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# Where Fossils Dare and Males Matter: combined morphological and molecular analysis untangles the evolutionary history of the spider ant genus *Leptomyrmex* Mayr (Hymenoptera : Dolichoderinae)

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**Abstract.** The distinctive ant genus *Leptomyrmex* Mayr, 1862 had been thought to be endemic to Australasia for over 150 years, but enigmatic Neotropical fossils have challenged this view for decades. The present study responds to a recent and surprising discovery of extant *Leptomyrmex* species in Brazil with a thorough evaluation of the Dominican Republic fossil material, which dates to the Miocene. In the first case study of direct fossil inclusion within Formicidae Latreille, 1809, we incorporated both living and the extinct *Leptomyrmex* species. Through simultaneous analysis of molecular and morphological characters in both Bayesian and parsimony frameworks, we recovered the fossil taxon as sister-group to extant *Leptomyrmex* in Brazil while considering the influence of taxonomic and character sampling on inferred hypotheses relating to tree topology, biogeography and morphological evolution. We also identified potential loss of signal in the binning of morphological characters and tested the impact of parameterisation on divergence date estimation. Our results highlight the importance of securing sufficient taxon sampling for extant lineages when incorporating fossils and underscore the utility of diverse character sources in accurate placement of fossil terminals. Specifically, we find that fossil placement in this group is influenced by the inclusion of male-based characters and the newly discovered Neotropical 'Lazarus taxon'.

Additional keywords: biogeography, character discretisation, genitalia, male morphology, paleontology, tip-dating, total evidence.

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### Introduction

Fossils are essential for identifying former distributions of living and extinct lineages, and are especially important in reconstructing the biogeographic history of taxa that now occupy only relictual distributions. Examples abound of fossil discoveries that have dramatically reshaped inferences of biogeographic history and ancestral areas. Early branching termites, for example, were thought to occur only in Australia on the basis of extant fauna until numerous specimens were recovered from present-day Neotropical and Palearctic fossil deposits (Emerson 1965; Grimaldi and Engel 2005). Other examples of fossils rewriting history include a lineage of dragonflies limited to Africa and Australia today but present as fossil species from Europe and South America (Petrulevičius and Nel 2009); modern Ginkgo trees occurring in present-day China but distributed throughout the northern hemisphere as recently as the Eocene (Royer et al. 2003); and diatomyid

rodents, represented by a single extant species in Laos, now known to have been distributed throughout Asia in the past (Dawson *et al.* 2006). Although the taxonomic assignment of many informative fossil species may be straightforward, this is not always the case. Here, we explore a more equivocal example, where taxonomic assignment was historically confounded by a highly disjunct biogeographic pattern. Through a combined morphological and molecular approach, we assess the placement of a key fossil in the phylogeny of the ant genus *Leptomyrmex* Mayr, 1862 (Dolichoderinae Forel, 1878), and demonstrate the effectiveness of 'total evidence' or 'tip-dating' phylogenetics in ants for the first time. Our findings help rewrite the biogeographic story of this remarkable ant lineage.

*Leptomyrmex* are among the most distinctive ant taxa; the majority of species are large, diurnal, and easily recognisable as well as behaviourally unique. Interestingly, most known species possess wingless queens. Colloquially, females are known as



**Fig. 1.** (*A*) A representative of the macro-*Leptomyrmex* clade, *L. darlingtoni* Wheeler, 1934, found on Cape York Peninsula, Queensland, Australia. Photo taken by Andrea Lucky, from www.AntWeb.org (*B*) Three-dimensional model of *L. neotropicus* specimen AMNHDR-13-85 from ca. 20 Ma Dominican amber reconstructed from CT-scan. (*C*) Dominican amber specimen AMNHDR-JVE 654 containing eleven *L. neotropicus* workers. Scale bars: A and B = 1.0 mm, C = 2.0 mm. Fig. 1. is available in colour online.

'spider ants' based on their lanky, gracile habitus (Fig. 1*A*) and the peculiar habit of raising the gaster over the mesosoma, giving the body the appearance of a stout arachnid. Extant *Leptomyrmex* 

are almost entirely limited to New Caledonia, New Guinea and eastern Australia, where species usually occur in wet forest and sclerophyll habitats. Recently, a remarkable new species was discovered from the Brazilian cerrado (i.e. savannah, Boudinot *et al.* 2016). With the inclusion of the Brazilian species, 28 extant species of *Leptomyrmex* are known and recognised as valid (Shattuck 2000; Lucky 2011; Boudinot *et al.* 2016). These 28 species are divided into three main groups: the 'micro', 'macro', and '*relictus*' clades (Lucky 2011; Boudinot *et al.* 2016). The micro clade consists of six small, compact species, which contrasts with the 21 large, elongate macro- species and the single, highly derived Brazilian species, *L. relictus* Boudinot, Probst, Brandão, Feitosa & Ward, 2016 (Smith and Shattuck 2009; Lucky and Ward 2010; Boudinot *et al.* 2016).

The paleontological history of Leptomyrmex has been a source of intrigue for more than a century. In 1891, one of the architects of modern ant classification, Carlo Emery, described the species Leptomyrmex maravignae based on a male ant in Sicilian amber dated to the Oligocene (Emery 1891, 1913). Emery attributed the species to Leptomyrmex owing to the peculiar male wing venation, which he interpreted as matching the extant members of the genus. At that time, only macro Leptomyrmex were known. The Mediterranean fossil was therefore considered to extend the range of these ants from Australasia to the Palearctic, and generated questions regarding their origin and prehistoric distribution well before acceptance of plate tectonics. The question emerged: were these ants distributed more widely in the past? Wheeler (1915) designated this male as a primitive relative of Leptomyrmex, and the species, now renamed Leptomyrmula maravignae, suggested that leptomyrmecine ants at one time occurred in Europe. If this proved true their current distribution and diversity reflected massive range contraction and extinction. A recently described extinct species from Eocene-aged Baltic amber, Usomvrma mirabilis Dlussky, Radchenko & Dubovikoff, 2014, was adduced as support for the European distribution (Dlussky et al. 2014).

A Eurasian range contraction hypothesis stood as the lone biogeographic explanation of Leptomyrmex until 1980, when several putative Leptomyrmex workers were reported in Dominican amber from the Miocene (Baroni Urbani 1980). Baroni Urbani posited a historical tropicopolitan distribution with range contraction for the genus based on Leptomyrmex *neotropicus* (Fig. 1B, C), which he described from a composite reconstruction of nine incomplete worker inclusions preserved within the same amber specimen - these fossil specimens were large and elongate, as in modern macro Leptomyrmex. Soon afterwards, Wilson (1985) disputed this designation on the basis of biogeographic improbability, and argued that L. neotropicus was a closer match to extant members of the genus Camponotus Mayr, 1861. Although some elongate Camponotus superficially resemble Leptomyrmex, the two genera belong to different subfamilies, the Formicinae Latreille, 1809 and Dolichoderinae, respectively. The most conspicuous differentiating feature of the Formicinae is the acidopore - a unique modification of the terminal abdominal sternum for chemical defence that bears a distinct margin of setae, termed the coronula. The presence or absence of an acidopore and coronula could not be ruled out given the original L. neotropicus reconstruction. Indeed, because Camponotus is a globally distributed and hyperdiverse genus, consisting of well over 1000 species (Ward et al. 2016), a case of misidentification or convergence seemed likely and could not be

ruled out. However, shortly thereafter, four intact Leptomyrmex workers from Dominican amber were discovered, revealing the clear lack of an acidopore and coronula. This discovery led Baroni Urbani and Wilson (1987: pg 2) to jointly declare: 'The additional, better preserved workers are identical or close to L. neotropicus. and prove to belong to the Leptomyrmecini [Emery, 1913] beyond any reasonable doubt'. This finding provided the first evidence that Leptomyrmex may have had a historical distribution in the Neotropics. Still, due to biogeographic incongruence, the exact relationship of L. neotropicus to extant Leptomyrmex species remained uncertain. The Dominican amber fossil was proposed as a stem relative of the closely related neotropical genera Forelius Emery, 1888 and Dorymyrmex Mayr, 1866 (Ward et al. 2010), a member of stem Leptomyrmex (Lucky 2011), or as sister-group to the recently discovered extant species from Brazil (Boudinot et al. 2016).

More recently, three studies were published addressing the questions of the placement of Leptomyrmex within the Dolichoderinae (Ward et al. 2010), the internal topology of Leptomyrmex (Lucky 2011), and the placement of the newly discovered L. relictus (Boudinot et al. 2016). These studies relied principally on molecular sequence data to infer dated phylogenies. Lucky (2011) found geographical structuring within the Australasian clade, while Ward et al. (2010) and Boudinot et al. (2016) recovered Leptomyrmex as sister-genus to a clade of Neotropical species; the latter study concluded that the European fossils are unrelated to Leptomyrmex, and that the genus was Neotropical in origin. The two studies that explicitly analysed the historical biogeography of Leptomyrmex were agnostic as to the phylogenetic placement of the Dominican amber fossils, conservatively treating L. neotropicus as a member of the stem group (Ward et al. 2010; Boudinot et al. 2016), and only experimentally treating the species as a crown group member (Boudinot et al. 2016). Moreover, the sole datum operationalised from L. neotropicus was the fossil age, which was used to set a minimum age constraint in fossil-calibrated divergence dating analyses; L. neotropicus was excluded altogether from biogeographic analyses. Although these studies radically change our biogeographic understanding of Leptomyrmex, the placement of L. neotropicus remains unresolved, and has the potential to influence biogeographic and divergence dating inferences drawn for this charismatic and fascinating genus.

While the role of fossils in the age of phylogenomics may be unclear, recently developed Bayesian methods allow for direct integration of fossils in phylogenetic inference through simultaneous analysis of morphological and molecular data (Ware et al. 2010; Pyron 2011; Ronquist et al. 2012). Although simultaneous analysis of morphological and molecular characters within a parsimony framework has been utilised to integrate fossil taxa for over two decades (e.g. Eernisse and Kluge 1993), the practice declined with the advent of model-based phylogenetic reconstruction. Bayesian 'tip-dating' analyses have the benefit of potentially placing fossils more accurately as well as improving temporal estimation and biogeographic reconstruction (Wood et al. 2013). This contrasts with the now traditional node-dating techniques, which include fossils in analyses as calibration points for divergence date estimates (Donoghue and Benton 2007). Node-dating typically relies on the assumed taxonomic placement of fossils, which can contribute to dating error (Rutschmann *et al.* 2007; Brady 2011). Furthermore, because fossils are not actually present in inferred phylogenies, their contribution to biogeographic reconstruction is limited, and ancient distributions may be excluded on the basis of ambiguous placement. For these reasons, we draw our attention to *L. neotropicus*.

To resolve the placement of L. neotropicus - the enigmatic fossil taxon at the heart of this story - we employ combined morphological and molecular analysis in both Bayesian and parsimony frameworks. In so doing, our objectives are to better contextualise the history of Leptomyrmex, to provide a case study for the targeted treatment of taxonomically controversial fossils, and to examine the phylogenetic implications of the 'total evidence' approach. Given the recent discovery of the 'Lazarus taxon', L. relictus, in Brazil, we also devote special attention to evaluating the impact of extant sampling on fossil placement. Further, we detail the effects of binning continuous morphological characters and the inclusion of male morphology, the latter of which is usually neglected from taxonomic and phylogenetic studies of ants. Ultimately, we aim to illuminate the history of L. neotropicus, the confounding fossil that Baroni Urbani (1980) and E.O. Wilson (1985) knowingly identified as a 'considerable biogeographic anomaly'.

#### Materials and methods

# Morphological and molecular data

To assess the phylogenetic position of L. neotropicus and the overall impact of taxonomic and character sampling, data were obtained for species across the genus *Leptomyrmex* and closely related groups, including workers and males. The fossil record includes males of two species that are putative relatives of Leptomyrmex from Sicilian and Baltic amber (Emery 1891; Dlussky et al. 2014), and the putative male of L. neotropicus (Baroni Urbani and Wilson 1987). The two Eurasian fossil taxa are not included owing to their morphological dissimilarity to Leptomyrmex (Boudinot et al. 2016), but the Dominican amber male is here treated as conspecific or near conspecific with L. neotropicus. The Lazarus taxon, L. relictus, was excluded in some analyses to explore the effect of this species on phylogenetic inference. In total, 27 terminals were included based on the worker caste, with males scored for 25 of them; this matrix comprises an outgroup taxon (Linepithema humile (Mayr, 1868)), the sister-group of *Leptomyrmex* (Dorymyrmex bicolor Wheeler, 1906 and Forelius pruinosus Roger, 1863), L. neotropicus, L. relictus and two micro- and 20 macro-Leptomyrmex species.

Previously available molecular data were used from Ward *et al.* (2010), Lucky (2011) and Boudinot *et al.* (2016). A matrix consisting of 11 loci was constructed: arginine kinase (*ArgK*), long wavelength rhodopsin (*LW Rh*), wingless (*Wg*), large ribosomal subunit gene (*28S* rRNA; *28S* hereafter), and mitochondrial cytochrome *c* oxidase subunit I (*COI*), small ribosomal subunit gene (*18S* rRNA), abdominal-A (*AbdA*), rudimentary (*CAD*), enolase (*EN*), elongation factor 1- $\alpha$  F1 copy (*F1*) and elongation factor 1- $\alpha$  F2 copy (*F2*). All but one of the extant taxa were represented by at least five genes: *ArgK*, *LW Rh*, *Wg*, *28S* and *COI* (this latter locus lacking for *L. relictus*);

the other loci were available for a subset of nine taxa. Specific taxon sampling and accession numbers are presented in Table S1, available as Supplementary Material to this paper. Loci were statically aligned in MUSCLE v3.8 (Edgar 2004) and concatenated, with the final molecular matrix totalling 9553 bp following excision of introns (Supplementary Data: molecular matrix). The concatenated matrix possesses 46.7% missing molecular character cells. It has been demonstrated that, in cases where missing data are confined to the same characters, not randomly distributed, the 'correct' topology is still recovered with up to 70% of data cells missing (Wiens 2003, 2006; Wiens and Morrill 2011). Moreover, this kind of missing data has been shown to have little impact on molecular dating estimates (Zheng and Wiens 2015).

Novel morphological characters were developed for workers and males, including 24 worker characters (8 continuous, 16 discrete; Table 1) and 45 male characters (all discrete; Table 2). The inclusion of continuous characters is significant; macro-clade spider ants are defined in part by a generalised elongation, which is difficult to capture in simple discrete character states. Worker and male characters were scored for all terminals, except the micro L. dolichoscapus Smith & Shattuck, 2009 and macro L. geniculatus Emery, 1914, for which males were unavailable. Continuous characters were obtained by taking the mean measurements of a minimum of four specimens per species. In the case of L. neotropicus, four worker specimens (AMNHDR-14-94, AMNHDR-13-85, AMNH-ANDL1, AMNHDR-JVE-654) from Dominican amber dated to 15-26 million years ago (Brody et al. 2001) were examined and measured. Specimen AMNHDR-JVE 654 is a remarkable assemblage of eleven workers, reported here for the first time (Fig. 1C). Examination of AMNHDR-13-85 and AMNHDR-1339, a male specimen, included 2- and 3-dimensional reconstruction performed at the American Museum of Natural History Microscopy and Imaging Facility utilising a GE (www.ge.com) Phoenix v|tome|x s 240 computed tomography system (Fig. 1B; Movie 1, available as Supplementary Material). X-ray data were analysed in ImageJ v1.48 (https://imagej.net) and Volume Graphics Studio Max v2.2 (www.volumegraphics.com).

# Morphological characters

#### Worker

Novel worker characters, descriptions, and definitions of character states are outlined in Table 1.

#### Male

Novel male characters, descriptions, and definitions of character states are outlined in Table 2.

# Bayesian analysis

MrBayes v3.2.6 (Ronquist and Huelsenbeck 2003) was used to conduct topological and divergence dating analyses on local machines or the CIPRES Science Gateway (Miller *et al.* 2010). The Mk model (Lewis 2001) was applied to morphological data. A molecular partitioning scheme with corresponding substitution models was identified using PartitionFinder v1.1.1

Character	States	Notes
1. Head capsule elongation (cephalic index)	Continuous (ratio)	All measurements from frontal view that maximises eye length. Maximum width of head, excluding eyes divided by maximum length of head measured as midline distance between anterior edge of clypeus to occipital foramen of head
2. Head lateral margin configuration	(0) parallel; (1) converging	From a frontal view taken at eye level, position of lateral margins of the head with respect to each other. Parallel indicating the lateral margins would not intersect if extended, while convergent configuration such that the sides of the head are directed towards each other
3. Postocular margin of head	<ul><li>(0) gradually rounded;</li><li>(1) broadly rounded;</li><li>(2) neck-like constriction</li></ul>	Shape of the head posterior to compound eyes when viewed from front view that maximises eye length. <i>Gradually</i> indicating the rounding continues to the posterior-most margin, whereas <i>broadly</i> refers to rounded lateral margins with a flattened posterior margin. <i>Neck-like</i> constriction refers to an abruptly narrowed postocular margin
4. Lateral eye position	<ul><li>(0) distant from lateral margin of head; (1) touching or overlapping head lateral margin</li></ul>	In a frontal view, position of the outer/lateral margin of the eye with respect to lateral margin of the head capsule. Either with outer/lateral margin of the eye distant from the lateral margin of the head (by $\geq 1/3$ width of eye) or with the lateral margin of the eye touching or overlapping lateral margin of head
5. Anteroposterior eye position	<ul><li>(0) posterodorsal;</li><li>(1) anteroventral</li></ul>	Eye position corresponding to either the posterodorsal or anteroventral half of head. Head length measured from anterior margin of clypeus to occipital foramen, overall eye position determined by position of eye centre (pinpointed as meeting point between eye height and width measured at greatest length and width)
6. Frontal carina shape	<ul><li>(0) straight, parallel;</li><li>(1) sinuous, convex</li></ul>	Anteroposterior shape of frontal carinae
7. Distance between frontal carinae	Continuous	Measured as minimum distance between carinae
8. Scape index	Continuous (ratio)	Total length of antenna scape, excluding radicle divided by head width at greatest
9. Eye length	Continuous	Total eye length measured in face-on view that maximises eye length
10. Anterior margin of clypeus	(0) flat; (1) convex	Overall shape of anterior margin of the clypeus
11. Clypeal margin	(0) emargination not present; (1) emargination present	Anterior margin of clypeus with notch or indentation along medial axis
12. Mandible dentition	<ul> <li>(0) less than 10;</li> <li>(1) greater than</li> <li>10 to 15; (2) greater</li> <li>than 15 to 20;</li> <li>(3) greater than 20</li> </ul>	Total number of teeth and denticles present on inner margin of mandibles
13. Colour banding or patterning on legs (red/black)	(0) absent; (1) present	Distinct colour banding visible on legs in any form (i.e. black to red, red to black to red, etc.)
14. Total mesosoma length	Continuous	'Weber's length' distance measured as a straight line between anterior margin of pronotum to posterior-most margin of propodeum in lateral view
15. Petiole scale	(0) rounded; (1) scale-like	Petiole scale defined as narrow, dorsally angular; contrasted with more broad, gradually rounded petiole
16. Hypostomal notch	(0) absent; (1) present	With head capsule in ventral view, the medial hypostoma is broadly notched. This is a previously recorded synapomorphy of <i>Leptomyrmex</i> (Shattuck 1992)
17. Transverse propodeal impression	(0) absent; (1) present	From lateral view, an indentation in the dorsal margin between the anterior suture and posterior face of the propodeum. Such an indentation is not merely a sheer declining face, but rather a distinct valley restricted to the propodeum
18. Maxillary palp length	<ul><li>(0) less than 3/4; head</li><li>length; (1) ≥3/4</li><li>head length</li></ul>	Fully extended, maxillary palp length as directed posteriad
19. Lateral/posterior margin of clypeus	(0) blended, as a rounded corner; (1) sharp, cornered	Meeting point of posterior and lateral margins of the clypeus. Either as a distinct, sharp corner, or gradually rounded
20. Anterior mesonotal ridge	(0) absent; (1) present	Dorsal ridge present abutting the promesonotal suture
21. Ventral surface of petiole	<ul><li>(0) flat; (1) concave</li><li>(2) convex</li></ul>	Shape of ventral margin of petiole from lateral view

# Table 1. Summary of Worker Characters

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Character	States	Notes
22. Head length	Continuous	Head length taken from frontal view that maximises eye length, from posterior margin of head to posterior clypeal margin
23. Scape length	Continuous	Total length of scape, excluding radicle
24. Eye width	Continuous	Taken from frontal view, which maximises eye length, measured perpendicular to maximum eye length line

#### Table 2. Summary of Male Characters

All venational characters apply to the forewing. Abscissa names follow Brown and Nutting (1950) (see also Boudinot *et al.* 2016). Genital terminology follows Boudinot (2013) with the following modifications: *genital foramen* replaces 'foramen genitale' for linguistic consistency, and *lateral carina* replaces lateral apodeme. Due to the complexity of male genitalia, these characters are illustrated in Figs 2 and 3. Material examined to develop these characters is the same as those from Boudinot *et al.* (2016). *L. neotropicus* determinations utilised images resulting from computed tomography scan of AMNHDR-1339 genitalia

Character	States	Notes
<ol> <li>Medial hypostomal notch</li> <li>Frontal carinae</li> </ol>	<ul><li>(0) absent; (1) present</li><li>(0) absent; (1) present</li></ul>	See description in worker character 16 The frontal carinae of male dolichoderines may be distinct and sharp (present) or effaced and indistinct (absent)
3. Antennomere 2 length relative to 3	<ul> <li>(0) 2 ≥3; (1) 2 &lt;3; (2) 2 &lt;&lt;3;</li> <li>(3) 2+3 fused. Unordered</li> </ul>	Length of antennomere 2 (pedicel) with antenna in medial view relative to length of antennomere 3 (flagellomere 1) in same view. There is a conspicuous difference between states 1 and 2. The 2nd and 3rd antennomeres of <i>L. puberulus</i> and <i>L. flavitarsus</i> are fused
4. Mandibular margins	<ul><li>(0) basal and masticatory margins distinct;</li><li>(1) basal and masticatory margins indistinct</li></ul>	With mandibles in dorsal view, basal and masticatory margins clearly offset (state 0) or forming a broad, even convexity or linear margin (state 1)
5. Mandibular form	<ul><li>(0) triangular to subtriangular;</li><li>(1) falcate</li></ul>	Some macro <i>Leptomyrmex</i> have falcate mandibles, which are elongate with hooked apices
<ol> <li>Masticatory mandibular margin</li> </ol>	(0) edentate; (1) dentate or serrate	Observed with mandibles in dorsal view
7. Malar space	(0) narrow; (1) broad	The malar space of male <i>Leptomyrmex</i> is conspicuously broadened in the macro clade due to anterior elongation of the head capsule
8. Occipital margin	<ul><li>(0) obscured by vertex or ocellar triangle in full-face view;</li><li>(1) visible in full-face view</li></ul>	With the head in full-face view, the posterior head margin constitutes either the vertex (state 0) or the occipital margin (state 1). Macro- <i>Leptomyrmex</i> males have state 1 due to posterior elongation of the head capsule
9. Pronotum	(0) dorsoventrally taller than anteroposteriorly long; (1) anteroposteriorly longer than dorsoventrally tall; (2) extremely elongate (ordered)	Observed with mesosoma in lateral view. Anteroposteriorly short pronota (state 0) are about as long as an ocellus; extremely elongate pronota (state 2) are considerably longer than the maximum eye diameter, while the long but not extreme pronota (state 1) are about as long as to shorter than the compound eye
10. Paired mesoscutal	(0) absent; (1) present	Best observed with mesoscutum in dorsolateral view. The sulcae are long and broad
longitudinal furrows 11. Transverse mesoscutal furrow	(0) absent; (1) present	impressions that are centred on the parapsidal lines Best observed with mesoscutum in lateral view. The mesoscutum may have a deep and broad transverse furrow somewhat anterior to the tegulae
12. Propodeal spiracles	<ul><li>(0) flush with propodeum;</li><li>(1) raised above propodeum</li></ul>	The margins of the propodeal spiracle may be raised above the surface of the propodeum in profile view
13. Petiole	<ul> <li>(0) length ≤ width in dorsal</li> <li>view; (1) length &gt; width</li> <li>in dorsal view</li> </ul>	Petiole anteroposteriorly shorter than lateromedially broad, or longer than broad
14. Pterostigma	(0) absent; (1) present	Pterostigma present or absent on anterior margin of forewing
15. Pterostigmal appendage	(0) absent; (1) present	A digitate to spherical lobe projecting from basal juncture of Rsf1. Synapomorphy of the macro- <i>Leptomyrmex</i> clade
16. Costal vein	(0) absent; (1) present	Costal vein present as a sclerotised tube or absent; for clarity, the macro- <i>Leptomyrmex</i> species examined lack the costal vein, but have the costal margin folded over, with secondary sclerotisation of the wing membrane

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Character	States	Notes
17. Rsf2+	(0) absent; (1) present	Second free abscissa (vein segment) and subsequent (distal) abscissae of the radial sector present or absent Rsf2+ occurs distal to Rs+M
18. Mf2–3	(0) absent; (1) present	Second and third abscissae of free M present or absent. Mf2–3 occurs between Rs+M and crossycin 2rs-m
19. Mf4–6	(0) absent; (1) present	Fourth through sixth abscissae of free M present or absent. Mf4–6 occurs after crossvein 2rs-m
20 2r-rs	(0) absent: (1) present	Second forewing crossvein joining the radius and radial sector present or absent
21. 2rs-m	(0) absent; (1) present	Second forewing crossvein joining the radial sector and media present or absent. The first radial sector-media crossvein was ancestrally lost in the Hymenoptera due to fusion of the radial sector and the media
22. 1m-cu	(0) absent; (1) present	First forewing crossvein joining the media and cubitus present or absent. This abscissa encloses the discal cell distally. Formicidae lack the second and third media–cubitus crossveins
23. Abdominal sternum IX anterolateral corners	(0) rounded; (1) produced anteriorly	The anterolateral corners of the ninth abdominal sternum may be produced as subtriangular lobes. There is notable variation in their orientation, but this variation was not scored
24. Spiculum	<ul> <li>(0) absent (Fig. 2<i>A</i>);</li> <li>(1) single (Fig. 2<i>B</i>);</li> <li>(2) paired (Fig. 2<i>C</i>);</li> <li>(3) trebled (Fig. 2<i>D</i>)</li> <li>(unordered)</li> </ul>	The spiculum is the anteromedial apodeme of the ninth abdominal sternum, which bears the origins of the cupular–sternal muscles. Remarkably, the ninth abdominal sternum of <i>Leptomyrmex</i> may have two (state 2), three (state 3), or no spicula (state 0). The spiculum is rarely lost in ants, and to date no other taxon has been observed to have more than one spiculum
25. Cupula dorsal and lateral surfaces	<ul> <li>(0) narrow (Fig. 2<i>I</i>, <i>J</i>, <i>M</i>, <i>O</i>, <i>P</i>);</li> <li>(1) broad (Fig. 2<i>F</i>, <i>G</i>, <i>H</i>, <i>N</i>, <i>R</i>, <i>T</i>)</li> </ul>	The cupulae of macro- <i>Leptomyrmex</i> species have anteroposteriorly elongated dorsal and lateral faces. In these species the anteroposterior length of the faces may be from about as long as to much longer than the lateromedial width of the cupula as measured from the middle of the genital foramen in ventral view
26. Cupula ventral disc	(0) absent (Fig. 2 <i>M</i> ); (1) present (Fig. 2 <i>F</i> , <i>Q</i> )	The ventral surface of the cupula may be produced posteriorly as a disc-like lobe, bearing the insertions of the abdominal sternum IX muscles. Present in macro <i>Leptomyrmex</i> as well as <i>Forelius</i> .
27. Cupula anterior margins	(0) unswollen (Fig. 2 <i>L</i> , <i>M</i> , <i>O</i> , <i>P</i> ); (1) grossly swollen (Fig. 2 <i>K</i> , <i>N</i> )	The anterior margins of the cupula, surrounding the genital foramen, may be grossly swollen, forming lip-like processes in some species
28. Genital foramen	(0) broad (Fig. 2 <i>M</i> , <i>O</i> ); (1) strongly constricted (Fig. 2 <i>K</i> , <i>L</i> , <i>N</i> )	The diameter of the genital foramen may be broad, about as wide as the aedeagus in dorsal view (state 0), or may be very strongly constricted, being just wide enough for the eiaculatory duct to pass through (state 1)
29. Cupula, dorsal view	<ul> <li>(0) anterior margin linear, convex, or V-shaped</li> <li>(Fig. 2H, J, M, O, P); (1) medially notched (Fig. 2N)</li> </ul>	With the genital capsule in dorsal view, the anterior margin of the cupula may be linear, convex, or V-shaped (state 0), or may be distinctly posteriorly impressed medially (state 1)
30. Basimere dorsomedial margins	(0) rounded and not parallel (Fig. 2 <i>P</i> ); (1) linear and parallel (Fig. 2 <i>H</i> , <i>N</i> )	With the genital capsule in dorsal view, the medial margins of the basimeres may be anteroposteriorly short and rounded (state 0), or long, linear, and clearly parallel
31. Basimere posterodorsal and posterolateral margins	(0) ecarinate (Fig. 2 <i>P</i> ); (1) carinate and strongly inflexed (Fig. 3 <i>A</i> , <i>B</i> )	The posterior margin of the basimere is carinate and strongly inflexed in macro <i>Leptomyrmex</i> , but is rounded (ecarinate) and weakly or not at all inflexed in other taxa
32. Basimere dorsal posteromedial margin	(0) lacking sclerotised ridge (Fig. 2 <i>J</i> , <i>P</i> ); (1) with sclerotised bridge (Fig. 2 <i>O</i> )	The basimeres of <i>L. relictus</i> are broadly fused, with the fused region depressed below the level of the remainder of the dorsal basimeral surface
<ol> <li>Basimere ventromedial margin</li> </ol>	<ul> <li>(0) lacking subrectangular medial process (Fig. 2<i>M</i>, <i>R</i>);</li> <li>(1) with subrectangular medial process (Fig. 2<i>Q</i>)</li> </ul>	The ventromedial basimeral margins of <i>Dorymyrmex</i> and <i>Forelius</i> each bear an anteroposteriorly narrow subrectangular process that projects medially to posteromedially
34. Basimere ventromedial margin	(0) ecarinate (Fig. 2 <i>Q</i> ); (1) laminate with lamella produced medially (Fig. 2 <i>R</i> )	The ventromedial basimeral margin of some species is anteroposteriorly broadly produced medially as a lamina, while others are not
35. Basimeral-penisvalvar corium (= membrane)	(0) membranous; (1) sclerotised	This is homologous with the penisvalvar bridge, character 43
36. Telomere, profile view	(0) triangular (Fig. 2 <i>G</i> , <i>I</i> ); (1) lobate (Fig. 2 <i>S</i> ); (2) bilobate (Fig. 2 <i>T</i> ) (unordered)	The telomere may be acutely triangular and somewhat thick (state 0), lobate and very thin (state 1), or bilobate (state 2)

Table 2. (continued)

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Table 2.	(continued)
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Character	States	Notes
37. Basivolsellar process	(0) absent (Fig. 2V); (1) present (Fig. 2U, W-Y)	The basivolsellar process is a dolichoderine synapomorphy (although homoplasious with the Amblyoponinae, Boudinot 2015), and is present at the posteroventral apex of the volsella, near the cuspis. The process may be present or absent
38. Cuspis	(0) absent (Fig. 2 <i>V</i> ); (1) present (Fig. 2 <i>U</i> , <i>X</i> – <i>Y</i> )	Near the posterior apex of the volsella, there may be a lobate to digitate cuspis, which is lateral (ectal) to the digitus
39. Digitus form	<ul> <li>(0) falcate, with ventral margin linear before arcing apicoventrally (Fig. 2<i>W</i>-<i>Y</i>);</li> <li>(1) strongly falcate, with ventral margin curving dorsally before arcing apicoventrally (Fig. 2<i>V</i>);</li> <li>(2) digitate, with ventral margin more-or-less linear (Fig. 2<i>U</i>) (unordered)</li> </ul>	Dolichoderine digiti vary considerably among and within the genera. Most genera have state 0, where the digital apex is strongly downturned after a linear, basal portion. The macro <i>Leptomyrmex</i> have been scored as 0 despite significant variation in digital form otherwise. Taxa with state 1 have an elongated falcate digitus that is directed posterodorsally before strongly arcing posteroventrally to the apex. Taxa with state 2 have digiti that are digitate and more-or-less linear, with the ventral margin only weakly concave or curved
40. Digitus apicodorsal	(0) absent (Fig. $2U$ , $V$ , $X$ ); (1) present (Fig. $2W$ , $Y$ )	Some macro <i>Leptomyrmex</i> bear an apicodorsal process on the digitus, near where the remainder of the digitus is downcurved
41. Digitus apical margin	<ul> <li>(0) evenly rounded (Fig. 2X);</li> <li>(1) truncate, linear to convex</li> <li>(Fig. 2W); (2) concave</li> <li>(Fig. 2I) (unordered)</li> </ul>	The posterior or posterodorsal margin of the digitus may be be continuously rounded with the anterodorsal margin (state 0), truncate or linear (state 1), or concave (state 2). State 2 occurs in taxa with strongly developed apicodorsal digital processes
42. Digitus apex	<ul> <li>(0) broadly tapering (Fig. 2<i>X</i>);</li> <li>(1) very narrowly tapering</li> <li>(Fig. 2<i>W</i>, <i>Y</i>)</li> </ul>	Some macro Leptomyrmex have extremely thin and narrowly tapering digital apices
<ol> <li>Dorsal sclerotic bridge of penisvalva</li> </ol>	(0) anteroposteriorly broad (Fig. 4 <i>P</i> ); (1) anteroposteriorly narrow (Figs 4 <i>J</i> , 5 <i>A</i> , <i>B</i> )	Presence of a dorsal sclerotic bridge that is part of and extends between the penisvalvae is a synapomorphy of the Leptomyrmecini as defined by Ward <i>et al.</i> (2010) (Boudinot <i>et al.</i> 2016; B. E. Boudinot in prep.). The sclerotic bridge of Australasian <i>Leptomyrmex</i> is strongly narrowed anteroposteriorly
44. Sclerotic bridge of penisvalva	<ul> <li>(0) thinly sclerotised (Fig. 4<i>J</i>);</li> <li>(1) very thickly sclerotised</li> <li>(Fig. 5<i>A</i>, <i>B</i>)</li> </ul>	The sclerotic bridge of the macro <i>Leptomyrmex</i> is very thickly sclerotised, forming a conspicuous, bulging bar
45. Valviceps, ventral to lateral carina	<ul> <li>(0) broadly sclerotised, subrectangular to ovate</li> <li>(Fig. 5<i>C</i>); (1) narrowly sclerotised, crescentiform</li> <li>(Fig. 5<i>D</i>); (2) broadly sclerotised, bent at two angles (Fig. 5<i>E</i>)</li> </ul>	Within the sampled dolichoderines, the valviceps (blade of the penisvalva, rather than the valvura, or anterodorsal apodeme) may be broadly sclerotised dorsoventrally, being subrectangular to ovate (state 0), or narrowly sclerotised dorsoventrally, with a concave dorsal margin, thus being crescentiform in lateral (ectal) view (state 1). <i>Leptomyrmex relictus</i> has an extremely modified valviceps, which is broadly sclerotised, with strongly convex sides, forming a tube that is bent at two angles, and bearing the ventral teeth on a short, distinctly offset blade (state 2). State 2 is unique in the Formicidae

(Lanfear *et al.* 2012) under a 'greedy' search program and assessed with the Bayesian Information Criterion (BIC). Four partitions were identified, with one modelled under Hasegawa–Kishino–Yano+invariable sites+gamma (HKY+I+G) and the other three HKY+G (Table 3). During the tree optimisation in MrBayes, Metropolis-Coupled Markov-Chain Monte Carlo searches employed two runs, each with four chains (one cold, three set to a default temperature of 0.2), sampling every 1000 generations. Searches were run for 50 million generations, with consensus trees generated after 25% of initial samples were removed as burn-in. Run convergence was assessed via the average standard deviation of split frequencies, which was found to be less than 0.01 in all runs. The resultant topology was used to constrain nodes in divergence estimation analyses.

To evaluate the influence of worker versus male morphological characters in our combined analyses, three additional treatments were conducted: (1) male characters excluded; (2) worker

characters excluded; and (3) characters from both sexes included. Molecular and morphological models were set as above. Nexus files were analysed for 25 million generations, sampling every 1000 generations, with nruns and nchains = 4. These four runs were manually combined for each treatment using a text editor then summarised using TreeAnnotator v2.3.1 (Rambaut and Drummond 2010) with a burn-in of 10%, as determined via Tracer v1.6 (Rambaut *et al.* 2013). Using Tracer, convergence was strongly inferred by the very high effective sample sizes for all parameters (>>300). FigTree v1.4.2 (Rambaut 2014) was used to visualise the resultant phylograms.

# Divergence dating

We conducted six divergence dating analyses: four node-dating and two tip-dating (Table 4), using two alternative root node



**Fig. 2.** Male genitalic characters. Taxa and specimens: (*A*) Leptomyrmex mjobergi (CASENT0011879); (*B*) L. rothneyi (CASENT0011911); (*C*, *W*) L. nigriventris (CASENT0011707); (*D*) L. flavitarsus (CASENT0127271); (*E*) Linepithema humile (CASENT0730303); (*F*, *Y*) L. erythrocephalus (CASENT0011675); (*G*, *H*) L. unicolor (CASENT0012016); (*I*, *J*, *M*) L. burwelli (CASENT0127142); (*K*, *N*) L. wiburdi (CASENT0011673); (*L*, *R*) L. tibialis (CASENT0011723); (*O*, *S*, *V*) L. relictus (LACMENT323015); (*P*, *Q*, *U*) Forelius pruinosus (CASENT0730402); (*T*) L. darlingtoni (CASENT0012029); (*X*) L. fragilis (CASENT0127229). Structures: (*A*, *B*, *C*, *D*) abdominal sternum IX, mesal (internal) view (in situ); (*E*) abdominal sterna VIII and IX, mesal view; (*F*) cupula, ventral view; (*G*, *I*, *S*, *T*) genital capsule, lateral view; (*U*, *W*, *Y*) volsella, mesal (internal/lateral) view; (*V*, *X*) volsella and paramere, medial view. Scale bars = 0.2 mm. Fig. 2. is available in colour online.



**Fig. 3.** Male genitalic characters. Taxa and specimens: (*A*, *B*) Leptomyrmex unicolor (CASENT0012016); (*C*) Forelius pruinosus (CASENT0730402); (*D*) L. nigriceps (CASENT0012140); (*E*) L. relictus (LACMENT323015). Structures: (*A*) genital capsule, posterior view; (*B*) genital capsule, posteriolateral oblique, with left paramere removed; (*C*) penisvalva, lateral view (*D*, *E*, valvura broken off). Scale bars = 0.2 mm. Fig. 3. is available in colour online.

 Table 3. Partitioning and model scheme identified with PartitionFinder applied to MrBayes and BEAST analyses

 HKY, Hasegawa–Kishino–Yano; I, invariable sites, G, gamma

Subset	Model applied	Partitions
1	HKY+I+G	18S, 28S, AA_pos1, AA_pos2, AK_pos1, AK_pos2, CD_pos1, CD_pos2, EL_pos1, EL_pos2, F1_pos1, F1_pos2, F2_pos1, F2_pos2, LR_pos1, LR_pos2, Wg_pos1, Wg_pos2
2	HKY+G	AA_pos3, AK_pos3, CD_pos3, EL_pos3, F1_pos3, F2_pos3, LR_pos3, Wg_pos3
3	HKY+G	COI_pos3
4	HKY+G	COI_pos1, COI_pos2

treatments for each. Node-dating optimisations utilised molecular-only datasets with partitions identified above while tip-dating optimisation included molecular and morphological data. Searches were performed in MrBayes for 50 million generations, sampling every 1000 generations with nruns=2 and nchains=4. Results were summarised as above. The root node in our phylogeny is equivalent to the crown node of the Leptomyrmecini (Ward et al. 2010; Boudinot et al. 2016), and was constrained using one of two a priori calibration ranges (in mega-annum, Ma). Calibration 1 ('Estimate Root'): 62 (95% max), 53 (mean), 43 (5% min), normally distributed (estimated range from Boudinot et al. 2016); Calibration 2 ('Fossil Root'): 75 (95% max), 45 (median), 20 (5% min), offset lognormal. The max and min of the second prior setting is explicitly informed from fossils: Chronomyrmex McKellar, Glasier & Engel, 2013, the oldest known dolichoderine (McKellar et al. 2013; Boudinot et al. 2016) was used for the maximum, and L. neotropicus for the minimum.

Differing from all previous studies, we employed a normal prior distribution of 57–48–39 for crown *Leptomyrmex* in

node-dating analyses based on the estimate of Boudinot et al. (2016); in the tip-dating analysis crown group, ages were allowed to be sampled freely, while the age of L. neotropicus was fixed at 20 Ma. Leptomyrmex neotropicus was excluded from all nodedating analyses to test the impact of including the fossil directly as a tip-dating calibration. Following Ronquist et al. (2012), four node- and all tip-dating analyses assumed a uniform clock with the clock prior set to an independent gamma rates (IGR) model (Lepage et al. 2007) where each branch is allowed an independent rate that is drawn from a gamma distribution (i.e. we employed a 'relaxed clock' model). Alternative to the 'relaxed clock' model assumption, we investigated the effect of the 'fossilisation' birth-death model on two additional node-dating analyses. To estimate a value for the clock-rate prior, a pilot lognormal relaxedclock run was performed in BEAST v1.8.2 under a birth-death tree model (Drummond and Rambaut 2007) with molecular data only. The MeanRate parameter estimated from this pilot analysis was subsequently applied to all dating analyses in MrBayes, which has been shown to be an effective method (Arcila et al. 2015).

		Node	dating		Tip d	ating	Prior analys	ses
	'Fossilisat	tion' clock	Unifor	n clock	Uniforn	n clock	Boudinot et al. (2016)	Lucky (2011)
	1: Estimate root	2: Fossil root	1: Estimate root	2: Fossil root	1: Estimate root <sup>A</sup>	2: Fossil root	Calibration 2	I
Root age	<b>52.8</b> [61.5–43.9]	<b>50.2</b> [62.9–38.1]	<b>54.2</b> [63.5–45.3]	<b>53.1</b> [68.6–39.9]	48.6 [57.9–40]	36.9 [49.7–27.5]	52 [61-43]	<b>48</b> [72–36]
Leptomyrmex	<b>45.6</b> [53.6–37.9]	43.8 [53.3–33.8]	44.4 [52.7–36.6]	43.7 [53.9–33.9]	43.8 [54-35.2]	33.7 [45.7–25.3]	46 [53–37]	I
Australasian clade	<b>34.9</b> [44.1–26.3]	<b>33.3</b> [43.7–22.7]	37.1 [45.8–28.7]	36.6 [47–27.1]	37.3 [48.1–28.4]	<b>28.9</b> [39.8–20.7]	<b>28</b> [40–18]	<b>31</b> [44–19]
Macro clade	22.6 [30.3–16.1]	21.8 [30.1–14.7]	<b>27.3</b> [36.2–19.7]	<b>26.9</b> [36.8–18.4]	<b>28.8</b> [38.5–20.6]	<b>22.7</b> [32–15.7]	12 [16–7]	<b>15</b> [22–8]
Micro clade	8.9 [15.0-4.6]	8.4 [14.5-4.2]	10.6 [18.4–3.9]	10.4 [18.7–3.8]	<b>12.5</b> [21–5.2]	<b>9.8</b> [17.3–3.8]	6 [11–2]	8 [15–3]

Vo age estimate for Lentonyrmer is available from Lucky (2011) as this study antedates the discovery of L relicitus. Bold value indicates mean age while bracketed range corresponds to 95% highest nosterior Table 4. Summary of divergence date estimates. Results are from all analyses for key nodes, and compared with the preferred calibration of Boudinot *et al.* (2016) and the results of Lucky (2011).

Parsimony analysis

Parsimony analyses were implemented in TNT v1.1 (Goloboff et al. 2008) to explore topology robustness and the influence of certain morphological characters. Tree searches employed sectorial searches, tree drifting, fusing and parsimony ratchet under the XMULT command until the best score was located 100 times. Nodes were evaluated with symmetric resampling (1000 pseudoreplicates; Goloboff et al. 2003). To explore the impact of discretising continuous characters, we experimented with character binning because it is not possible to include nonwhole-number character states with MrBayes (all combined analyses performed under Bayesian optimisation utilised discrete character states). To bin the eight continuous worker caste characters, state ranges were plotted to assess natural discontinuities in the data. In cases where no breaks were readily identifiable, the standard deviation of character states was used to create 'binnable' ranges beginning from the lowest character state value (Supplementary Data: morphological matrices). Potential loss of signal due to binning was evaluated through parsimony analyses of two morphological matrices, one including the eight raw continuous characters and another with those characters discretised. These matrices, both with the same total number of characters (69), were analysed alone and together in combined analyses with molecular data. To evaluate the relative roles of worker and male characters in combined and morphology-only analyses, we ran data exclusion experiments, using either worker or male characters only.

# Results

# Topology

Leptomyrmex was recovered as monophyletic across all analyses using both Bayesian and parsimony methods. Within Leptomyrmex, the placement of L. neotropicus and L. relictus varied across treatments. In Bayesian analyses using combined data, the two Neotropical species were consistently recovered as a highly supported clade (Fig. 4A, B), except when male morphology was excluded (Fig. 4C). This Neotropical clade was similarly recovered in all parsimony analyses employing male morphology (Fig. 5), but L. neotropicus and L. relictus are recovered as a grade in worker-only analyses, with or without molecular data (Supplementary Data: additional trees). In all combined analyses including male characters, the Neotropical clade is sister-group to the micro and macro Leptomyrmex (Figs 4A, B, 5A), but is nested within the Australasian clade as sister-group to the macro clade in morphology-only parsimony analyses (Fig. 5C). The position of L. neotropicus is, moreover, highly contingent on L. relictus: when L. relictus is excluded, L. neotropicus is recovered as sister-group to macro Leptomyrmex in combined analyses (Fig. 5B). The relationships among macro Leptomyrmex in combined Bayesian analyses are identical to those revealed by Lucky (2011), except that L. wiburdi Wheeler, 1915 is found as sister-group to L. nigriventris Guérin-Méneville, 1831+L. tibialis Emery, 1895 with high support (Fig. 4A), rather than L. erythrocephalus (Fabricius, 1775) +L. cnemidatus Wheeler, 1915. Topologies within the macro clade vary significantly among the different parsimony treatments (Supplementary Data: additional trees).



Fig. 4. Bayesian phylograms resultant from combined morphological and molecular analysis, with three different morphological treatments. (A) Both worker and male characters included. (B) Male characters included, worker excluded. (C) Worker characters included, male excluded. Fig. 4. is available in colour online.

Bayesian analyses produced trees with overall higher support relative to parsimony analyses, and had the highest posteriors when both worker and male characters were included (Fig. 4A). Support values decreased when worker characters were excluded (Fig. 4B), but were still higher than the worker-only combined analysis (Fig. 4C). Discretisation of the worker morphological characters resulted in less-resolved trees in parsimony searches, appearing to reduce signal in both morphology-only and combined analyses. Among resolved nodes, however, relationships recovered were highly similar, and in most cases identical, in continuous and discrete topologies. This indicates that while signal was effectively masked, it was not altered otherwise by binning. Regardless of caste sampling or discretisation, no morphology-only analysis recovered the Neotropical clade (L. neotropicus and L. relictus) as sister-group to the Australasian clade (Fig. 5C).

# Divergence dating

Tip-dating results recovered in our analyses are largely congruent with those generated from a 5-gene dataset spanning 25 taxa (Lucky 2011) and a 10-gene dataset based on nine of the taxa utilised in this study (Boudinot et al. 2016). Our dating analysis resulted in a topology that conflicts with that of Lucky (2011), although the support at these nodes in our study is negligible (Fig. 6). In particular, the macro-Leptomyrmex clade is divided into two monophyletic lineages that are distinct from those recovered in Lucky (2011), while more shallow relationships relating to the monophyly of species occupying the same local distribution (i.e. south-eastern Australia or New Caledonia) are conserved. In addition, deeper macro-Leptomyrmex nodes remain unresolved as a polytomy of three lineages in node-dating analyses that exclude L. neotropicus (Supplementary Data: additional trees). This topology disagrees with the results of other analyses in this study as well as with the results of Lucky (2011). Possible causes for this are our constraints on the monophyly of *Linepithema humile* + *Dorymyrmex bicolor*, all Leptomyrmex terminals, and micro Leptomyrmex following the results of an agnostic Bayesian combined analysis. Ultimately, the dataset analysed here is distinct from those utilised to generate previous hypotheses - with greater taxonomic and character sampling, as well as greater missing data for some genes (Table S1) - and therefore can be expected to produce novel results.

Tip-dating with *L. neotropicus* did not appear to have a major impact on divergence date estimates. Rather, our results indicate sensitivity of root constraints when a single terminal-based internal calibration is used. This is demonstrated by the tip-dating analysis that employed the stratigraphic age of *Chronomyrmex* for point calibration (Table 4, 'Tip Dating 2: Fossil Root'), which resulted in a reduction of the median ages of the root and crown *Leptomyrmex* by 10 My. Results for these nodes from other analyses are in accord with one another and with Boudinot *et al.* (2016) and Lucky (2011), with broadly overlapping 95% highest probability densities (HPD; Table 4). Notably, the estimated age of the micro clade is in agreement across all treatments and previous studies.

The sole unanimous disagreement between our results and those from the literature is the crown age of the macro clade,



Fig. 5. Topological summaries of three alternative analyses, underscoring the importance of combined analyses and taxon sampling in placement of *Leptomyrmex neotropicus*. (*A*) Combined analysis, *L. relictus* included. (*B*) Combined analysis, *L. relictus* excluded. (*C*) Morphology only, *L. relictus* included. Fig. 5. is available in colour online.

which we estimate between 39 and 15 Mya (Table 4) versus 22 and 7 Mya in Boudinot *et al.* (2016) and Lucky (2011). Excluding the more extreme ages from the tip-dating 'fossil root' analysis, the 'fossilisation' clock model resulted the greatest overlap for the macro clade across studies, in our case resulting in a median age of 23–22, which corresponds with oldest age of the 95% HPD in Lucky (2011). Surprisingly, the tip-dating *Chronomyrmex* analysis agrees most closely with Boudinot *et al.* (2016) and Lucky (2011) in comparison with our other analyses for the crown age of the Australasian clade, demonstrating that convergent results may be produced given differing datasets and calibration regimes, in part possibly owing to somewhat overlapping prior distributions.

# Discussion

# Phylogenetic relationships

On the basis of the combination of molecular and morphological data, our results strongly support placement of the extinct species *L. neotropicus* in the crown group of *Leptomyrmex*, together with Brazilian *L. relictus* as sister-group to the extant Australasian species. Overall, our results highlight the joint importance of complete taxon sampling and the inclusion of morphological data from both sexes. In most studies of ant systematics, males are excluded; however, we find that male morphological data – especially of the genitalia – contribute to resolution of the phylogeny. Perhaps most importantly in the case of spider ants, male characters alone recover the sister-group

relationship between *L. neotropicus* and *L. relictus* whereas worker characters do not. This is attributable to characters 37:1 and 39:1, which are uniquely shared between these two taxa; the strongly arching falcate volsella of *L. neotropicus* and *L. relictus* is particularly striking.

With respect to taxon sampling, the newly discovered relictual species has a substantial impact on the placement of L. neotropicus. As described above, the inclusion of L. relictus results in the recovery of a Neotropical clade, which is in turn sister-group to the Australasian clade. When L. relictus is excluded, however, L. neotropicus is recovered as sister-group to the Australasian macro *Leptomyrmex* (Fig. 4B). Whether the morphological similarity between the gracile Neotropical species and the macro clade is due to convergence or common inheritance, the potential for these data to misinform the analysis is only mitigated by the inclusion of molecular data (Fig. 5). Finally, we note that binning (i.e. discretising) continuous morphometric characters does reduce overall signal and appears to erode phylogenetic resolution. We therefore recommend that the impact of character binning be explored as a source of low support, in particular when discretising characters due to the current limitations of phylogenetic frameworks.

#### Biogeography

The presence of *L. neotropicus* in Dominican amber provides us with an opportunity to evaluate biogeographic history that is as yet unique in the Formicidae. Specifically, by conducting



Fig. 6. Preferred chronogram resulting from combined Bayesian analysis of 11 genes and 69 male + worker characters across 27 taxa. Search employed a uniform clock, root constraints informed from previous analyses and *Leptomyrmex neotropicus* as the sole internal calibration point. Unless otherwise noted, nodes are recovered with 1.0 Bayesian posterior probability. Minimum and maximum confidence intervals for key nodes displayed in Table 4; detailed error ranges for each node for this tree, as well other trees resulting from other analyses available as Supplementary Material. Fig. 6. is available in colour online.

divergence dating analyses in a 'total evidence' or tip-dating framework, we are able to jointly evaluate placement of key fossil taxa while improving estimates of crown group origins. Buttressing the conclusions of the 'phylogenetic relationships' section above, we find that including male morphology and the recently discovered species *L. relictus* supports a single dispersal event to Australia ~35 Mya, in accord with the results of Boudinot *et al.* (2016). Contrastingly, when we exclude male data or *L. relictus*, either two dispersals to Australia from the Neotropics or one dispersal and one backdispersal are equally parsimonious explanations for the known distribution of crown *Leptomyrmex*.

As discussed by Boudinot *et al.* (2016), the most plausible scenarios for a present-day distribution within both Australasia and the Neotropics are terrestrial dispersal through Antarctica or passive oceanic rafting. Our results cannot definitively dismiss either hypothesis; however, our divergence dates are in line with what would be expected given a case of geodispersal. Trans-Antarctic dispersal has been frequently interpreted in other animal groups (Sanmartín and Ronquist 2004): South America and Antarctica are estimated to have remained in contact until 30–28 Ma, while Antarctica and Australia were connected until

~35 Ma. Antarctica was warm during this time period, as glaciation did not take place until 33–34 Ma following the opening of Drake's passage (DeConto and Pollard 2003; Katz *et al.* 2008), but see full discussion of Boudinot *et al.* (2016). It is worth noting that this scenario is not unique to *Leptomyrmex*, but is a dispersal pattern also echoed in other taxa.

While trans-Antarctic dispersal is plausible for several ant lineages (Boudinot *et al.* 2016), most Dominican amber species have close relationships with extant taxa in the Americas. However, there are at least 18 arthropod groups represented in this amber with distinct Australasian connections (reviewed in Grimaldi *et al.* 2013). Among them are two Dominican species of the apterygotan silverfish genus *Trinemurodes*, a genus with two sole extant species in Indonesia (Sturm and Mendes 1998). Another example is the stag beetle tribe Syndesini, which comprises two genera: *Psilodon* with seven Neotropical species and *Syndesus* known from four Australasian species, the latter now also known from Dominican amber (Woodruff 2009; Grossi and Aguiar 2014).

An interesting clue to the history of *Leptomyrmex* lies in the particular morphology of the genus: extant macro-*Leptomyrmex* queens are apterous (Wheeler 1934), and therefore have limited

dispersal ability relative to winged species. It is possible that the loss of wings in female reproductives occurred relatively recently, as micro-*Leptomyrmex* queens retain wings, and with comparatively complete venation (Boudinot *et al.* 2016). No queens have yet been described from Dominican amber specimens or the Lazarus taxon *L. relictus*; therefore, discovery of a winged New World *Leptomyrmex* queen, whether in amber or extant, would reveal that prehistoric dispersal ability in this lineage was much greater than previously considered possible by exclusively wingless queens. Such a finding would support an historical overland dispersal scenario as much more likely.

### Conclusions

Uncertainty regarding the taxonomic assignment of fossils is widespread among plants and animals. Most fossil assignments are made based on researchers' interpretation of morphology in light of other fossils or extant taxa. More recently, the approach of combining morphology and molecular data in phylogenetic analyses has proven useful in placing controversial fossils. In the present study, this approach is combined with remarkable new evidence: morphological and molecular data from a living member of a lineage thought to be long extinct. These new data have allowed us to examine how well our methods performed with and without the Lazarus taxon, and remind us that excluding fossil taxa on the basis of apparent disjunct ranges represents a potential pitfall that can obscure historical reconstruction. Moreover, we find that males are crucial for unravelling the evolutionary history of spider ants.

We suggest that as combined analyses and terminal-informed divergence dating methods are developed, extinct taxa should be critically evaluated beyond their taxonomic attribution. In addition to detailing the narratives of individual groups, the accumulation of biogeographic data across lineages will allow for greater synthesis of dispersal and extinction patterns throughout the history of life. We hope this study serves to remind that fossils are a fundamental record of evolutionary change through time and across space, however implausible a story they may tell on first glance. Additionally, we encourage the evaluation of males in systematic studies of ants whenever possible. It is wise to incorporate all available data for taxa both living and extinct into our understanding of evolutionary and biogeographic history.

### **Conflicts of interest**

The authors declare no conflicts of interest.

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#### References

- Arcila, D., Pyron, R. A., Tyler, J. C., Ortí, G., and Betancur-R, R. (2015). An evaluation of fossil tip-dating versus node-age calibrations in tetraodontiform fishes (Teleostei: Percomorphaceae). *Molecular Phylogenetics and Evolution* 82, 131–145. doi:10.1016/j.ympev.2014. 10.011
- Baroni Urbani, C. (1980). The first fossil species of the Australian ant genus Leptomyrmex in amber from the Dominican Republic. Stuttgarter Beiträge zur Naturkunde, Serie B 62, 1–10.
- Baroni Urbani, C., and Wilson, E. O. (1987). The fossil members of the ant tribe Leptomyrmicini (Hymenoptera: Formicidae). *Psyche* 94, 1–8. doi:10.1155/1987/38760
- Boudinot, B. E (2013). The male genitalia of ants: musculature, homology, and functional morphology (Hymenoptera, Aculeata, Formicidae). *Journal of Hymenoptera Research* **30**, 29–49. doi:10.3897/jhr.30.3535
- Boudinot, B. E. (2015). Contributions to the knowledge of Formicidae (Hymenoptera, Aculeata): a new diagnosis of the family, the first global male-based key to subfamilies, and a treatment of early branching lineages. *European Journal of Taxonomy* **120**, 1–62.
- Boudinot, B. E., Probst, R. S., Brandão, C. R. F., Feitosa, R. M., and Ward, P. S. (2016). Out of the Neotropics: newly discovered relictual species sheds light on the biogeographic history of spider ants (*Leptomyrmex*, Dolichoderinae, Formicidae). *Systematic Entomology* **41**, 658–671. doi:10.1111/syen.12181
- Brady, S. G. (2011). Effects of fossil calibration uncertainty on divergence dating of ants and bees. *American Entomologist* 57, 56–57. doi:10.1093/ ae/57.1.56
- Brody, R. H., Edwards, H. G., and Pollard, A. M. (2001). A study of amber and copal samples using FT-Raman spectroscopy. *Spectrochimica Acta. Part A: Molecular and Biomolecular Spectroscopy* 57, 1325–1338. doi:10.1016/S1386-1425(01)00387-0
- Brown, WL, Jr., and Nutting, WL (1950). Wing venation and the phylogeny of the Formicidae. *Transactions of the American Entomological Society* 75, 113–132.
- Dawson, M. R., Marivaux, L., Li, C. K., Beard, K. C., and Métais, G. (2006). Laonastes and the "Lazarus effect" in recent mammals. Science 311, 1456–1458. doi:10.1126/science.1124187
- DeConto, R. M., and Pollard, D. (2003). Rapid Cenozoic glaciation of Antarctica induced by declining atmospheric CO<sub>2</sub>. *Nature* 421, 245–249. doi:10.1038/nature01290
- Dlussky, G., Radchenko, A., and Dubovikoff, D. (2014). A new enigmatic ant genus from late Eocene Danish amber and its evolutionary and zoogeographic significance. *Acta Palaeontologica Polonica* 59, 931–939.
- Donoghue, P. C., and Benton, M. J. (2007). Rocks and clocks: calibrating the Tree of Life using fossils and molecules. *Trends in Ecology & Evolution* 22, 424–431. doi:10.1016/j.tree.2007.05.005
- Drummond, A. J., and Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7, 214. doi:10.1186/1471-2148-7-214
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797. doi:10.1093/nar/gkh340
- Eernisse, D. J., and Kluge, A. G. (1993). Taxonomic congruence versus total evidence, and amniote phylogeny inferred from fossils, molecules, and morphology. *Molecular Biology and Evolution* 10, 1170–1195.
- Emerson, A. E. (1965). A review of the Mastotermitidae (Isoptera), including a new fossil genus from Brazil. *American Museum Novitates* 2236, 1–46.
- Emery, C. (1891). Le formiche dell'ambra Siciliana nel Museo Mineralogico dell'Universita di Bologna. Accademia delle Scienze dell'Istituto di Bologna 5, 141–165.

- Emery, C. (1913). Hymenoptera family Formicidae subfamily Dolichoderinae. Genera Insectorum 137, 1–50.
- Goloboff, P. A., Farris, J. S., Källersjö, M., Oxelman, B., and Szumik, C. A. (2003). Improvements to resampling measures of group support. *Cladistics* 19, 324–332. doi:10.1111/j.1096-0031.2003.tb00376.x
- Goloboff, P. A., Farris, J. S., and Nixon, K. C. (2008). TNT, a free program for phylogenetic analysis. *Cladistics* 24, 774–786. doi:10.1111/j.1096-0031.2008.00217.x
- Grimaldi, D., and Engel, M. S. (2005). 'Evolution of the Insects.' (Cambridge University Press: New York.)
- Grimaldi, D., Engel, M. S., Nascimbene, P. C., and Singh, H. (2013). Coniopterygidae (Neuroptera: Aleuropteryginae) in amber from the Eocene of India and the Miocene of Hispaniola. *American Museum Novitates* 3770, 20–39. doi:10.1206/3770.2
- Grossi, P. C., and Aguiar, N. O. (2014). Discovery of a third stag beetle genus in the Amazonian region, with description of a new species of *Psilodon* Perty (Coleoptera: Lucanidae: Syndesinae: Syndesini). *Coleopterists Bulletin* 68, 83–90. doi:10.1649/0010-065X-68.1.83
- Katz, M. E., Miller, K. G., Wright, J. D., Wade, B. S., Browning, J. V., Cramer, B. S., and Rosenthal, Y. (2008). Stepwise transition from the Eocene greenhouse to the Oligocene icehouse. *Nature Geoscience* 1, 329–334. doi:10.1038/ngeo179
- Lanfear, R., Calcott, B., Ho, S. Y. W., and Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29, 1695–1701. doi:10.1093/molbev/mss020
- Lepage, T., Bryant, D., Philippe, H., and Lartillot, N. (2007). A general comparison of relaxed molecular clock models. *Molecular Biology and Evolution* 24, 2669–2680. doi:10.1093/molbev/msm193
- Lewis, P. O. (2001). A likelihood approach to estimating phylogeny from discrete morphological character data. *Systematic Biology* 50, 913–925. doi:10.1080/106351501753462876
- Lucky, A. (2011). Molecular phylogeny and biogeography of the spider ants, genus *Leptomyrmex* Mayr (Hymenoptera: Formicidae). *Molecular Phylogenetics and Evolution* **59**, 281–292. doi:10.1016/j.ympev.2011. 03.004
- Lucky, A., and Ward, P. S. (2010). Taxonomic revision of the ant genus Leptomyrmex Mayr (Hymenoptera: Formicidae). Zootaxa 2688, 1–67.
- McKellar, R. C., Glasier, J. R., and Engel, M. S. (2013). New ants (Hymenoptera: Formicidae: Dolichoderinae) from Canadian Late Cretaceous amber. *Bulletin of Geosciences* 88, 583–594. doi:10.3140/ bull.geosci.1425
- Miller, M. A., Pfeiffer, W., and Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In 'Proceedings of the Gateway Computing Environments Workshop (GCE)', 14 November 2010, New Orleans, LA. pp. 1–8. (Institute of Electrical and Electronics Engineers: Piscataway, NJ.) Available at http://ieeexplore.ieee.org/document/5676129/?tp=&arnumber=5676129 &filter%3DAND(p\_IS\_Number:5676117) [Verified July 2017]
- Petrulevičius, J. F., and Nel, A. (2009). First Cordulephyidae dragonfly in America: a new genus and species from the Paleogene of Argentina (Insecta: Odonata). *Comptes Rendus. Palévol* 8, 385–388. doi:10.1016/ j.crpv.2008.12.004
- Pyron, R. A. (2011). Divergence time estimation using fossils as terminal taxa and the origins of Lissamphibia. *Systematic Biology* **60**, 466–481. doi:10.1093/sysbio/syr047
- Rambaut, A. (2014) FigTree. Available at http://tree.bio.ed.ac.uk [Verified July 2017].
- Rambaut, A., and Drummond, A. J. (2010) TreeAnnotator. Available at http://beast.bio.ed.ac.uk [Verified July 2017].
- Rambaut, A., Suchard, M., and Drummond, A. J. (2013) Tracer. Available at http:/tree.bio.ed.ac.uk [Verified July 2017].

- Ronquist, F., and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574. doi:10.1093/bioinformatics/btg180
- Ronquist, F., Klopfstein, S., Vilhelmsen, L., Schulmeister, S., Murray, D. L., and Rasnitsyn, A. P. (2012). A total-evidence approach to dating with fossils, applied to the early radiation of the Hymenoptera. *Systematic Biology* 61, 973–999.
- Royer, D. L., Hickey, L. J., and Wing, S. L. (2003). Ecological conservatism in the "living fossil" Ginkgo. *Paleobiology* 29, 84–104. doi:10.1666/0094-8373(2003)029<0084:ECITLF>2.0.CO;2
- Rutschmann, F., Eriksson, T., Salim, K. A., and Conti, E. (2007). Assessing calibration uncertainty in molecular dating: the assignment of fossils to alternative calibration points. *Systematic Biology* 56, 591–608. doi:10.1080/10635150701491156
- Sanmartín, I., and Ronquist, F. (2004). Southern hemisphere biogeography inferred by event-based models: plant versus animal patterns. *Systematic Biology* 53, 216–243. doi:10.1080/10635150490423430
- Shattuck, S. O. (1992). Generic revision of the ant subfamily Dolichoderinae (Hymenoptera: Formicidae). Sociobiology 21, 1–181.
- Shattuck, S. (2000). 'Australian Ants: their Biology and Identification. Vol. 3.' (CSIRO Publishing: Melbourne.)
- Smith, D. J., and Shattuck, S. (2009). Six new, unusually small ants of the genus *Leptomyrmex* (Hymenoptera: Formicidae). *Zootaxa* 2142, 57–68.
- Sturm, H., and Mendes, L. F. (1998). Two new species of Nicoletiidae (Zygentoma, "Apterygota," Insecta) in Dominican amber. *American Museum Novitates* 3226, 1–11.
- Ward, P. S., Brady, S. G., Fisher, B. L., and Schultz, T. R. (2010). Phylogeny and biogeography of dolichoderine ants: effects of data partitioning and relict taxa on historical inference. *Systematic Biology* 59, 342–362. doi:10.1093/sysbio/syq012
- Ward, P. S., Blaimer, B. B., and Fisher, B. L. (2016). A revised phylogenetic classification of the ant subfamily Formicinae (Hymenoptera: Formicidae), with resurrection of the genera *Colobopsis* and *Dinomyrmex. Zootaxa* **4072**, 343–357. doi:10.11646/zootaxa.4072.3.4
- Ware, J. L., Grimaldi, D. A., and Engel, M. S. (2010). The effects of fossil placement and calibration on divergence times and rates: an example from the termites (Insecta: Isoptera). *Arthropod Structure & Development* 39, 204–219. doi:10.1016/j.asd.2009.11.003
- Wheeler, W. M. (1915). The Australian honey-ants of the genus Leptomyrmex Mayr. Proceedings of the American Academy of Arts and Sciences 51, 255–286. doi:10.2307/20025576
- Wheeler, W. M. (1934). A second revision of the ants of the genus Leptomyrmex Mayr. Bulletin of the Museum of Comparative Zoology 77, 69–118.
- Wiens, J. J. (2003). Missing data, incomplete taxa, and phylogenetic accuracy. Systematic Biology 52, 528–538. doi:10.1080/10635150390218330
- Wiens, J. J. (2006). Missing data and the design of phylogenetic analyses. Journal of Biomedical Informatics 39, 34–42. doi:10.1016/j.jbi.2005. 04.001
- Wiens, J. J., and Morrill, M. C. (2011). Missing data in phylogenetic analysis: reconciling results from simulations and empirical data. *Systematic Biology* 60, 719–731. doi:10.1093/sysbio/syr025
- Wilson, E. O. (1985). Ants of the Dominican amber (Hymenoptera: Formicidae). 3. The subfamily Dolichoderinae. *Psyche* 92, 17–37. doi:10.1155/1985/20969
- Wood, H. M., Matzke, N. J., Gillespie, R. G., and Griswold, C. E. (2013). Treating fossils as terminal taxa in divergence time estimation reveals ancient vicariance patterns in the palpimanoid spiders. *Systematic Biology* 62, 264–284. doi:10.1093/sysbio/sys092
- Woodruff, R. E. (2009). A new fossil species of stag beetle from Dominican Republic amber, with Australasian connections (Coleoptera: Lucanidae). *Insecta Mundi* 98, 1–10.