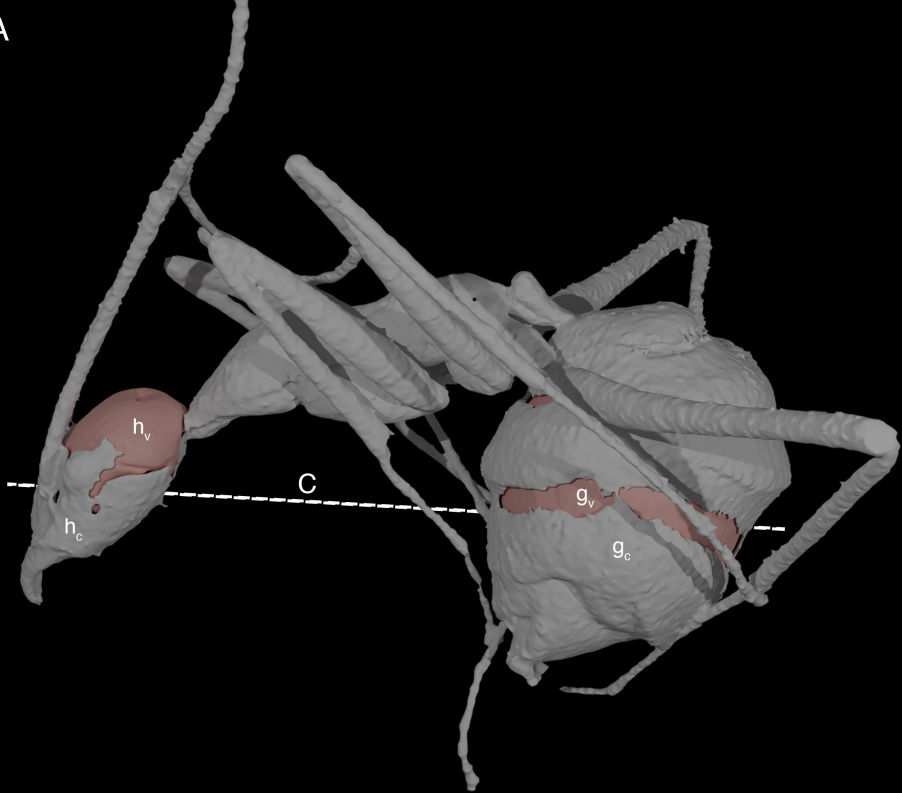


Formicinae

Dolichoderinae



A



B



C

