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Biogeography and evolution of social parasitism in Australian *Myrmecia* bulldog ants revealed by phylogenomics

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ABSTRACT

Studying the historical biogeography and life history transitions from eusocial colony life to social parasitism contributes to our understanding of the evolutionary mechanisms generating biodiversity in eusocial insects. The ants in the genus *Myrmecia* are a well-suited system for testing evolutionary hypotheses about how their species diversity was assembled through time because the genus is endemic to Australia with the single exception of the species *M. apicalis* inhabiting the Pacific Island of New Caledonia, and because at least one social parasite species exists in the genus. However, the evolutionary mechanisms underlying the disjunct biogeographic distribution of *M. apicalis* and the life history transition(s) to social parasitism remain unexplored. To study the biogeographic origin of the isolated, oceanic species *M. apicalis* and to reveal the origin and evolution of social parasitism in the genus, we reconstructed a comprehensive phylogeny of the ant subfamily Myrmeciinae. We utilized Ultra Conserved Elements (UCEs) as molecular markers to generate a molecular genetic dataset consisting of 2,287 loci per taxon on average for 66 out of the 93 known *Myrmecia* species as well as for the sister lineage *Nothomyrmecia macrops* and selected outgroups. Our time-calibrated phylogeny inferred that: (i) stem Myrmeciinae originated during the Paleocene ~ 58 Ma ago; (ii) the current disjunct biogeographic distribution of *M. apicalis* was driven by long-distance dispersal from Australia to New Caledonia during the Miocene ~ 14 Ma ago; (iii) the single social parasite species, *M. inquilina*, evolved directly from one of the two known host species, *M. nigriceps*, in sympatry via the intraspecific route of social parasite evolution; and (iv) 5 of the 9 previously established taxonomic species groups are non-monophyletic. We suggest minor changes to reconcile the molecular phylogenetic results with the taxonomic classification. Our study enhances our understanding of the evolution and biogeography of Australian bulldog ants, contributes to our knowledge about the evolution of social parasitism in ants, and provides a solid phylogenetic foundation for future inquiries into the biology, taxonomy, and classification of Myrmeciinae.

1. Introduction

1.1. Biology, evolution, and classification of Myrmeciinae

Ants in the subfamily Myrmeciinae Emery, 1877, colloquially known as bull ants, bulldog ants, or jack jumper ants, are conspicuous predators endemic to Australia and New Caledonia. Myrmeciinae encompasses a total of 11 genera, of which nine are extinct and only two genera are extant: *Myrmecia* Fabricius, 1804 which is the most species-rich genus in the subfamily, encompassing 93 described species (Bolton, 2022), and *Nothomyrmecia* Clark, 1934, consisting of the single species *Nothomyrmecia macrops* Clark, 1934.

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Unlike many other ants, myrmeciines are large insects and workers forage solitarily using visual cues (Greiner et al., 2007; Ward and Brady, 2003). The morphology of bulldog ants reflects their predatory habits. Their body sizes are usually large with more than 20 mm in length. They have long, strongly toothed mandibles and possess very large, convex, anteriorly positioned eyes. The workers have ocelli, the pronotum is separated and freely movable with regard to the mesonotum, and the mid and hind tibiae present a pair of apical spurs. *Myrmecia* ants also possess a powerful functional sting which they employ for killing prey and in self-defense (Ogata and Taylor, 1991; Ward and Brady, 2003). In addition, the workers are not highly differentiated into morphological castes, such as workers and soldiers, as can be observed in some other ants that are part of the formicoid clade (Hölldobler and Wilson, 1990; Jaisson et al., 1992; Ward and Brady, 2003; Wheeler, 1933; Wilson, 1971).

The phylogenetic placement of the Myrmeciinae was the subject of controversy for decades. In the past, biologists believed that myrmeciines represented morphologically and behaviorally "primitive" species that could elucidate earlier stages of social evolution, suggesting that the Myrmeciinae was one of the earliest diverging branches of the ant tree of life (Haskins and Haskins, 1950; Haskins, 1970). In contrast, molecular phylogenetic and cladistic analyses inferred the Myrmeciinae as part of the formicoid clade, recovered the two extant genera *Myrmecia* and *Nothomyrmecia* as sister taxa, and established the Pseudomyrmecinae Smith, 1952 as the closest relative to Myrmeciinae (Borowiec et al., 2019; Brady et al., 2006; Moreau et al., 2006; Romiguier et al., 2022; Ward and Brady, 2003).

Myrmecologists have studied *Myrmecia* taxonomy in detail (Browning, 1987; Clark, 1951; Ogata and Taylor, 1991) and the most recent taxonomic revision of the genus was carried out by Ogata and Taylor in 1991, who recognized 89 species and provided a taxonomic key for morphological identification. Subsequently, Taylor (2015) described four new species from the *Myrmecia pilosula* species complex. Currently, 9 species groups are recognized within *Myrmecia,* including the *aberrans, cephalotes, gulosa, mandibularis, nigrocincta, picta, pilosula, tepperi,* and *urens* species groups.

The evolutionary relationships of the species groups were first inferred in part using morphological characters by Ogata (1991). However, the internal topology was unstable depending on the chosen outgroup (Ogata, 1991). Hasegawa and Crozier (2006) inferred the phylogenetic relationships among the 9 species groups using two nuclear markers and 38 *Myrmecia* species. The authors found the *gulosa, nigrocincta, picta*, and *mandibularis* species groups to be monophyletic but they also suggested that the resolution of the other species groups was unclear due to limited gene and taxon sampling (Hasegawa and Crozier, 2006).

1.2. Historical biogeography of Myrmeciinae

Bulldog ants are a unique element of the Australian entomofauna. The extant *Myrmecia* and *Nothomyrmecia* ants are currently geographically restricted to Australia (Heterick, 2009; Shattuck, 1999; Ogata and Taylor, 1991). Only a single species, *Myrmecia apicalis* Emery, 1883 is found outside the Australian mainland, inhabiting the oceanic island New Caledonia (Ogata and Taylor, 1991; Wilson, 1971).

The fossil taxa are paramount for understanding the subfamily's historical biogeography and evolution. Except for the Baltic amber fossils of the genus *Prionomyrmex* Mayr, 1868, the fossil record of Myrmeciinae consists only of impression fossils for which the phylogenetic placement has been debated (Table 1). Numerous fossils that have been attributed to the Myrmeciinae are known from what today is North and South America, Europe, and Asia (see Table 1). *Tyrannomecia inopinata* Jouault and Nel, 2021 was recently described as a ~ 60 Ma fossil from France and constitutes the oldest confirmed myrmeciine fossil to date (Jouault and Nel, 2022). Interestingly, *Cariridris bipetiolata* Brandão and Martins-Neto, 1990 could potentially represent an earlier fossil of the Myrmeciinae from the Lower Cretaceous Crato Formation in Brazil, which is ~ 110–112 Ma old (Brandão et al., 1990), but due to the poor preservation of the holotype, the taxonomic classification of *Cariridris* and the question whether it is an ant remain unresolved and therefore, we excluded this fossil species from our analyses (Barden, 2017; Grimaldi et al.1997; Jouault and Nel, 2022; Ohl, 2004, Verhaagh, 1996).

All extant *Myrmecia* species exclusively occur on the Australian mainland (Heterick, 2009; Ogata and Taylor, 1991; Shattuck, 1999) except for *Myrmecia apicalis* which is the only bulldog ant species endemic to New Caledonia (Ogata and Taylor, 1991; Wilson, 1971). The disjunct biogeographic distribution of *Myrmecia* ants is curious because New Caledonia is located ~ 1,300 km east of Australia in the South Pacific Ocean. New Caledonia is of continental origin and the island separated from the northeastern margin of Australia during the Late Cretaceous ~ 80 Ma ago (Grandcolas, 2017; Grandcolas et al., 2008; Morat, 1993). Historically, New Caledonia was considered a Gondwanan biota refuge (Lowry, 1998; Morat, 1993; Murienne et al., 2005), and its species richness was attributed to long-term cladogenesis rather than long-distance dispersal events (Grandcolas et al., 2008; Nattier et al., 2017). More recent geological evidence suggested that New Caledonia was under strong tectonic activity during the Paleocene when the island was deeply submerged below the sea level for more than 20 Ma until the island re-emerged above sea level during the Eocene ~ 37 Ma ago (Grandcolas, 2017; Grandcolas et al., 2008; Murienne, 2009; Murienne et al., 2005; Paris, 1981; Pelletier, 2006). Most of the current biota of New Caledonia is hypothesized to have arrived on the island after its re-emergence as a result of long-distance dispersal events (Grandcolas, 2017; Grandcolas et al., 2008; Murienne et al., 2005; Myers et al., 2000; Nattier et al., 2017) and only a few taxa were inferred to be older than the oldest existing island in the area, suggesting that those clades could have extinct members on vanished islands or nearby continents (Nattier et al., 2017). It remains untested how and when the ancestor of *M. apicalis* arrived in New Caledonia and its relationship to the mainland Australian *Myrmecia* species is unresolved.

1.3. Social parasitism in Myrmeciinae

Studying the phylogenetic relationships of *Myrmecia* is also expected to provide insights into the evolution of social parasitism in ants because at least one social parasite species, *Myrmecia inquilina* Douglas and Brown, 1959 is known from the myrmeciines and the ecological and evolutionary factors contributing to the parasite's origin remain unexplored.

Social parasitism is a form of brood parasitism in which one social species exploits the social behavior of another social species for survival and reproduction (Buschinger, 1986, 1990, 2009; Hölldobler and Wilson, 1990; Rabeling, 2020). Despite their highly specialized life history traits and behaviors, ant social parasites are remarkably diverse with approximately 400 known species, and they originated at least 91 times across the ant tree of life (Gray and Rabeling, 2022; Rabeling, 2020). Understanding the convergently evolved life history transitions from a cooperative colony life to a socially parasitic behavior has intrigued evolutionary biologists for centuries (Buschinger, 2009; Darwin, 1859; Emery 1909; Hölldobler and Wilson, 1990; Kutter, 1968; Müller, 1872; Rabeling, 2020; Wasmann, 1891; Wheeler, 1919), and empirical studies repeatedly addressed the sociobiological, genetic, ecological, and behavioral factors associated with the origins of parasitic lineages (Bourke and Franks, 1991; Buschinger, 1986, 2009; Dahan and Rabeling, 2022; Hölldobler and Wilson, 1990; Rabeling, 2020). Early entomologists observed that social parasites and their hosts are usually closely related (Emery, 1909; Müller, 1872; Wheeler, 1919) and noted that social parasites display similar ecological, behavioral, and physiological traits, all of which are crucial for the social parasite to be fully integrated into the host colony. Emery's observations were later coined Emery's Rule (Le Masne, 1956) and in the context of modern evolutionary biology, Emery's rule can be divided into two testable

Table 1
Fossil record of the subfamily Myrmeciinae. Table modified from Jouault and Nel (2022) and https://www.antcat.org.

Taxonomic classification	Genus	Species	Reference	Age and locality/formation	Estimated age in Ma [*sensu* Barden, 2017]	Country
Incertae sedis in Myrmeciinae	*Archimyrmex*	*piatnitzkyi*	Viana and Rossi, 1957	Eocene (Ventana Formation)	47.7 [48.6–40.4]	Argentina
	Archimyrmex	*rostratus*	Cockerell, 1923	Middle Eocene (Green River Formation)	51 [53.5–48.5]	USA
Incertae sedis in Myrmeciinae	*Archimyrmex*	*smekali*	de Garcia, 1983	Eocene (Ventana Formation)	47.7 [48.6–40.4]	Argentina
	Archimyrmex	*wedmannae*	Dlussky, 2012	Middle Eocene (Messel Formation)	47 [48.6–40.4]	Germany
Incertae sedis in Myrmeciinae	*Avitomyrmex*	*elongatus*	Archibald et al., 2006	Eocene (McAbee)	51 [55.8–48.6]	Canada
Incertae sedis in Myrmeciinae	*Avitomyrmex*	*mastax*	Archibald et al., 2006	Eocene (McAbee)	51 [55.8–48.6]	Canada
Incertae sedis in Myrmeciinae	*Avitomyrmex*	*systenus*	Archibald et al., 2006	Eocene (McAbee)	51 [55.8–48.6]	Canada
Excluded from Formicidae	*Cariridris*	*bipetiolata*	Brandão et al., 1990	Lower Cretaceous (Crato, Santana Formation)	110–112	Brazil
Incertae sedis in Myrmeciinae	*Macabeemyrma*	*ovata*	Archibald et al., 2006	Eocene (McAbee)	51 [55.8–48.6]	Canada
	Myrmeciites	*goliath*	Archibald et al., 2006	Eocene (McAbee)	51 [55.8–48.6]	Canada
	Myrmeciites	*herculeanus*	Archibald et al., 2006	Eocene (McAbee)	51 [55.8–48.6]	Canada
	Myrmeciites	*tabanifluviensis*	Archibald et al., 2006	Eocene (Black Creek Road)	51 [55.8–48.6]	Canada
Prionomyrmecini	*Prionomyrmex*	*gusakovi*	Radchenko and Perkovsky, 2020	Eocene (Baltic amber)	44.1 [47.8–41.3]	Russia
Prionomyrmecini	*Prionomyrmex*	*janzeni*	Baroni Urbani, 2000	Eocene (Baltic amber)	44.1 [47.8–41.3]	Russia
Prionomyrmecini	*Prionomyrmex*	*longiceps*	Mayr, 1868	Eocene, (Rovno amber, Baltic amber), Late Eocene-Early Oligocene (Bitterfeld amber)	Rovno: 35.6 [37.2–33.9]; Baltic: 44.1 [47.8–41.3]; Bitterfeld: 24.6 [25.3–23.8] Total: 47.8–23.8	Baltic Sea Region
Prionomyrmecini	*Prionomyrmex*	*wappleri*	Dlussky, 2012	Late Oligocene (Rott)	24 [23.8–24.2]	Germany
	Propalosoma	*gutierrezae*	Dlussky and Rasnitsyn, 1999	Early Eocene (Klondike Mountain Formation)	49 [55.8–48.6]	USA
	Tyrannomecia	*inopinata*	Jouault and Nel, 2022	Paleocene (Old quarry/Menat)	60	France
	Ypresiomyrma	*bartletti*	Archibald et al., 2006	Eocene (McAbee)	51 [55.8–48.6]	Canada
	Ypresiomyrma	*orbiculata*	Archibald et al., 2006	Eocene (McAbee)	51 [55.8–48.6]	Canada
	Ypresiomyrma	*orientalis*	Dlussky et al., 2015	Late Eocene (Bol'shaya Svetlovodnaya)	35.6 [37.2–33.9]	Russia
	Ypresiomyrma	*rebekkae*	Rust and Andersen, 1999	Early Eocene (Ølst and Fur Formation)	55–56	Denmark

hypotheses, the strict and loose versions of Emery's Rule (Ward, 1996). The strict version implies that social parasite and host species are sister taxa and that the social parasite speciated from the host population in sympatry via the intraspecific route of social parasite evolution. The loose version suggests that the social parasite and host species are congeneric but not each other's closest relatives, and under such an evolutionary scenario, the social parasite would have originated via the interspecific route of social parasite evolution in allopatry (Borowiec et al., 2021; Bourke and Franks, 1991; Buschinger, 2009; Rabeling, 2020; Ward, 1996). Studies that support the intraspecific route of social parasite evolution are rare and of general interest to evolutionary biology because they provide empirical evidence for how reproductive isolation can evolve in the presence of gene flow (Bourke and Franks, 1991; Buschinger, 1990; Coyne and Orr, 2004; Rabeling et al., 2014, 2019; Savolainen and Vepsäläinen, 2003).

Myrmecia inquilina is a workerless, inquiline social parasite and the only known social parasite species in the ant subfamily Myrmeciinae (Douglas and Brown, 1959). *Myrmecia inquilina* was discovered inside the nest of its host *Myrmecia vindex* Smith, 1858 in Western Australia (WA) (Douglas and Brown, 1959). Neither the host nor the parasite queens showed aggressive behavior towards each other, indicating that the social parasite is tolerant of the host queen(s) (Douglas and Brown, 1959). *Myrmecia inquilina* also exhibits morphological traits typical of the anatomical parasitic syndrome such as the reduction in body size with respect to the host, reduced body sculpture and pilosity as well as smoother and shinier mandibles (Douglas and Brown, 1959). In 1964, Haskins and Haskins (1964) identified *Myrmecia nigriceps* Mayr, 1862 as a second host species. Inquiline workers have not been identified and therefore *M. inquilina* is presumed to be a workerless social parasite (Douglas and Brown, 1959; Haskins and Haskins, 1964).

One additional population of a *Myrmecia* social parasite was discovered in 1979 by Bede Lowery, who found a parasitized colony of presumably *M. nigriceps.* Strikingly, this parasite population was located in New South Wales (NSW) on the east coast of Australia. The disjunct biogeographic distribution of the parasite populations begs the question of whether the populations from the east and west coasts correspond to two distinct inquiline species or whether these disjunct populations belong to a single social parasite species with a broad biogeographic distribution.

1.4. Study objectives

Here, we infer a detailed molecular phylogeny of the ant subfamily Myrmeciinae using phylogenomic markers to better understand the evolutionary history of Australian bulldog ants. Our integrative phylogenetic framework includes fossil evidence and paleogeographic records

to reveal the subfamily's biogeographic history and to understand the current geographic distribution of the New Caledonian endemic *M. apicalis*. Furthermore, we aim at reconstructing the phylogenetic relationships between the social parasite *M. inquilina* and its hosts to reveal the evolution of social parasitism in the genus. In addition, we aim to resolve the evolutionary relationships among the *Myrmecia* species groups, which will inform future taxonomic studies.

2. Materials and methods

2.1. Taxon sampling

We newly sequenced 132 samples belonging to 66 species (71 %) out of the 93 currently described *Myrmecia* species representing 8 out of the 9 established *Myrmecia* species groups, and two samples corresponding to the monotypic sister genus *Nothomyrmecia*. DNA was extracted predominantly from museum vouchers from the Museum of Comparative Zoology at Harvard University (MCZC), the Australian National Insect Collection (ANIC), the Bohart Museum of Entomology Collection at the University of California Davis (UCDC), as well as S. Pratt's (ASU), and C. Rabeling's personal collections. Fresh samples of *M. apicalis* were collected on the Isle of Pines in New Caledonia in 2011. As outgroups, we included 30 previously sequenced ant samples (Branstetter et al., 2017) plus 4 newly sequenced samples. Collection data associated with sequenced samples and outgroups used in our phylogenomic reconstruction can be found in Tables S1 and S2.

To test if the social parasite renders any of the two known host species (i.e., *M. nigriceps* and *M. vindex*) paraphyletic, as was observed in other ant social parasite species (Rabeling et al., 2014; Ward and Branstetter, 2022), we included multiple populations for each host species. We sampled at least nine *M. nigriceps* and eight *M. vindex* populations (Table S1). For the host species, we sequenced two host individuals that were collected in direct association with the social parasite, including one sample previously identified as *M. vindex* (DNA extraction code: 12745) and one previously identified as *M. nigriceps* (DNA extraction code: 11557). For the social parasite, *M. inquilina*, we sampled three populations, two from Western Australia (DNA extraction codes: 13404,12746/13405) and the disjunct population from New South Wales (DNA extraction code: 11556) (Table S1). Previous authors pointed out that *M. vindex* and its close relatives may constitute a complex of cryptic species (Browning, 1987; Ogata and Taylor, 1991). Therefore, we included species closely related to *M. inquilina*, *M. nigriceps*, and *M. vindex*, such as *M. desertorum* Wheeler, 1915, *M. fulgida* Clark, 1951, *M. fuscipes* Clark, 1951 , and *M. pavida* Clark, 1951 in our sampling (Table S1). Moreover, we utilized these data to conduct species delimitation analyses in the larger "*M. vindex*" species subgroup using morphological, population genetic, phylogenetic, and biological information (see below).

2.2. Molecular data generation

We used Ultra Conserved Elements (UCEs) as molecular markers. UCEs have been widely used as a reliable source for inferring molecular phylogenies in several taxa including ants (Barrera et al., 2022; Borowiec et al., 2021; Branstetter et al., 2017; Camacho et al., 2022; Faircloth et al., 2015; Hanisch et al., 2022; van Elst et al., 2021; Zhang et al., 2019). UCEs can be extracted from museum vouchers that are old and/or have not been preserved under optimal conditions (Blaimer et al., 2016; Borowiec et al., 2021).

Ant DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, GmbH) following the manufacturer's protocol. We extracted DNA from the three right legs of the ants when the specimens were longer than 15 mm. When the ants were smaller, we extracted DNA non-destructively without removing any appendages. After the DNA extractions, the specimens were re-mounted and preserved as vouchers in their corresponding biological collections.

50–100 ng of DNA were sheared by sonication (Qsonica) to an average fragment size of ~ 600 bp and used for the library preparation (Kapa Hyper Prep Library Kit, Kapa Biosystems) incorporating "with-bead" (SeaPure) cleaning steps (Fisher et al., 2011). Each library was associated with a unique combination of iTru adapters (Gnirke et al., 2009). We followed the protocol for library preparation as described in Faircloth et al. (2015) and modified in Branstetter et al. (2017).

Libraries were pooled for target enrichment using the hym-v2 set of ant-specific RNA probes (MYcroarray), which target 2,590 UCE loci in ants (Branstetter et al., 2017). The enrichment protocol followed Faircloth et al. (2015) and modifications described in Branstetter et al. (2017) and Prebus (2021).

After the enrichment, we performed quantitative PCR to reliably estimate the DNA concentration for the enriched pools. We used a SYBR® FAST qPCR kit (Kapa Biosystems) and a Bio-Rad CFX96 RT-PCR thermal cycler (Bio-Rad Laboratories). Finally, we combined all samples into an equimolar final pool. In total, we sequenced two lanes submitted as two final pools of samples. One of the lanes was sequenced at the High Throughput Genomics Core Facility at the University of Utah on an Illumina HiSeq 2500 PE-125, and the second lane was sequenced at the Novogene Corporation Inc. on an Illumina HiSeq 2500 PE-150.

2.3. Data processing

Samples were processed using the standard PHYLUCE v1.7.1 protocol for processing UCEs in preparation for phylogenomic analyses (as in Faircloth et al., 2015 and Prebus, 2021). Samples were downloaded from the sequencing facility, cleaned, and posteriorly assembled using SPAdes (Bankevich et al., 2012). Assembled contigs were matched to the UCE probes, and then we obtained the monolithic fasta file to be aligned as well as the statistics on UCE assemblies for individual taxa.

2.3.1. Alignment

We captured an average of 2,287 UCEs contigs per sample with a mean contig length of 880 bp. The fasta data were aligned using MAFFT (Katoh and Standley, 2013). The resulting alignment was trimmed with Gblocks v0.91b (Castresana, 2000). Locus names were removed from the taxon labels. Our alignment had a length of 1,704,012 bp and consisted of 2,523 loci with a mean length of 675 bp each. We extracted a matrix with 80 % as the minimum percentage of taxa contained by each locus. The matrix included 2,214 loci with a total of 1,559,993 sites of which 884,980 were informative and 18 % missing data. Detailed information about the UCEs sequenced can be found in Table S3. Finally, we concatenated the selected loci into a single alignment to generate a phylip file used as input for the phylogenetic analyses.

2.3.2. Data partitioning

To account for variation in rates of molecular evolution among sites (Tagliacollo and Lanfear, 2018), we utilized four different partitioning schemes to verify the robustness of the results. The first scheme employed was the unpartitioned data as obtained directly from the alignment. Second, we prepared a concatenated alignment file and a partition file as input files for a partitioning scheme using the Sliding-Window Site Characteristics based on the site entropies (SWSC-EN) method (Tagliacollo and Lanfear, 2018). The third scheme partitioned data by locus. Lastly, we used ModelFinder (Kalyaanamoorthy et al., 2017) in IQ-TREE v.2.1.2 (Minh et al., 2020) to group loci sharing a similar rate of evolution and to identify the best-fitting model for each group of loci.

2.4. Phylogenetic inference

First, we conducted a maximum likelihood analysis for the unpartitioned alignment for which the model of molecular evolution was selected as GTR + F using IQ-TREE v.2.1.2 (Minh et al., 2020). Node support was estimated using 1,000 ultra-fast bootstrap approximations

and we set 200 for the number of unsuccessful iterations to stop (n-stop) (Hoang et al., 2018). The resulting tree had a negative log-likelihood value of 27,194,283. Then, we conducted the model-selection and maximum likelihood inference analyses for the different partitioning schemes in IQ-TREE v.2.1.2 (Minh et al., 2020; Chernomor et al., 2016). We obtained a total of 343 partitions for the SWSC-EN partitioning, 2,214 partitions for the partitioning by loci, and 240 partitions for the partitioning by best groups of loci. The negative log-likelihood values from the trees obtained from these analyses were SWSC-EN partitions = 24,984,693, partition by locus = 25,235,796, and partition by best groups of loci = 25,351,421. We also compared our defined partitioning schemes using PartitionFinder v.2.1 (Lanfear et al., 2017; Stamatakis, 2014).

We tested for violations of the assumptions of stationarity and homogeneity of the UCE loci by conducting a matched-pair test of symmetry (Hanisch et al., 2022; Naser-Khdour et al., 2019) implemented in IQ-TREE v.2.1.2 (Minh et al., 2020). This test allowed us to remove the uninformative or "bad" partitions potentially violating assumptions. We prepared new alignment files containing the informative or "good" partitions only, and then we performed the corresponding phylogenetic analyses in IQ-TREE v.2.1.2 (Minh et al., 2020). The negative log-likelihood values of the trees obtained from these analyses were SWSC-EN partitions = 12,894,462, partition by locus = 11,598,616, and partition by best groups of loci = 5,496,643.

The resulting topologies were congruent using the different partitioning schemes for maximum likelihood analyses before and after removing the uninformative partitions. The support values for the nodes in the resulting trees displayed maximum ultrafast bootstrap approximation support (UFBS) (higher than 95) except for a few nodes between closely related taxa where the UFBS values were below 95. Different partitioning schemes did not affect the resulting topology of the inferred tree. Since the most appropriate partitioning scheme for our dataset was the SWSC-EN partitioning (Lanfear et al., 2017; Stamatakis, 2014), we selected this resulting tree for subsequent analyses and description of the results. All obtained phylogenetic trees are available in the Supplementary Materials (Figs. S1–S6).

Finally, we estimated species trees using the software package ASTRAL III (Zhang et al., 2018). We generated gene trees for each of the UCE loci in our matrix. Each of the gene trees was obtained in IQ-TREE v.2.1.2 (Minh et al., 2020). Our resulting species tree displays most of the nodes with maximum local posterior probabilities (LPP above 0.9) and the topology is congruent with the topologies obtained from the maximum likelihood analyses with only a few discordances at the species level (Fig. S7).

2.5. Divergence dating inference

We used the Bayesian phylogenetic software MCMCTree from the package PAML v.4.9 (Yang, 2007; Yang and Rannala, 1997) to estimate divergence times using fossil constraints and different molecular clock models (dos Reis et al., 2016; Inoue et al., 2010; Rannala and Yang, 2007; Yang and Rannala, 2006).

We used information from six confirmed fossil taxa to calibrate our phylogeny. A detailed list of the fossils and calibrations used can be found in Table S4. We conducted six independent MCMCTree analyses with sampling frequency of 500, nsample 50,000 and burn-in of 5,000,000. We confirmed convergence among different runs if ESS values surpassed 200 in Tracer v1.7.1 (Rambaut et al., 2018).

2.6. Morphological identification of species in the M. "vindex" subgroup

To identify the social parasite *M. inquilina* and its host species in the *M. "vindex"* species subgroup, we used taxonomic identification keys to *Myrmecia* species written by Ogata and Taylor (1991), Clark (1951), and Browning (1987). We also compared our samples to images of the type specimens that are available on AntWeb.org. We observed continuous variation between supposedly diagnostic traits which caused difficulties at discriminating between closely related species. Based on traits proposed by Browning (1987), Clark (1951), and Ogata and Taylor (1991), we identified the following morphological species in the *M. "vindex"* subgroup:

Myrmecia inquilina can be recognized by its socially parasitic life history. This species is presumably a host-tolerant, workerless inquiline social parasite (Douglas and Brown, 1959; Haskins and Haskins, 1964). *Myrmecia inquilina* queens are approximately 15 mm long, which is significantly smaller than the host queens, which are approximately 25 mm long. The body sculpture of the parasite is smoother and shinier compared to its host, in which it is strongly striated and dull. To date, *M. inquilina* is known from the type locality at Badjanning Rocks in Western Australia (WA), Boddington, and Chittering in Western Australia (Douglas and Brown, 1959; Haskins and Haskins, 1964), as well as from a distant locality in Coonabarabran in New South Wales (NSW). The main morphological difference between the disjunct populations from WA and NSW is that the population from NSW presents abundant long-standing hairs on the head, mesosoma and metasoma, including petiole and postpetiole, whereas the populations from WA does not present long standing hairs its body.

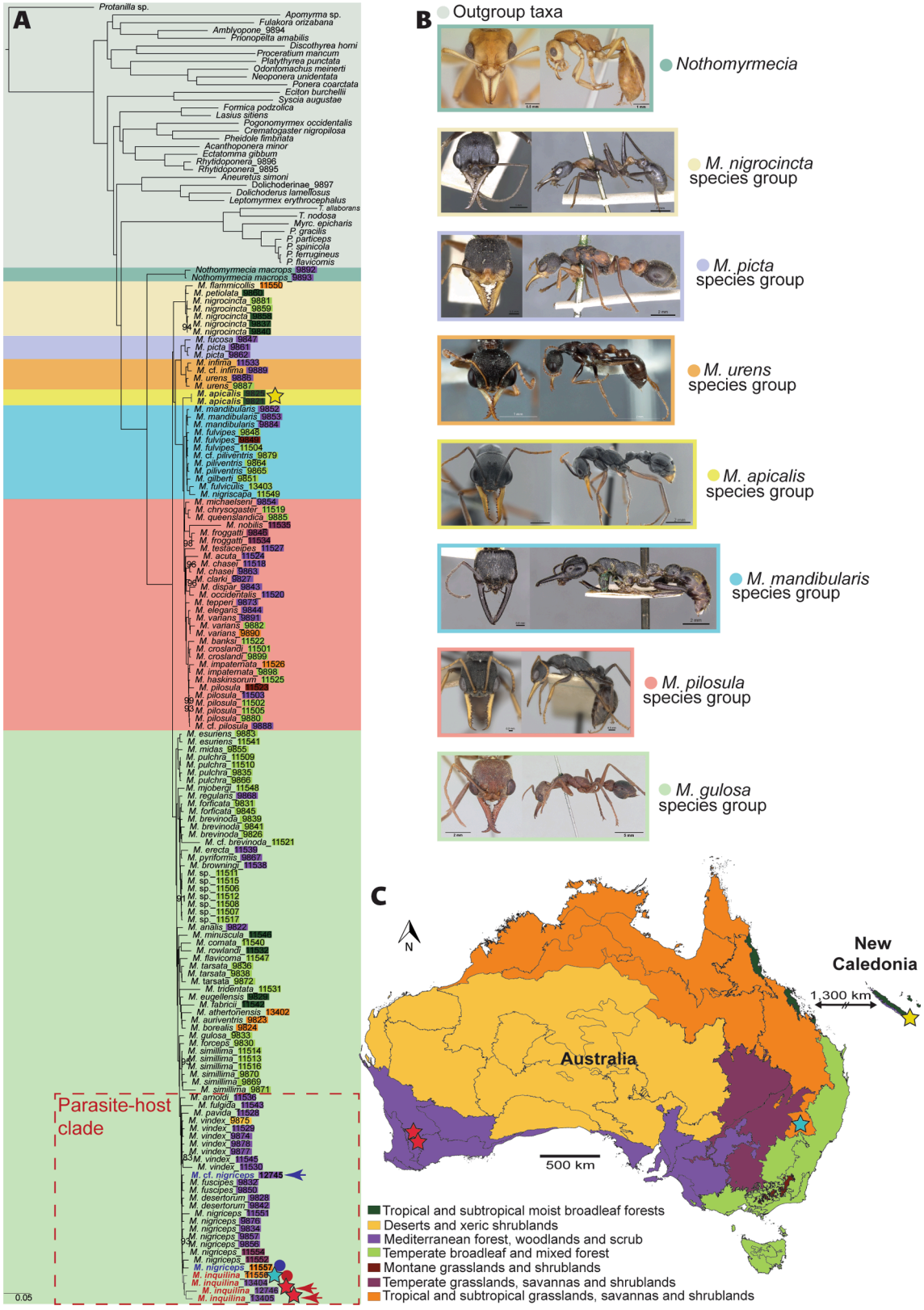
Myrmecia fuscipes can be differentiated from all other species in the "*vindex*" subgroup by its black or brown legs which are always darker than the mesosoma. In contrast, other species in the "*vindex*" subgroup have legs that are yellow or yellowish-brown, and the leg color is similar to that of the mesosoma. Like closely related species such as *M. desertorum* and *M. nigriceps*, *M. fuscipes* presents a dark coloration of the head, which is almost as dark as the black metasoma. Mesosoma, petiole, and postpetiole are brown or yellow, distinctly lighter than head and metasoma.

Myrmecia fulgida can be identified by long erect hairs on the head, mesosoma, and metasoma. The rest of the species in the "*vindex*" subgroup lack this type of pilosity except for the *M. inquilina* individual from NSW, which is different from *M. fulgida* in that it is workerless, smaller in size, and has a parasitic life history.

Myrmecia nigriceps can be recognized by the dark brown coloration of the head, which is almost as dark as the metasoma. The mesosoma, petiole, and postpetiole are brown or yellow, lighter than the head and metasoma. The apex of the antennal scape presents at least 3 erect long hairs. *Myrmecia nigriceps* is widely distributed in the southern half of Australia. The main trait that was used to separate *M. nigriceps* from *M. desertorum* is a slight difference in coloration of the head (see Ogata and Taylor, 1991). We recognized that the samples in our study that were previously identified as *M. desertorum* have a slightly different head color than our *M. nigriceps* samples. However, we wonder if this trait is truly diagnostic and a future taxonomic study needs to test whether *M. desertorum* is a diagnosable species or whether *M. desertorum* represents a junior synonym of *M. nigriceps*. Moreover, we determined that both host populations sampled in our study (DNA extraction codes: 11,557 and 12745; see Fig. 1; Table S1) have morphological features that are consistent with the diagnosis of *M. nigriceps*. One of the hosts samples (DNA extraction code 12745) was previously identified as *M. vindex* and was inferred as the sister taxon to a clade of two *M. fuscipes* samples in our phylogeny (see Fig. 1, 3 & discussion), however, the morphological characters are consistent with the diagnosis of *M. nigriceps*.

Myrmecia pavida can be identified by its dark coloration of the clypeus, which is as dark as the head. The rest of the species in the "*vindex*" subgroup present a clypeus that is yellow in coloration, more similar in coloration to the mandibles.

Myrmecia vindex is very similar to *M. nigriceps*, and both species have brown heads. However, *M. vindex* differs from *M. nigriceps* in that the head of *M. vindex* is not as dark as the head of *M. nigriceps*, which is almost as black as the metasoma. Also, in *M. vindex*, the mesosoma, petiole, and postpetiole are reddish-brown and lighter than the metasoma. The apex of the antennal scape has some short appressed hairs



(caption on next page)

Fig. 1. Evolution, diversity, and biogeography of the bulldog ants in the subfamily Myrmeciinae. **A.** Maximum-likelihood phylogeny produced in IQ-TREE from the 80% complete SWSC-EN partitioned UCE matrix. Ultrafast bootstrap values are 100 for all nodes, except when values of less than 100 are mentioned specifically at the respective nodes. Taxa are listed by species names and DNA extraction codes. Small color tags next to the taxon names correspond to the Australian bioregion where the specimens were collected (Fig. 1C). Boxes in different colors in the phylogeny indicate the outgroup taxa, the genus *Nothomyrmecia,* as well as the taxonomic species groups inferred in this study. Different populations of the social parasite included in our study are indicated by blue and red stars (Fig. 1A,C), and the social parasite-host clade is highlighted by a red dotted box. Social parasite and host species sampled in our study are marked by red and blue font colors, respectively. Social parasite and host pairs sampled from the same colony are highlighted by the same symbols next to the taxon names. The New Caledonian endemic *M. apicalis* is indicated by a yellow star (Fig. 1 A,C). **B.** Ant images show the head in frontal view and the body in lateral view of a specimen from each of the taxonomic species groups inferred in the phylogeny. The color code corresponds to the colors used in the phylogeny (Fig. 1A). Ant photos from top to bottom: *N. macrops* (CASENT0172002), *M. nigrocincta* (CASENT0902805), *M. picta* (CASENT0902807), *M. urens* (JDM32-002547), *M. apicalis* (CR111126-01), *M. mandibularis* (CASENT0907099), *M. pilosula* (CASENT0217500), *M. gulosa* (CASENT0103310). Images by April Nobile, Zach Lieberman, Brian Heterick, Shannon Hartman; available from AntWeb. Photo of *M. apicalis* by authors. **C.** Map of Australian ecoregions; modified from Ecoregions (2017). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

whereas *M. nigriceps* has at least 3 long erect hairs. This species is widely distributed in southwestern Australia, where it occurs in sympatry with *M. nigriceps*.

2.7. Species delimitation analyses

Using the software package 3s, we tested for gene flow among the putative species in the *M. "vindex"* species subgroup to find out if they belong to the same species under the biological species concept (Dalquen et al., 2017; Mayr, 1942; Zhu and Yang, 2012). A list of the triplets analyzed along with their phylogenetic configurations and critical values are provided in Table S5.

In addition, we used the species discovery software bPTP (Carstens et al., 2013; Zhang et al., 2013). We performed the analysis on the bPTP web server https://species.h-its.org/ptp/ with 500,000 generations and burn-in of 10 % to ensure convergence.

Lastly, we used a validation method for species delimitation implemented in the Bayesian program BPP (Flouri et al., 2018; Yang, 2015; Yang and Rannala, 2010). We first used the BPP analysis A00 to estimate the parameter priors. Then, we performed the BPP analysis A10 for species delimitation using a user-specified guide tree (Yang and Rannala, 2010; Rannala and Yang, 2013). We used the delimitation algorithm 1 (species delimitation = 1 1 2 0.5). We performed six independent analyses with sampling frequency of 2, burn-in of 8,000 and n sample of 250,000. We verified convergence in Tracer v1.7.1 (Rambaut et al., 2018). We considered nodes as strongly supported when they had a posterior probability equal or higher than 0.8.

Finally, we integrated the results from our approaches using morphology, population genetics, phylogenetics, and biology to identify the social parasite species and their respective hosts, as well as to infer the evolutionary history of social parasitism in the genus. Analyses were performed on the Agave Computing Cluster at Arizona State University and the Smithsonian High Performance Cluster (SI/HPC), Smithsonian Institution.

3. Results

3.1. Evolution of Myrmecia bulldog ants

To infer the evolutionary history and biogeography of the diverse Australian bulldog ants, we reconstructed a comprehensive molecular phylogeny of the ant subfamily Myrmeciinae and selected outgroups using Ultra Conserved Elements (UCEs) as genetic markers (Fig. 1). The obtained phylogeny includes a total of 168 taxa, of which 134 taxa are part of the subfamily Myrmeciinae and 34 are outgroup taxa. Our ingroup includes 132 taxa representing 66 of the total 93 described species in the genus *Myrmecia* from 8 out of the 9 previously established *Myrmecia* species groups and 2 samples of *Nothomyrmecia macrops* (Fig. 1 A,B). Our taxon sampling covers the geographic distribution of the subfamily across Australia and includes the New Caledonian endemic *Myrmecia apicalis* (Fig. 1A,C). Our analyses recovered the subfamily Myrmeciinae as monophyletic with maximum statistical support (Fig. 1). *Myrmecia* was also recovered as monophyletic (UFBS = 100) and the monotypic genus *Nothomyrmecia* was inferred as its sister lineage.

Our divergence dating analysis inferred a crown group age of ~ 164 Ma (95 % highest posterior density [HPD]: 149–174 Ma) for the family Formicidae, suggesting that the ants might have originated during the Late Jurassic (Fig. 2; Table 2). We also inferred that the closest extant relative of *Myrmecia* and *Nothomyrmecia* is the subfamily Pseudomyrmecinae, and these two lineages split during the Paleocene ~ 58 Ma ago (95 % HPD: 55–60 Ma). *Myrmecia* diverged from a common ancestor with its sister lineage *Nothomyrmecia macrops* during the Eocene ~ 44 Ma ago (95 % HPD: 34–53 Ma) (Fig. 2; Table 2). The crown group of the genus *Myrmecia* originated during the Oligocene ~ 29 Ma ago (95 % HPD: 21–38 Ma) (Fig. 2; Table 2).

3.2. Disjunct biogeographic distribution of Myrmecia apicalis

Myrmecia apicalis is the sole extant species in the genus that occurs outside the Australian mainland and is endemic to New Caledonia. To study the disjunct biogeographic distribution of *M. apicalis*, we inferred the time-calibrated phylogeny of the genus *Myrmecia* including *M. apicalis*. Our results indicate that the ancestral lineage of *M. apicalis* diverged from its sister clade during the Miocene ~ 14 Ma ago (95 % HPD: 9–18 Ma) (Fig. 2). Considering the geological history of New Caledonia (see introduction), our fossil calibrated phylogeny is consistent with the hypothesis that the disjunct biogeographic distribution of *M. apicalis* is a result of long-distance dispersal across the already existing oceanic barrier between Australia and New Caledonia.

3.3. Evolution of social parasitism in bulldog ants

To reveal the evolutionary history of the socially parasitic species in the genus *Myrmecia* and to inform the speciation mechanism by which the social parasite originated, we included representatives of two populations of the social parasite *M. inquilina* from Western Australia, which includes the type locality of *M. inquilina,* and one sample from a population in New South Wales (Fig. 1A,C). We also included individuals from 17 populations of the two previously recognized host species, *M. vindex* and *M. nigriceps*, into our phylogenetic reconstruction (Fig. 1A, 3A). Images of all the social parasite specimens included in this study are shown in Fig. 4.

Our maximum likelihood phylogeny recovered all social parasite samples as monophyletic with maximum statistical support (UFBS = 100) suggesting that social parasitism originated once in the "*vindex*" species subgroup (Fig. 1A, 3A). Interestingly, the socially parasitic clade was nested within the *M. nigriceps* clade (UFBS = 100) (Fig. 3A) rendering the host species paraphyletic.

In contrast to the maximum likelihood phylogeny, our species tree reconstruction did not recover all social parasite samples as monophyletic (Fig. S7) (LPP = 1). Instead, three *M. inquilina* samples (DNA extraction codes: 13405, 11556, and 13404) from both Western Australia and New South Wales were inferred as monophyletic and this

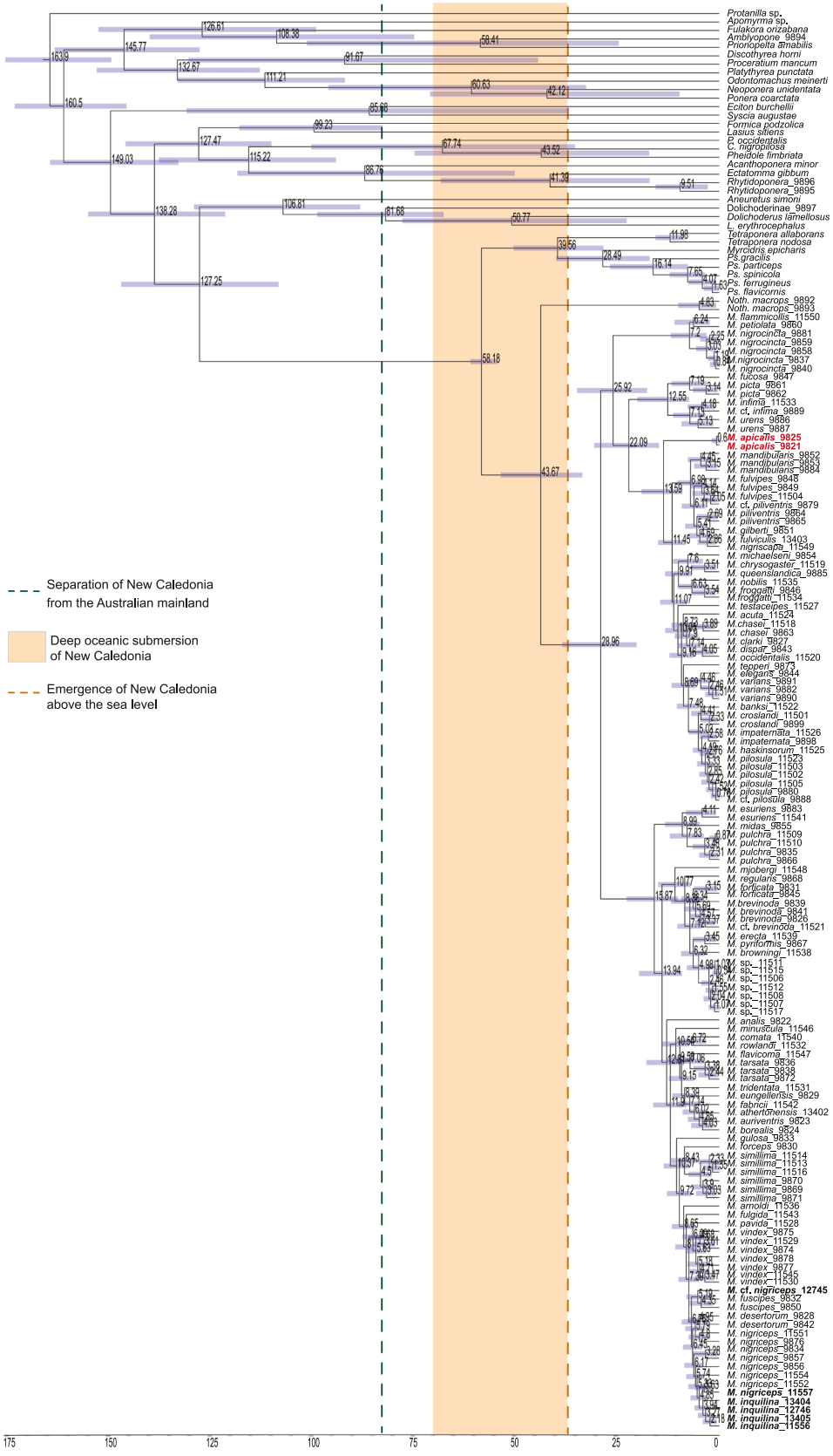


Fig. 2. Divergence dating of the ant subfamily Myrmeciinae based on Bayesian analyses conducted in MCMCTree. Numbers at the nodes indicate the estimated mean age in Ma and the blue bars represent 95 % highest posterior densities (HPD). The green dotted line indicates the time when New Caledonia was separated from the Australian mainland ~ 80 Ma ago. The orange dotted line indicates the time when New Caledonia re-emerged above the sea level after marine submersion ~ 37 Ma ago. The orange shade between the dotted lines indicates the time period when New Caledonia was submerged below sea level. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Divergence dating estimates (crown group ages) in Ma for the family Formicidae, the subfamily Myrmeciinae, and the genus *Myrmecia*.

Reference	Formicidae	Myrmeciinae	*Myrmecia*
Ward and Brady, 2003		74 (53–101)	
Brady et al., 2006	111–137		
Moreau et al., 2006	141–169		
Moreau and Bell, 2013	128–169		
Schmidt, 2013	111–152		
Brady et al., 2014		49 (46–52)	
Blanchard and Moreau, 2017	120–145		
Economo et al., 2018	154–169		
Borowiec et al., 2019	103–123	45 (37–57)	
Romiguier et al., 2022	150		
Boudinot et al., 2022	125–145		
This study	164 (149–174)	44 (34–53)	29 (21–38)

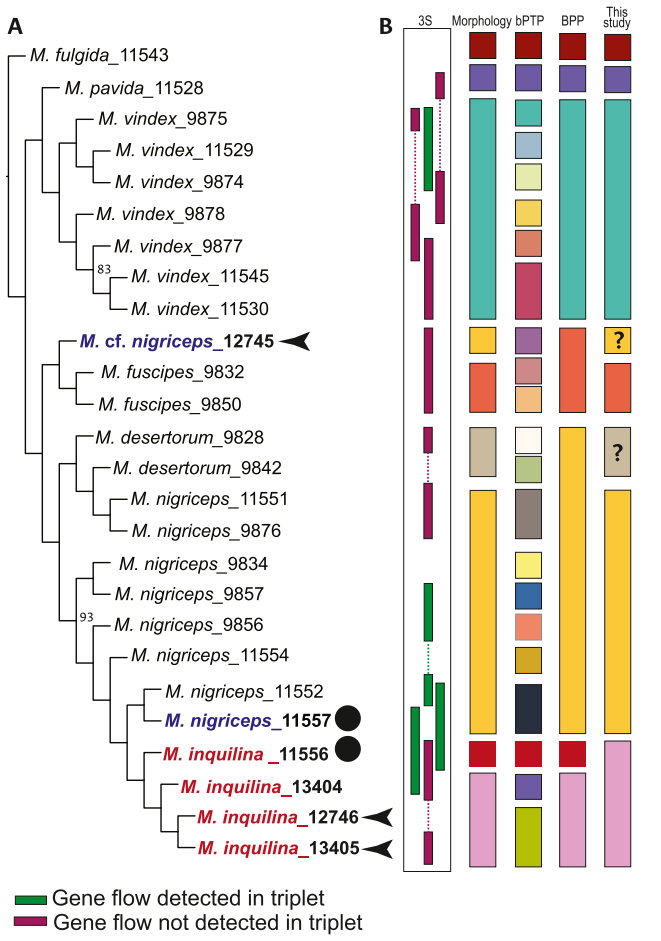


Fig. 3. Delimitation of species in the *Myrmecia* "*vindex*" species subgroup. **A.** Maximum-likelihood phylogeny produced in IQ-TREE from the 80 % complete SWSC-EN partitioned UCE matrix. Ultrafast bootstrap values are 100 for all nodes, except when values of less than 100 are mentioned specifically at the respective nodes. Taxa are listed by species names and DNA extraction codes. Social parasite and host species sampled in our study are marked by red and blue font colors, respectively. Social parasite and host pairs sampled from the same colony are highlighted by the same symbols next to the taxon names. **B.** Species delimitation analyses. Different columns show results from different species delimitation approaches. From left to right: Reproductive isolation 3s, morphological identification, bPTP, BPP, and our interpretation of the consensus identification in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

clade was nested within *M. nigriceps*. The fourth *M. inquilina* sample (DNA extraction code: 12746) was inferred as the sister to the larger *M.* "*vindex*" species subgroup including *M. desertorum*, *M. fulgida*, *M. fuscipes*, *M. nigriceps*, *M. pavida*, and *M. vindex*. Considering that the sample *M. inquilina* 12746 yielded a low amount of UCE loci (1733 UCE loci of which only a single locus was longer than 1 kb) and that *M. inquilina* 12746 was sampled from the same colony as 13405, we suggest that the phylogeetic placement as sister to the larger *M.* "*vindex*" species subgroup is an analytical artifact due to poor data quality. The paraphyly of *M. nigriceps* with regard to *M. inquilina* is consistent with the result from our maximum likelihood analyses, which we interpret as an indicator of incomplete lineage sorting. Moreover, the split of the parasite populations from the host lineage is not well supported in our species tree reconstruction in ASTRAL (LPP = 0.58) (Fig. S7), suggesting discordance between gene and species trees.

The divergence dating analysis estimated that *M. inquilina* and its closest non-parasitic relative shared a common ancestor during the Pliocene ~ 4.8 Ma ago (95 % HPD: 3.4–6.4 Ma). The crown group age of *M. inquilina* indicates that the populations from Western Australia and New South Wales also shared a common ancestor during the Pliocene ~ 3.9 Ma ago (95 % HPD: 2.6–5.4 Ma) (Fig. 2).

Considering that the studied parasite populations stem from opposite ends of the maximum possible species distribution range in Australia (Fig. 1A,C), we also tested if the disjunct population from New South Wales could represent a new social parasite species. We integrated population genetic, molecular phylogenetic, ecological, morphological, and biogeographic data to delimit these putative species (Figs. 1, 3, 4). The results of our analyses are incongruent. (1) Morphologically the parasites from the two disjunct populations differ in that *M. inquilina* individuals from Western Australia do not present long standing hairs on their bodies, whereas the single individual from New South Wales presents abundant long-standing hairs on the head, mesosoma and metasoma, including petiole and postpetiole (Fig. 4). (2) Our maximum likelihood phylogeny and species tree reconstruction recover all parasite samples as monophyletic (Figs. 1, 3), which is consistent with the hypothesis that *M. inquilina* is a single, widely distributed species. (3) Using species delimitation methods, we tested for the presence of gene flow between social parasite populations (Fig. 3; Table S5). Using 3s, we could neither reject nor support the null hypothesis of reproductive isolation (i.e., absence of gene flow) between *M. inquilina* populations from WA and NSW (Fig. 3). Using Bayesian and coalescence models, the WA populations were inferred as a species that is likely separate from the NSW population (BPP: BPP = 0.86; bPTP: inverse BPP = 0.80) (Fig. 3; Figs. S8, S9). In summary, our morphological and species delimitation analyses suggest that the NSW population could be a new social parasite species that is reproductively isolated from *M. inquilina* on the west coast. In contrast, the monophyly of the *M. inquilina* lineages and the dependence on *M. nigriceps* as a host on both east and west coasts are at least consistent with the hypothesis that the social parasite is a single, widely distributed species.

3.4. Implications for Myrmecia classification and taxonomy

The current taxonomic classification of *Myrmecia* ants recognizes 93 extant species, which were placed into 9 species groups (Ogata and Taylor, 1991). In our phylogenetic study we included 66 *Myrmecia* species representing 8 species groups (Fig. 1A,B). Our results indicate that the genus *Myrmecia* first diverged into two large clades, where the *gulosa* species group is sister to all other species groups that are included in our study (UFBS = 100, LPP = 1) (Fig. 1). Our phylogeny revealed that only the *aberrans, nigrocincta, picta,* and *urens* species groups are monophyletic (Fig. 1; Table 3). In contrast, our phylogeny recovered the *gulosa, pilosula*, and *tepperi* species groups as polyphyletic and the *mandibularis* species group as paraphyletic (Fig. 1; Table 3), indicating that the internal taxonomic classification of the genus *Myrmecia* needs to be updated to reflect the evolutionary relationships among the species

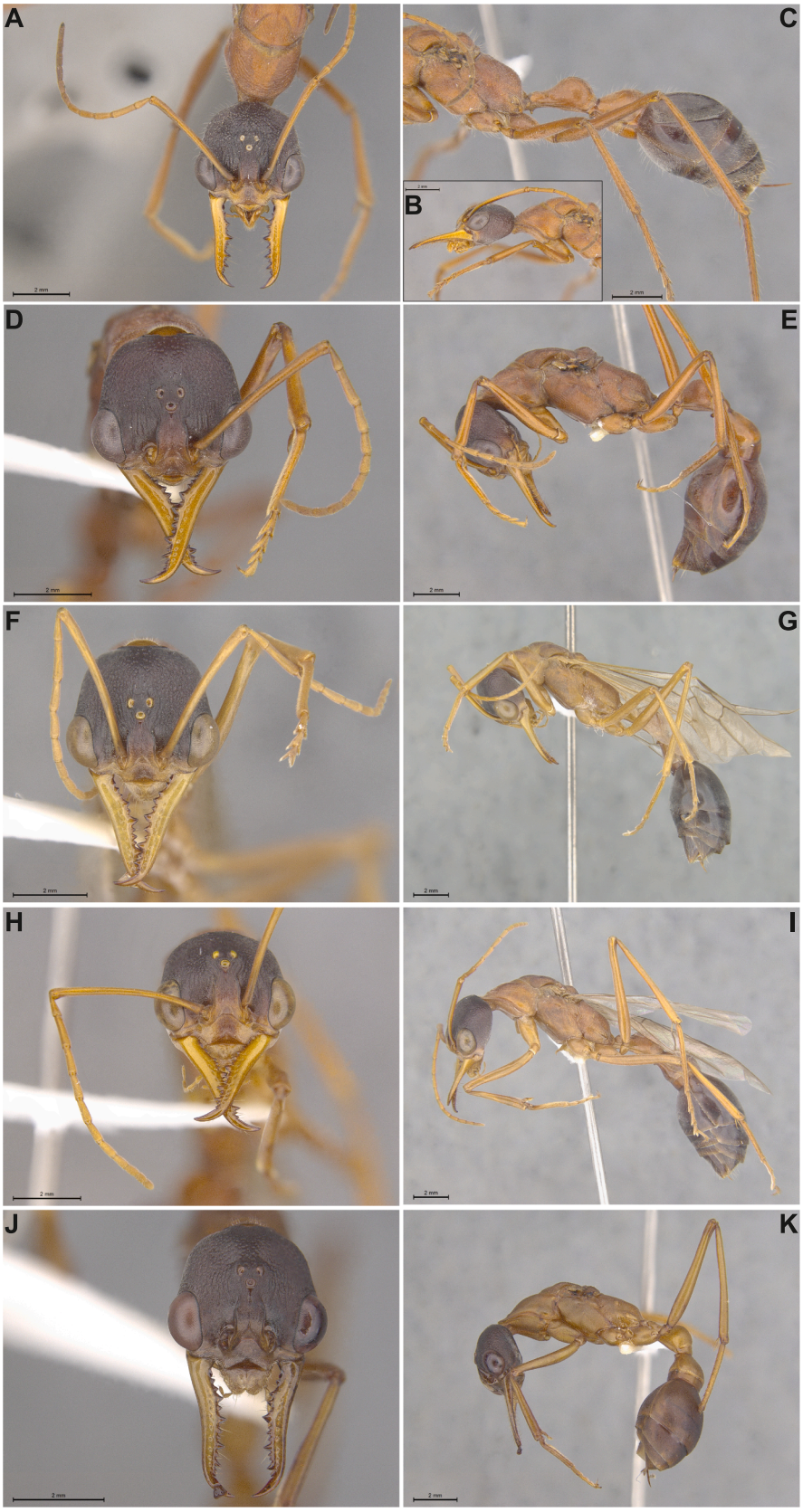


Fig. 4. Gynes of the studied *Myrmecia inquilina* social parasites from different populations. *M. inquilina*_11556 (A–C) from Coonabarabran in New South Wales, *M. inquilina*_13404 (D–E) from Boddington in Western Australia, *M. inquilina*_12746 (F–G) from the type locality Badjanning Rocks in Western Australia, *M. inquilina*_13405 (H–I) from the type locality Badjanning Rocks in Western Australia, *M. inquilina*_11553 (J–K) from Chittering in Western Australia. Full-face (A, D,F,H,J) and lateral (B,C,E,G,I,K) views.

Table 3
Taxonomy and phylogenetic classification of *Myrmecia* ants across currently and previously recognized species group. Please refer to Table S6 for a revised species list of the *Myrmecia* species groups.

Species group	Previous classification by Ogata and Taylor (1991)		Suggested classification based on this study			
	Species number	Phylogenetic inference of the previous classification	Species number	Species included in this study	Phylogenetic inference of the suggested classification	Taxonomic action suggested
aberrans	5	monophyletic	n/a	n/a	n/a	Synonymize under *pilosula* group
apicalis	n/a	n/a	1	1	monotypic	New monotypic species group
cephalotes	3	unknown	3	0	unknown	Unchanged (not studied)
gulosa	42	polyphyletic	41	34	monophyletic	Species group remains, but *M. nigriscapa* transferred to *mandibularis* group (see Table S6)
mandibularis	7	paraphyletic	8	6	monophyletic	Species group remains, but now includes *M. nigriscapa* (see Table S6)
nigrocincta	3	monophyletic	3	3	monophyletic	Species group remains unchanged
picta	2	monophyletic	2	2	monophyletic	Species group remains unchanged
pilosula	19	polyphyletic	28	18	monophyletic	Species group remains, but now includes species from *abberans* and *tepperi* groups; *M. apicalis* was transferred to the monotypic *apicalis* group (see Table S6)
tepperi	5	polyphyletic	n/a	n/a	n/a	Synonymize under *pilosula* group
urens	7	monophyletic	7	2	monophyletic	Species group remains unchanged
total	93		93	66		

(Fig. 1; Tables 3; S6). We were unable to determine the phylogenetic placement of the *cephalotes* species group due to the lack of samples of any of the three known species in that group (Table 3).

The New Caledonian endemic *M. apicalis* was inferred as the sister lineage to a larger clade that includes members of the non-monophyletic *aberrans, gulosa, mandibularis, pilosula,* and *tepperi* species groups (UFBS = 100, LPP = 1) (Fig. 1A,B).

The social parasite species as well as its host species are phylogenetically placed in the *M.* "*vindex*" species subgroup as part of the *gulosa* species group (UFBS = 100, LPP = 1). The *M.* "*vindex*" species subgroup consists of *M. desertorum, M. inquilina, M. fulgida, M. fuscipes, M. nigriceps, M. pavida*, and *M. vindex* (Ogata and Taylor, 1991) (Fig. 3A). *Myrmecia arnoldi* Clark, 1951 was traditionally not included in the *M.* "*vindex*" species subgroup (Browning, 1987). In our analysis, however, *M. arnoldi* was inferred as part of the "*vindex*" clade being sister to all other species in the *M.* "*vindex*" subgroup. The non-socially parasitic species in the *M.* "*vindex*" subgroup were not consistently recovered as reciprocally monophyletic in our maximum likelihood phylogeny nor in our species tree reconstruction (Figs. 1A, 3A, S7).

To correctly identify the host species of *M. inquilina*, we integrated our morphological taxonomic identification of the species in the *M.* "*vindex*" species subgroup with tests for monophyly and gene flow as well as species delimitation analyses (Fig. 3B). Using morphological traits, we identified a total of eight species (Fig. 3B). Using 3s, we tested for the presence of gene flow among triplets of closely related species (Fig. 3B; Table S5) detecting 16 species in the *M.* "*vindex*" subgroup (Fig. 3B). Using bPTP, a total of 22 species were hypothesized to be part of the *M.* "*vindex*" subgroup (Fig. 3B). Bayesian support values for the species hypotheses ranged from 0.45 to 1.0 (Fig. S8). BPP delimited 7 well-supported species in the *M.* "*vindex*" species subgroup (Fig. 3B) (but see discussion). Bayesian support values for the species hypotheses ranged from 0.86 to 1.0 (Fig. S9).

In previous studies, *M. nigriceps* and *M. vindex* were identified as hosts of *M. inquilina* (Douglas and Brown, 1959; Haskins and Haskins, 1964). The host population from Boddington, WA represented by the sample *M.* cf. *nigriceps*_12745 was previously identified as *M. vindex*. However, using morphological characters, we identified the specimen as *M. nigriceps* (see Section 2.6). Interestingly, the host *M.* cf. *nigriceps*_12745 is sister to a clade consisting of two *M. fuscipes* samples, which is sister to a clade consisting of *M. desertorum*, *M. inquilina*, and *M. nigriceps* (Fig. 3A). If this host (12745) would be classified as *M. fuscipes* or a previously unrecognized species in a subsequent study, it would add a new host species record for *M. inquilina*. Alternatively, the larger *M. nigriceps* clade could represent a single species with high degrees of intraspecific morphological variation, which would render *M. desertorum* and *M. fuscipes* junior synonyms of *M. nigriceps*.

Considering the available evidence, we conclude that *M. inquilina* parasitizes two closely related host species: *M. nigriceps* and *M. vindex*. Importantly, we could not examine and confirm *M. vindex* as a host of *M. inquilina* from the type locality at Badjanning Rocks, and therefore, we relied on the identification of *M. vindex* as a host species by Douglas and Brown (1959). It would be reassuring, though, if future studies could confirm *M. vindex* as a host of *M. inquilina* in Western Australia.

4. Discussion

Our study provides a comprehensive molecular phylogenetic framework for studying the evolution of the diverse and biologically fascinating bulldog ants of the subfamily Myrmeciinae, which are endemic to Australia and New Caledonia. This phylogenetic reconstruction allows for testing hypotheses regarding the biogeographic origin of the New Caledonian endemic *M. apicalis* and the evolution of social parasitism in bulldog ants. In addition, the phylogeny provides novel information about the internal taxonomic classification of the genus *Myrmecia*, and we suggest minor alterations to the classification at the species-group level that allow for reconciling the evolutionary history with the current taxonomic classification of the genus.

4.1. Evolution of Myrmecia bulldog ants

Our phylogeny inferred the genus *Myrmecia* and the subfamily Myrmeciinae as monophyletic supporting results from previous studies (Borowiec et al., 2019; Hasegawa and Crozier, 2006; Moreau and Bell, 2013; Ogata, 1991; Ward and Brady, 2003). We estimated that extant *Myrmecia* and *Nothomyrmecia* species shared a common ancestor during the Eocene ~ 44 Ma ago (95 % HPD: 34–53 Ma), which is roughly consistent with previous estimates of 49 Ma (46–52 Ma) (Brady et al., 2014) and 45 Ma (37–57 Ma) (Borowiec et al., 2019) (Table 2). However, our estimate is considerably younger than a previous estimate that was based on the analysis of 18S and 28S ribosomal genes as well as morphological characters suggesting a crown group age for Myrmeciinae of ~ 74 Ma (53–101 Ma) (Ward and Brady 2003) (Table 2). Our estimate indicates that *T. inopinata,* the oldest fossil attributed to Myrmeciinae (~60 Ma) from the Menat repository in France is part of the stem group Myrmeciinae. Similarly, most of the other fossils currently classified as Myrmeciinae are potentially stem lineages and only

Prionomyrmex wappleri Dlussky, 2012 and *Ypresiomyrma orientalis* Dlussky et al., 2015 (Table 1) are putative representatives of crown Myrmeciinae.

The subfamily Myrmeciinae was previously hypothesized to have originated in Gondwana, followed by a secondary separation of ancestral lineages via continental drift onto different southern continents from which they then dispersed to Europe (Ward and Brady, 2003). This hypothesis was supported by the apparent absence of myrmeciine fossils from parts of the northern hemisphere other than Europe (Ward and Brady, 2003). However, more recent studies discovered putative myrmeciine fossils in North America and at additional localities in Europe (Fig. 5; Table 1). Moreover, Ward and Brady (2003) provided evidence that *Nothomyrmecia* shared a more recent common ancestor with the extinct genus *Prionomyrmex*, which is only known from Europe, rather than with *Myrmecia*. Our calibrated phylogeny estimates that the subfamily Myrmeciinae is younger than the relative timing of the final stage of the break-up of Gondwana during the late Cretaceous. This estimate, combined with the recent evidence that fossils attributed to the Myrmeciinae from the Paleogene are currently known from every continent except for Africa, Antarctica, and Australia (Fig. 5; Table 1), suggests that the ancestral Myrmeciinae might have originated in Laurasia and dispersed to Australia more than once during their evolutionary history. Our study focuses on the phylogenetic relationships of extant taxa and cannot resolve the biogeographic origin of the Myrmeciinae, but future studies need to revisit the phylogenetic relationships among the diverse myrmeciine fossils and infer the paleobiogeography of both extinct and extant Myrmeciinae to resolve this biogeographic puzzle.

We estimated the crown age of *Myrmecia* to be ~ 29 Ma old indicating that extant *Myrmecia* ants likely originated during the Oligocene in a period of global climate change shifting Earth's greenhouse conditions to global cooling episodes (Hutchinson et al., 2021; Zachos et al., 2001). The diversification of the genus *Myrmecia* and the radiation of the main lineages in the genus coincided with the late Oligocene warming period and the extension of arid habitats on the Australian continent (Byrne et al., 2011). The majority of today's 93 known *Myrmecia* species are adapted to desert and semiarid environments and only a small number of species inhabit mesic and rainforest habitats (Shattuck, 1999). Interestingly, similar diversification patterns were inferred for northern hemisphere ants, such as the Holarctic genera *Formica* and *Myrmica* (Borowiec et al., 2021; Jansen et al., 2010). It would be interesting to further explore the effects that paleoclimatic events had on the diversification patterns of speciose ant genera on the northern versus the southern hemispheres (Byrne et al., 2011; Hutchinson et al., 2021; Zachos et al., 2001).

In summary, it seems well-supported that the subfamily Myrmeciinae originated during the Paleocene or earlier, was globally distributed during the Eocene, and then most myrmeciine lineages became extinct. Extant myrmeciines shared a common ancestor during the Eocene, and the genus *Myrmecia* diversified only in Australia during the Oligocene and later. The sister lineage of *Myrmecia*, encompassing *Nothomyrmecia* and the extinct Baltic amber *Prionomyrmex*, evidently had a much more widespread distribution in the past, but survives today only as a single relict species in southern Australia (Archibald et al., 2006; Jouault and Nel, 2022; Ogata and Taylor, 1991; Ward and Brady, 2003). It remains to be understood why the myrmeciines survived and diversified exclusively in Australia and New Caledonia.

4.2. Disjunct biogeographic distribution of Myrmecia apicalis

We inferred a time-calibrated phylogeny for the bulldog ants in the genus *Myrmecia* and its relatives to better understand the disjunct biogeographic distribution of *M. apicalis,* which is the only extant Myrmeciinae species that lives outside of Australia and is endemic to New Caledonia. Our study supports the hypothesis that long-distance dispersal caused the current disjunct distribution of *M. apicalis*. The ancestral lineage of *M. apicalis* diverged from its sister clade during the

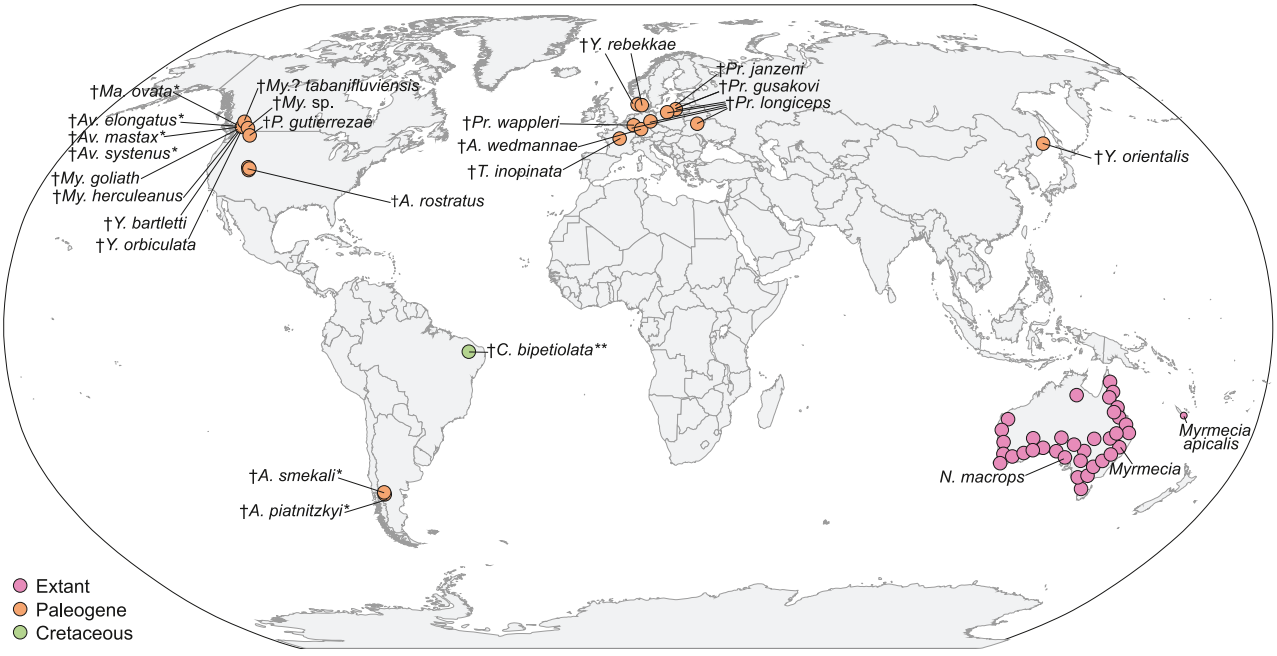


Fig. 5. Biogeographic distribution of extant and extinct ant lineages in the subfamily Myrmeciinae. The occurrence of extinct taxa is indicated by a dagger (†). Taxa included in the subfamily Myrmeciinae as *incertae sedis* indicated by an asterik (*). Taxa excluded from Formicidae that were previously included in Myrmeciinae are indicated by a double asterisk (**). Extant taxa are marked by a pink circle, Paleogene taxa are marked by an orange circle, and Cretaceous taxa are marked by a green circle. Genus abbreviations: †*Archimyrmex* = *A.*, †*Avitomyrmex* = *Av.* †*Cariridris* = *C.*, †*Myrmeciites* = *My.*, *Nothomyrmecia* = *N.*, †*Prionomyrmex* = *Pr.*, †*Propalosoma* = *P.*, †*Tyrannomecia* = *T.*, †*Ypresiomyrma* = *Y.* The fossil occurrence data were modified from Jouault and Nel, 2022, Paleobiology Database (2022), and https://www.antcat.org. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Miocene ~ 14 Ma ago (Fig. 2), which is significantly more recent than the separation of New Caledonia from the Australian mainland ~ 80 Ma ago as well as the re-emergence of the island from marine submersion ~ 37 Ma ago (Grandcolas, 2017). Therefore, a long-distance dispersal hypothesis is consistent with both the recent evolutionary origin of *M. apicalis* and the geological history of New Caledonia (Fig. 2). New Caledonia is the oldest oceanic island known in the South Pacific region and there is no evidence for the existence of nearby islands that could have facilitated short-distance dispersal to New Caledonia (Grandcolas, 2017; Nattier et al., 2017). Accordingly, our results add further support to the interpretation that the biotas of New Caledonia were assembled during the last 37 Ma (Grandcolas, 2017; Grandcolas et al., 2008; Murienne, 2009; Murienne et al., 2005; Nattier et al., 2017). Other biogeographic studies about the endemic fauna of the island also suggest long-distance dispersal events in at least 36 taxa (as reviewed in Nattier et al., 2017) including other groups of insects such as ants, caddisflies, cockroaches, and flies (Couri et al., 2010; Espeland and Johanson, 2010; Lucky, 2011; Murienne, 2009; Murienne et al., 2005; Nattier et al., 2017; Sinclair, 2008), as well as shrimps (Page et al., 2005), centipedes (Murienne et al., 2011) and lizards (Smith et al., 2007; Nattier et al., 2017).

Long-distance dispersal of arthropods to isolated oceanic islands is known to be facilitated by wind (anemochory), bird-mediated transfer (zoochory), or transport of vegetation rafts by water (hydrochory) (Leitch et al., 2021; Pfingstl, 2013). Evidence of anemochory was presented for some flying insects such as moths and butterflies, which were transported by airflow across large distances from Australia to New Zealand or further to the distant oceanic Island Macquarie (Fox, 1978; Greenslade et al., 1999). In general, many ant species perform nuptial flights where queens and males mate before the newly inseminated queens found new colonies (Helms, 2018; Hölldobler and Wilson, 1990), and during these nuptial flights, winged ants could be blown out to distant islands. *Myrmecia* ants usually do not perform mass nuptial flights and non-inseminated queens leave the maternal nest singly or in small groups (Clark, 1951). Therefore, long-distance dispersal of the ancestor of *M. apicalis* to New Caledonia by anemochory seems less likely. Similarly, records of bird migratory routes in the South Pacific do not support the hypothesis that the ancestor of *M. apicalis* was carried to New Caledonia by migratory birds (Gillespie et al., 2012). Alternatively, the ancestor of *M. apicalis* and other ant clades endemic to New Caledonia that have wingless queens, such as *Leptomyrmex* Mayr, 1862, *Leptogenys* Roger, 1861, and *Lioponera* Mayr, 1879, could have arrived on New Caledonia via hydrochory, i.e., floating on vegetation rafts. Numerous plant, vertebrate, and invertebrate taxa were reported to have dispersed across the ocean using vegetation rafts (Abe, 1984; reviewed in Thiel and Gutow, 2005; Gillespie et al., 2012), including the transoceanic rafting of primates from Africa to South America across the Atlantic during the Eocene-Oligocene transition (Seiffert et al., 2020). Currently, *M. apicalis* is known to nest in the soil at the base of small trees (H. Jourdan, personal observation), and closely related species are known to nest in rotten logs (Clark, 1951). Records of oceanic currents in the South Pacific show some episodic rafting routes that could have directed vegetation rafts from the Australian mainland to New Caledonia (Gillespie et al., 2012). Therefore, it seems possible that the ancestor of *M. apicalis* and other endemic ant species could have arrived in New Caledonia as a result of long-distance, transoceanic rafting.

4.3. Evolution of social parasitism in bulldog ants

To test competing hypotheses regarding the evolutionary origins of ant social parasites, we reconstructed a detailed phylogeny of *Myrmecia* including multiple populations of the social parasite *M. inquilina* as well as the two previously identified host species *M. nigriceps* and *M. vindex*. Our phylogeny recovered a social parasite clade that was nested within one of the host species, *M. nigriceps*, rendering the host species paraphyletic (Fig. 1, 3). This phylogenetic pattern suggests that the most recent common ancestor (MRCA) of the social parasite shared a common ancestor with a subset of *M. nigriceps* populations but does not share a MRCA with all *M. nigriceps* populations, indicating that the social parasite speciated secondarily from its host population after *M. nigriceps* had originated and dispersed in Australia. The host paraphyly also suggests that ancestral polymorphisms are still retained across the speciation event (i.e., incomplete lineage sorting) (Philippe et al., 2011; Warnow, 2015; Zhang et al., 2018) (Fig. S7), and that occasional gene flow might occur between host and parasite or did occur in the recent past (Fig. 3B; Table S5). Moreover, a small effective population size of the parasite species might also contribute to the host paraphyly as genetic diversity is low and ancestral polymorphisms likely became fixed.

Our result provides phylogenetic evidence for the intraspecific hypothesis of social parasite evolution via sympatric speciation (Fig. 3A). The common ancestor of *M. inquilina* likely diverged directly from the *M. nigriceps* host population, and secondarily *M. inquilina* shifted to additional host species (see discussion on host species below) (Douglas and Brown, 1959; Haskins and Haskins, 1964). Our study adds another empirical example of an ant inquiline social parasite that diverged directly from its host species evolving along the intraspecific route of social parasite evolution via sympatric speciation. The intraspecific route was documented for a few inquiline social parasites species from distantly related ant genera including *Acromyrmex* Mayr, 1865 (Rabeling et al., 2018; Schrader et al., 2021), *Ectatomma* Smith, 1858 (Nettel-Hernanz et al., 2015), *Mycocepurus* Forel, 1893 (Rabeling et al., 2014), *Myrmica* Latreille, 1804 (Savolainen and Vepsäläinen, 2003; Jansen et al., 2010; Leppänen et al., 2015), *Plagiolepis* Mayr, 1861 (Degueldre et al., 2021), and *Pseudomyrmex* Lund, 1831 (Ward and Branstetter, 2022). In contrast, recent empirical studies inferred the speciation mechanism of temporary and dulotic social parasites as allopatric speciation evolving via the interspecific route of social parasite evolution (Borowiec et al, 2021; Fischer et al., 2020; Prebus, 2017). In general, and from a growing body of empirical studies, the impression emerges that dulotic and temporary social parasites speciate via the interspecific, allopatric route of social parasite evolution whereas sympatric speciation has so far only been observed in some distantly related inquiline social parasites (Rabeling, 2020). Although it would be fascinating to better understand how reproductive isolation evolves when incipient parasites and hosts co-exist within the same colony, it seems that the bulk of social parasite biodiversity originated via the interspecific trajectory of social parasite evolution.

In our study, we included *M. inquilina* samples from the parasite's type locality and a nearby locality in Western Australia as well as a geographically distant population in New South Wales representing at least two disjunct parasite populations that are approximately 3,100 km apart from each other (Fig. 1C). For the time being, we conclude that the social parasites from the disjunct populations belong to *M. inquilina* because samples from both social parasite populations were recovered as monophyletic (Fig. 1A, 3B) and because species delimitation software based on the multispecies coalescent model are prone to overestimate species numbers by delimiting population structure rather than species (Sukumaran and Knowles, 2017; Sukumaran et al., 2021). Hence, we cannot reject the hypothesis that the genetic differentiation detected by the species delimitation analyses is a result of isolation by distance. Moreover, the social parasite could be continuously distributed along the southern coast of Australia, as is its host *M. nigriceps*, and under such a scenario gene flow would be expected to be significantly reduced among distant populations, as indicated by our population genomic analyses (Fig. 3B).

In addition, we only know of a single *M. inquilina* individual from NSW. We could identify some morphological differences between the individuals from the east and west coast populations (see results and Fig. 4). However, we could not test whether these traits mark diagnostic morphological characters that are indicative of two distinct species or whether they vary continuously throughout the biogeographic distribution of *M. inquilina* (Fig. 1C, 4). Currently, there is simply not enough

evidence to support the hypothesis that the east and west coast parasite populations correspond to two distinct species. If additional populations of *M. inquilina* were discovered, future studies would need to revisit this question and test whether *M. inquilina* is a single widely distributed species or whether *M. inquilina* is in fact a group of multiple reproductively isolated species.

The evolutionary origins of social parasitism cannot always be inferred from reconstructing the phylogenetic relationships among extant social parasites and their hosts. In some cases, secondary evolutionary events, such as host shifting, speciation and/or extinction of the parasite and/or the host might obscure the conditions under which social parasitism originated (Borowiec et al., 2021; de Vienne et al., 2013; Parker and Rissing, 2002). For instance, host shifting is known to have a significant effect on plant and insect diversification (Forbes et al., 2017), and in speciose clades where plants and insects co-evolved for a long time, it becomes difficult to infer the evolutionary relationships of the original host-parasite lineages (de Vienne et al., 2013). *Myrmecia inquilina* seems to exploit two closely related host species: *M. nigriceps* and *M. vindex*. Our phylogeny inferred *M. inquilina* as rendering its host *M. nigriceps* paraphyletic suggesting that the social parasite originated as a parasite of *M. nigriceps* and shifted secondarily to *M. vindex* with whom *M. nigriceps* has a partially overlapping biogeographic range in Western Australia. Interestingly, *M. inquilina* populations on the east and west coasts both exploit the same host species, i.e., *M. nigriceps*. Therefore, even if *M. inquilina* from Western Australia and New South Wales represented two distinct species, parasite speciation would not be associated with a host shifting event, which contrasts with studies of more speciose ant social parasite clades in *Acromyrmex*, *Pheidole* Westwood, 1839, and *Pogonomyrmex* Mayr, 1868, where shifting to a new host species was suggested as a mechanism of secondary social parasite speciation (Fischer et al., 2020; Parker and Rissing, 2002; Rabeling et al., 2018).

4.4. Implications for Myrmecia classification and taxonomy

We present a comprehensive molecular phylogeny for the *Myrmecia* bulldog ants including 66 of the 93 currently described species from 8 of the 9 currently recognized species groups (Fig. 1) increasing phylogenetic resolution at both the gene and the taxon sampling levels with regard to previous phylogenetic studies (Hasegawa and Crozier, 2006; Ogata, 1991; Ward and Brady, 2003). Our results indicate that the internal classification of the genus *Myrmecia* needs to be revised taxonomically to reflect the evolutionary relationships among the extant species (Fig. 1A,B; Table 3). To reconcile phylogeny and taxonomic classification, we propose three minor changes to the taxonomic classification of the species groups.

First, *M. apicalis* is currently considered a member of the *pilosula* species group. Our phylogeny recovered *M. apicalis* as sister to a diverse clade of *Myrmecia* bulldog ants (Fig. 1A), thus rendering the *pilosula* group polyphyletic. We propose to exclude *M. apicalis* from the *pilosula* species group so it constitutes its own monotypic species group reflecting its phylogenetic position and unique phylogeographic origin (Fig. 1C).

Second, the *aberrans* and *tepperi* species groups are nested within the *pilosula* species group (Fig. 1A,B), rendering the *pilosula* group paraphyletic. Synonymizing the *aberrans* and *tepperi* species groups with the *pilosula* species group and excluding *M. apicalis* from the *pilosula* group would render the larger *pilosula* species group monophyletic.

Third, *M. nigriscapa* is currently a member of the *gulosa* species group. In our phylogeny, it was recovered as part of the *mandibularis* clade (Fig. 1A,B). We suggest to transfer *M. nigriscapa* to the *mandibularis* species group because as a result both the *mandibularis* and the *gulosa* species groups would become monophyletic.

The proposed reclassification would leave *Myrmecia* with 8 monophyletic species groups including the *apicalis, cephalotes, gulosa, mandibularis, nigrocincta, picta, pilosula,* and *urens* species groups (Table 3; Table S6). It is important to note that the *cephalotes* species group remains unaltered because its members were not included in our study. Similarly, the species that were not included in our phylogenetic reconstruction could make additional taxonomic changes necessary.

The lack of support for the monophyly of the taxonomic species groups in *Myrmecia* (Browning, 1987; Hasegawa and Crozier, 2006; Ogata, 1991; Ogata and Taylor, 1991) indicates that variation in rates of morphological evolution and convergence of morphological traits that evolved in distantly related *Myrmecia* lineages might have affected the work taxonomists have done to identify the species in the genus (Lee and Palci, 2015; Wake et al., 2011; Wyss, 1989; Zou and Zhang, 2016). Considering the biological relevance of *Myrmecia* bulldog ants for understanding Australian biodiversity, a taxonomic revision of the genus *Myrmecia* at the species level is needed to incorporate the evolutionary history into the taxonomic classification as well as to delimit species boundaries in recently diverged clades.

5. Conclusions

Our study provides a robust phylogenomic framework that allows for studying (i) the evolution of the diverse Australian bulldog ants in the genus *Myrmecia* and its relatives, (ii) the biogeographic origin of the New Caledonian endemic *M. apicalis*, and (iii) the evolution of social parasitism in the genus. We show that the subfamily Myrmeciinae split from their sister lineage, the Pseudomyrmecinae, in the Paleocene and that the ancestral (stem) *Myrmecia* diverged during the Eocene, representing a relatively young ant genus that diversified into a species-rich and ecologically dominant group in Australia. We conclude that the ancestral lineage of *M. apicalis* diverged from mainland congeners during the Miocene ~ 14 Ma ago, supporting the hypothesis that the ancestor of *M. apicalis* arrived in New Caledonia via long-distance dispersal from the Australian mainland across the already existing oceanic barrier. We also conclude that social parasitism had a single origin during the evolutionary history of the myrmeciines. *Myrmecia inquilina* likely evolved in sympatry from its free-living host *M. nigriceps* supporting the intraspecific hypothesis of ant social parasite evolution. Subsequently, *M. inquilina* likely started exploiting *M. vindex* as a second host species. Our study tentatively inferred *M. inquilina* as the single social parasite species in the bulldog ants. We also show that the genus *Myrmecia* would benefit from a detailed taxonomic revision at the species level. Although the taxon sampling available to us is incomplete, we suggest some minor changes to the current species groups as a first step to place the taxonomic classification in an evolutionary context. Future studies need to investigate the evolutionary relationships among the fossil taxa in the Myrmeciinae to elucidate the historic biogeography of the subfamily. We hope that our results will encourage future comparative studies about the fascinating biology of bulldog ants in Australia.

CRediT authorship contribution statement

Daniela Mera-Rodríguez: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Visualization. **Hervé Jourdan:** Resources, Data curation. **Philip S. Ward:** Resources, Data curation. **Steven Shattuck:** Resources, Data curation. **Stefan P. Cover:** Resources, Data curation. **Edward O. Wilson:** Conceptualization, Resources, Data curation. **Christian Rabeling:** Conceptualization, Methodology, Resources, Investigation, Data curation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All new raw Illumina reads generated for this study have been deposited at the NCBI Sequence Read Archive (SRA), BioProject PRJNA936453. All UCE contigs generated for this study have been deposited at the Dryad Digital Repository (https://doi.org/10.5061/dryad.qnk98sfkp).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2023.107825.

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