

Deep-time parallel evolution of myrmecoid syndrome in rove beetle symbionts of army ants

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Recent adaptive radiations provide striking examples of convergence, but the predictability of evolution over much deeper timescales is controversial, due to a scarcity of ancient clades exhibiting repetitive patterns of phenotypic evolution. Army ants are ecologically dominant arthropod predators of the world's tropics, with large nomadic colonies housing diverse communities of socially parasitic myrmecophiles. Remarkable among these are many species of rove beetle (Staphylinidae) that exhibit ant-mimicking “myrmecoid” body forms and are behaviorally accepted into their aggressive hosts' societies: emigrating with colonies and inhabiting temporary nest bivouacs, grooming and feeding with workers, but also consuming the brood. Here we demonstrate that myrmecoid rove beetles are strongly polyphyletic, with this novel adaptive morphological and behavioral syndrome having evolved at least twelve times during the evolution of a single staphylinid subfamily, Aleocharinae. Each independent myrmecoid clade is restricted to one zoogeographic region and highly host-specific on a single army ant genus. Dating estimates reveal that myrmecoid clades are separated by substantial phylogenetic distances—as much as 105 million years (My). All such groups arose in parallel during the Cenozoic, as army ants are proposed to have risen to ecological dominance. This work uncovers a rare example of an ancient system of complex morphological and behavioral convergence, with replicate beetle lineages following a predictable phenotypic trajectory during their parasitic coevolution with army ants.

Results and discussion

The degree to which biological evolution is idiosyncratic or predictable is a fundamental question in evolutionary biology. Convergence—the acquisition of similar traits in different taxa evolving under comparable selective regimes—provides a compelling argument for predictability in evolutionary change. The most striking convergent systems are recent adaptive radiations, in which independent lineages have followed seemingly parallel evolutionary trajectories. Darwin’s finches [1], Caribbean anoles [2], *Heliconius* butterflies [3], African lake cichlids [4] and sticklebacks [5] are natural experiments, where exposure to common selection pressures has led to near-identical phenotypes in populations adapting to similar niches. Although predictable evolution is manifestly demonstrated in these systems, the likelihood of convergence is nevertheless enhanced by their young ages: the close genetic relatedness of lineages is expected to bias the production of genetic variation, enhancing the probability that similar traits will evolve repeatedly [6,7]. Molecular studies of such recently descended convergent taxa support this notion, often revealing selection acting on the same loci or signaling pathways [8,9]. With increasing phylogenetic divergence between taxa, however, the likelihood of such marked convergence decreases [10]. Ancient clades displaying equivalently conspicuous repeated evolution are rare, lending apparent credence to Gould’s view that evolution is inherently contingent [11], and that adaptive responses to a given selection pressure are likely to be different in distantly related taxa.

Here, we report a novel example of predictable evolution of a highly complex phenotype that has occurred over a deep timescale. We have explored the evolutionary origins of specialized rove beetles (Staphylinidae) that live symbiotically with army ants, uncovering an ancient system of striking parallel evolution. Army ants are dominant eusocial predators of the world’s tropics: their colonies are nomadic, with hundreds of thousands of workers that emigrate between temporary nest sites and engage in group foraging (raiding) to harvest invertebrate prey [12-14]. Although notoriously aggressive, army ant colonies represent major concentrations of resources, attracting numerous myrmecophiles that form obligate symbioses with their hosts [15-17]. Diverse taxa including mites, silverfish, flies, wasps and beetles exploit this resource, employing either defensive morphologies, or behavioral and chemical strategies to evade worker hostility.

A dramatic manifestation of this lifestyle occurs in numerous genera of the staphylinid subfamily Aleocharinae, where the beetles anatomically mimic their host ants and are recognized and accepted by them [15,18-20]. Such species live as behaviorally integrated social parasites—appearing at least partially assimilated into colony life, but simultaneously feeding on the ants’ brood and raided food. In contrast to the majority of the ~16,000 species of Aleocharinae, which are mainly free-living species with “generalized” staphylinid morphology and extremely similar in body form [21], ant-like “myrmecoid” aleocharines are heavily modified (Fig 1A), with a petiolate abdomen (a narrowed waste and expanded gaster), elongate appendages, geniculate (elbowed) antennae, and further similarities to host ant body size, thorax shape and cuticle sculpturation. The myrmecoid ecomorph is thought to mediate tactile mimicry of nestmate recognition cues [18,20,22-24], and is accompanied by a suite of behaviors, including frequent grooming and licking of workers [25,26], cohabitation of temporary nesting bivouacs, and synchronicity with the colony where the beetles emigrate with hosts and join them on raids, sometimes being carried by or phoretically attaching to workers [27,28]. Where known, the beetle’s cuticular hydrocarbons match those of the host [28], and novel glands on the beetles’ cuticles are thought to facilitate chemical integration into the ant society [20]. In species associated with day-raiding army ants, mimicry of host body color also occurs, performing a possible role in Batesian mimicry to protect against predators in [29].

The myrmecoid morphological and behavioral syndrome presents an evolutionary puzzle: because the morphology of these beetles is so strongly modified, their phylogenetic relationships to other aleocharines are obscure. Prominent aleocharine authors have proposed conflicting evolutionary scenarios: Seevers [18,19] argued for a single principal origin of these beetles within Aleocharinae, forming the large tribe Dorylomimini [30,31], and posited an ancient association with army ants followed by codiversification with hosts throughout the tropics. In contrast, Kistner and co-workers argued for multiple origins [23,24,32,33], splitting Dorylomimini into numerous small tribes and invoking potentially widespread—and extraordinary—morphological and behavioral convergence. Neither scenario has been tested phylogenetically, and to date, the relationships of these beetles are unknown. A molecular approach is essential but has

been practically unimaginable due to difficulties in obtaining specimens. The beetles rank among the rarest and most challenging of insects to find in nature, with many known only from a small number of museum specimens. In this study, we present the outcome of efforts to collect these beetles and explore their evolutionary relationships. Over the course of a decade, we observed army ant raids and emigrations across the World's tropics, accumulating aleocharines associated with all known army ant genera. In reconstructing the evolutionary history of these specialized myrmecophiles, we uncovered evidence of conspicuous, repeated evolution over deep time that runs counter to the notion of evolutionary contingency, and represents a new paradigm for understanding interspecies relationships.

Parallel evolution of myrmecoid syndrome in Aleocharinae

Army ants include the genera *Eciton*, *Labidus*, *Neivamyrmex*, *Nomamyrmex* in the New World and *Aenictus* and *Dorylus* in the Old World. These “true” army ants exhibit classical nomadic biology [14], and are split into separate Old and New World clades within the ant subfamily Dorylinae [30,31]. We collected aleocharines associated with all of these genera, as well as from two distantly related ants that display army-ant-like group foraging behavior: *Carebara* (Myrmicinae) and *Liometopum* (Dolichoderinae). Both generalized and myrmecoid aleocharines were collected, and to avoid subjectivity on our part, we defined myrmecoid species as those with petiolate abdomens and long legs that have been judged by previous authors to be myrmecoid [19,23,24,32-34]. Because most species and many genera we collected were new to science, we extracted DNA from specimens non-destructively [35,36] so they could be taxonomically described [37-39 and ongoing efforts], and sequenced the five loci that have previously been used successfully in aleocharine phylogenetics: nuclear *28s rRNA*, *18s rRNA* and *Topoisomerase I*; mitochondrial *COI* and *16s rRNA* [40-42]. Army ant myrmecophile data were integrated with sequences from free-living, morphologically generalized taxa representing a broad taxonomic spectrum of Aleocharinae including all major tribes, and outgroups belonging to the tachyporine-group of staphylinid subfamilies to which Aleocharinae belongs [43-45]. We performed Bayesian phylogenetic inference on the resulting 181-taxon matrix (see Materials and Methods in Supplemental Online

Information). The topology produced by this analysis, along with exemplar beetle and host ant images, is shown in Figure 2.

The pattern of morphological convergence is dramatic and clear to the eye. Generalized aleocharines form an ancestral backbone to the tree, from which numerous elaborate myrmecoid lineages have emerged in parallel across the subfamily (Fig 2, Fig S1). Each independent origin of the myrmecoid ecomorph is represented by a small clade or single taxon that is host-specific on a single army ant genus. All potential host genera are targeted: each of the “true” army ants belonging to Dorylinae have their own, dedicated symbiont clade(s), and so too do the group-foraging ants *Liometopum* and *Carebara*. We estimated the number of times this has arisen across the tree using parsimony optimization and Bayesian ancestral state reconstruction. For parsimony we assumed dollo-type irreversibility of myrmecoid morphology, which may be a valid assumption in this system: the “tippy” distribution of this myrmecoid lineages across the tree is consistent with it being a terminal phenotype, and an improbably large number of regains of primitive characters would be required for a lineage to lose myrmecoid morphology and restore generalized aleocharine morphology (together with a reversion to ancestral ecology). Such a model of evolution produces the fifteen instances of myrmecoid evolution depicted in Figure 2. However, for a more conservative estimate that takes branch lengths and support values into account, and includes the possibility of trait reversal, ancestral states were calculated over a tree distribution using BAYESTRAITS [46], which gave an estimate of 12 independent origins (Fig S1). We think that 12–15 origins is surely an underestimate: there remain numerous myrmecoid genera associated with both Old and New World army ants that we have thus far been unable to collect, some of which—given the polyphyletic evolution of this syndrome—likely represent additional independent origins. A detailed morphological study of myrmecoid clades and their generalized sister groups revealed aspects of mouthpart and genitalia morphology that provide supporting characters for some of our molecular groupings (see “Systematic Relationships of Myrmecoid Aleocharinae” in Supplemental Discussion, which also includes a summary of the known behavior of each clade; a full systematic review will be published elsewhere).

Importantly, we see no evidence in any of the myrmecoid clades of a beetle promiscuously switching to a different host genus, indicating that all of these relationships are highly host specific. The converse of this relationship does not hold, however, with some ant genera—*Aenictus* and *Dorylus* in particular—playing host to more than one myrmecoid beetle lineage. The stringency with which each beetle clade associates with a single ant genus likely extends to the species level, since individual beetle species have generally been recorded living with a single army ant species [18,19]. From this evolutionary pattern we determine the following: i) separate lineages of Aleocharinae have evolved to socially parasitize each of the different host ant genera; ii) during subsequent coevolution of these lineages with ants, they have specialized and become host-specific; iii) most dramatically, their morphology and aspects of behavior have followed a predictable evolutionary trajectory, leading to an overtly stereotyped symbiosis. Cumulatively the outcome is an extraordinary system of parallel evolution in the classical sense, where multiple ancestral taxa sharing a relatively conserved body plan have each evolved in the same direction [47]. To gauge the time scale over which this parallel system has emerged, we dated the tree using a Bayesian lognormal relaxed clock in BEAST 2 [48]. Nodes were calibrated with fossils in Cretaceous Burmese and Middle Eocene Baltic ambers, and a compression fossil of the Jurassic Talbragar Fish Bed (see Materials and Methods in Supplemental Online Information). We varied the stringency of our calibration densities to evaluate the impact of our priors on nodes dates, and also performed analyses without certain fossils of questionable placement.

Our analyses consistently show that virtually all myrmecoid clades arose in parallel during the Cenozoic (Fig 3). This temporal window is consistent with when ants in general (including army ants) are thought to have risen to ecological dominance [49-51], promoting the diversification of myrmecophiles [20,52,53]. Although army ant dating estimates are problematic due to limited fossils for calibration (only a single, Miocene Dominican amber *Neivamyrmex* is known [54]) recent dating estimates hypothesize that stem-groups of army ant clades within Dorylinae date to the Upper Cretaceous (~80 Mya) [31] or Palaeocene-Eocene boundary (~57–56 Mya) [55]. These clades subsequently radiated into crown-group genera between ~35 and 20 Mya, broadly consistent with the origins of myrmecoid clades inferred in this study, although we see no

clear relationship between the age of each ant genus and its corresponding myrmecoid lineage(s) (data not shown). Of foremost interest, however, is that we estimate the most recent common ancestor of all myrmecoid clades to have existed in the Early to mid-Cretaceous. In our focal analysis, this ancestor lived ~105 Mya (Fig 3). There is thus an ancient and inherent potential for Aleocharinae to evolve symbioses with army ants, which was realized by multiple lineages in parallel during the Cenozoic, and which has led to the repeated evolution of symbionts with matching ecomorphologies and similar behaviors over an extraordinarily deep timescale. Even though evolutionary instances of myrmecoid syndrome are not evenly distributed across the subfamily, this pattern appears to hold true for the subfamily at large: twelve of the fifteen myrmecoid lineages are clustered within a clade, “APL” (Fig 2, 3), comprised of the tribes Pygostenini, Lomechusini and the vast, paraphyletic Athetini, where myrmecoid lineages occur among the “False Lomechusini” group of athetines in particular (clade F) [40,41,56]—a clade that consists of multiple New World athetine genera formerly placed in Lomechusini [57]. This bias probably represents the tropical dominance of the APL clade relative to other aleocharine lineages, with ecological coexistence with army ants acting to elevate the likelihood of APL lineages evolving this type of symbiosis (in contrast, within the speciose but mostly temperate tribe Oxypodini (Clade O), we see no myrmecoid lineages emerging; Fig 3). Despite this lineage clustering, the APL clade is itself still comparatively ancient, dating to 84.77 My, and the three sporadic instances seen outside the APL clade attest to a potential for evolving myrmecoid syndrome that extends broadly across the Aleocharinae. These instances are the Neotropical *Labidus*-associated tribe Mimecitini [32], the *Liometopum*-associated *Sceptobius schmitti* in the Western USA [58], and the Indomalayan *Aenictus*-associated *Myrmecosticta* that emerges within Aleocharini (Clade A) [38]—a tribe that is moderately prevalent in the tropics.

What circumstances permitted this deep-time parallel evolutionary system to arise? We deduce that historical selection pressures imposed by different army ant genera on separate aleocharine lineages were likely similar; so too were the adaptive responses of the beetles as they coevolved with their hosts. This inherency in the outcome of selection begs the question of why myrmecoid syndrome has evolved repeatedly in Aleocharinae in particular, as opposed to all other groups of beetles, including the 31

other staphylinid subfamilies numbering some 45,000 species—most of which have generalized staphylinid morphology similar to aleocharines. We have previously argued that in contrast to other beetles, aleocharines are ancestrally preadapted for social parasitism of ants in general, and morphologically preadapted to evolve myrmecoid shape in particular when exposed to specific selection pressures imposed by army ants or other group-foraging hosts [20]. Aleocharinae's combination of predatory habits, small body size, and major defensive capacity in the form of a tergal gland on the dorsal abdomen constitute a groundplan unique among Coleoptera; this suite of characters predisposes members of the subfamily to successful entry and exploitation of ant colonies [20], providing the basis for why myrmecophily has evolved an inordinate number of times [15,18,20], including repeated associations with army ants [19]. Based on our present findings, we further posit that aleocharines that are specialized on army ants commonly gain a selective advantage from social integration, with many such lineages experiencing intense selection on body shape to conform to an ant-like morphology that enables the beetles to pass tactile assessment by workers [18,20,22-24]. If the narrow niche of social acceptance demands an ant-like shape, then the generalized aleocharine body form, comprising short elytra and an exposed, flexible abdomen, is especially conducive to this developmental modification [19-21]. Consequently, aleocharines are evolutionary poised for myrmecophily and also for becoming myrmecoid as the predominant socially parasitic strategy when specializing on army ants. We propose that such a near-clade-wide preadaptive ground state may underlie the repeated evolution of myrmecoid syndrome across the aleocharine tree.

Documented examples of deep-time convergence are mostly limited to the evolution of single traits with few instances of repeated evolution, and where a narrow range of alternative functional solutions are available. The independent origin of wings for flight in birds, bats and insects is a prime example. Similarly, although an expanding body of work has shown that parallel genetic changes can occur in extremely widely separated taxa [59,60], such examples are typically functionally equivalent mutations in single, broadly conserved genes that govern relatively simple traits, such as pigmentation [61-63] or toxin resistance [64]. In contrast, we have presented evidence that a complex morphological and behavioral syndrome has evolved recurrently over >100 My, across a

clade approximately equal in species richness to mammals and birds combined. Such a system of predictable evolution challenges Gould's view that if the tape of life were replayed, an entirely different assemblage of life would exist [11]. On the contrary, our results argue that the tape of life is highly predictable whenever aleocharines ecologically coexist with army ants—a consequence we think of ancestral, near-clade-wide inherency for specialization on army ants and evolving the myrmecoid body shape. We note, however, that despite this overarching deterministic pattern, there is nevertheless an element of contingency: as Seevers noted [19], the construction of the abdominal petiole differs among myrmecoid aleocharines, from a weak narrowing of the abdominal base (e.g. *Wasmannina*, *Sceptobius*) to a strong constriction (*Pseudomimeciton*, *Myrmecosticta*), to a thin waste (*Diploeciton*, *Giraffaenictus*) to taxa with distinct petiolar and postpetiolar segments (*Ecitocryptus*, *Weissfloggia*); additionally, not all species have fully geniculate antennae, and some have unique, dramatic specializations, such as the gland-associated abdominal lobes of *Aenictoteras*, and the complete loss of eyes and elytra in *Pseudomimeciton*. Hence, evolution appears to have followed slightly different routes in achieving the ant-like shape, and there are likely further differences in the behavioral interactions of different myrmecoid taxa with hosts [25]. Whether this spectrum of variation represents an evolutionary continuum of specialization is unclear, but we posit that at least some apparently idiosyncratic elements in this otherwise parallel system stem from clade-specific peculiarities: differences between ancestral genotypes and phenotypes of each myrmecoid lineage, disparities in selection pressures imposed by distinct army ant taxa, as well mutational and environmental stochasticity. Future studies exploiting the parallel nature of this system promise to reveal much about the genetic and evolutionary forces shaping intricate symbioses in the animal kingdom.

Acknowledgements

We are grateful to numerous people for assistance with fieldwork or permitting to collect army ant myrmecophiles, in particular Rosli Hashim, Takashi Komatsu, Taku Shimada, Christoph von Beeren, Taro Eldredge, Stefan Harrison and Ian Butler. Alfred Newton and Margaret Thayer (Field Museum of Natural History), Taro Eldredge (University of

Kansas), Terry Ord (University of New South Wales) and Christoph von Beeren (TU Darmstadt) provided important feedback on the manuscript. This work was supported by Grant-in-aid # XXXXX to MM, and a Sir Henry Wellcome Postdoctoral Fellowship and NIH support to JP.

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Figure Legends

Figure 1. Myrmecoid Syndrome in aleocharine rove beetles. A: Examples of free-living Aleocharinae with generalized morphology, *Oxypoda* and *Atheta*. **B:** Examples of army ant social parasites with myrmecoid morphology, *Ecitocryptus* (associated with *Nomamyrmex*) and the eyeless, elytra-less *Pseudomimeceton* (associated with *Labidus*). **C–E:** Living myrmecoids with host ants: *Ecitophya* with *Eciton* host (Peru), *Aenictoteras* with *Aenictus* host (Malaysia), *Beyeria* with *Neivamyrmex* host (Ecuador).

Figure 2. Bayesian consensus tree of Aleocharinae. Myrmecoid clades are highlighted in orange, with representative taxa shown along with their respect host army ant genera. Clade/lineage numbers indicate independent instances inferred from Dollo-type parsimony optimization and correspond to the numbers in Figure 3 and in the Supplemental Discussion. Generalized, free-living species that embody the ancestral morphology in Aleocharinae are also shown for comparison. Circles on nodes signify posterior probability (PP) values: open circles: PP > 0.95; closed circles, PP > 0.9. “APL” marks the “Athetini, Pygostenini, Lomechusini” clade; “F” labels the “False Lomechusini” clade.

Figure 3. Dating the evolution and ancestry of myrmecoid clades. Dated phylogeny produced by BEAST 2 and 8 calibration points under a Bayesian Lognormal Relaxed clock, with separate clocks for nuclear and mitochondrial data. Myrmecoid clades are highlighted in orange. Clade numbers corresponding to those in Figure 2. The APL, F, O (Oxypodini) and A (Aleocharini) clades are highlighted.





