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RESEARCH ARTICLE

Biology of the relict fungus-farming ant *Apterostigma megacephala* Lattke, including descriptions of the male, gyne, and larva

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Abstract Fungus-farming "attine" ant agriculture consists of five distinct agricultural systems characterized by a remarkable symbiont fidelity in which five phylogenetic groups of ants faithfully cultivate five phylogenetic groups of fungi. Across-system garden switching experiments result in colony decline and death, indicating that attine ant-fungus symbiont fidelity is enforced by poorly understood biological constraints. The most dramatic violation of this pattern of symbiont fidelity occurs in the relict species *Apterostigma megacephala*, the only lower-attine ant known to cultivate a higher-attine fungus. *Apterostigma megacephala* is the sole surviving representative of an ancient lineage that diverged from all other *Apterostigma* fungus-farming ants ~39 million years ago, yet it cultivates *Leucoagaricus gongylophorus*, a highly domesticated fungal species that originated in the gardens of the recently evolved leaf-cutting ants 8–11 million years ago. Understanding the biology of *A. megacephala*, therefore, may provide important clues about the biological mechanisms that constrain the otherwise seemingly obligate ant-fungus associations that characterize attine ant agriculture. Here, based on field work in the Floresta Nacional de Carajás in the state of Pará in Brazil, we report the previously unknown biology of *A. megacephala*, including nest architecture, colony demography, foraging behavior, and the morphologies of the previously undescribed gyne, male, and larva.

Keywords Attini · Coevolution · *Leucoagaricus gongylophorus* · Nest architecture · Symbiosis

Introduction

Fungus-farming ants (subfamily Myrmicinae, tribe Attini, subtribe Attina; henceforth "attine" ants) are a monophyletic group of exclusively New World ants descended from a common ancestor that transitioned from hunting-gathering to agriculture ~55–65 million years ago (Ješovnik et al. 2016; Mueller et al. 2001; Nygaard et al. 2016; Rabeling et al. 2011; Schultz and Brady 2008; Ward et al. 2015; Wilson 1971). Since that origin, attine ant agriculture has diversified into five known agricultural systems characterized by nearly monolithic patterns of symbiont fidelity between broad phylogenetic groups of attine ants, fungal cultivars (Basidiomycota: Agaricales: Agaricaceae [*Leucoagaricus* and *Leucocoprinus*] and Pterulaceae), and *Escovopsis* fungal parasites (Ascomycota: Pezizomycotina: asexual Hypocreales). These five agricultural systems include: (i) *lower agriculture*, the ancestral system, in which a diverse, paraphyletic group of ants cultivates a diverse group of agaricaceous fungi, which, so far as is

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known, are facultative symbionts, capable of living freely apart from the ants; (ii) *yeast agriculture*, a derived form of lower agriculture in which ants in a derived clade in the *Cyphomyrmex rimosus* group cultivate a derived clade of agaricaceous fungi that are also capable of living freely apart from the ants; (iii) *coral fungus agriculture*, in which ants in a derived clade in the *Apterostigma pilosum* group cultivate a clade of fungi in the Pterulaceae, and in which the facultative/obligate status of the fungal partner remains unknown; (iv) *generalized higher agriculture*, in which two genera of "higher" attine ants cultivate a derived clade of agaricaceous fungi that are specialized, obligate symbionts, incapable of life without the ants; and (v) *leaf-cutter agriculture*, a derived form of higher agriculture, in which most ant species in the ecologically dominant genera *Acromyrmex* and *Atta* cultivate a single, highly derived higher-attine fungal species, *Leucoagaricus gongylophorus* (Aylward et al. 2013; De Fine Licht et al. 2013; Kooij et al. 2015; Nygaard et al. 2016). In contrast to most of their fungal cultivars, all attine ants are obligate symbionts, incapable of surviving without their fungus gardens. Seal et al. (2012) and Seal and Mueller (2014) showed that, when the generalized higher-attine ant species *Trachymyrmex septentrionalis* is forced to cultivate the leaf-cutter fungus *L. gongylophorus*, colonies suffer no ill effects in the short term (i.e., 6 weeks), but over longer periods of time colonies decline and die, indicating that cultivar switching across agricultural systems is associated with significant fitness costs.

Although attine ant and fungal cultivar species are remarkably faithful at the level of the five agricultural systems, *within* each of the agricultural systems, associations between species are apparently uncorrelated with phylogeny, i.e., within a given agricultural system, an ant species may be associated with multiple distantly related fungal cultivar species and vice versa. Based on this lack of congruence across symbiont phylogenies within agricultural systems, the prevailing paradigm for attine symbiotic evolution is one of diffuse coevolution (Mikheyev et al. 2010; Mikheyev et al. 2007; Mikheyev et al. 2008; Mueller et al. 1998). Contrary to this paradigm, however, an intensive study of ant-fungus associations in the *Cyphomyrmex wheeleri* clade found that ant-fungus species pairs have persisted for 5–10 million years and that rare switches to new cultivars are correlated with and may even have caused ant speciation events (Mehdiabadi et al. 2012), suggesting that diffuse coevolution may apply to some but not all attine agricultural symbioses.

Prior to 2008, *Apterostigma megacephala* was known from only four specimens collected in 1988, 1989, 1990, and 1992 on the western peripheries of the Amazon basin in southern Peru and eastern Andean Colombia (Lattke 1999; Schultz et al. 2015). Initial morphological studies suggested that *A. megacephala* was a relict species, retaining a combination of traits more primitive than those found in *Apterostigma* species fossilized in Dominican amber (Lattke 1999; Schultz 2007). Based on its hypothesized phylogenetic position, *A. megacephala* was fully expected to cultivate a lower-attine fungus (Schultz 2007). In 2008, da Silva and Brandão discovered a single specimen in a litter sample taken at the opposite end of the Amazon basin in the Floresta Nacional de Carajás (FLONA-Carajás) in Pará, Brazil, ~800 km south of Belém. Based on this information, two of us (JS-C and CTL) discovered a population of *A. megacephala* in Carajás in 2009, leading to subsequent field investigations in 2010, 2011, and 2014.

Based on phylogenetic analyses of molecular data from ants and fungi obtained in those field investigations, Schultz et al. (2015) reported that *A. megacephala* is the very distant sister taxon of the remainder of the genus, the sole surviving representative of an ancient lineage that diverged ~39 million years ago from the ancestor of all other known extant and extinct *Apterostigma* species. It is, in fact, the oldest known single-species (i.e., relict) fungus-farming ant lineage. Surprisingly, based on molecular data from its fungal cultivar, *A. megacephala* is the only lower-attine ant known to cultivate a higher-attine fungus. Even more surprisingly, *A. megacephala* cultivates the most highly derived and recently evolved higher-attine fungal cultivar, *Leucoagaricus gongylophorus*, the species cultivated by most leaf-cutter ants (Schultz et al. 2015). The cultivation of *L. gongylophorus* by *A. megacephala* in eastern Amazonian Brazil can only be due to a horizontal transfer event because *L. gongylophorus* is estimated to have originated ~8–10 million years ago (Nygaard et al. 2016). The ability of *A. megacephala* to cultivate *L. gongylophorus* is likely to be important for understanding the currently unknown biological constraints enforcing symbiont fidelity at the level of the five attine agricultural systems, in particular the constraints preventing lower-attine ants from cultivating higher-attine fungi.

Here, based on data and specimens gathered during the previously mentioned 2010, 2011, and 2014 field studies, we describe the previously unknown reproductive forms (i.e., males and gynes), larva, nest architecture, and other aspects of the biology of this remarkable fungus-farming ant species.

Materials and methods

Study area, field observations, and nest excavations

Field work was conducted at Floresta Nacional de Carajás (FLONA-Carajás) located in southern Pará state, Brazil, from March 30 to April 07, 2010; from September 08 to September 13, 2011; and from October 01 to October 05,

2014. The FLONA-Carajás encompasses an area of approximately 400,000 hectares (Gumier-Costa and Sperber 2009) located on a large plateau covered by a mixture of semi-deciduous rain forest and, in areas of rock outcrops, scrub forest. The plateau lies within the Central Amazon province of the larger Amazonian Craton, an Archean (2.75 Ga) tectonic area of >4.4 million km^2 situated in northern South America (Cordani et al. 2009; Teixeira et al. 2007). It is estimated that the Serra dos Carajás, of which FLONA-Carajás is a part, contains the largest deposit of iron ore in the World (Cordani et al. 2009; Teixeira et al. 2007; Trendall et al. 1998). Although FLONA-Carajás is an area of environmental conservation protected by the Brazilian government, it is extensively exploited for iron ore, copper, gold, manganese, and nickel, and it is surrounded by disturbed habitat consisting of lower-elevation pastures and plantations. A detailed description of the site can be found in Gumier-Costa and Sperber (2009).

Nests of *Apterostigma megacephala* were located by generously spreading Cream of Rice® cereal on the leaf litter, then following bait-laden foragers returning to their nest entrances. Eighteen separate nests were located at the boundary of a small patch of secondary forest and a grass-covered clearing adjacent to a paved service road, some in the forest and some in the grass, at the Parque Zoobotânico de Carajás (06.06326°S 50.05774W ± 5 m, elevation 658 m). Excavations were conducted following Sosa-Calvo et al. (2015) and Schultz (1993). When a nest chamber was encountered, the fungus garden was carefully removed using a flame-sterilized spoon and/or forceps. Both ants and fungus garden were then maintained alive by placing them in plastic nest boxes with plaster-lined floors that were kept saturated with water. When the ants had reconstructed their fungus garden and removed any soil or debris that had become mixed with the garden during excavation, series of workers, alate gynes, and males, as well as portions of the gardens, were preserved in 2-ml Sartstedt micro tubes (Sartstedt Inc., Newton, NC, U.S.A.) filled with 95% ethanol. All colonies collected in the 2010 field study were preserved in 95% ethanol after the ants reconstituted their gardens, whereas colonies collected in the 2011 and 2014 field studies were kept alive in plastic containers. Following excavation, chamber measurements were recorded.

Morphology and standard measurements and indices

Images of the worker, gyne, and male were generated in the SI NMNH Ant Lab using a JVC KY-F75U digital camera mounted on a Leica Z16 APO stereomicroscope attached to a Dell Optiplex GX620 computer. Composite images were assembled using Auto-Montage Pro® (Version 5.03.0061 BETA) software (Synoptics Ltd.). Two worker prepupae (i.e., post-feeding last-instar larvae) were dehydrated sequentially through a series of ethanol concentrations to 100% absolute and then critical point dried in a Balzers CPD-030 using liquid CO_2 at the scanning electron microscopy (SEM) lab in the SI NMNH Laboratories of Analytical Biology. Once the ethanol was replaced with CO_2, the samples were slowly heated to the critical point, slowly depressurized back to atmospheric pressure, dried, and mounted on aluminum stubs. The two prepared larvae were sputter-coated with 60:40 wt% gold:palladium alloy on a Cressington Scientific 108 auto/SE sputter coater to a thickness of 20–25 nm. Scanning Electron Micrographs (SEMs) of these specimens were generated using a Philips XL-30 ESEM with a Lanthanum Hexaboride (LaB6) source and with a backscatter detector.

For examination of the fungal cultivar of *A. megacephala*, mycelia were removed from the fungus garden using sterilized needles and tweezed apart to prepare wet mounts. Two types of slide mounts were prepared: (i) fresh mounts with sterilized water and (ii) fresh mounts with lactoglycerol cotton blue as the mounting fluid. Gongylidia were examined under a compound microscope (Leica, DM750) using bright field for stained gongylidia and phase contrast for unstained gongylidia. Images were generated with a Leica ICC50-HD camera. All images were edited using Photoshop CS5® (Version 12.0) (Adobe Inc.).

Measurements are represented in mm. Indices, abbreviations, and morphological terminology follow Gauld and Bolton (1988); Ješovnik et al. (2013); Klingenberg and Brandão (2009); Rabeling et al. (2007a); (2015); Serna and Mackay (2010); Sosa-Calvo and Schultz (2010); Sosa-Calvo et al. (2013); and literature cited therein, with modifications where noted. Characters and terminology used in the description of the larvae are based on Schultz and Meier (1995).

Specimen deposition

Specimens examined and specimen vouchers are deposited in the following institutions:

CRC	C. Rabeling Collection, Arizona State University, Tempe, AZ, U.S.A.
DZUP	Coleção Entomológica "Padre Jesus Santiago Moure", Departamento de Zoologia, Universidade Federal do Paraná, Curitiba, Paraná, Brazil.
IAvHC	Departamento de Entomología, Instituto "Alexander von Humboldt," Villa de Leyva, Boyacá, Colombia.
ICN	Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, Colombia.

INPA	Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas, Brazil.
MCZC	Museum of Comparative Zoology, Harvard University, Cambridge, MA, U.S.A.
MPEG	Museu Paraense Emilio Goeldi, Belem, Pará, Brazil.
MZSP	Museu de Zoologia, Universidade de São Paulo, São Paulo, Brazil.
MBC–UFU	Universidade Federal de Uberlândia, Minas Gerais, Brazil.
USNM	National Museum of Natural History, Washington, DC., U.S.A.

Results

Taxonomic treatment

Apterostigma megacephala Lattke 1999

Figures 1, 2, 3, 4, 5 and 6. *Apterostigma megacephala* Lattke 1999: 2, Figs. 1–4. Holotype worker: PERU, Madre de Dios, Cuzco Amazónico, 15 km NE Puerto Maldonado, Río Tambopata, 200 m, 21.vi.89, (SP Cover, JE Tobin), CA-357, Plot IE 24 [MCZC] (examined).

Geographic range Brazil (states of Pará and Mato Grosso), Colombia, and Peru; from 200 to 650 m elevation, lowland mesic forest to semi-deciduous Amazonian rain forest (Fig. 7).

Description, gyne (Fig. 2a–c)

Measurements EL 0.37–0.50; FLD 1.02–1.09; GL 2.11–2.52; HL 1.77–1.95; HFL 2.88–2.98; HTL 1.89–2.04; HW 1.64–1.71; ML 1.09–1.34; PL 0.93–1.12; PPL 0.71–0.82; PPW 0.83–0.95; SL 1.67–1.83; TL 9.49–10.7; WL 2.82–3.02; CI 88–93; MI 59–69; OI 22–29; PPI 111–133; RFLI1 54–60; RFLI2 61–64; SI 102–107 ($n = 6$).

Similar to worker except for differences typical of caste. *Head*: subquadrate; in full-face view, excluding mandibles, nearly as broad as long; cephalic corners convex; cephalic margin almost straight and medially weakly emarginate. Mandibles triangular; inner (masticatory) margin of mandibles 8–9-toothed, apical and subapical teeth larger than rest; outer margin of mandibles slightly sinuous; dorsum of mandibles longitudinally striate and with appressed hairs. Clypeal apron present, shiny and horizontally striate (shared with members of *A. pilosum* group), broadly convex and interrupted medially by conspicuous projection; clypeal apron with long, thick, unpaired median setae; posterior margin of clypeus extending between frontal lobes. In lateral view, eyes large with ~21 ommatidia in longest row, surmounted on subconical tubercle as in worker. Frontal carina present but inconspicuous, blending with rugae present on head. Dorsum of head and frontal lobes rugose. Median ocellus round (width 0.073, length 0.072 mm). In lateral view, occipital collar conspicuous and subquadrate. Antennal scape surpassing occipital corner by ~0.62 mm; dorsum of antennal scape rugulose, leading edge of antennal scape with erect simple hairs, posterior margin with suberect or subdecumbent simple hairs.

Mesosoma Pronotum with reduced humeral denticles or tubercles, and pair of low lateral tubercles; dorsum of pronotum rugose; inferior corner of pronotum forming an obtuse angle, lacking tooth or spine. In lateral view, anterior margin of mesoscutum angulate; parapsidal lines conspicuous. In dorsal view, notauli as deep grooves, anterior margin of mesoscutum strongly concave. In dorsal view, mesoscutum rugose. Deep and conspicuous pits in laterodorsal margin of scutellum, connected by shallow groove. Posterior margin of scutellum with pair of blunt tubercles. In lateral view, propodeum armed with long triangular spines.

Metasoma Node of petiole vestigial, very low; in profile, rounded; in dorsal view, node with lateral shallow rugae, remainder smooth; antero-ventral portion of petiole with inconspicuous process (tooth). Postpetiole broader than long (PPL 0.71–0.82, PPW 0.83–0.95, PPI 111–133), and weakly rugose dorsally. Ventral margin of postpetiole with anterior and posterior tooth-like projections. First gastral tergite and sternite strongly rugo-reticulate, each cell with a thick suberect simple hair.

Head and dorsum of mesonotum with long simple erect hairs. Eyes, dorsum of pronotum, and sides of mesosoma with spatulate (i.e., flattened at the tip) hairs. Legs with short, subdecumbent or decumbent hairs.

Wings Forewing (length 5.7 mm) smoky, with five closed cells. Area below the marginal cell with a patch (Fig. 4d), darker anteriorly and lighter apically, homologous to the fenestrae present in forewings of other Paleoattini; and as in other Paeoattini, this patch is absent in the males (Fig. 4e). Hindwing (length 4.1 mm) with three closed cells and nine hamuli (Fig. 4d).

Palpal formula: 4,2 (Fig. 4c).

Description, male (Fig. 3a–c)

Measurements EL 0.41–0.69; FLD 0.43–0.47; GL 1.35–2.05; HL 1.00–1.24; HFL 2.11–2.39; HTL 1.66–1.83; HW 0.94–1.15; ML 0.40–0.57; PL 0.58–0.97; PPL

Fig. 1 Worker of *Apterostigma megacephala*, **a** full-face view, **b** dorsal view, **c** lateral profile

0.47–0.59; PPW 0.61–0.69; SL 0.42–0.53; TL 5.98–7.72; WL 1.88–2.45; CI 83–115; MI 34–48; OI 36–64; PPI 116–142; RFLI1 37–47; RFLI2 41–46; SI 40–48 ($n = 7$).

Head: Dorsum rugose. In full-face view, cephalic margin convex. Inner margin of mandibles without teeth; dorsum of mandibles strongly reticulate and covered with appressed hairs. Clypeal apron thin and dorsally reticulate. Median clypeal seta long and thick (length 0.15 mm), originating at the edge between the clypeal apron and the clypeus. Anterior tentorial pits conspicuous, each located in the middle of a deep groove (grooves connected by shallow depression forming posterior clypeal margin). In lateral view, frontal lobes extending forward and, in full-face view, failing to cover antennal insertions. Antennal scape thick and shorter (SL 0.42–0.53) than the length of funicular segments I–III combined (1.03–1.21 mm); dorsum of antennal scape with long, suberect simple hairs. Median ocelli rounded (width 0.08, length 0.08 mm). In lateral view, lacking hypostomal teeth.

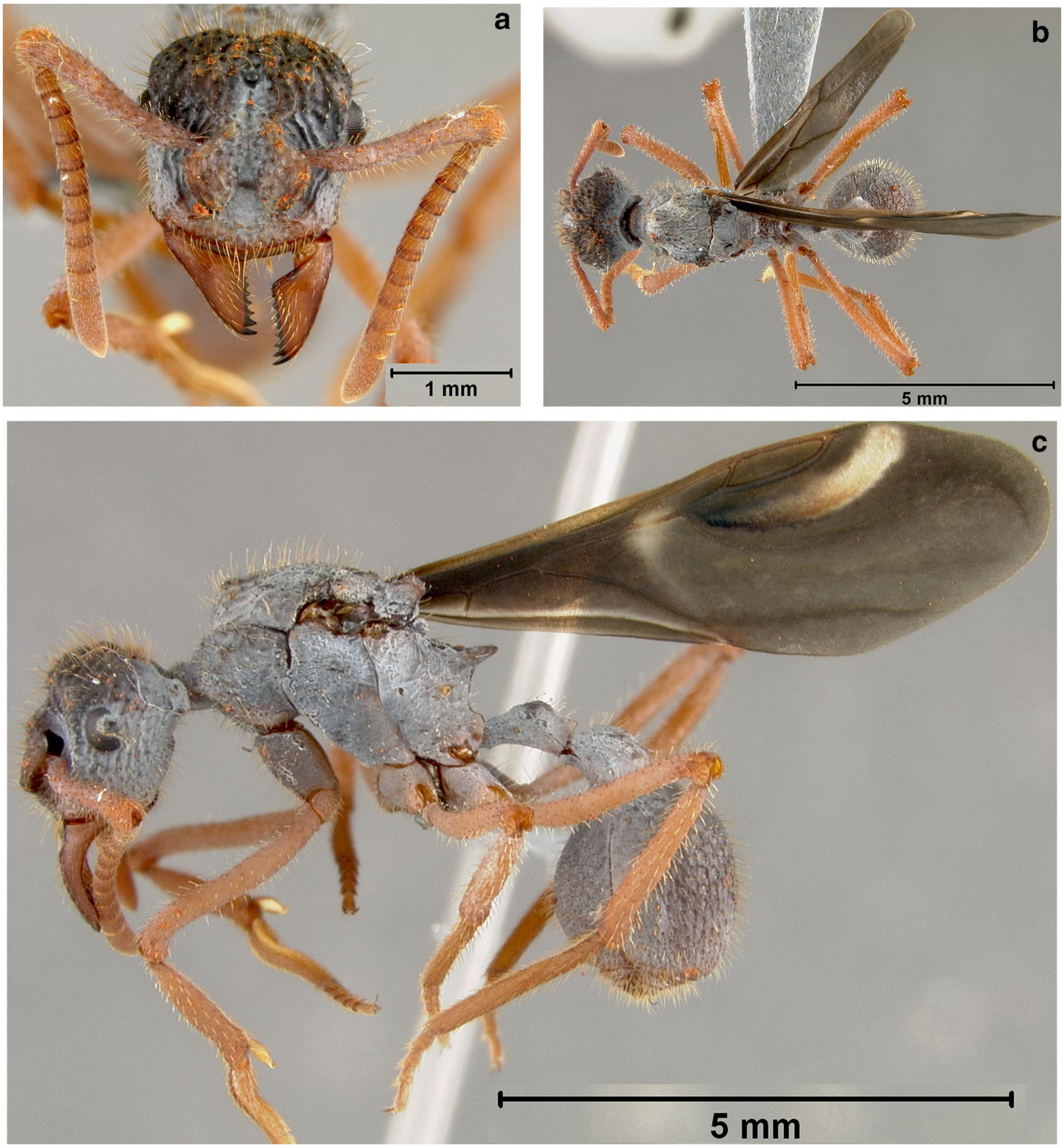

Fig. 2 Alate queen of *Apterostigma megacephala*, **a** full-face view, **b** dorsal view, **c** lateral profile

Mesosoma Pronotum lacking humeral tubercles; lateral tubercles of pronotum reduced to raised ridges. Mesonotum like that of gyne, but with posterior medial groove (seen in dorsal view). Scutellum like that of gyne. Metanotum small and smooth. Katepisternum separated from anepisternum by deep, conspicuous, groove. In profile, midposterior portion of mesonotum and scutellum somewhat raised; lateral margins smooth and somewhat shining. Dorsum of propodeum smooth and with pair of lateral carina connecting pair of long, thick, triangular propodeal spines. Declivity of propodeum smooth with thin lateral carina. Metapleural gland absent, prominent carina present instead.

Metasoma In profile, node of petiole low and convex; in dorsal view, node of petiole with thin lateral carinae and weakly concave medially. Petiolar spiracles positioned approximately equidistant between anterior and posterior ends of the petiole, conspicuously projecting and directed backwards. Anterior ventral process small, inconspicuous. Postpetiole broader than long (PPL 0.47–0.59, PPW 0.61–0.69, PPI 116–142); in lateral view, postpetiole dorsally convex; ventral margin of postpetiole with pair of conspicuous, almost equally sized, triangular teeth, one located anteriorly, another posteriorly; dorsum of postpetiole rugose. Dorsum of gaster weakly rugo-reticulate. First

Fig. 3 Male of *Apterostigma megacephala*, **a** full-face view, **b** dorsal view, **c** lateral profile

gastral tergum with low lateral carinae that extend longitudinally almost entire length of segment; first gastral tergum covered with suberect simple hairs.

Color: Body dark brown, antennae ferrugineous, legs light ferrugineous to yellowish. Wings: Forewing (length 3.35 mm) with five closed cells. Hindwing (length 2.52 mm) with one closed cell and 7–9 hamuli (Fig. 4e). Both wings are slightly darker anteriorly than posteriorly, and covered with minute pubescence. The forewing of the male lacking the fenestra underneath marginal cell, differing from the forewing of the gyne.

Palpal formula: 4,2 (Fig. 4b).

Genitalia (Figs. 4f–i, 5a–e): sternum of abdominal segment IX posteriorly rounded, with a thin spiculum anteriorly (Fig. 5a); parameres long, cup-like; telomeres narrow, medially curved, and apically rounded (Figs. 4f, g, i, 5c, e); in ventral view, outer portion of telomeres with

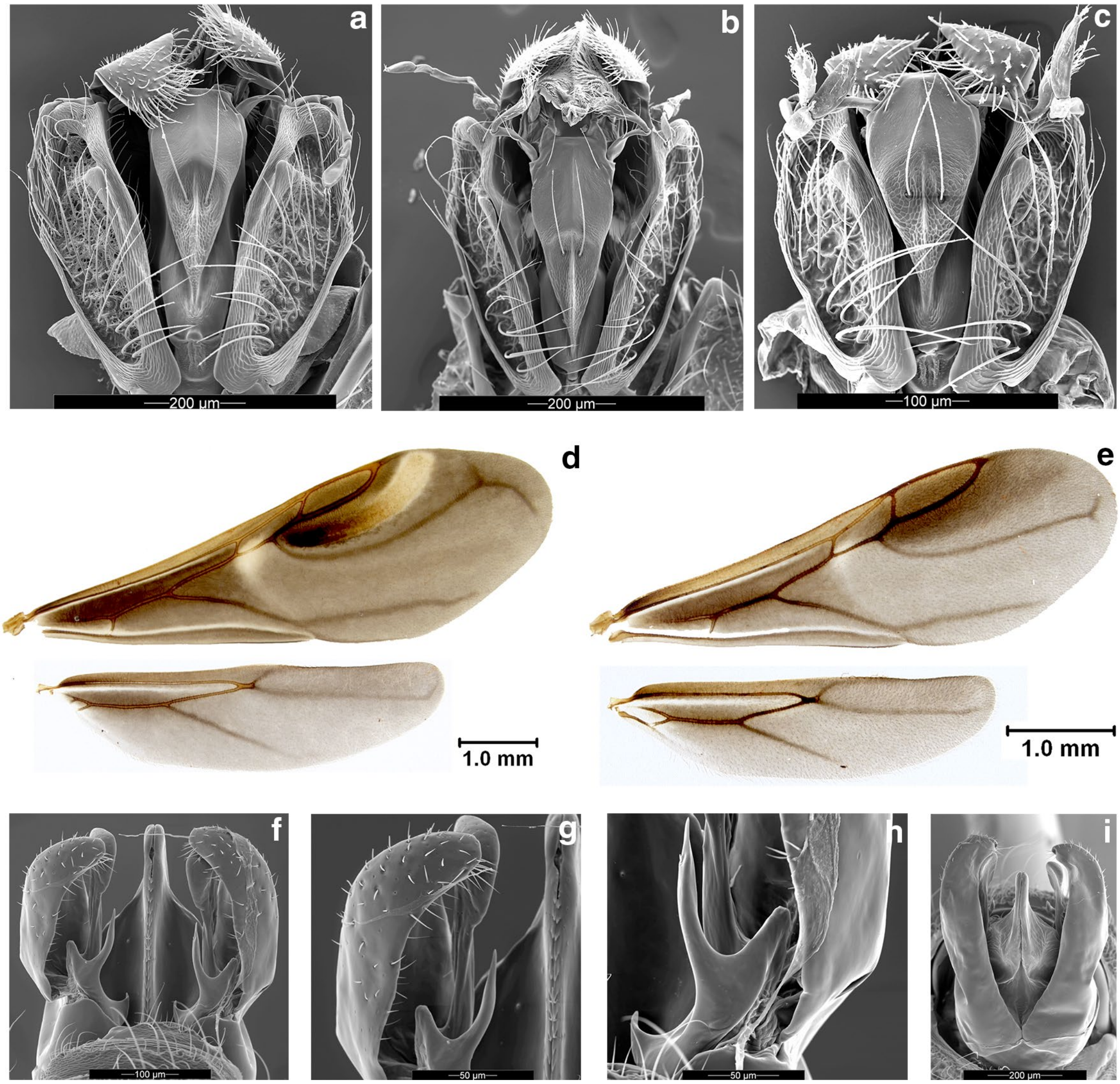

Fig. 4 Internal mouthparts of **a** worker, **b** male, and **c** queen, illustrating the 4,2 palpal formula. Fore and hind wings of **d** queen and **e** male. Male genitalia, **f** ventral view of genital capsule, **g** close-up of paramere and volsella (cuspis and digitus), **h** close-up of digitus, composed of two processes, one needle-like and longer, the other blunt and shorter, **i** dorsal view of genital capsule

short simple hairs, inner portion with somewhat larger simple hairs (Fig. 4f, g); in dorsal view, parameres glabrous except for apical portion (Fig. 4i). In ventral view, volsella with digitus club-like (broader at apex) and with sparse foveae (Figs. 4f, g, 5c, d); cuspis with two processes, a short tooth dorsally (2) and a needle-like, long process ventrally (1), approximately half the height of the digitus (Figs. 4f–h, 5c, d). There is, however, uncertainty regarding the homologies of these two structures; specifically, it is unclear if both are part of the cuspis. Base of volsella with an additional tooth-like process [proximal basivolsellar process (3)] (Fig. 5c, d); valviceps of penisvalve, ventrally, with 19–20 denticles (Figs. 4f, g, 5b).

Description, larva (Fig. 6a–f)

Based on two worker prepupae from nest collection JSC110910-03. Profile "attoid" *sensu* Wheeler and Wheeler (1976), i.e., with a moderately curved, ventrally shortened profile (Fig. 6a); in this regard differing from most other studied *Apterostigma* species, including *A. auriculatum*, which have in common extremely curved profiles in which the ventral region is so shortened that the mouthparts are nearly in contact with the anus. As in all other Attina, thoracic-abdominal articulation absent, thoracic intersegmental constrictions superficial, deep lateral depressions associated with abdominal spiracles absent, and leg vestiges present as open slits. As in most other Attina, dorsal and lateral body surfaces devoid of setae. Ventral hair

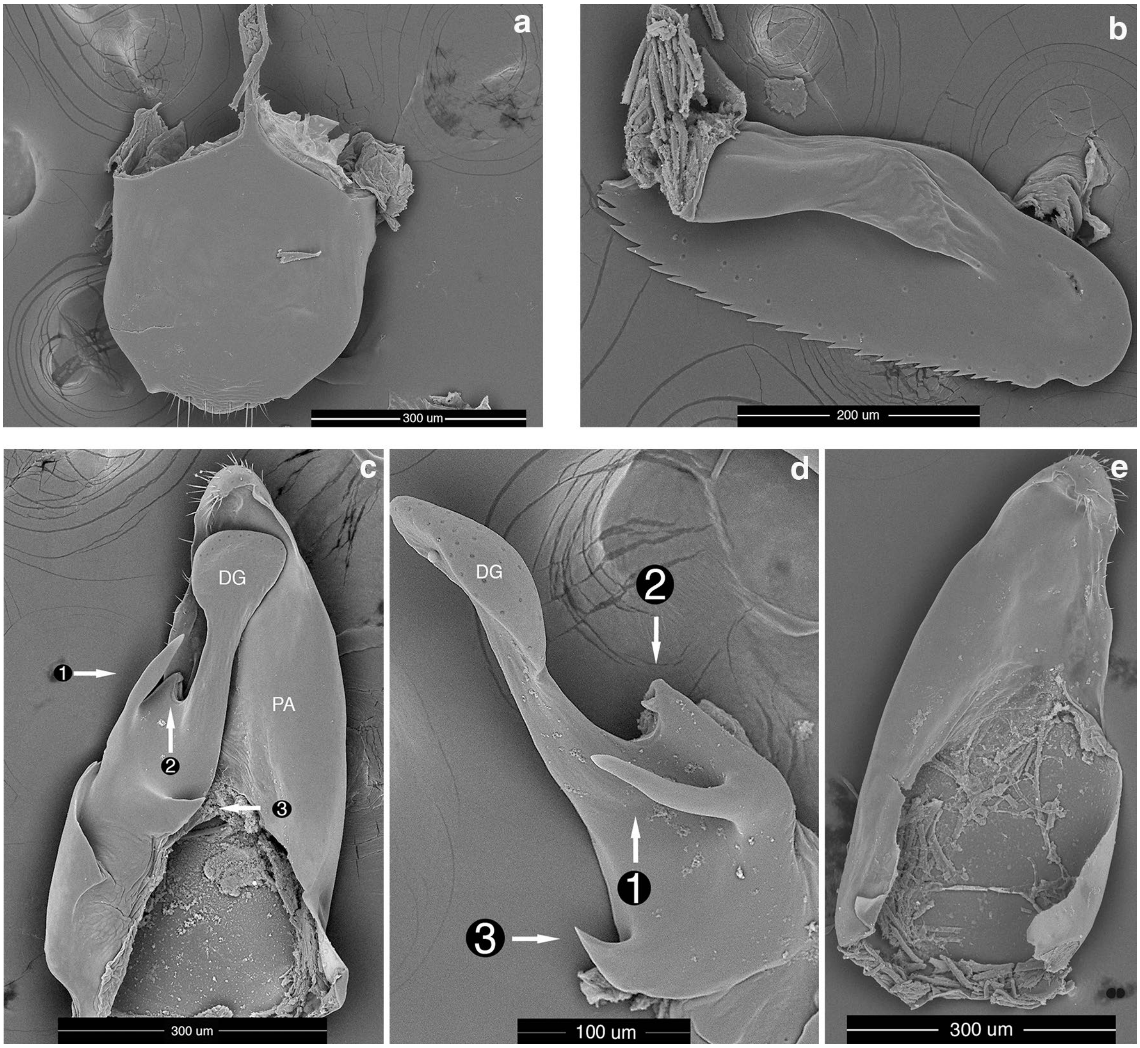

Fig. 5 Male genitalia of *Apterostigma megacephala*, **a** abdominal sternum IX, **b** penisvalve, **c** parameres and volsella; **d** volsella; **e** paramere. *DG* digitus, *PA* paramere

"feeding organ" on first thoracic segment present, but ventromedian lobe on the first thoracic segment, shared by all studied *Apterostigma* species except *A. dentigerum*, absent (Fig. 6b). Spinules on thoracic segment I confined to venter and mostly serrate/denticulate. Thoracic and abdominal ventromedian bosses absent. Ventral setae present on all thoracic and abdominal segments. As in other Paleoattini and in *Mycetarotes* species, genal lobes absent. Head with three supra-antennal setae overarching each antenna, as in other studied *Apterostigma* species; with two supraclypeal setae, a feature shared with *Apterostigma auriculatum* but absent in *A. pilosum* group species; and with five setae on each gena, more than in other studied *Apterostigma* species, which have three or four per gena. Two clypeal setae present. Two setae of uncertain homology present, one adjacent to each maxilla (Fig. 6c). Papilliform spinules present on the head on the genae and vertex, absent from the occipital area, and very sparse on the clypeus. As in other fungus-farming ants, anal setae arranged to form a basket or brush, apparently for the purpose of holding a drop of anal liquid (Lopes et al. 2005; Schneider et al. 2000), most closely conforming to the "*Pheidole* pattern" (Schultz and Meier 1995), with six setae ventrally and eight setae dorsally. Long and abundant ventral setae occurring on adjacent abdominal segments (Fig. 6d) may also play a role in forming the anal brush or, possibly, a food anchor, although it is difficult to imagine how the larva could be capable of flexing its head far enough posterad to feed on any fungal hyphae positioned in this region. Ventral anal lip absent. Labrum monolobate, narrow, and bulging, a synapomorphy for the fungus-farming ants; at least four anterior labral setae present, setiform. Mandibles fleshy and subconical as in other Attina, but unusually long and protruding and with the tips noticeably curved anterad (Fig. 6b); differing

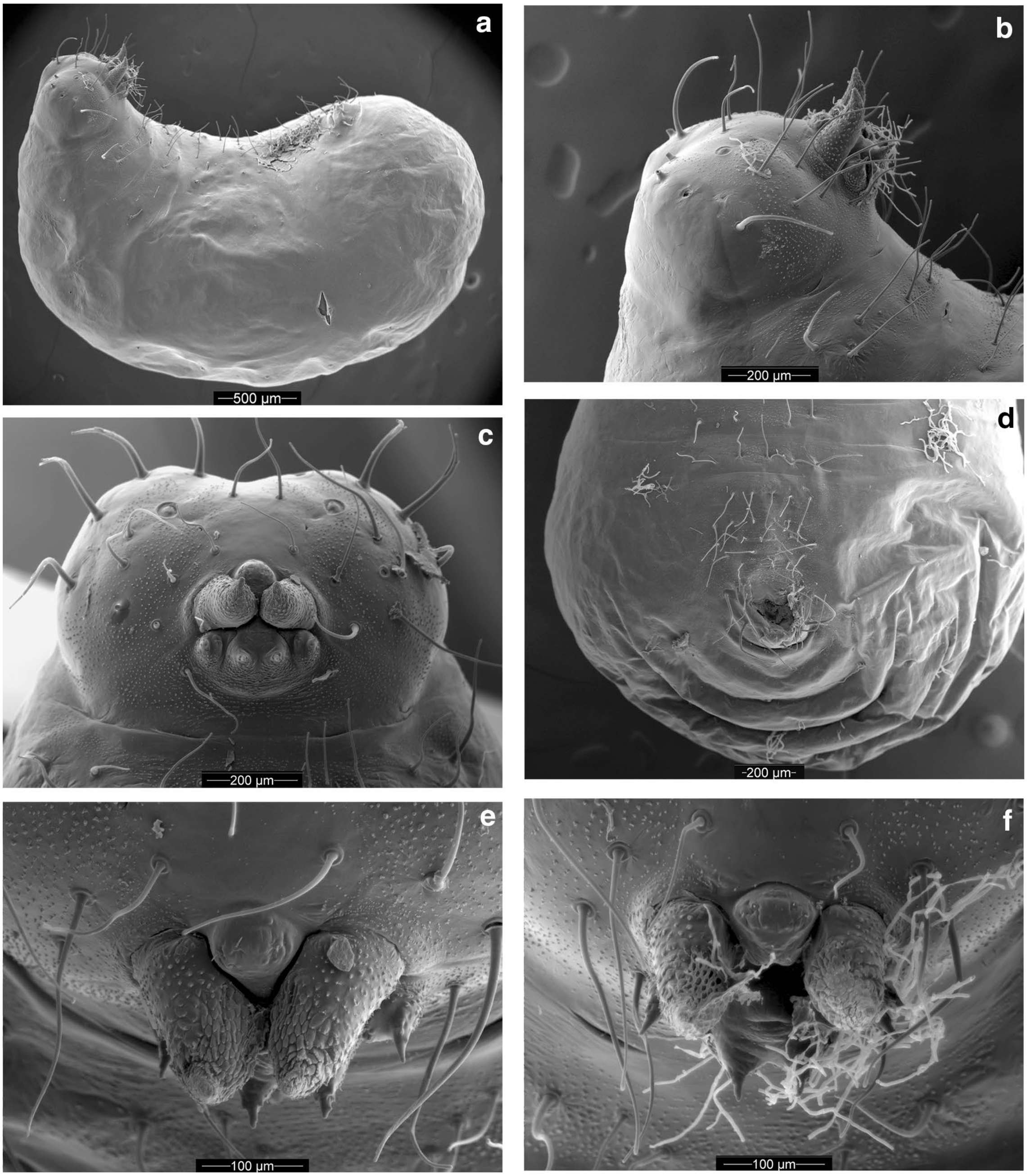

Fig. 6 Larva of *Apterostigma megacephala*, **a** lateral profile, **b** head in lateral view, **c** head in full-face view, **d** anal region in ventral view, **e** mandibles in dorsal view, **f** mandibles in dorsal view with mycelium present

from all other known *Apterostigma* species larvae, which have very short mandibles. A distinct, undivided apical mandibular tooth and no subapical teeth; spinules densely distributed on the distal half of the mandibles, particularly medially; less densely in the proximal half (Fig. 6e); differing markedly from *A. auriculatum*, which has no spinules on the mandible, and from most studied *A. pilosum* group species (including *A. dentigerum* and *A. mayri*, but not *A. collare*), which lack spinules on the mandibular apex. Mandibular gnathobases absent (present only in *Mycocepurus* species). Basal portions of maxillae fused with head capsule. As in other Paleoattini, maxillary palp and galea closely approximated (Fig. 6c). Galea reduced, as in other attines. Maxillary palp strikingly digitiform (Fig. 6e), in this way differing from all other *Apterostigma* species, for which a reduced maxillary palp is a synapomorphy, but resembling the condition in other attines, particularly other paleoattines. Maxillary accessory palpal sensillum

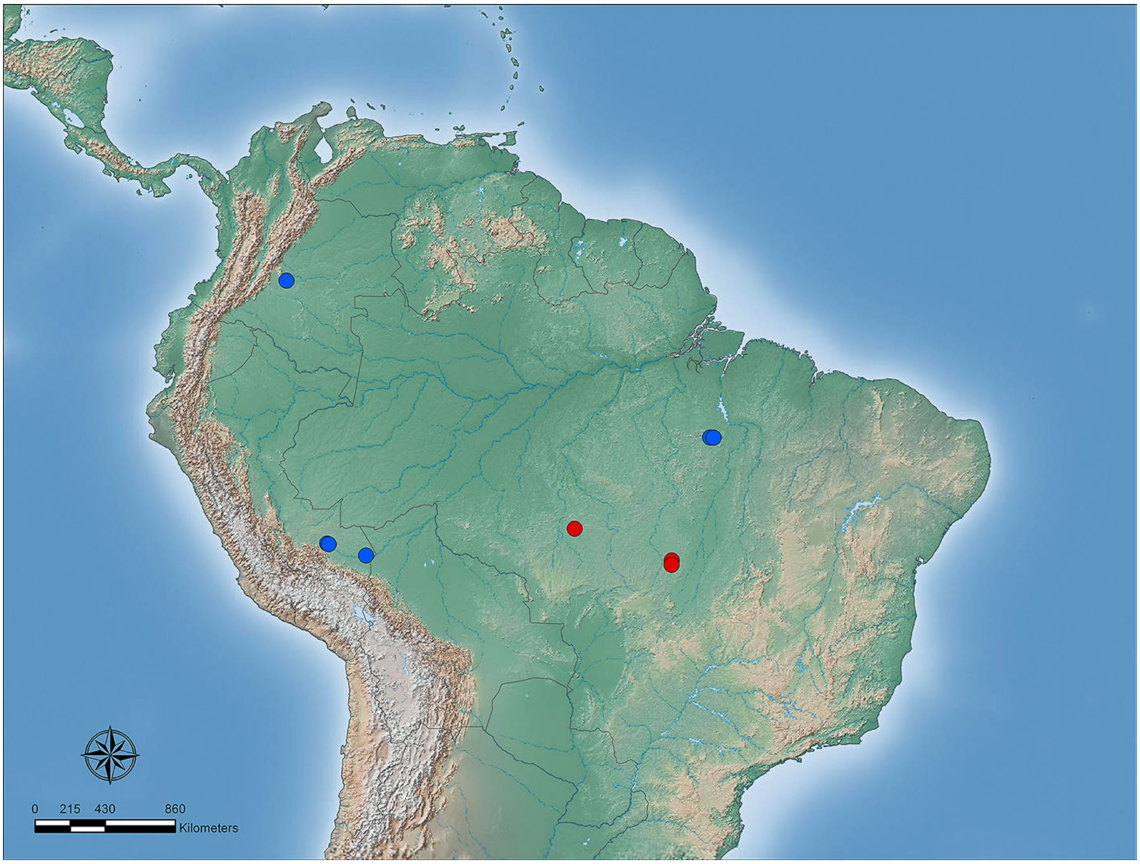

Fig. 7 Known distribution of *Apterostigma megacephala* in (*blue circles*) Serrania de La Macarena, Colombia; Madre de Dios, Peru; and Floresta Nacional de Carajás, Pará, Brazil; and (*red circles*, new records) Mato Grosso, (Canarana and Juara), Brazil. (Color figure online)

not observed. Maxillary setae apparently absent unless the two setae of uncertain homology mentioned above, each adjacent to a maxilla (Fig. 6c), are homologous with those occurring laterally on the maxillae of *Mycocepurus* species, one on each maxilla in *M. smithii* and two on each maxilla in *M. goeldii* (Schultz and Meier 1995). Labium very unusual for an attine, bilobed, and strongly protruding and with long, digitiform labial palps (Fig. 6f), most similar to the form observed in some *Myrmicocrypta* and *Mycocepurus* species rather than in other *Apterostigma* species. Labial spinules absent or nearly absent both basad and distad of the sericteries, suggesting that, unlike in other *Apterostigma* species, the mandibles are the primary organs employed in fungal maceration. Hypopharyngeal spinules unobservable.

Material examined

BRAZIL: Pará, Parauapebas, Floresta Nacional de Carajás, Parque Zoobotánico; 658 m; 50.05777°W, 6.06323°S; 13.ii.2009, (*CT Lopes & J Sosa-Calvo*), forest edge, worker foraging on leaf litter, JSC090213-01; same locality as previous entry, but 1.iv.2010, (*TR Schultz, J Sosa-Calvo, CT Lopes*), nest series, forest edge, in ground, fungus garden, TRS100401-09; TRS100401-10; same locality as previous entry, but 672 m, 50.05774°W, 6.06321°S, 2.iv.2010, (*J Sosa-Calvo, TR Schultz, CT Lopes*), nest series, forest edge, in ground, fungus garden, TRS100402-02; same locality as previous entry, but 663 m, 50.05762°W, 6.06313°S, 2.iv.2010, (*CT Lopes, TR Schultz, J Sosa-Calvo*), nest series, forest edge, in ground, fungus garden, TRS100402-03; same locality as previous entry, but 650 m, 50.05781°W, 6.06325°S, 2.iv.2010, (*J. Sosa-Calvo, TR Schultz, CT Lopes*), nest series, 2° forest edge, in soil, JSC100402-11; same as previous entry, but 659 m, 50.05758°W, 6.0632°S, 2.iv.2010, (*J. Sosa-Calvo, TR Schultz, CT Lopes*), nest series, 2° forest edge, under ground, JSC100402-12; same as previous entry, but 641 m, 50.05785°W, 6.06321°S, 10.ix.2011, (*J. Sosa-Calvo, CT Lopes*), nest series, forest edge, under ground, [=CTL110910-01] JSC110910-01; JSC110910-04; JSC110910-05; same as previous entry, but 676 m, 50.05776°W, 6.06321°S, 10.ix.2011, (*J. Sosa-Calvo, CT Lopes*), nest series, 2° forest edge, in soil, JSC110910-03;

JSC110911-16; JSC110912-02; CTL110911-01; same as previous entry, but 12.ix.2011, nest series, 2° forest edge, leaf litter/soil interface, JSC110912-03; same as previous entry, but 632 m, 50.05761°W, 6.06321°S, 2.x.2014, (*J Sosa-Calvo, TR Schultz, A Ješovnik, CT Lopes*), nest series, forest edge, under ground, JSC141002-04; JSC141002-06; TRS141002-02; Mato Grosso, ~70 km from Canarana, 390 m, 52.38068°W, 13.07660°S, vii.2013, (*L Paolucci & ML Bicalho*); Juara, [350 m], 57.7184861°W 11.0902028°S, ii.2015, (*RCL Santos*) [not examined].

Natural history

Macrohabitat and foraging behavior

Specimens of *Apterostigma megacephala* have been collected in lowland (from 200 to 350 m elevation) mesic forests of Colombia and Peru (Lattke 1999). In the Floresta Nacional de Carajás (FLONA-Carajás) in Pará, Brazil, we collected this species at relatively higher elevation (650 m) in semi-deciduous, Amazonian rain forest. Recently, five workers of *A. megacephala* were collected in Winkler leaf-litter samples in patches of lowland forest (390 m) in Mato Grosso state, Brazil (J Chaul, pers. comm.).

Field observations suggest that workers of *Apterostigma megacephala* forage individually for substrate (Fig. 8a, b), which consists of insect frass and small inflorescences. Individuals observed in FLONA-Carajás were carrying leaflets, probably of *Newtonia suaveolens* (Fabaceae), into their nests, but the leaflets were not incorporated into their fungus gardens. Instead, they were used to line the garden chamber floors. In the field, workers of *A. megacephala* resemble workers of species of the ant genus *Ectatomma* Smith in size, behavior, and superficial habitus. When workers are disturbed, they feign death and remain motionless for a few minutes, then return to normal activity. Workers of *A. megacephala* seemed to be more active during the afternoon hours. In the study area, a dense layer of leaf litter had accumulated at the boundary of the forest and the grassy area. Individual foragers were far more conspicuous when this layer of leaf litter was removed.

Nest architecture

Nest entrances of *Apterostigma megacephala* consist of an irregular hole in the ground, ~2 cm wide, hidden under a thick layer of leaf litter. Eighteen colonies of *Apterostigma megacephala* were excavated and censused (Table 1). Nests consisted of a relatively shallow single chamber located from <1 to 16.5 cm below the surface (Table 1).

Fig. 8 Workers of *Apterostigma megacephala* **a, b** foraging in leaf litter and **c, d** tending their fungus garden

Nest chambers were subspherical and measured 5–20 cm in diameter and 3.5–9.5 cm in height. They contained the fungus garden, workers, brood, queen and, in some cases, alate reproductives (Fig. 8c, d), which, based on collections made both in April and September, seem to be produced continuously throughout the year (Table 1). Nests of *Apterostigma megacephala* excavated in or near the grassy area with little (<2 cm thick) leaf litter were consistently deeper than those excavated in forest fragments where the leaf-litter layer was ~20-cm thick. The ceilings of the chambers were usually covered with rootlets from which the garden was suspended. The floors of the chambers were usually covered with the previously mentioned small, uncut leaflets brought in by the workers, probably of *Newtonia suaveolens* (Fabaceae); however, these leaflets were never observed to be incorporated into the gardens.

Fungus gardens

Apterostigma megacephala cultivates the leaf-cutting ant fungal symbiont *Leucoagaricus gongylophorus* and all examined gardens possessed gongylidia [nutritious swollen hyphal tips; (Fig. 9b, d)], known to consistently occur in the obligate fungal symbionts of higher-attine ants in the genera *Trachymyrmex, Sericomyrmex, Acromyrmex*, and *Atta* (Fig. 9a, c) (De Fine Licht et al. 2014). Rarely, gongylidia occur in the fungus gardens of lower-attine ants, but the fungi are distantly related to *L. gongylophorus* and the convergent occurrence of gongylidia is not fully understood (Masiulionis et al. 2014). The consistent occurrence of gongylidia in the gardens of *A. megacephala* is consistent with the discovery that it is the only lower-attine ant known to cultivate a higher-attine fungus.

Demography

Colonies of *Apterostigma megacephala* are relatively small, containing less than 100 workers (Table 1). Dealate queens were found in 13 out of the 18 colonies excavated. When a colony was disturbed in the course of the excavation, the queen was observed to quickly escape from the nest, a frequent observation in nest excavations of the so-called “lower” fungus-farming ants (Rabeling et al. 2007b). Therefore, queen escape is the probable explanation for the five queenless colonies. All queen-right nests consistently contained a single queen, and therefore colonies of *A. megacephala* can be considered monogynous (Table 1). Brood was encountered in all colonies but brood demographics are reported here only for the colonies collected in April 2010, which were preserved directly into 95% ethanol. The colonies collected in September 2011 and October 2014 were maintained alive in artificial plastic nests [following Sosa-Calvo et al. (2015) and Schultz (1993)]. Alate queens and males were found in 10 out of 18 colonies collected both during the rainy season (December to April) and the dry season (June to October). Colonies maintained alive in the laboratory continued to sporadically produce alate reproductives.

Ant morphology

Morphologically, *Apterostigma megacephala* possesses a unique combination of character states from the two main species groups (*auriculatum* and *pilosum*) within the genus *Apterostigma*. For example, it possesses the presumably plesiomorphic shining border of the clypeal apron found in the *A. pilosum* but absent in the *A. auriculatum* species group, whereas it possesses the more pronounced sculpture typical of the *A. auriculatum* species group. In some character states, however, *A. megacephala* differs from all other *Apterostigma* species. For example, all other known species in the genus *Apterostigma* have a palpal formula of 3,2, whereas *A. megacephala* (Fig. 4a–c) retains the ancestral attine condition of 4,2. (This corrects the palp formula reported for *A. megacephala* by Lattke (1999), who, due to the few specimens available to him at the time of his study, was unable to perform dissections.) The reduced palpal formula in *Apterostigma* has previously been considered to be a synapomorphy for the genus, convergently derived in the distantly related social parasites *Pseudoatta argentina* and *Mycocepurus castrator* (Lattke 1999; Rabeling and Bacci 2010; Schultz 2007).

Molecular characters

All other *Apterostigma* species for which DNA sequence data are available have a unique codon deletion at position corresponding to site 473 in the *Apis mellifera* mitochondrial *cytochrome c oxidase 1* (COI) gene (Genbank accession number KJ396181) (Schultz 2007), whereas *A. megacephala* retains the ancestral condition in which the codon is present.

Discussion

The relict species *Apterostigma megacephala* is a plesiomorphic fungus-farming ant, i.e., it retains a suite of character states that are believed to be ancestral to all fungus-farming ants, including pronounced sculpture (retained in the *A. auriculatum* group but vestigial in the *A. pilosum* group); palpal formula 4,2 (3,2 in all other *Apterostigma* species, shared with most other attine ants); distal margin of the clypeal apron smooth and shining (lost in the *A. auriculatum* group); head quadrate (head narrowed posteriorly in all other *Apterostigma* species); larval maxillary

Table 1 Nest measurements and colony demographics of 18 excavated nests of *Apterostigma megacephala* in Floresta Nacional de Carajás (FLONA-Carajás)

Nest	Locality	Coll. code	Date	Chamber no.	Depth (cm)	Chamber dimensions (cm)			Notes
						Height	Width	Depth	
1	FLONA	TRS100401-09	April 01, 2010	1	NA	NA	NA	NA	Chamber encountered by accident. 29w, 2ag, 3L, and garden present. In addition, 1 snail shell and bee head. **Queen present**
2	FLONA	TRS100401-10	April 01, 2010	1	~ 5	6	8.5	NA	Nest chamber was located in loose rootlet layer. 71w, 2ag, 3 m, 8L, 31p, 10e. **Queen present**
3	FLONA	TRS100402-02	April 02, 2010	1	3	7.5	11.5	7	Garden hanging from rootlets. Sides of chamber lined with small leaflets (*Newtonia suaveolensis*). Large colony: 50w, 3ag, 4 m, 10L, 10p, 11e. Queen not found
4	FLONA	TRS100402-03	April 02, 2010	1	3	7	11	NA	Chamber ovate. Garden suspended from roots. Large colony: 68w, 7ag, 14 m, 41L, 25p, 35e. Queen not found. One *Strumygenys* sp worker in garden
5	FLONA	JSC100402-11	April 02, 2010	1	<1	9.5	11	5	16w, 2L, 1p, 4e. Males and queen absent
6	FLONA	TRS100402-12	April 02, 2010	1?	NA	NA	NA	NA	Encountered by accident. Medium-sized colony: 39w, 3ag, 7 m, 4L, 19e. **Queen present**
7	FLONA	JSC110910-01	September 10, 2011	1	~6	7	7	11	Rounded chamber. Several workers were collected. **Queen present**
8	FLONA	JSC110910-03	September 10, 2011	1	16.5	6	10	12.5	Large nest chamber. Fungus garden hanging from rootlets at the ceiling of chamber. Bottom of chamber covered with leaflets (*Newtonia suaveolensis*) and other dry leaf-litter material. The nest chamber was located approximately 45° from nest entrance. 1ag, 2 m. **Queen present**
9	FLONA	JSC110910-04	September 10, 2011	1	4	6	6	10	Small rounded chamber. Nest chamber located underneath a big root. The ceiling of the chamber with abundant rootlets and the garden seeming to hang from them. Floor of chamber flattened and with dry pieces of leaf litter. No alates found. **Queen present**
10	FLONA	JSC110910-05	September 10, 2011	1	NA	6	5	20	Rounded chamber. Chamber located underground immediately below leaf litter. Ceiling of chamber with lots of rootlets. **Queen present**
11	FLONA	JSC110911-15	September 10, 2011	1	7	4	8	18.5	Large nest chamber. Ceiling of chamber covered by rootlets. Fungal cultivar hanging from rootlets in ceiling. Thick layer (~ 20 cm) of leaf litter above soil. 3ag, 4 m. **Queen present**
12	FLONA	JSC110911-16	September 10, 2011	1	2	4	6	10	Small chamber and colony. Ceiling of chamber covered with rootlets. 1 m. **Queen present**

Table 1 (continued)

Nest	Locality	Coll. code	Date	Chamber no.	Depth (cm)	Chamber dimensions (cm)			Notes
						Height	Width	Depth	
13	FLONA	CTL110911-01	September 10, 2011	1	3	8	9	9	Medium-sized chamber. Queen not collected
14	FLONA	JSC110912-01	September 10, 2011	1	4	5	9	11	Large rounded nest chamber. Ceiling of chamber with abundant rootlets; chamber floor flattened and with several leaf pieces and dry leaf litter. 5ag. **Queen present**
15	FLONA	JSC110912-03	September 10, 2011	1	NA	5	16	10.5	Large nest chamber located underneath leaf litter–soil interface. Garden hanging from rootlets in the ceiling. 4 m. **Queen present**
16	FLONA	JSC141002-04	October 02, 2014	1	NA	3.5	13	5	Large nest chamber located underneath leaf litter and soil layer. No alates present. Several workers and brood. **Queen present**
17	FLONA	JSC141002-06	October 02, 2014	1	NA	4	10	5	Large, very shallow chamber. Chamber located between the leaf litter and soil layers. Garden suspended from rootlets and bottom of chamber covered with small leaves
18	FLONA	TRS141002-02	October 02, 2014	1	5	7	11	8	Large nest chamber. Chamber lined with leaflets from a legume tree, probably *Newtonia suaveolensis*. No alates present. **Queen present**

ag alate gyne, *e* eggs, *L* larvae, *m* male, *p* pupae, *w* worker

palp digitiform (reduced in all other *Apterostigma* species); larval labial palp digitiform (lost in all other *Apterostigma*, but shared with some other Paleoattini); larval profile moderately curved [“attoid” of Wheeler and Wheeler (1976)] (extremely curved in all other *Apterostigma* species); and mitochondrial *COI* DNA sequence with a codon triplet present in all other known ants, including non-attine ants, but absent in all other *Apterostigma* species. In addition, *A. megacephala* possesses a number of unique, derived character states, most notably the cultivation of *Leucoagaricus gongylophorus*, otherwise cultivated only by some species in the leaf-cutting ant genera *Acromyrmex* and *Atta* (Schultz et al. 2015).

The biological constraints enforcing strong symbiont fidelity at the level of the five attine agricultural systems demonstrably do not apply to *A. megacephala*. Although the constraints remain unknown, it is plausible that they are associated with a primitive retention of nutritional independences that have been lost in other fungus-farming ants. An example of such a nutritional independence is the ability to synthesize the amino acid arginine, which is present in all ants for which genomic data are available with the notable exception of fungus-farming ants, which lack two of the enzymes (argininosuccinate lyase and argininosuccinate synthase) in the arginine synthesis pathway, presumably creating a nutritional dependence on their fungal cultivars for this amino acid (Ješovnik et al. 2016; Nygaard et al. 2016; Nygaard et al. 2011; Suen et al. 2011). The absence of argininosuccinate lyase and argininosuccinate synthase in *A. megacephala* indicates that this loss happened early in the evolution of attine ants and that it might have been one of the factors causing the ants to become obligate symbionts, dependent on the fungus. If similar nutritional dependences (or, in the case of higher-attine agriculture, co-dependences) are responsible for the symbiont fidelity characterizing each of the five attine agricultural systems, then perhaps *A. megacephala*, although also presumably unable to synthesize arginine (Ješovnik et al. 2016), otherwise retains a primitive ability to utilize a wide range of fungi. If so, and if the recently evolved *L. gongylophorus* provides *A. megacephala* with a fitness advantage relative to lower-attine fungi, then perhaps *A. megacephala* has been able to take advantage of *L. gongylophorus* in a way that all other lower-attine as well as many other higher-attine ants cannot.

Further study of *A. megacephala* at Carajás, as well as at the other localities where it is known to occur, may provide important insights into our broader understanding of

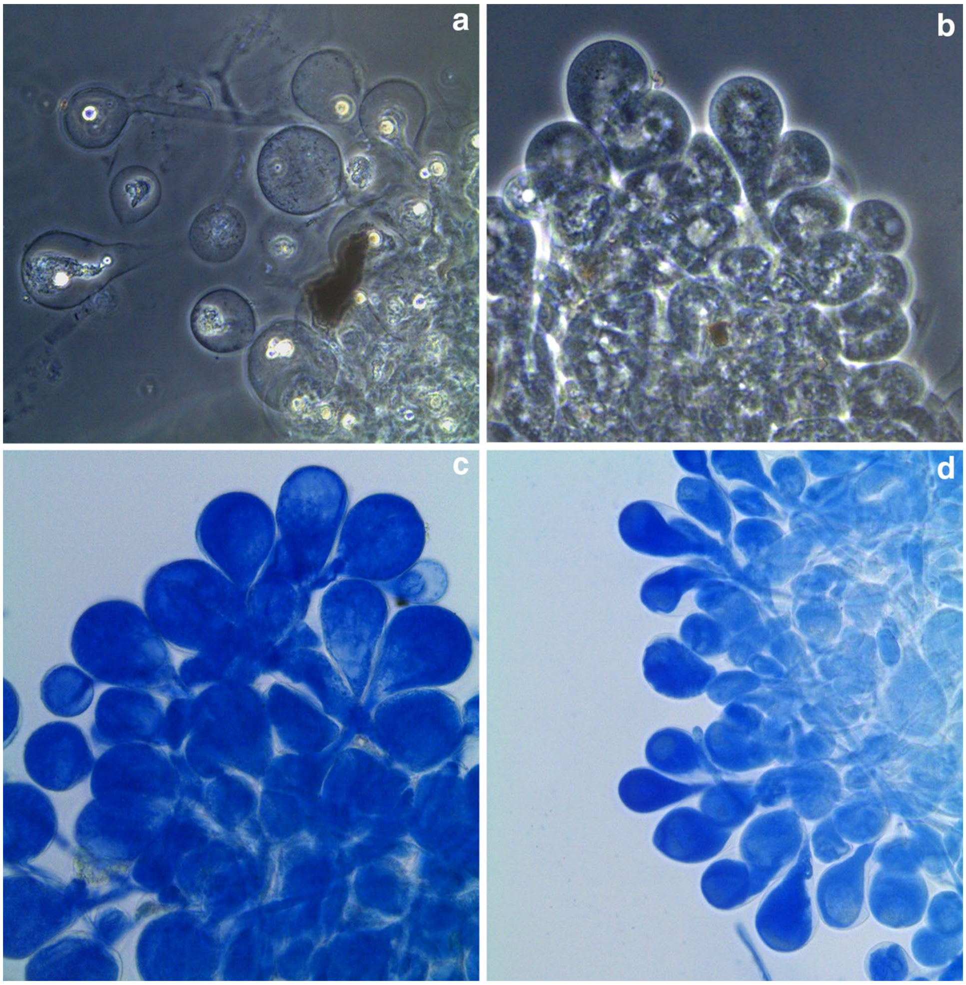

Fig. 9 Nutritious swollen hyphal tips (gongylidia) typical of higher-attine fungal cultivars, **a, c** gongylidia of *L. gongylophorus* from the fungus garden of an *Atta* sp., **b, d** gongylidia of *L. gongylophorus* from the fungus garden of *Apterostigma megacephala*. **a, b** Gongylidia not stained, **c, d** gongylidia stained with lactoglycerol cotton blue

the biological mechanisms that constrain symbiont fidelity in the five attine agricultural systems. If *A. megacephala* is consistently found to cultivate *L. gongylophorus* wherever it occurs, this would indicate that, since the association began, it has become obligate, whereas variation in cultivar association across localities would indicate that *A. megacephala* is able to utilize both lower- and higher-attine fungi for food, making it unique among known fungus-farming ants.

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