

How common is trophobiosis with hoppers (Hemiptera: Auchenorrhyncha) inside ant nests (Hymenoptera: Formicidae)? Novel interactions from New Guinea and a worldwide overview

Petr KLIMES, Michaela BOROVANSKA, Nichola S. PLOWMAN & Maurice LEPONCE



Abstract

Trophobiotic interactions between ants and honeydew-providing hemipterans are widespread and are one of the key mechanisms that maintain ant super-abundance in ecosystems. Many of them occur inside ant nests. However, these cryptic associations are poorly understood, particularly those with hoppers (suborder Auchenorrhyncha). Here, we study tree-dwelling ant and Hemiptera communities in nests along the Mt. Wilhelm elevational gradient in Papua New Guinea and report a new case of this symbiosis between *Pseudolasius* EMERY, 1887 ants and planthoppers. Furthermore, we provide a worldwide review of other ant-hopper interactions inside ant-built structures and compare their nature (obligate versus facultative) and distribution within the suborder Auchenorrhyncha. The novel interactions were observed in nests located at the tree trunk bases or along the whole trunks. Only immature planthopper stages were found inside nests, so full species identifications were not possible. However, nymph morphology and molecular data (18S and COI genes) indicated four related species of the family Flatidae (infraorder Fulgoromorpha) associated with *Pseudolasius*. Ant-planthopper occurrences were relatively rare (6% of all trophobiotic interactions) and peaked at mid-elevation (900 m above sea level). *Pseudolasius* was the only genus associated with planthoppers in the communities, with most cases monopolised by a single species, *P. breviceps* EMERY, 1887. In contrast, all other ant genera tended various scale insects (Sternorrhyncha: Coccoidea). This apparent partner-specificity is rare: Worldwide, there are only about ten reported cases of obligate symbiosis in ant nests, distributed in five of the thirty-three Auchenorrhyncha families. Those trophobioses are randomly dispersed across the Auchenorrhyncha phylogeny, and thus likely originated multiple times independently. Further research on both adult and nymph hopper life history is needed to answer how these symbioses, notably rare in hoppers compared with other hemipterans, are maintained.

Key words: Ants, Fulgoroidea, Formicinae, molecular identification, myrmecophily, specialisation, trophobionts, trophic networks, tropical forest.

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Petr Klimes (contact author), Biology Centre of the Czech Academy of Sciences, Institute of Entomology, Branisovska 1160/31, 370 05 České Budějovice, Czech Republic; New Guinea Binatang Research Center, P.O. Box 604, Madang, Papua New Guinea. E-mail: peta.klimes@gmail.com

Michaela Borovanska, Biology Centre of the Czech Academy of Sciences, Institute of Entomology, Branisovska 1160/31, 37005 České Budějovice, Czech Republic.

Nichola S. Plowman, Biology Centre of the Czech Academy of Sciences, Institute of Entomology and University of South Bohemia, Faculty of Science, Branisovska 31, 37005 České Budějovice, Czech Republic.

Maurice Leponce, Royal Belgian Institute of Natural Sciences (RBINS), Operational Directorate Natural Environment, Rue Vautier 29, 1000 Brussels, Belgium; Université Libre de Bruxelles, Behavioural & Evolutionary Ecology, Av. F.D. Roosevelt 50, 1050 Brussels, Belgium.

Introduction

Trophic interactions among ants and other arthropods are very diverse and have important consequences for the functioning of ecosystems (STYRSKY & EUBANKS 2007, CHOMICKI & RENNER 2017). Ants are one of the most ecologically diverse and abundant invertebrate groups, notably in tropical rainforests where their roles range from predatory to omnivorous and herbivorous in food webs (BLÜTHGEN & al. 2003, DAVIDSON & al. 2003). One of the key mechanisms maintaining the high abundance of ants and their ability to

build up huge colonies, which can dominate whole trees or forest patches, is their trophobiotic interactions with honeydew-providing insects (DELABIE 2001, BLÜTHGEN & al. 2004, DEJEAN & al. 2007).

In trophobiosis, ant partners usually provide protection to herbivorous insects (trophobionts) from enemies (predators, parasitoids), while the herbivores provide the ants energy-rich food resources in the form of honeydew, via gland secretion from special organs (FIEDLER 2006, KA-

MINSKI & al. 2010) or more often as anal exudates (WAY 1963, DELABIE 2001). These interactions have evolved repeatedly among various ant and herbivorous insect lineages from several insect orders (CHOMICKI & RENNER 2017) including Lepidoptera (e.g., FIEDLER 2006), Heteroptera (e.g., DEJEAN & al. 2000, SILVA & FERNANDES 2016) and various groups of Hemiptera (BOURGOIN 1997, DELABIE 2001, STADLER & DIXON 2008, IVENS 2015). Special attractants to ants are offered also by many other invertebrates (e.g., Coleoptera, Zygentoma), but those interactions, while myrmecophilous, are not considered trophobiotic since the attractants are used to manipulate ants into a commensal or parasitic relationship (HÖLDOBLER & WILSON 1990, PARKER 2016, MOLERO-BALTANÁS & al. 2017). Trophic symbioses range from facultative, where a trophobiont is tended by multiple ant species usually outside of the nest (sometimes only collecting the fallen honeydew), to obligate interactions with a single or few ant species, where an ant is always in direct communication (tactile, chemical) with the symbiont (DELABIE 2001, STADLER & DIXON 2008). Obligate interactions can occur either in ant territory or inside ant-built structures (nest chambers or ant-built satellite shelters in plants), where the symbiont is prevented from escaping (BOURGOIN 1997) and is less likely or unable to survive without ants (GULLAN 1997, IVENS 2015). The ecological importance of both facultative and obligate trophobiotic interactions is high due their direct and indirect impacts not only on ant populations but other insects and plants, although benefits among the partners can vary (CUSHMAN & al. 1998, STYRSKY & EUBANKS 2007, ZACHARIADES & al. 2009).

Ant-tending of “herds” of trophobionts, especially hemipterans, outside of their nests is common (PFEIFFER & LINSÉNMAIR 2007, STADLER & DIXON 2008, IVENS 2015). However, trophobiosis in ant-built structures is also widespread, notably in the suborder Sternorrhyncha with aphids (Aphidoidea) and scale insects (Coccoidea) (e.g., GULLAN 1997, ANDERSON & MCSHEA 2001, DELABIE 2001, IVENS 2015). This is likely due to the fact that Sternorrhynchans are less mobile: They lack a jumping apparatus to escape from predators and can substantially benefit from sheltered living. In contrast, most trophobionts from the suborder Auchenorrhyncha (e.g., cicadas, leafhoppers, planthoppers) have well developed jumping abilities and their occurrence inside of ant-built structures has been only occasionally documented (BOURGOIN 1997, DELABIE 2001, MEZGER & BLÜTHGEN 2007). Despite the commonness of interactions inside ant-built structures in some systems (GULLAN 1997, ANDERSON & MCSHEA 2001), they are studied less because they are difficult to access and observe. This is particularly true of trophobiosis in nests built on tropical trees, where even the ant communities themselves are usually not well known (FLOREN & LINSÉNMAIR 2005, KLIMES & al. 2015). Although there is a large body of literature on trophobiosis that includes some information on interactions with Hemiptera inside nests, e.g., scale insects and aphids in particular (reviews by DELABIE 2001, IVENS 2015), no study thus far has focused on ant-Auchenorrhynchan symbioses inside nests and other ant-built structures.

New Guinea (NG) is one of the most diverse regions in the world, with notably high levels of diversity and endemism of plants and insects (LEPONCE & al. 2016), including ants (WILSON 1959). Yet, most insect species there remain unstudied and information on ant trophobiotic interactions is scarce, with no information on tending of hoppers. The

few studies available have shown that NG arboreal ant species exploit honeydew mainly from scale insects (GULLAN & al. 1993, KLIMES & McARTHUR 2014). Symbiosis with scale insects is likely also prominent in ground-dwelling fauna, such as *Acropyga* ROGER, 1862 ants (Hymenoptera: Formicidae: Formicinae) which are known to tend mealybugs (LAPOLLA & al. 2008). This genus is common in NG, and all its species are thought to perform trophophoresy, an obligate symbiosis where a founding queen carries in its mandibles a symbiotic scale insect before establishing a new colony in the soil (LAPOLLA & al. 2006). Another common genus, *Pseudolasius* EMERY, 1887, related to *Acropyga*, is also known to tend honeydew-producing insects (WILSON 1959). However, in this case the symbiosis is facultative, as ants visit common scale insects, and sometimes aphids, of several families feeding on the roots of the plants underground (HECKROTH & al. 2004). Queens are not known to exhibit trophophoresy (LAPOLLA & al. 2010), although workers can transport symbionts to the plant roots (MALSCH & al. 2001) or to new satellite colonies created by budding (KAUFMANN & al. 2003). Currently, 13 species and subspecies of *Pseudolasius* are described from NG region (JANDA & al. 2016), but none have been reported to be associated with Auchenorrhyncha.

Here, we report the first case of the symbiosis between the ant genus *Pseudolasius* and planthoppers of the family Flatidae, which were repeatedly found in close association in forests in Papua New Guinea. Using morphological and molecular data from planthoppers and their ant hosts, and background data on the arboreal ant communities examined along the Mt. Wilhelm elevational gradient (up to the tree line at 3700 m above sea level, a.s.l. hereafter) (LEPONCE & al. 2016), we focus on the following questions: (I) How many *Pseudolasius* species and planthopper species are involved in the interaction, and how is this symbiosis affected by elevation? (II) How unique is the trophobiosis compared to other ant-trophobiont interactions in NG forests? (III) How common is ant-hopper trophobiosis inside nests in general? For this last question, we review all other ant-Auchenorrhyncha interactions known to occur in ant nests and other ant-built structures worldwide, and discuss the evolution of the symbiosis within this group.

Materials and methods

Study site and field methods

Interactions among ants and hemipterans were documented during ecological surveys in Madang province in Papua New Guinea (PNG) in collaboration with New Guinea Binatang Research Center (NGBRC) and the international biodiversity survey “Our Planet Reviewed” (OPR). A census of all ant nests that occurred in trees of DBH (diameter at breast height) ≥ 5 cm was conducted in a 0.5-ha plot of primary forest near Wanang village (200 m a.s.l.) and a 0.2-ha plot of primary forest near Numba village (900 m a.s.l.) (KLIMES & al. 2015, NGBRC 2017). All trees were felled and inspected immediately for ants and their nest sites; details of the methods are given in KLIMES & al. (2015) and KLIMES (2017). Each ant nest was visually inspected for symbiotic insects (including trophobionts). Additionally, another ten elevational sites were sampled for arboreal-dwelling dominant ants during the OPR survey in the same region (LEPONCE & al. 2016). These other sites were sampled from 10 m to 3700 m a.s.l., with one or two plots per site of size 2800 m² each (i.e., up to 0.56 ha per site; for details on all localities, see Tab. S1 in

Tab. 1: Overview of the sampled interactions among Flatidae nymphs and the ants in New Guinea rainforests. Ant partners: PK - *Pseudolasius karawajewi*; PB - *Pseudolasius breviceps*. NA: data not available, DBH: diameter at breast height. For more details on the elevational sites, see Table S1. Codes of the sequences refer to the symbionts (for Wg and COI sequences of the ant partners, see text).

Sample codes	Date	Elevation (m a.s.l.)	Ant sp.	Symbiont (Flatidae)	18S seq. code (GenBank)	COI seq. code (GenBank)	Height (m)	N of nests	Host tree species family	Size (DBH)
HP0711	26.06.2007	200	PK	Sp. 1	MF627430	MF627439	0.5	1	<i>Pimelodendron amboinicum</i> , Euphorbiaceae	11.4
HP0729	27.05.2006	200	PB	Sp. 1	MF627431	MF627440	0.5	1	<i>Allophylus cobe</i> , Sapindaceae	23.0
ML10477, ML10479	28.10.2012	700	PB	Sp. 2	MF627427, MF627428	MF627438, NA	0.5	1	NA, Icacinaceae	NA
NA0233-NA0235	08.07.2013	900	PB	Sp. 2	MF627433	MF627442	1.6 - 15.6	3	<i>Gomphandra montana</i> , Stemonuraceae	18.8
NA0236	02.08.2013	900	PB	Sp. 2	MF627434	NA	1.6	1	<i>Elaeocarpus dolichodactylus</i> , Elaeocarpaceae	57.0
NA0232	14.06.2013	900	PB	Sp. 3	MF627432	MF627441	4.8	1	<i>Dysoxylum arborescens</i> , Meliaceae	11.7
NA0237	17.07.2013	900	PB	Sp. 4	MF627435	MF627443	8.0	1	<i>Sloanea sogerensis</i> , Elaeocarpaceae	37.0
ML47119	20.03.2012	1200	PB	Sp. 4	MF627429	NA	0.5	1	NA	NA

Appendix, as digital supplementary material to this article, at the journal's web pages). Ants occurred only up to 2700 m a.s.l. In the OPR plots, trophobionts were also collected, but only from ground level, as no trees were felled. A voucher of several ant workers and Hemiptera was collected from each nest and stored in a vial with 99% ethanol for later identification.

Identification and photo-documentation of morpho-species

All ants were first sorted to genus and morphospecies. Species boundaries were then re-assessed using additional data from DNA barcoding of representative workers (IBOL 2017; next section), GenBank database (BENSON & al. 2013) and online specimen images for the New Guinea region (ANTWEB 2017). In this study, we focus only on the genus *Pseudolasius* and their trophobionts at species level. The planthopper nymphs were first sorted to morphospecies and the material was sent to taxonomists, who suggested that multiple families and species of planthoppers were perhaps involved (Fulgoromorpha: Flatidae, Issidae, or Tropiduchidae), but could not identify the material further without adults (T. Bourgoïn & M. Wilson, pers. comm.). Therefore, we performed molecular analysis of all nymph samples to clarify the status of the family and morphospecies of the fulgoroids (see next section). Each ant and nymph morphospecies was photodocumented using a Leica DFC450 camera fitted with macroscope Leica Z16APO (at Biology Centre CAS) or a Leica DFC290 and macroscope Leica Z6APO (at RBINS).

Molecular and phylogenetic analysis for taxonomic identification

Isolations and PCR: Representative individuals of each *Pseudolasius* species and of all planthopper samples were chosen for sequencing. Total genomic DNA was extracted from the whole body (ants, two samples) or two legs (planthoppers, nine samples) using the Genomic DNA Mini Kit Tissue (Geneaid Biotech Ltd., Taiwan). The Geneaid manufacturer protocol was followed until the elution step, for which only 50 µl of Elution Buffer was used. Ants: two

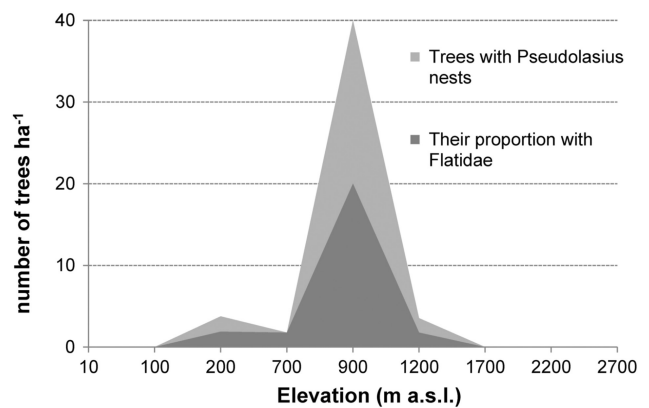


Fig. 1: Changes in the density of trees hosting *Pseudolasius* ants and planthopper trophobionts (Fulgoromorpha: Flatidae) in their nests along the Mt. Wilhelm elevational gradient.

markers were used: one nuclear gene Wingless (Wg, 400 bp) and one mitochondrial gene cytochrome oxidase subunit 1 (COI barcoding region, 658 bp). The PCR products were amplified using the published primers WG578F, WG1032R and LCO1490, HCO2198 for Wg and COI respectively (MOREAU & al. 2006). Twenty-five microlitre amplification reactions produced DNA product under following conditions: 12.5 µl of Combi PPP Master Mix (Top-Bio s.r.o., Czech Republic), 1 µl of 0.1 µM each primer, 9.5 µl of PCR H₂O and 1 µl of DNA genomic template. Standard PCR procedure was used for Wg, in which an initial denaturation cycle of 94 °C for 4 min was followed by 35 cycles of 92 °C for 60 s, 52 °C for 1 min, 72 °C for 90 s, and concluded by 10 min at 70 °C. For COI, a thermocycling profile of initial denaturation steps of 5 min at 95 °C was followed by 40 cycles of 94 °C for 30 s, 47 °C for 30 s, and 72 °C for 90 s, followed by 10 min at 72 °C. Planthoppers: we first aimed to amplify COI using the same primers and protocols as for the ants but products failed in all cases and PCR optimization did not help. We instead used an 18S rRNA fragment (SSU loci, 566 bp), used previously for other hemipterans, which is usually easily amplified (LIN & al. 2013), to confirm the preserva-

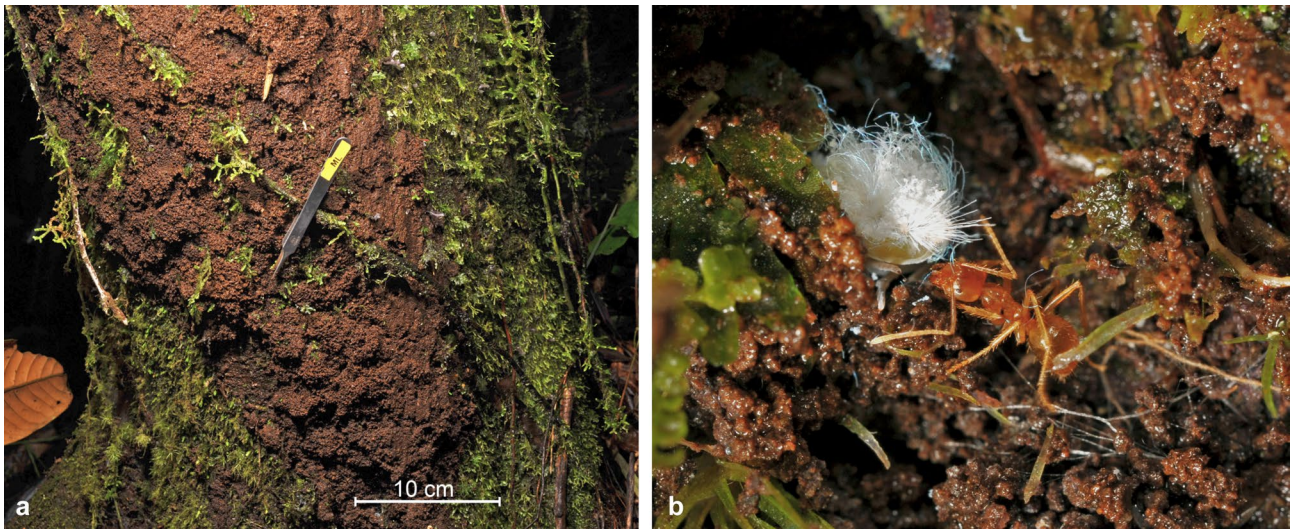


Fig. 2: Photo of a typical nest structure and habitat, where the interactions among the *Pseudolasius* ants and the planthoppers occur: (a) nest on tree trunk, (b) detail of ant and symbiont interaction (for a video, see information in Fig. S3). Partners: flatid “Sp. 4” sample ML47119 and worker of *Pseudolasius breviceps*.

tion of the species DNA in all samples. Finally, COI was successfully amplified in most symbiont samples too (see Tab. 1), but using the primers LCO1490puc and HCO2198puc (WANG & al. 2016). All PCRs were run on a TProfessional TRIO Thermocycler (Biometra GmbH, Germany). Samples were purified and standard sequencing was performed on automated ABI 3730XL sequencer (Applied Biosystems) at Macrogen Inc. (Korea).

Alignments and molecular tree construction: The aim was to reveal at least family (and eventually genus) of the unknown planthopper symbionts, and also to assess the number of families and species interacting with *Pseudolasius* species in the elevational gradient. Raw sequences of the ants and symbionts were proofread, folded and aligned in Geneious R6 (version 1.6.8) and checked with online databases for preliminary taxonomic identifications and any contaminations (BLAST 2017). The subsequent phylogenetic analyses of symbionts were limited to the infraorder Fulgoromorpha of Auchenorrhyncha. First, we analysed the sequences for both genes separately, as our search of the literature and GenBank (BENSON & al. 2013) revealed that most of the representative species and genera with available DNA information differ among the 18S and COI fragments we used here, even within family. For each gene fragment (530 and 615 after alignment respectively), we constructed a 50% majority-rule consensus tree using Bayesian inference in MrBayes version 3.2.1. *Philaenus spumarius* (LINNAEUS, 1758) (Cicadomorpha: Cercopoidea) was used as the outgroup in both analyses. GTR substitution model, variation rate “invgamma”, four Markov chains, and one million cycles were used for both trees. The first 10% of cycles were discarded as burn-in. Most of the comparative 18S sequences that overlapped with our gene segment were used from (URBAN & CRYAN 2007, SONG & LIANG 2013), which covered the highest proportion of extant Fulgoromorpha families (19 of 21); plus a few other sequences available from GenBank were added to increase the taxa sampling (see Appendix Figs. S1, S2). For COI, we used representatives of 15 of the 19 families as some do not have COI gene data yet available or previous authors sequenced different regions of the gene (CRYAN & URBAN 2012). Finally, a concatenated tree

of the both 18S and COI gene sequences was created using the same methods but only for *Pseudolasius* symbionts. The closest relative revealed by the broader analysis above was used as an illustrative outgroup. In addition, as NG ant fauna is poorly known at species level, an analogous phylogenetic analysis was performed on *Pseudolasius* ant species using COI barcodes available from this study and GenBank to assess the ant species identity. All trees were adjusted in FigTree ver.1.3.1 and Inkscape ver. 0.91. The novel sequences from this study are available in GenBank under codes MF627425 - MF627443.

Ecological and evolutionary comparative analyses

Changes in the density of trees occupied by *Pseudolasius* along the elevational gradient and the presence of the symbionts were first explored using the observed tree densities with *Pseudolasius* nests (and the symbionts) in the total area sampled (i.e., 0.2 – 0.56 ha per elevation, Tab. S1) and recalculated per one-ha of the forest. An interaction network was then constructed with the R package “bipartite” (DORMANN & al. 2009, R DEVELOPMENT CORE TEAM 2017), to visualize the quantity and specificity of the interactions among planthoppers and *Pseudolasius* ants: (i) between all ant and planthopper species along the Mt. Wilhelm gradient, (ii) among all ant genera and the major groups of symbiotic Hemiptera encountered in all nests sampled (i.e., all data from all sites pooled). We did not calculate numeric food-web parameters (DORMANN & al. 2009) due to the small network size and pooling across multiple elevational sites. The symbiosis was also compared with previous records in the literature of ant-Auchenorrhyncha interactions in ant-built structures, using past reviews (BOURGOIN 1997, DELABIE 2001 and literature there) and new searches in online databases (Web of Science, Zoological Records, FORMIS). All known cases of trophobiosis in ant nests were then mapped into the phylogeny of families of the suborder Auchenorrhyncha using information from previously published phylogenetic trees (URBAN & CRYAN 2007, CRYAN & URBAN 2012), which covered 19 of 21 extant families of the infraorder Fulgoromorpha (BOURGOIN 2017) and all 12 families of the infraorder Cicadomorpha (31 families in total). MEGA 7 software was

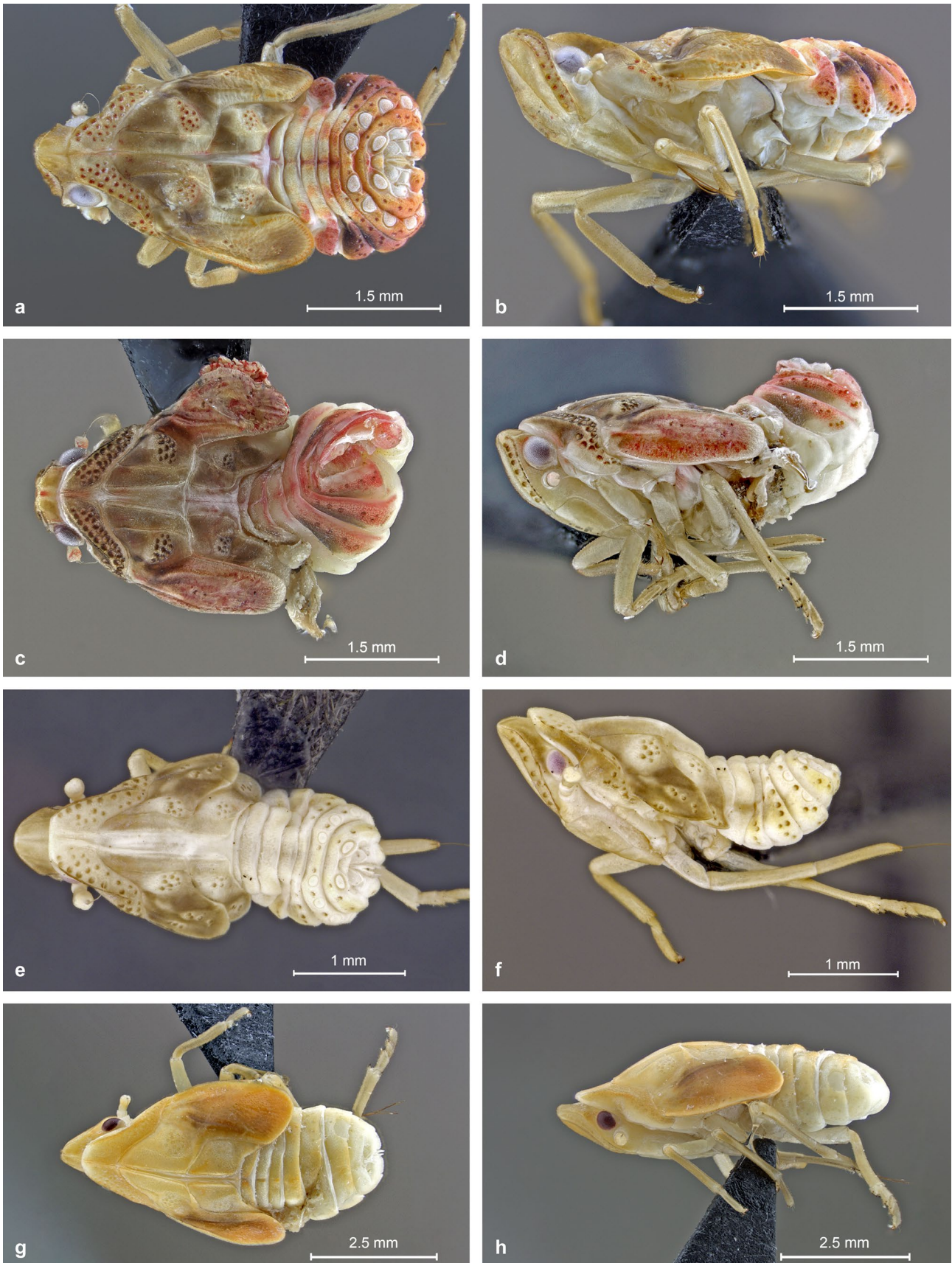


Fig. 3: Planthopper morphospecies (Fulgoromorpha: Flatidae; nymphs) discovered inside *Pseudolasius* nests: Sp. 1 sample HP0711 – dorsal (a), lateral (b); Sp. 2 ML10479 – dorsal (c), lateral (d); Sp. 3 sample NA0232 – dorsal (e), lateral (f); Sp. 4 sample ML47119 – dorsal (g), lateral (h). Note that all live specimens carry whitish wax structures dorsally and on abdomen (e.g., Fig. 2b) which are lost here due to preservation in ethanol. For more information on samples, see Table 1. For photos of ant host species see Appendix (Fig. S4).

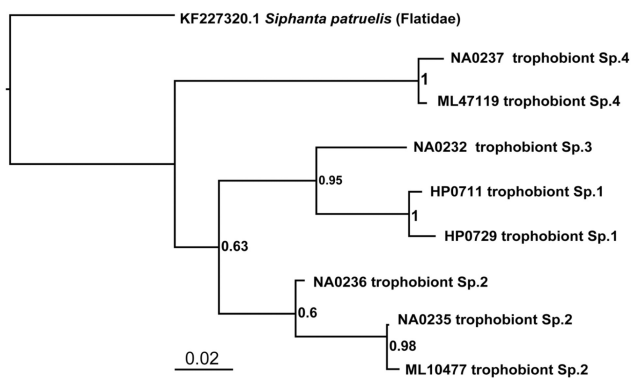


Fig. 4: Phylogenetic analysis of the planthopper symbionts of *Pseudolasius* in New Guinea rainforests. The 50% majority-rule consensus tree is based on 18S and COI gene fragments (1196 bp in total; except samples NA0236 and ML47119 where only 18S available). Posterior probabilities of the branches are noted at each resolved node (> 0.5). Each branch represents a sample and its code (see Tab. 1 for more on the samples, and Figs. S1 and S2 for trees constructed for each gene separately which include also the symbiont position within the Fulgoromorpha).

used to create the family-level hopper cladogram (KUMAR & al. 2016) and the distribution of the number of studies which found the trophobiosis was mounted to it using the online tool iTOL (LETUNIC & BORK 2016). As there are competing hypotheses for a higher-level phylogeny of Hemiptera and evolution of Auchenorrhyncha (SONG & al. 2012), a similar comparison was conducted using the alternative phylogenetic tree of Fulgoromorpha from SONG & LIANG (2013). To assess if the distribution of the symbiosis is random or phylogenetically dependent across Auchenorrhyncha, we calculated mean pairwise distance (mpd) between lineages ($n = 33$), while each of them was scored by presence-absence of an ant trophobiosis in the family-level cladogram. The analysis was performed three times using scoring as (I) any trophobiosis (sensu lato) or (II) trophobiosis inside ant nest structures or (III) obligate trophobiosis inside ant nests present. Null models were generated by swapping tip labels across the tree and implemented in the R package “picante” (KEMBEL & al. 2010), using 999 randomisations for each of the three null-model tests (mpd.obs.z, two-tailed at $\alpha = 0.025$ and 0.975 respectively).

Results

Observations and distribution of the *Pseudolasius*-planthopper association

In total, 15 trees were occupied by *Pseudolasius* nests across all plots. Of those, eight trees had planthopper nymphs present in nests (Tab. 1). Interactions were observed from 200 m up to 1200 m a.s.l., with a mid-elevational peak (900 m a.s.l.), where four trees were occupied in only 0.2 ha by the planthopper symbionts, and some with multiple nests (Fig. 1, Tab. 1). The density of trees with the symbiosis varied from four to 20 trees per ha. No *Pseudolasius* nests were found above 1200 m a.s.l. Nests in which we did not discover any symbionts after breaking the structure (seven trees) were usually only small structures under epiphyte roots or nests expanding from soil in hollow trunks of dry trees. In the

other trees, soil structures built by ants covered most of the trunk width and the symbionts were found under these structures (Fig. 2). Only ant workers were present with them, typically in the structures. However, in the case of a *Gomphandra montana* tree with multiple nests along the trunk and multiple cases of the symbionts (Tab. 1), numerous ant larvae and pupae were found in the same nest structures as the nymphs. The vertical height at which symbionts were found ranged from 0.2 m (soil nest at tree bases) up to 15.6 m (soil nest under epiphytic roots). Interactions were not specific to particular tree families or species (Tab. 1).

At the highest elevation (1200 m a.s.l.), the nest and ants interacting with planthoppers were observed, photographed and filmed (see Fig. 2b and Fig. S3). Two cases of a nymph and a worker interacting were observed for a few minutes. After breaking the nest, the nymph stood still on the trunk, while an ant worker ran in excited circles around it, antennating both at its back and front. The worker then attempted to grasp the nymph in its mandibles. This attempt failed, probably due to the presence of waxy structures on the dorsal side of the nymph and the large body size relative to the ant. After these attempts, the ant pushed the nymph back to the shelter by repeatedly running around it and touching it. Direct observation of trophobiosis was not possible as ants and nymphs were highly disturbed by the breaking of the nest and some of the nymphs skipped away. However, the nymphs were in all cases observed only inside of the soil structures on the bark, never outside of the nests. No adult hoppers were found.

Identity of the ants and their trophobionts

In total, four species of planthopper nymphs (Fig. 3) and two *Pseudolasius* species (Fig. S4) were found at the studied sites. An overview of samples is given in Table 1. The nymphs were mostly found with a locally common species of the genus, *Pseudolasius breviceps* EMERY, 1887 (Fig. S4a, b), except for one case of *P. karawajewi* DONISTHORPE, 1942 (Fig. S4c, d) tending the nymph of planthopper morphospecies Sp. 1. Our study is the first to barcode *Pseudolasius karawajewi* (sample HP0711, GenBank codes MF627426 - Wg, MF627436 - COI) and our specimen fits morphologically to its syntype (CASENT0903170, Indonesia: Waigeu, 1.V.1938, leg. L.E.Cheesman, 1 worker). The morphology and barcode data for our *P. breviceps* specimens were very close to published data for *P. australis* FOREL, 1915 (sample ML47119, Genbank codes MF627425 - Wg, MF627437 - COI; 98% similarity in COI compared to CASENT0106005 Genbank code FJ982482.1, Australia: Queensland, 24.VIII.2004, leg. P.S. Ward, UCDC, Davis, CA, USA, 1 worker). However, morphological comparison of more individuals of the two species and the construction of a *Pseudolasius* phylogeny revealed that past studies have likely misidentified specimens sequenced as *P. australis* (Fig. S5). Photographs of the sequenced workers (e.g., GenBank code FJ982482.1) do not correspond morphologically with the type specimen of *P. australis* (CASENT0910972, Australia: ?, leg. Mjoberg, coll. Forel, MHNG, Geneva, Switzerland, 1 worker) but with *P. breviceps* (CASENT0905653, syntype, Indonesia: Ambon island, 31.XII.1873, leg. O. Becari, MSNG, Genoa, Italy, 1 worker). This evidence suggests that all published sequences for *P. australis* in GenBank are in fact *P. breviceps*, the same common species we studied (Figs. S4, S5).

Phylogenetic analysis of both 18S and COI planthopper data obtained similar results (compare Figs. 4, S2, S4) and

fitted with the nymph morphospecies (Fig. 3). Four species from a single related group of planthoppers were found in *Pseudolasius* nests. We obtained COI and 18S sequences for all planthopper samples except two for COI (Tab. 1, Fig. 4). Phylogenetic analysis comparing our hopper species with representative species from extant Fulgoromorpha families revealed Australian Flatidae as the closest relatives in both gene datasets: specifically *Siphanta patruelis* (STÅL, 1859) and *Falcophantis westcotti* FLETCHER, 1988 for the COI tree, and *Massila* WALKER, 1862 sp. for the 18S tree (all Flatidae; Figs. S1, S2). Overall, the closest taxon to our planthoppers was *S. patruelis*, an Australian species with a mean of 97% sequence similarity in COI (BLAST 2017). Neither gene fragments were informative enough to reveal monophyletic patterns within some of the Fulgoromorpha families, including Flatidae. Nevertheless, this family was consistently the best fit for the planthoppers, and it was also one of those suggested by experts (see Methods).

Interaction networks and partner shifts with elevation

There was a strong turnover of our Flatidae species with elevation: Sp. 1 was found only in a lowland site (Wanang, 200 m), while Sp. 2 dominated the mid elevation (700 - 900 m a.s.l.) and Sp. 3 and Sp. 4 the high elevations (≥ 900 m a.s.l.) (Fig. 5a). In total, there were 163 nests recorded in the sampled plots, in which ants of 13 genera kept trophobionts directly in their nests (Tab. 1). Notably, all genera were associated with scale insects (Sternorrhyncha: Coccoidea, four families) with the exception of *Pseudolasius* which was associated exclusively with Flatidae (10 nests, 6% of the interactions) (Fig. 5b). Outside of the nests, *Pseudolasius* ants were not observed tending any trophobionts. Overall, 1725 nests were sampled; of those 0.6% hosted planthoppers and 8.9% scale insects.

A review of ant-Auchenorrhyncha trophobioses inside ant-built structures and their distribution

Our search for other cases of hoppers (Auchenorrhyncha) known to be actively kept inside nest structures revealed that there have been only a few such symbioses described, and in a relatively small proportion of hopper families. Specifically, only five of 33 families are known to contain specialised / obligate cases (see Tab. 2 for the overview and all literature). This is in contrast when trophobioses *sensu lato*, i.e., those including facultative interactions inside nests or that were reported to occur commonly outside nests, are all considered (then at least 13 families interacting with ants; Fig. 6). For the infraorder Cicadomorpha we found only two obligate cases, both in the family Cicadellidae, although two other families (Membracidae, Cercopidae) are occasionally in a facultative relationship with dominant arboreal ant species that build temporal small shelters around them on vegetation, or silk carton nests (Tab. 2). Obligate interactions occurring in nest structures are more common in Fulgoroidea hoppers, where at least eight different cases have been reported across four different planthopper families (including this study; Fig. 6). While ant interactions with Cicadomorpha seem to be limited to tropical vegetation, within Fulgoromorpha the interactions are more diverse, reported from both the tropical and the temperate zone and from both terrestrial and arboreal nests (Tab. 2). The phylogenetic distribution of Auchenorrhyncha families interacting with ants suggests that the symbiosis evolved multiple times in several groups (Figs. 6, S6). This has likely occurred independently even within some families (e.g., in both Australian and neotropical Cicadellidae, Tab. 2).

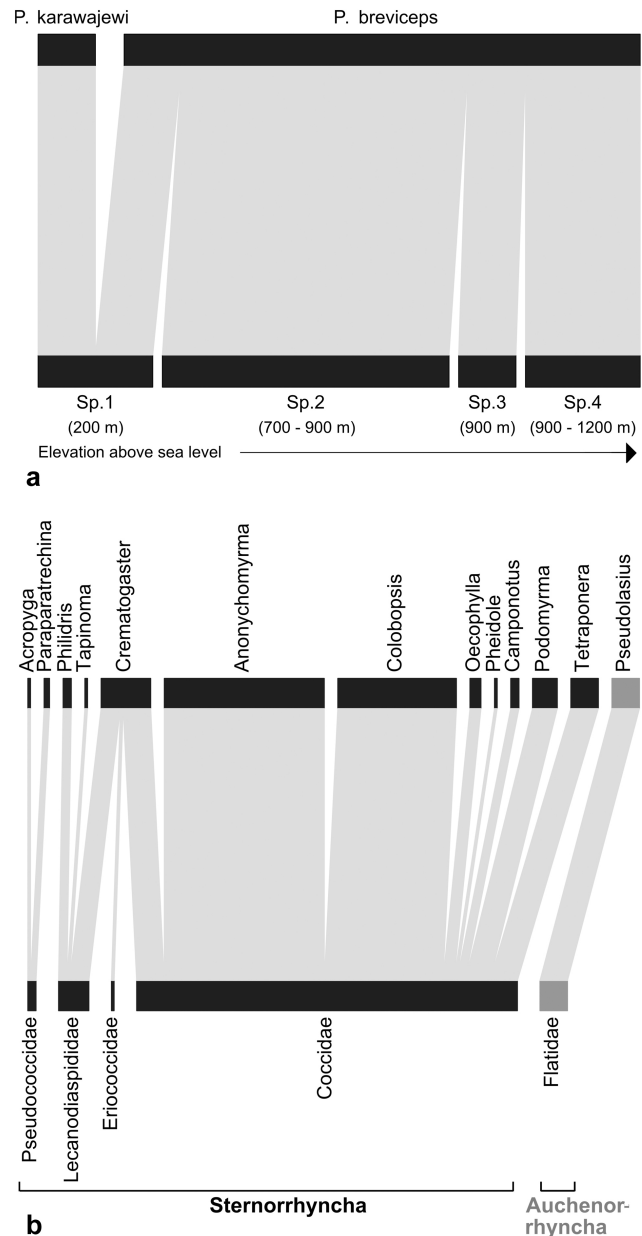


Fig. 5: Interaction networks of the arboreal ant communities (upper bars) and trophobionts kept in their nests (lower bars) in trees in New Guinea rainforests. Length of the bars and strength of the links correspond to the number of nests found in the trees (see Tab. 1 and the methods section for the data used). (a) Interaction network among planthoppers (Auchenorrhyncha: Flatidae) and *Pseudolasius* ants. Symbiont species (Tab. 1, Fig. 3) are ordered by the elevational spans at which they occurred. (b) Interactions between all ant genera and the main groups (families) of trophobionts. Different colours distinguish Sternorrhyncha (i.e., *Pseudolasius*-flatids; grey bar) and Auchenorrhyncha (scale insects; black bars).

The occurrence of obligate symbiosis inside nests was not significantly different from random when considering the family-level Auchenorrhyncha phylogeny (mpd.obs.z = -0.5, $p = 0.28$). Similar results were obtained for families with any trophobiosis in ant-structures, or with trophobiosis *sensu lato* (i.e., including the families that interact with ants outside nests, see Tab. S2 for more).

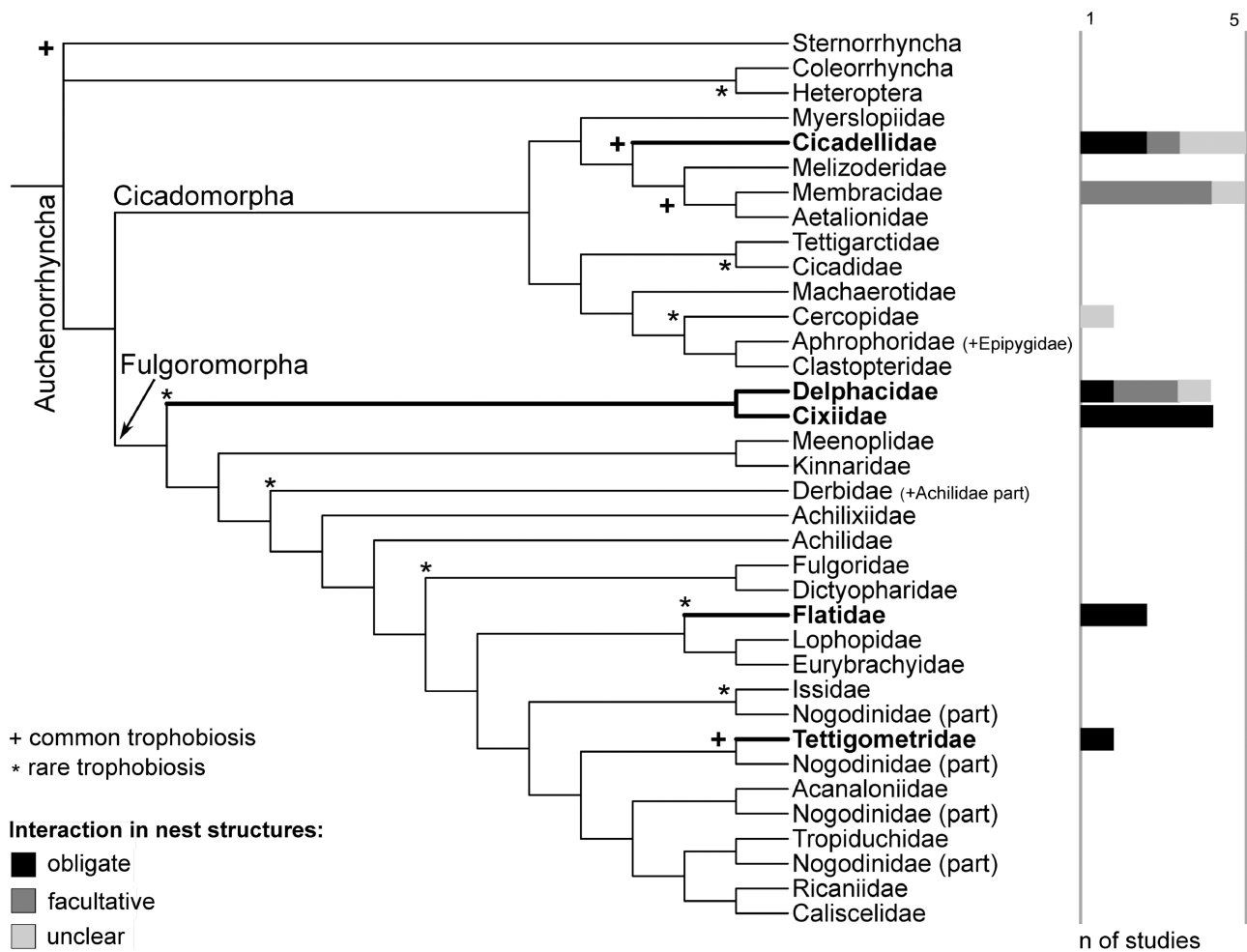


Fig. 6: Cladogram of Auchenorrhyncha families based on previous several studies of nuclear markers (URBAN & CRYAN 2007, CRYAN & URBAN 2012), and distribution of mutualistic interactions with ants in their nests by number of case studies (from Tab. 2). The lineages with the obligate association with ants in their nests in some species are in bold; those relatively commonly or rarely involved in any trophobiosis with ants (BOURGOIN 1997, DELABIE 2001) are marked by crosses or asterisks, respectively. Presences of all categories of trophobiosis across the cladogram are distributed randomly (999 randomisations of mean phylogenetic pairwise distances, $p > 0.025 < 0.0975$, see Tab. S2 for more).

Discussion

Trophobiotic interactions between ants and Hemipteran symbionts are very common on tropical rainforest vegetation (BUCKLEY 1987, DELABIE 2001, DEJEAN & al. 2007), but relatively little is known about the cryptic interactions inside of ant-nest structures. Our study is the first to look at a community of exclusively nest-dwelling Auchenorrhynchan trophobionts in rainforests (Figs. 1, 5), and the first to review similar cases not only across tropics, but worldwide (Tab. 2).

Novel trophobiosis from New Guinea and its comparison to other similar symbioses

Previous studies have noted that some ecologically dominant ants occasionally build shelters to cover symbiont colonies, including some groups of Auchenorrhyncha, but they rarely quantify such interactions at the community level (BOURGOIN 1997, GULLAN 1997, BLÜTHGEN & al. 2006). One exception is a study by MEZGER & BLÜTHGEN (2007), which included quantitative data on symbionts kept in ant-built shelters on the liana *Dinochloa trichogona* S.DRANSF., 1981 in Borneo.

They found that in 11 of 31 trophobioses observed with Delphacidae, ants built covers from soil over the herds. Similarly, the cases of keeping Membracidae inside ant nests are rare as they are normally tended on branches, but they can occasionally occur in the silk-nest pavilions of *Oecophylla* (see BLÜTHGEN & FIEDLER 2002). These are examples of a facultative symbiosis with hoppers, where the shelters are usually small and temporary with typically multiple ant species and multiple taxonomical groups of insect herds interacting, and thus not exclusive to Auchenorrhyncha (McKAMEY 1992, DEJEAN & BOURGOIN 1998, ANDERSON & McSHEA 2001) (Tab. 2). Only in some cases, have more specific obligate interactions evolved, where the symbiont is dominated by a single or a few ant species, which always build the shelters over them, and sometimes even carry ant larvae and / or honeydew-providing partners to these satellite nests (DOROW & MASCHWITZ 1990, DEJEAN & al. 1996, ANDERSON & McSHEA 2001, MOYA-RAYGOZA & LARSEN 2008).

Our study presents a new case of this kind of specialised and likely obligate trophobiosis inside nests, which

Tab. 2: Review of the trophobiotic interactions among Auchenorrhyncha and ants that occur in nest structures based on the literature. **In bold**: obligate relationships, where symbiont species kept always with ant workers closed in the core nests or their satellites (shelters). In grey: little studied observations (uncertain symbioses).

Family Genus / species	Host ant	Host plant	Interaction	Habitat	Region	References
Cicadomorpha (3 of 11 families)						
Cercopidae						
not determined	<i>Aphaenogaster relicta</i> WHEELER, W.M. & MANN, 1914	not specified	not specified (single occurrence)	two nymphs collected in the nest, uncertain habit	tropical (Central America)	MANN (1915)
Cicadellidae						
<i>Chunroides knighti</i> MALDONADO-CAPRILES, 1975	<i>Azteca</i> sp.	not specified	unclear (single occurrence)	shelter on plant stem	tropical (S America)	DIETRICH & MCKAMEY (1990)
<i>Dalbulus quinque-notatus</i> DELONG & NAULT, 1983	various; dominated by <i>Pheidole</i> spp., <i>Solenopsis geminata</i> (FABRICIUS, 1804)	<i>Tripsacum</i> spp.	obligate (myrmecophilous)	always in ant shelters in stem/leaves near ground	tropical (Central America)	MOYA-RAYGOZA & LARSEN (2008)
Pogonoscopini (Eurymelinae): five species	<i>Camponotus</i> spp.	<i>Eucalyptus</i> spp.	obligate (myrmecophilous), probably species-specific among both partners	in nests underground, symbionts move from nest at night to feeding sites in trees, where attended by workers	subtropical (Australia)	DAY & PULLEN (1999)
<i>Myrmecophryne formiceticola</i> (KIRKALDY, 1906)	not specified	unknown	not specified	in a nest underground	subtropical (Australia)	KIRKALDY (1906)
Membracidae						
not specified	<i>Camponotus brutus</i> FOREL, 1886	<i>Bridelia micrantha</i> (HOCHST.) BAILL.	facultative (the ant tends many other symbionts; sometimes not in shelters)	shelters at trunk bases	tropical (Africa)	DEJEAN & BOURGOIN (1998)
<i>Sextius</i> sp.	<i>Oecophylla smaragdina</i> (FABRICIUS, 1775)	various, dominantly on lianas	facultative (the ant tends many other symbionts; only occasionally in shelters)	pavilions from folded leaves (satellites for symbionts)	SE Asia	BLÜTHGEN & FIEDLER (2002)
<i>Eunusa</i> , <i>Erechtia</i>	<i>Azteca</i> spp.	various, understorey	facultative (also freely on plants; sometimes multiple species including scale insects in the shelters)	small shelters at stems (1-3 cm)	tropical (S America)	MCKAMEY (1992)
not determined	<i>Temnothorax sallei</i> (GUÉRIN-MÉNEVILLE, 1852)	not specified	not specified	in ant nest, single nymph only, prey or symbiont	tropical (Central America)	MANN (1915)
Membracidae / Cicadellidae						
Various	<i>Camponotus acvapimensis</i> MAYR, 1862, <i>Pheidole megacephala rotundata</i> FOREL, 1894 (Africa) and various arboreal ants (S.E. Asia)	various	facultative (the ant tends many other symbionts; sometimes not in shelters)	shelters in plants	tropical (Africa), SE Asia	LAMBORN (1914), BLÜTHGEN & al. (2006)
Fulgoromorpha (5 of 21 families)						
Cixiidae						
<i>Mnemosyne cubana</i> MYERS, 1929	<i>Odontomachus insularis</i> GUÉRIN-MÉNEVILLE, 1844	not specified	obligate (nymphs only, and inside or close to nest chambers)	nests in rotten trunk, feeds underground on plant roots	tropical (Central America)	MYERS (1929)
<i>Oliarus vicarius</i> WALKER, 1851	<i>Solenopsis invicta</i> BUREN, 1972, <i>Paratrechina vividula</i> (NYLANDER, 1846), <i>Aphaenogaster carolinensis</i> WHEELER, 1915	not specified	obligate (nymphs only, and inside or close to nest chambers)	nest mounds underground	subtropical (N America)	SHEPPARD & al. (1979), THOMPSON & al. (1979)

<i>Oecleus borealis</i> Van DUZEE, 1912	<i>Paratrechina arenivaga</i> (WHEELER, 1905)	<i>Pinus clausa</i> (ENGELM)	obligate (nymphs only, and inside of nest chambers)	nest mounds underground	subtropical (N America)	THOMPSON (1984)
<i>Reptalus panzeri</i> (LÖW, 1883)	<i>Aphaenogaster subterranea</i> (LATREILLE, 1798)	<i>Fraxinus ornus</i> L.	obligate (nymphs kept in the nests)	nests under stones and in soil, feeds on roots of the plant	temperate (Central Europe)	LÖRINCZI (2012)
Delphacidae						
<i>Peregrinus maidis</i> (ASHMEAD, 1890)	<i>Pheidole megacephala</i> (FABRICIUS, 1793), <i>Crematogaster</i> spp., <i>Paratrechina</i> spp.	<i>Zea mays</i> L.	obligate (closed always in shelters and actively protected, larvae transported there)	shelters and nests in stem/leaves	tropical (Africa)	DEJEAN & al. (1996)
not specified	<i>Pheidole</i> sp., <i>Crematogaster</i> sp., <i>Myrmecaria opaciventris</i> EMERY, 1893	<i>Bridelia micrantha</i> (HOCHST.) BAILL.	facultative (the ants tend many other symbionts on the plant)	occasional shelters in trees	tropical (Africa)	DEJEAN & BOURGOIN (1998)
not specified	<i>Camponotus</i> cf. <i>arrogans</i> , (F. SMITH, 1858), <i>Lophomyrmex bedoti</i> EMERY, 1893, <i>Crematogaster</i> spp.	<i>Dinochloa trichogona</i> S.DRANSF., and others	facultative (the ants tend many other symbionts on the plants)	occasional shelters in trees	tropical (SE Asia)	BLÜTHGEN & al. (2006), MEZGER & BLÜTHGEN (2007); N. Blüthgen, pers. comm.
<i>Notuchus</i> spp.	<i>Paratrechina</i> sp.	not specified	obligate? (adults endogeic and with troglomorphy)	only one of the species found under stone, where ants present; unclear if trophobiotic	tropical (New Caledonia)	HOCH & al. (2006)
Flatidae						
<i>Budginmaya eulae</i> FLETCHER, 2009	<i>Camponotus terebrans</i> (LOWNE, 1865)	feeding unknown	obligate (transported by ants and myrmecophilous body modification)	in nest, underground	subtropical (Australia)	FLETCHER & MOIR (2009)
not determined (four morpho-species)	<i>Pseudolasius</i> spp.	various	obligate (only in <i>Pseudolasius</i> nests)	shelters and nests on trunks	tropical (New Guinea)	this study
Hypochthonellidae						
<i>Hypochthonella caeca</i> CHINA & FENNAH, 1952	<i>Dorylus fulvus</i> (WESTWOOD, 1839) and other spp.	various crop plants	obligate? (adults endogeic and with troglomorphy)	feeding in roots underground (unclear if kept in the nest or random foraging)	tropical (Africa)	CHINA & FENNAH (1952)
Tettigometridae						
<i>Tettigometra laetus</i> HERRICH-SCHÄFFER, 1835	various (three species of different genera)	various	obligate (carried by workers between feeding sites and ant nests)	in nests underground and under herbs/grass	temperate (N Europe)	LEHOUCQ & al. (2004)

is rare in Auchenorrhyncha (Tab. 2). As expected for the New Guinea region, most insects kept directly in ant nests belonged to various groups of scale insects (GULLAN & al. 1993). The same has been observed in other tropical regions, with Auchenorrhyncha making up only a small proportion of the interactions (BLÜTHGEN & FIEDLER 2002, BLÜTHGEN & al. 2006, MEZGER & BLÜTHGEN 2007) or not present in arboreal ant nests (DOROW & MASCHWITZ 1990, WEISSFLOG 2001, ROHE & MASCHWITZ 2003). Furthermore, the novel interaction between *Pseudolasius* ants and several planthopper species was a notable exception in the NG ant-trophobiont community: No other ant genera were observed tending Auchenorrhyncha in nests. Notably, the only Hemiptera that were visited by ants outside of nests on sampled trees were scale insects, and *Pseudolasius* was not seen to interact with them (P. Klimes, unpubl.). The hopper nymphs were always found inside of nest structures (never outside), sometimes even in the same chambers as the ant brood. Although these

relationships were not species-specific (i.e., two ant species and four planthopper species were involved), our comparisons using molecular data and the available phylogenies of the hopper families support that all the planthoppers came from a single group, related to Australian Flatidae.

Flatidae is one of the largest hopper families worldwide with nearly 1500 species (BOURGOIN 2017), but they are rarely involved in interactions with ants. Even facultative visitation of their nymphs and adults sucking on vegetation outside nests is rare compared to other Auchenorrhyncha such as Cicadellidae and Membracidae (DELABIE 2001, STAAB & al. 2015). One exception is the mutualism between the flatid *Bythopsyrna circulata* (GUÉRIN-MÉNEVILLE, 1844) and *Dinomyrmex gigas* (LATREILLE, 1802): in this trophobiosis, ant workers forage to herds of the hoppers attached to the bark of a tree, and antennate the abdomen or head of the planthopper to induce honeydew-dropping; but they do not build shelters or carry the symbionts into nests (PFEIFFER & LINSSENMAIR

2007). Only one study has reported a close relationship of a flatid species with ant nests, but underground (FLETCHER & MOIR 2009). In this case the trophobiont was found under a stone in a *Camponotus* nest in Australia, perhaps kept in a similar manner as some Cixiidae and Tettigometridae in the temperate zone (LEHOUCK & al. 2004, LŐRINCZI 2012) (Tab. 2). It is unknown how specific this relationship is to *Camponotus* ants, and the general ecology of the planthopper species remains unstudied (FLETCHER & MOIR 2009). However, specialised relationships have been observed in some other Auchenorrhyncha groups: Several species of *Camponotus* are associated with Australian Eurymelinae hoppers (Cicadomorpha), similar to the *Pseudolasius*-flatids relationship, but underground (DAY & PULLEN 1999). In some cases, these hoppers leave the nest at night to feed openly on vegetation, accompanied by the ants, and behavioural confrontation tests suggest that the symbiosis might be partner species-specific (DAY & PULLEN 1999). Whether there are any similar behaviours of the flatids in New Guinea remains unclear. We did not observe any nymphs or adults outside of nest shelters, but we did not conduct any nocturnal or behavioural experiments.

Although not species-specific, there was a considerable turnover of symbiont species with elevation, so multiple species of hoppers rarely co-existed in the same sites. This implies a very close association among the ant genus and the symbiont group, but our dataset and field observations are too scarce to test the hypothesis of possible coevolution. Another support for a close association here is, however, observations that other ant species (e.g., some from the genera *Crematogaster*, *Philidris*, *Paratrechina*) frequently build soil-nest covers on the bark of trees similar to those of *Pseudolasius* ants (KLIMES 2017), but they never contained any planthoppers. The interaction observed in New Guinea is thus very unique for *Pseudolasius* ants, as the genus only tends scale insects and aphids elsewhere (see MALSCH & al. 2001, KAUFMANN & al. 2003). Although this symbiosis seems to be relatively rare in rainforest ecosystems, it may be more frequent in mid-elevational pristine forests, where greater cover of mosses and epiphytes over trunks probably facilitate the nesting of *Pseudolasius breviceps* above ground on tree trunks. However, as the ant species is also relatively common in the lowlands and predominantly nests underground, we cannot exclude the possibility that our study on tree-dwelling communities may have missed the cases of trophobiosis taking place on plant-roots underground, including possibly those with scale insects (MALSCH & al. 2001). Also, as we have sampled a limited number of plots per elevation and over a single mountain range, elevational patterns need to be further evaluated in future observations.

Knowledge of life-history and evolution of cryptic trophobioses in Auchenorrhyncha

Our study brings an important contribution to the knowledge of an intimate symbiosis among ants and planthoppers, in terms of occurrences and interactions across a wide elevational range and whole ant community. Yet, the life-history of the flatid symbiosis inside the nest is still little known. Further research should focus more on the nesting habits of the host ants, the ethology and ecology of the trophobiosis, and on seeking the adult stages. It is not clear how nymphs get into the nest to begin the trophobiosis; whether the youngest instars are brought in by workers, as has been observed in *Pseudolasius* with scale insects (MALSCH & al.

2001). Or alternatively, if nymphs actively seek out the ant nests themselves. In addition, because we did not observe direct trophobiotic feeding of ants on nymphs (probably due to the disturbance caused by breaking into nests), the exact way in which ants collect honeydew is unknown, though it may be similar to that of *D. gigas* ants and flatid *B. circulata* (see PFEIFFER & LINSENMAIR 2007). Furthermore, the lack of material and information on adults as well as being a taxonomic impediment, means that the feeding ecology, lifecycle, and habitat requirements of these Flatids remain mysterious. The lack of above information is, however, very common for cryptic trophobioses taking place in nests. Most other reports of these symbioses within Auchenorrhyncha consist of single-case studies and taxonomical reports, rather than detailed ecological data. In particular, information on whole ant and symbiont communities (as provided here) or evidence from behavioural experiments are rare (but see DEJEAN & al. 1996, DAY & PULLEN 1999, LEHOUCK & al. 2004, MOYA-RAYGOZA & LARSEN 2008). Despite this, certain studies with limited evidence (Tab. 2: e.g., endogeic Delphacidae, Hypochthonellidae, Cicadellidae) have been cited as examples of obligate myrmecophily (DIETRICH & MCKAMEY 1990, BOURGOIN 1997, HOCH & al. 2006) while it may not be the case. For instance, that hoppers might be have been accidentally collected in those studies near nest or ant foraging trail cannot be excluded.

Current knowledge of the evolution of these symbioses is also relatively poor. Previous studies have focused more on ancestral preferences of the lineages for particular vegetation stratum and general ant-attendance than on interaction with ant nests (BOURGOIN 1997). As even the basic phylogeny of Auchenorrhyncha families and monophyly of the whole group is under debate (CRYAN & URBAN 2012, SONG & al. 2012; J.R. Cryan, pers. comm.) (Figs. 6, S5) and for most families only a handful of taxa have been sequenced so far from this very species-rich insect group, it is not yet feasible to test the evolutionary hypothesis of trophobiotic traits with ants in this group at species or genus level. However, given the rarity of obligate hopper trophobiosis in ant nests compared to other Hemiptera groups, and its wide phylogenetic and geographical distribution across five different families and both Cicadomorpha and Fulgoromorpha (Tab. 1, Fig. 6), there are likely independent origins within each family. This is supported by a lack of phylogenetic clustering of any trophobioses across the family-level cladogram (Fig. 6, Tab. S2). In extreme cases, in certain species, the myrmecophilous habit of the hoppers in the nests may lead to morphological changes in the adults that are typical for antinquilines (HOCH & al. 2006, FLETCHER & MOIR 2009), although it is not clear if that is a consequence of true coevolution or rather an adaptation to subterranean life (HOCH & al. 2006).

Reasons for the rarity of ant-Auchenorrhyncha associations inside nests

An interesting question emerges, why the obligate symbiosis in ants with Auchenorrhyncha is so scarce in ant nests compared to other hemipterans (Fig. 5, Tab. 2). The advantages for trophobionts living in nests and shelters are relatively well known, particularly the benefits of protection against parasites and predators (GULLAN 1997, DELABIE 2001, STYRSKY & EUBANKS 2007). In return, the ants get from the symbionts a stable food-resource (ROHE & MASCHWITZ 2003, BLÜTHGEN & al. 2006). Furthermore, keeping the trophobionts inside nests enables some ant species to avoid costly direct com-

petition with other ant colonies and species and increase their dominance (e.g., HÖLLDOBLER & WILSON 1990, LIEFKE & al. 1998, BLÜTHGEN & FIEDLER 2002, DEJEAN & al. 2007). The stable resources of honeydew significantly contribute to the ecological dominance of certain ant species and hence drive the spatial distribution of the species in trees (BLÜTHGEN & al. 2004, 2006, DEJEAN & al. 2007). Despite these advantages, keeping symbionts in ant-built structures appears to be less common than tending them externally, where they are more vulnerable to predation. In the case of Auchenorrhyncha, a combination of relatively larger body-size and higher mobility, represents assembly of traits which may make the evolution of the closer mutualism less likely and too costly compared to aphids and scale insects. Indeed, facultative tending of large herds of Tettigometridae that have lost the ability to jump represents one of the most common trophobioses in Auchenorrhyncha in some tropical regions (BOURGOIN 1997). Jumping ability may also compel ants to keep hoppers inside nest structures to prevent them escaping and consequently lead to a specialised mutualism. This may explain why Tettigometridae are not the most common group with cryptic symbiosis (i.e., inside nests, Tab. 2). Another factor which may contribute to the rarity of obligate trophobiosis of ants with Auchenorrhyncha hoppers is the relatively narrow specialisation of the group on particular plant taxa (Tab. 1), rather typical for phloem suckers (NOVOTNY & al. 2010), since specialisation to particular plants may mediate such symbiosis.

Future perspectives

Our literature review of trophobiosis with hoppers inside ant nests shows that it is not exclusive to the tropics, although in the temperate zone it might be limited to nests underground rather than on vegetation (Tab. 2). Our study and previous works suggest, however, that some of these symbioses might have been overlooked compared to those with externally-feeding hoppers (DELABIE 2001, STADLER & DIXON 2008). Although studying and observing the cryptic interactions is difficult, future studies should focus more on distinguishing basic characteristics of the symbiosis such as the frequency and specificity of interactions, and whether they occur inside nests, or externally. As demonstrated here, a search for nests and examining them in the context of complete arboreal communities can be helpful in this respect. From an ecological perspective, it may influence the nature of the symbiosis if the ants build small and temporal shelters (facultative relationship) versus building large long-term structures for both brood and trophobionts (obligate relationship), but this is rarely investigated for honeydew-providing insects (but see McKAMEY 1992, GULLAN 1997, ANDERSON & McSHEA 2001). More insights are needed into the life-history and ecology of the cryptic symbiosis in nests, including behavioural experiments, to assess the advantages of these unusual relationships between ants and hopper partners.

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Digital supplementary material to

KLIMES, P., BOROVANSKA, M., PLOWMAN, N.S. & LEPONCE, M. 2017: How common is trophobiosis with hoppers (Hemiptera: Auchenorrhyncha) inside ant nests (Hymenoptera: Formicidae)? Novel interactions from New Guinea and a worldwide overview. – Myrmecological News 26: 31-45.

Tab. S1: List of all localities and plot sizes sampled for the *Pseudolasius* and their trophobionts in New Guinea (for the list of the occupied sites and trees see Tab. 1); a.s.l. = above sea level; GPS = geographic coordinates (latitude, longitude) as gathered using a global positioning system.

Elevation (m a.s.l.)	GPS	Total area sampled (ha)	Area per plot (ha)	N of plots	N of trees with <i>Pseudolasius</i> nests	N of trees with plant-hoppers	Hand collecting method	Source
10	-5.1382, 145.75367	0.28	0.28	1	0	0	Ant mosaic study in trees	M. Leponce, unpubl.
100	-5.2254, 145.0808	0.56	0.28	2	0	0	Ant mosaic study in trees	M. Leponce, unpubl.
200	-5.2317, 145.1788	0.50	0.50	1	4	2	Tree felling	KLIMES & al. (2015)
200	-5.7398, 145.3297	0.56	0.28	2	0	0	Ant mosaic study in trees	M. Leponce, unpubl.
700	-5.7319, 145.2521	0.56	0.28	2	1	1	Ant mosaic study in trees	M. Leponce, unpubl.
900	-5.72167, 145.2700	0.20	0.20	1	8	4	Tree felling	N.S. Plowman, unpubl.
1200	-5.7208, 145.2694	0.56	0.28	2	2	1	Ant mosaic study in trees	M. Leponce, unpubl.
1700	-5.7592, 145.2356	0.56	0.28	2	0	0	Ant mosaic study in trees	M. Leponce, unpubl.
2200	-5.7589, 145.1860	0.28	0.28	1	0	0	Ant mosaic study in trees	M. Leponce, unpubl.
2700	-5.8152, 145.1564	0.28	0.28	1	0	0	Ant mosaic study in trees	M. Leponce, unpubl.

Tab. S2: Results of comparisons between observed and random phylogenetic mean pairwise distance (mpd) between families containing trophobioses using the Auchenorrhyncha cladogram (Fig. 6, see methods section for details). No trophobiosis is statistically clustered across the cladogram (i.e., phylogenetically clustered $p < 0.025$, or overdispersed $p > 0.975$); mpd.obs = observed mpd, mpd.rand.mean = mean random mpd, mpd.rand.sd = standard deviation of the random mpd, mpd.obs.rank = rank of observed vs. random mpd, mpd.obs.z = standardized effect size of observed vs. random mpd, mpd.obs.p = p-value (quantile) of observed vs. random mpd.

Dataset (presence / absence)	N of taxa (lineages)	mpd. obs	mpd. rand. mean	mpd. rand. sd	mpd. obs. rank	mpd. obs. z	mpd. obs. p	N of runs
All trophobioses (sensu lato)	13	9.56	10.21	0.65	157.00	-1.00	0.16	999
All trophobioses inside ant structures	7	10.00	10.20	1.15	401.00	-0.17	0.40	999
Obligate trophobioses inside ant nests	5	9.40	10.18	1.56	278.50	-0.50	0.28	999

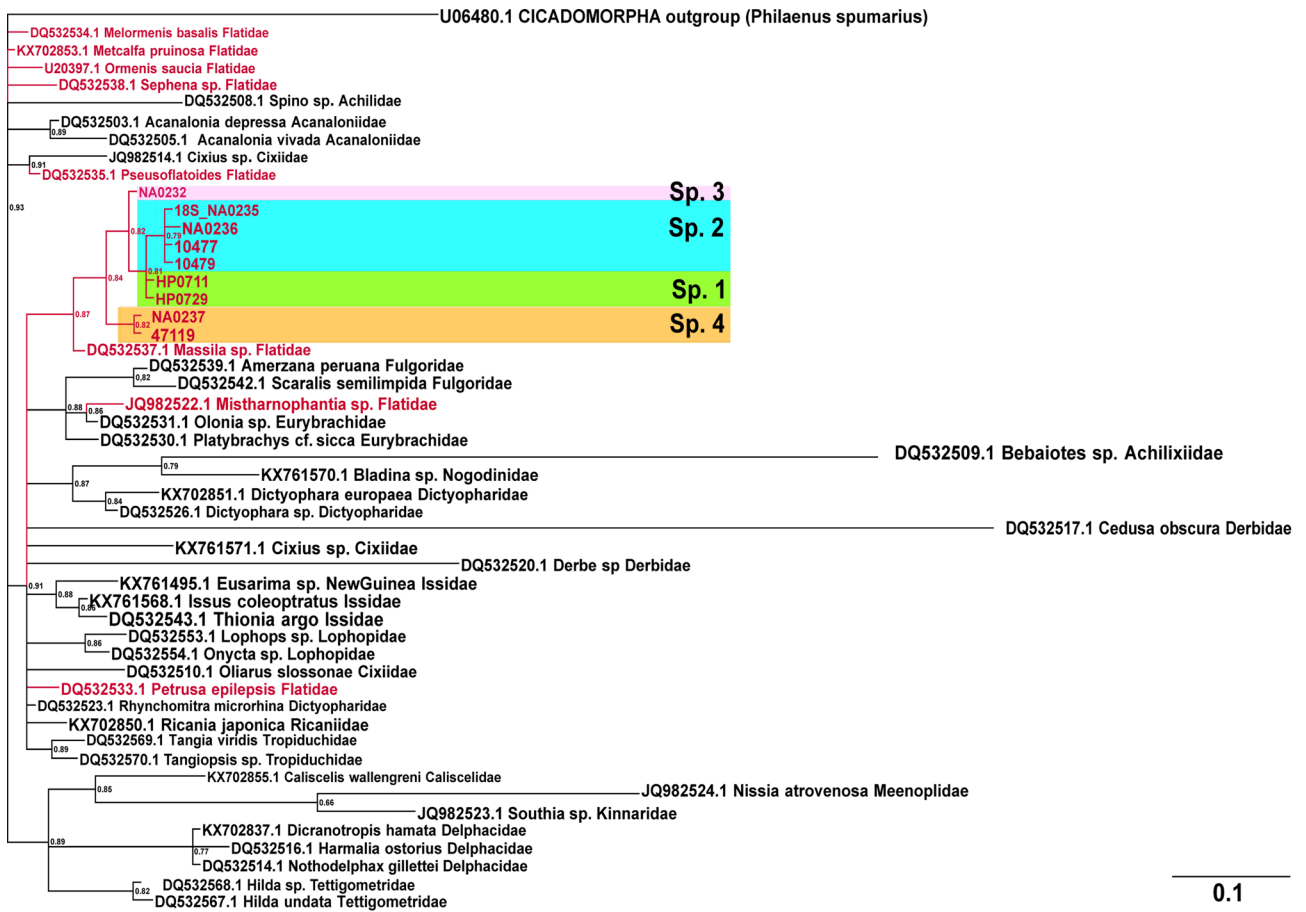


Fig. S1: The 50% majority-rule consensus tree of Fulgoromorpha estimated from the Bayesian analysis based on 18S gene (530 bp fragment). Posterior probabilities of the branches are noted at each resolved nodus (> 0.5). Each branch represents a species (with its GenBank / sample code, species and family name). Representatives of 19 families of Fulgoroidea are included, with multiple species per family where possible. The new samples of Fulgoroidea symbionts of *Pseudolasius* are highlighted by the respective morphospecies; family Flatidae marked in red. See methods section for details.

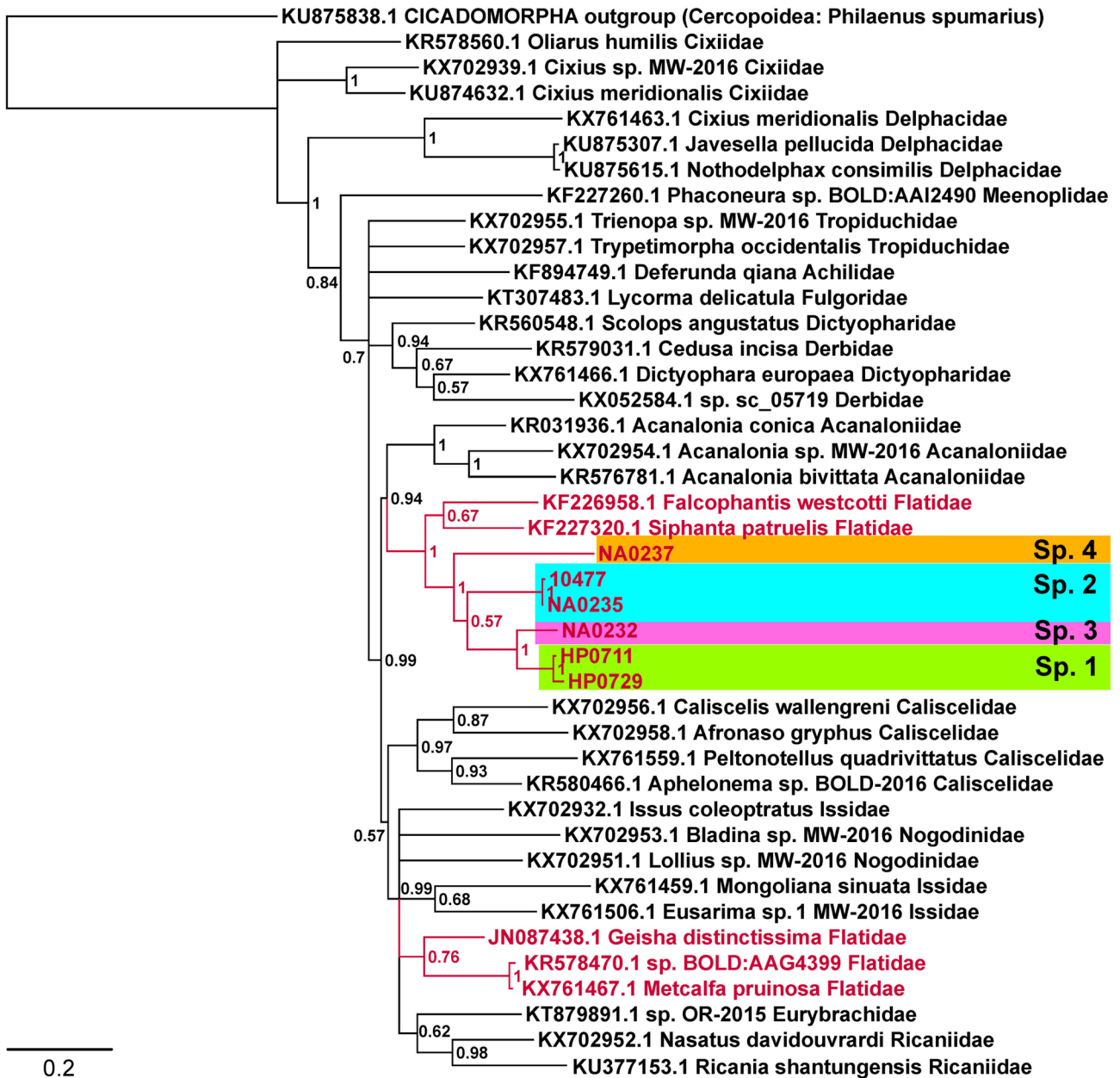


Fig. S2: The 50% majority-rule consensus tree of Fulgoromorpha estimated from the Bayesian analysis based on COI gene (615 bp fragment). Posterior probabilities of the branches are noted at each resolved nodus (> 0.5). Each branch represents a species (with its GenBank / sample code, species and family name). Representatives of 15 families of Fulgoroidea are included, with multiple species per family where possible. The new samples of Fulgoroidea symbionts of *Pseudolasius* are highlighted by the respective morphospecies; family Flatidae marked in red. See methods section for details.



Fig. S3: Video records of interaction of flatid nymph (Sp. 4: sample ML47119) with ant worker (*Pseudolasius breviceps*) after the soil cover of the nest has been broken on tree trunk. Openly accessible video-record at: <https://www.youtube.com/watch?v=c74H-AFbG0I&feature=youtu.be>

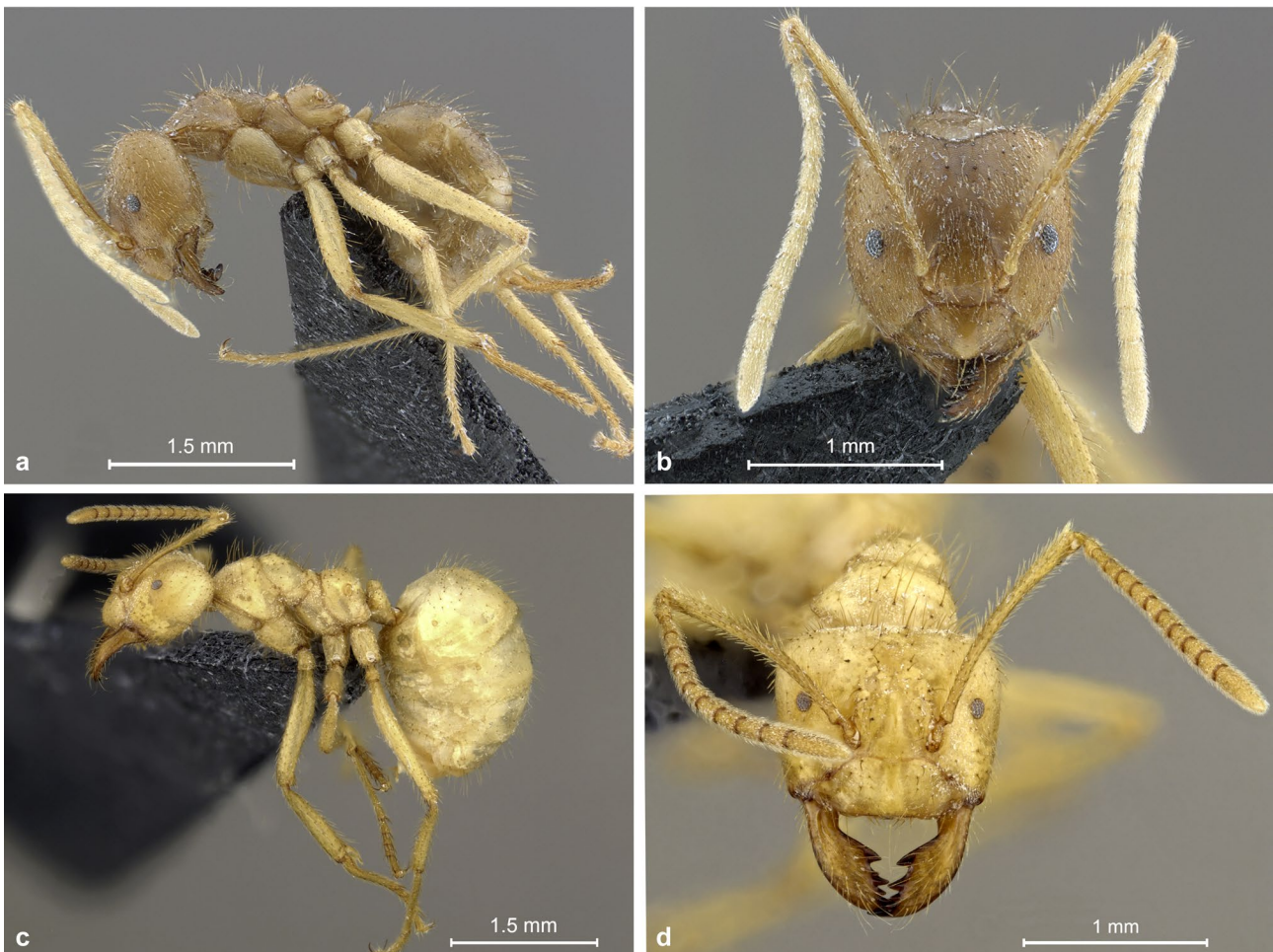


Fig. S4: *Pseudolasius* ant species that interact in their nests with planthopper nymphs (Flatidae). *Pseudolasius breviceps* sample 47119: (a) lateral, (b) frontal; *P. karawajewi* sample HP0711: (c) lateral, (d) frontal (see Tab. 1 for more information on the samples).

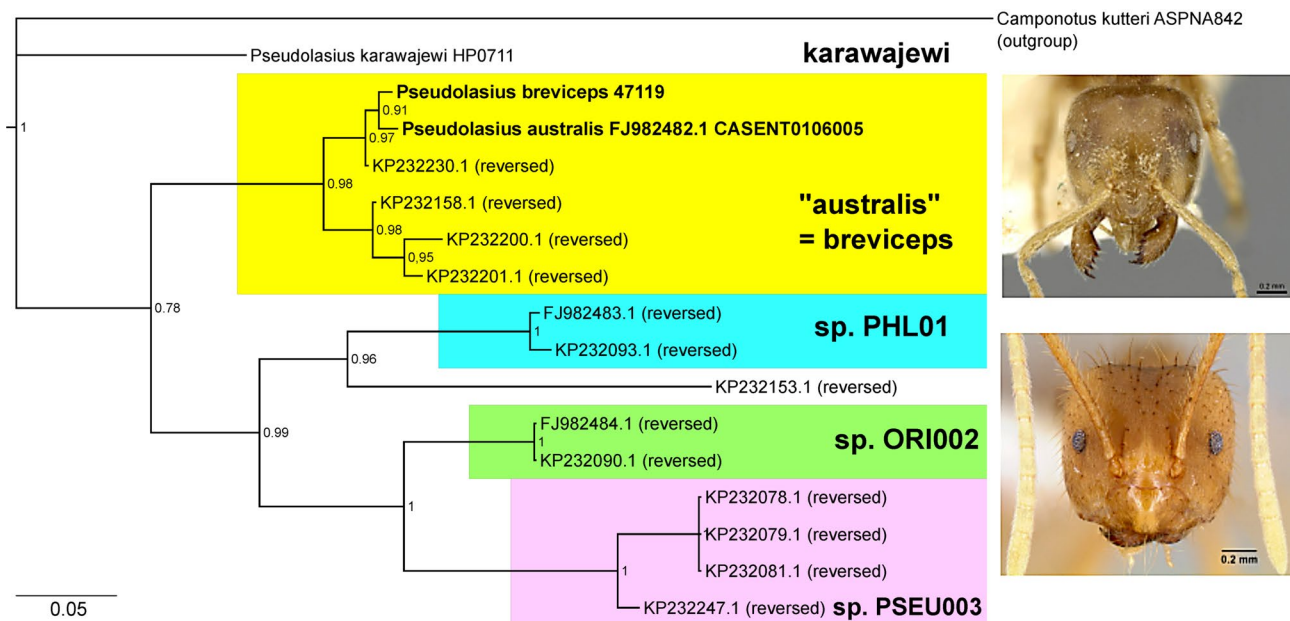


Fig. S5: The 50% majority-rule consensus tree of Australasian *Pseudolasius* estimated from the Bayesian analysis based on COI gene (658 bp fragment). The sequences were loaded from GenBank (BENSON & al. 2013) and compared with COI barcode obtained in this study (samples ML47119 and HP0711: Fig. S3). On the right are frontal head images from Antweb (ANTWEB 2017) for *P. australis* type (top) and CASENT0106005 specimen (bottom), with the latter matching morphologically *P. breviceps* from our study. The “australis” clade in GenBank database thus refers to *P. breviceps*, not *P. australis*. The latter species has not been thus likely sequenced previously, including this study. The clades are highlighted and named by species or morphospecies from GenBank.

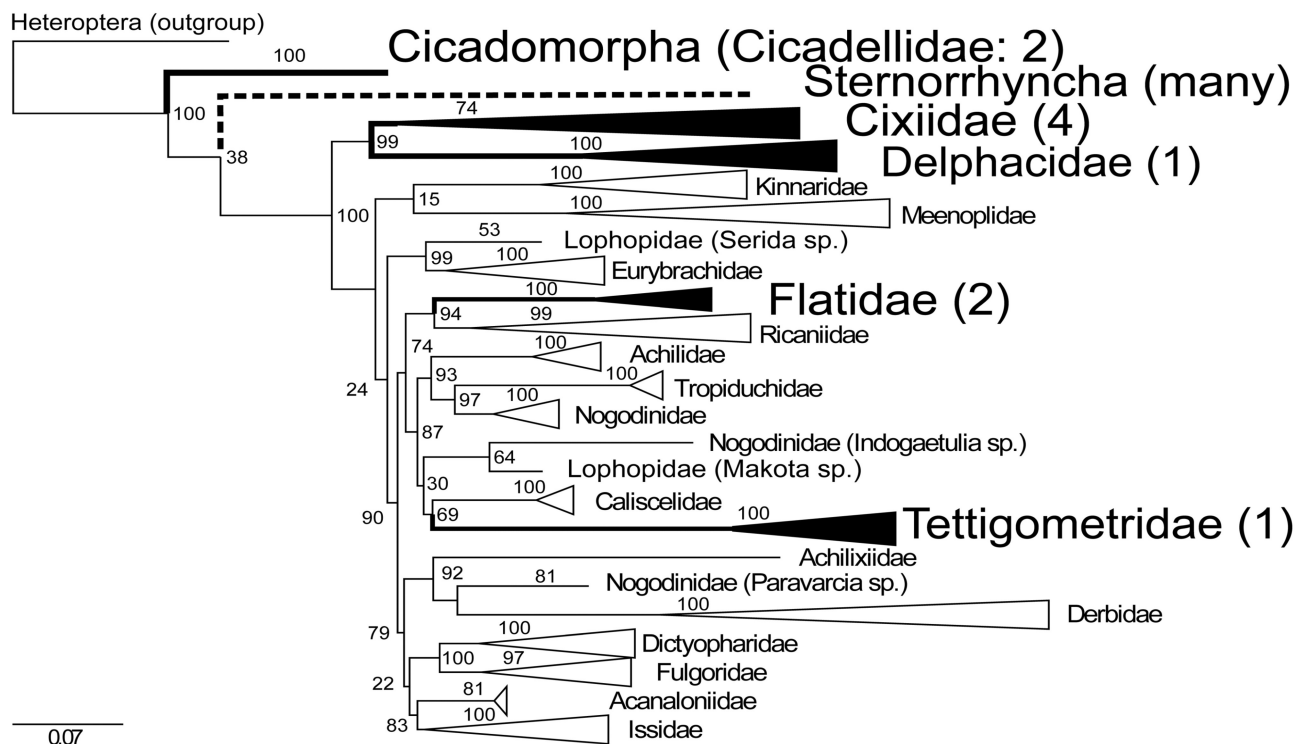


Fig. S6: ML tree of the Hemiptera main groups and 19 Fulgoromorpha families (Cixiidae to Issidae) based on several mitochondrial and nuclear genes adapted from SONG & LIANG (2013). The branches (families) based on more than one species are collated to triangles; digits present their bootstrap support. The groups with known trophobiosis with ants in their nests are marked by enlarged letters in bold with the number of the studies on obligate symbiosis in brackets (see Tab.2 for more). Sternorrhyncha are marked in dashed line due its uncertain phylogenetic position depending on the molecular data used (see Fig. 5 for alternative phylogeny based on nuclear genes only but a wider sampling across whole Auchenorrhyncha).

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