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Research

Partner choice through concealed floral sugar rewards evolved with the specialization of ant–plant mutualisms

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Summary

• Obligate mutualisms require filtering mechanisms to prevent their exploitation by opportunists, but ecological contexts and traits facilitating the evolution of such mechanisms are largely unknown.

• We investigated the evolution of filtering mechanisms in an epiphytic ant–plant symbiotic system in Fiji involving Rubiaceae and dolichoderine ants, using field experiments, metabolomics, X-ray micro-computed tomography (micro-CT) scanning and phylogenetics.

• We discovered a novel plant reward consisting of sugary sap concealed in post-anthetic flowers only accessible to *Philidris nagasau* workers that bite through the thick epidermis. In five of the six species of Rubiaceae obligately inhabited by this ant, the nectar glands functioned for 10 d after a flower's sexual function was over. Sugar metabolomics and field experiments showed that ant foraging tracks sucrose levels, which only drop at the onset of fruit development. Ontogenetic analyses of our focal species and their relatives revealed a 25-fold increase in nectary size and delayed fruit development in the ant-rewarding species, and Bayesian analyses of several traits showed the correlated evolution of sugar rewards and symbiosis specialization.

• Concealed floral nectar forestalls exploitation by opportunists (generalist ants) and stabilizes these obligate mutualisms. Our study pinpoints the importance of partner choice mechanisms in transitions from facultative to obligate mutualisms.

Introduction

How does cooperation among species remain stable over time and escape exploitation by non-reciprocators that do not pay back for what they gain? Understanding this puzzling question is a fundamental research goal in ecology and evolutionary biology (Axelrod & Hamilton, 1981; Sachs *et al.*, 2004; Sachs & Simms, 2006; Frederickson, 2013). Exploiters can have higher fitness than mutualists as they gain the benefits of a mutualistic interaction without incurring the associated costs (Yu, 2001), which can ultimately lead to mutualism breakdown (Sachs & Simms, 2006). Two types of exploitation are distinguished. Cheaters or 'cheater mutants' are exploiters that evolved from mutualistic ancestors (Emery, 1909; Bronstein, 2001; Bull & Rice, 1991; Sachs *et al.*, 2004). Despite being predicted by theory (Trivers, 1971; Axelrod & Hamilton, 1981), there are only a few well-documented examples (Sachs & Simms, 2006), for example in bees (Schaefer & Renner, 2008; Litman *et al.*, 2013), but a majority of parasites are nested within non-mutualistic clades (Sachs & Simms, 2006; Chomicki *et al.*, 2015). The other class of exploiters is referred to as 'parasites of mutualisms' *sensu* Yu (2001), and comprises unrelated opportunistic species that invade mutualisms; some invaders are specialized parasites, such as the ant *Cautalacus*, which exploits the mutualism between *Leonardoxa africana* and *Petalomyrmex phylax* (Gaume & McKey, 1999). Three types of mechanism are generally considered in mutualism stabilization, namely by-product mutualism, partner fidelity feedback and partner choice (Sachs *et al.*, 2004). By-product mutualism occurs when the mutualistic behaviour is cost-free (i.e. involving by-products of other traits), and selection for cheating is thus unlikely to arise (Sachs *et al.*, 2004; Foster & Wenseleers, 2006). Partner fidelity feedback posits that the positive feedback between host and symbiont finesses is sufficient to prevent exploitation, a mechanism that has gained recent theoretical support from economic contract theory (Weyl *et al.*, 2010; Archetti *et al.*, 2011). Finally, partner choice consists in excluding non-cooperative partners by preferentially, or only, rewarding cooperative ones (Bull & Rice, 1991). Individuals choosing cooperative partners enhance their own fitness, and the filtering (choice), in turn, promotes the maintenance of cooperation in the cooperative partner (Sachs *et al.*, 2004). The ecological contexts and traits facilitating the evolution of partner choice mechanisms, however, remain poorly understood (Sachs *et al.*, 2004; Frederickson, 2013).

Ant–plant symbioses involve plants with specialized structures (domatia) in which ants nest, sometimes with the same plant also offering food rewards (e.g. extrafloral nectar (EFN), food bodies),

in return for defence against herbivores, extra nutrients and occasionally the physical or chemical removal of competing plant species (Huxley, 1978; Davidson & McKey, 1993; Renner & Ricklefs, 1998; Frederickson *et al.*, 2005). The evolutionary specialization of such mutualisms could involve an increased investment in rewards, so as to maintain the desired symbiont, for example, by increasing the amount of EFN offered. However, increasing reward levels also increase the interest of opportunists, and partner choice mechanisms should thus evolve to exclude the less desired partners. Three such mechanisms, all involving food rewards, have been documented in myrmecophytic Mesoamerican *Vachellia* (Fabaceae) that host *Pseudomyrmex* (Pseudomyrmecinae) ants (Heil *et al.*, 2005, 2014; Orona-Tamayo *et al.*, 2013), illustrating the importance of rewards as a substrate for the evolution of partner choice in ant–plant symbioses.

The family richest in ant-housing species is the Rubiaceae, which includes over 160 species that develop domatia regardless of the presence of ants. Surprisingly, no ant-plant species in this family has extrafloral nectaries (Weber & Keeler, 2013). Within Rubiaceae, a clade of *c.* 100 epiphytic species from the Australasian region (Psychotriae subtribe Hydnophytinae) is characterized by large hypocotyl domatia with networks of galleries (Fig. 1). The domatia are inhabited by ants, frequently of the dolichoderine genera *Philidris* and *Anonychomyrma*, that feed the plants by defecating inside the cavities, and, in some instances, also provide antiherbivore defence (Huxley, 1978). During fieldwork on rubiaceous ant-plants in Fiji, however, we discovered a novel type of exclusive food reward, when we noticed the more than week-long persistence of old (post-anthetic) flowers visited by the ant mutualist. Our system consists of a clade of nine species from the genus *Squamellaria* (Rubiaceae, Psychotriae, Hydnophytinae), three of which form facultative symbioses with a wide range of ants, and six of which are obligately associated with the dolichoderine ant *Philidris nagasau*. In addition to this Fijian study system, we produce here a phylogeny for the whole subtribe Hydnophytinae and reconstruct the evolutionary histories of symbiosis specialization and partner choice mechanisms. Based on behavioural experiments, three-dimensional reconstructions of nectar gland ontogeny, sugar metabolomics and phylogenetics, we describe the new type of food reward and then address the following questions: By which developmental steps did the new partner choice mechanism evolve? And did the partner choice mechanisms evolve concurrently with increasing symbiosis specialization?

Materials and Methods

Collection of material on Fiji and study sites

In September 2014 and March 2015, we conducted fieldwork on the islands of Viti Levu, Vanua Levu and Taveuni, and collected all nine species of the genus *Squamellaria* that occur on these islands (Chomicki & Renner, 2016). The study sites in Viti Levu were Colo-i-Suva Forest Reserve in the south of the island (18°1′46.808″S, 178°24′0.4175″E) and forest around Navai in the centre of the island (17°37′ 49.5979″S, 177°58′34.9315″E). In Vanua Levu, the collection sites were Waisali Forest Reserve (16°38′19.8″S, 179°13′19.7″E) and along the Cross Island road before the bifurcation to Nabouwalu and Labasa. In Taveuni, the collections were made along the trail to DesVoeux peak and Mt. Manuca on the western side of the island (16°48′ 25.8133″S, 179°56′36.6843″E) and at the end of Lavena coastal walk, Bouma heritage park, on the eastern side of the island (16°51′45.4433″S, 179°54′ 6.5149″E). All collections were made in collaboration with Alivereti Naikatini and Marika Tuiwawa from the University of South Pacific, Suva, and vouchers have been deposited in the herbaria of Suva (SUVA) and Munich (M). For DNA extraction, we collected young leaves and dried them in silica gel. Except for a few cases, *Squamellaria* plants were accessed by tree climbing, using a rope secured by a partner on the ground. This technique allowed long stays in the canopy with minimal disturbance of the ant colony.

Cafeteria experiments addressing the attraction of *Philidris nagasau* to different sugars

To determine whether a decrease in the concentration of sucrose, glucose or fructose affected *P. nagasau* attendance, we conducted 'cafeteria'-style experiments. During these experiments, we synchronously offered different sugar solutions to ants. All experiments were performed without displacements of ants and without artificial platforms, as a pilot had shown that transport and platforms affected ant behaviour. Distilled water was used as a negative control. For each cafeteria, 10 replicates were performed, and five independent ant colonies were used. At each single site, three droplets (10 µl) of each sugar solution were placed on the host tree bark (close to the epiphytic plants), and the order of each solution was randomized, with all drops present at *c.* 10 cm from each other. Ants feeding on each solution were counted twice, at 4 and 6 min following droplet placement, as droplets generally dried out in *c.* 15 min. Droplets were replaced three times and the same procedure was repeated (so that each individual cafeteria consisted of a triplicate, itself performed 10 times on different ant colonies, days and time of the day). Their numbers were summed to calculate the relative numbers of ants that had been attracted to the respective sugar solution. Statistical evaluation was performed by summing the ant numbers attracted to one particular sugar solution for each replicate, and subjected to one-way ANOVA and Tukey's *post-hoc* test, all performed in R v3.2.0 (R Core Team, 2015).

Experiments addressing ant behaviour on young and old *Squamellaria* flowers

To determine whether post-anthetic *Squamellaria* flowers (i.e. floral cups without the petals) were attractive to opportunistic ants, we offered *S. imberbis* (in Vanua Levu) inflorescences to the opportunistic ant species *Camponotus chloroticus*, *Colobopsis polynesica* (*Camponotus polynesicus*; Ward *et al.*, 2016), *Pheidole* sp. 1 and *Pheidole* sp. 2, which live in non-specialized species of *Squamellaria*. As these ants showed no interest in the post-anthetic flowers (different from *P. nagasau* workers, below), we decided to test whether this was caused by the absence of any

Fig. 1 Facultative and obligate symbioses in Fijian epiphytic ant-plants. (a) *Squamellaria imberbis*, Taveuni, an obligate ant-epiphyte species. (b) *Squamellaria wilkinsonii*, Vanua Levu, a facultative ant-epiphyte. (c) Foraging of the (single) ant symbiont of *Squamellaria* (*Philidris nagasau*) inside post-anthetic nectaries. Inset: scars of *P. nagasau* bites after a few days. (d) *Philidris nagasau* exploiting the concealed nectar of *S. wilsonii* by biting into the nectary disc. Bars: (a) 20 cm; (b) 12 cm; (c) 2 cm; (d) 6 mm.

secretion in post-anthetic flowers. We therefore offered the same four opportunistic ant species as well as workers of *P. nagasau* the following: (1) intact floral cups of post-anthetic flowers; (2) floral cups of post-anthetic flowers in which the epidermis had been scratched to expose the accumulated nectar; and (3) intact floral cups bearing a drop of aqueous solution with a similar concentration in sucrose, glucose and fructose (~2400 ng μg^{-1} dry mass), mimicking secreted nectar. Each experiment was replicated five times, each time on a different ant colony. Ants were counted at 5, 7 and 10 min. The numbers of ants attracted to any particular sugar solution were summed and subjected to one-way ANOVA and Tukey's *post-hoc* test, all performed in R.

Philidris nagasau monitoring

We also monitored *P. nagasau* foraging on anthetic and post-anthetic *Squamellaria* flowers throughout the day, focusing on five ant colonies (each living on a different tree) over a 3-d rolling basis. For each colony, behaviour on the floral cups was recorded once an hour for 10 min during daylight. To relate foraging to flower age, we marked and observed 53 flowers of *S. imberbis* (on Taveuni) from the time at which they had just opened to 20 d after anthesis, returning to each flower for 10-min periods between 13:00 and 15:00 h. We also monitored the location of *P. nagasau* workers on *Squamellaria* plants, by counting all ants on the domatia, stems, leaves, post-anthetic nectaries and fresh flowers. We monitored the worker distribution from 20 *Squamellaria wilsonii* (Taveuni) and *S. imberbis* (Vanua Levu) plants, by counting every worker present on all plant parts, for a total of 534 different workers.

Fruit phenology

To test whether *Squamellaria* species characterized by the production of ant-addressed post-anthetic rewards show delayed fruit development compared with closely related species without such rewards, we measured ovary diameter (in the middle) daily from anthesis to 20 d after anthesis in at least 10 flowers of each of the nine species of this genus that occur on Viti Levu, Vanua Levu and Taveuni.

Metabolomics and absolute sugar measurements

Metabolites for gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) were extracted and derivatized using a modified version of the method described in Roessner *et al.* (2001), Lisec *et al.* (2006) and Erban *et al.* (2007). We determined the metabolomic composition of post-anthetic floral rewards in all five rewarding species (*Squamellaria huxleyana*, *S. imberbis*, *S. major*, *S. thekii*, *S. wilsonii*). For each species, we selected a healthy specimen, with similar sun exposure, and

collected its ant-rewarding nectaries (i.e. 2–6 d post-anthesis). To measure the absolute concentration of sugars in each of the key phases of nectary development, we collected *S. imberbis* (Vanua Levu) nectaries from three stages: (1) at anthesis; (2) 2–6 d post-anthesis (i.e. in the phase in which they were actively rewarding ants); and (3) after the onset of fruit development, when the ovary had just started to bulge (i.e. non-ant-rewarding, 14–16 d post-anthesis). In all cases, nectaries were immediately dissected and microwave dried, a method that preserves metabolites (Popp *et al.*, 1996) and is ideal under field conditions. For the extraction, ~5 mg of plant material (dry weight) was ground in 300 µl of cold (–20°C) methanol (80%) containing 15 µl of ribitol (0.1 mg ml^{-1} in water) and 15 µl of ^{13}C-sorbitol (0.1 mg ml^{-1} in water), which were added as internal standards for the quantification of metabolite abundances. After incubation at 70°C for 15 min, 30 µl of the extract was dried *in vacuo*. The pellet was re-suspended in 10 µl of methoxyaminohydrochloride (20 mg ml^{-1} in pyridine) and derivatized for 90 min at 37°C. After the addition of 20 µl of BSTFA (*N,O*-bis[trimethylsilyl] trifluoroacetamide) containing 5 µl of retention time standard mixture of linear alkanes (n-decane, n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotriacontane), the mix was incubated at 37°C for a further 45 min. A volume of 1 µl of each sample was injected into a GC-TOF-MS system (Pegasus HT, Leco, St Joseph, MI, USA). Samples were derivatized and injected by an autosampler system (Combi PAL, CTC Analytics AG, Zwingen, Switzerland). We used helium as carrier gas at a constant flow rate of 1 ml min^{-1}. We performed GC on an Agilent GC system (7890A, Agilent, Santa Clara, CA, USA) using a 30-m VF-5 ms column with a 10-m EZ-Guard column. The injection temperature of the CIS injector (CIS4, Gerstel, Mühlheim, Germany) increased with a rate of 12°C s^{-1} from an initial temperature of 70°C to 275°C. Transfer line and ion source temperatures were set to 250°C, with an initial oven temperature of 70°C gradually increased by 9°C min^{-1} to a final temperature of 320°C. To avoid solvent contamination, the solvent delay was set to 340 s. Metabolites that passed the column were released into the TOF-MS. The transfer line connecting the GC and the TOF-MS was set to 250°C. The ion source at which the in-streaming metabolites were ionized and fractionated by an ion pulse of 70 eV was also set to 250°C. Mass spectra were recorded at 20 scans s^{-1} with an *m/z* 35–800 scanning range. Chromatograms and mass spectra were evaluated using ChromaTOF 4.5 and TagFinder 4.1 software (Luedemann *et al.*, 2008). Absolute quantitative estimation was performed using external standards of each compound. Relative values are the specific ratios of the metabolite intensity multiplied by the intensity of the internal standard compound, normalized by the amount of dry weight. The full list of metabolites is given in Supporting Information Table S1.

DNA extraction, phylogenetic analyses and molecular clock dating

We generated two phylogenies for this study. First, a nine-marker phylogeny for the nine Fijian *Squamellaria* species using six plastid regions (*trnL* intron, *trnL-trnF* spacer, *ndhF*, *rps12-rpl20*, *trnS*-trnG and *rps16*) and three nuclear regions (18S, ITS and ETS), which have been proven to be useful in Rubiaceae phylogenetics (e.g. Barrabé *et al.*, 2014). The primers used are reported in Table S2. All accessions of Fijian *Squamellaria* were extracted from silica-dried leaves collected by GC and are all linked to herbarium specimens deposited in the herbaria SUVA and M (Table S3). Outgroups (in the tribe Psychotrieae) were selected based on Barrabé *et al.* (2014). Second, a six-marker phylogeny for the whole subtribe Hydnophytinae, sampling 50% of their *c.* 100 species (55 ingroup plus 22 outgroup) for two nuclear markers (ITS and ETS) and three plastid markers (*ndhF*, *trnL* intron and *trnL-trnF* spacer), obtained from a combination of herbarium material, material collected in Fiji by the first author and vouchered cultivated material. The outgroup sequences were downloaded from GenBank and came from Barrabé *et al.* (2014). Vouchers, geographical origin and GenBank accession numbers are reported in Table S3.

Total genomic DNA was extracted from *c.* 20 mg of leaf tissues using a commercial plant DNA extraction kit (NucleoSpin; Macherey-Nagel, Düren, Germany) according to the manufacturer's protocols. Polymerase chain reaction (PCR) was performed using Taq DNA polymerase (New England Biolabs, Cambridge, MA, USA) and a standard protocol (39 cycles, annealing temperature of 56°C). PCR products were purified using the ExoSap clean-up kit (Fermentas, St Leon-Rot, Germany), and sequencing relied on Big Dye Terminator kits (Applied Biosystems, Foster City, CA, USA) on an ABI 3130 automated sequencer (Applied Biosystems, Perkin-Elmer). Sequences were edited in Sequencher 5.1 (Gene Codes, Ann Arbor, MI, USA). All new sequences were Blast searched in GenBank. Sequence alignment was performed in Mafft v.7 in the online server (http://mafft.cbrc.jp/alignment/server; Katoh & Standley, 2013) under standard parameters, except for the ITS region which was aligned under Q-INS-i optimization, which takes rRNA secondary structure into consideration. Minor alignment errors were corrected manually in Mesquite v.2.75 (Maddison & Maddison, 2011).

In the absence of statistically supported incongruence (i.e. maximum likelihood (ML) bootstrap support > 75) between the plastid and nuclear data partitions, we concatenated all DNA matrices, yielding an alignment of 9346 bp for the *Squamellaria* matrix and 5895 bp for the Hydnophytinae matrix. ML inference relied on RAxML v.8.0 (Stamatakis *et al.*, 2008) with 100 ML bootstrap replicates and the analysis partitioned by gene region, all under the GTR + Γ substitution model, with empirical nucleotide frequencies and 25 gamma rate categories. We also conduced Bayesian inference in MrBayes v.3.2 (Ronquist *et al.*, 2012), using the default two runs and four chains (one cold and three heated), with the uniform default priors. Model parameters were unlinked, and posterior probabilities of the tree topologies were estimated from all 10 partitions, each running under its best-fitting model according to the Akaike information criterion (AIC) as determined in jmodeltest2 (Darriba *et al.*, 2012). We set a 10×10^6 Markov chain Monte Carlo (MCMC) chain, sampling trees every 1000th generation. Split frequencies approaching

zero indicated convergence. We used the 50% consensus tree to assess the posterior probabilities for the nodes of interest. Molecular clock dating was performed in BEAST 2 (Bouckaert *et al.*, 2014) and used Yule tree priors, with an MCMC chain length of 20 million, sampling every 10 000th generation, with the chain length depending on convergence, as determined by examining the log files in TRACER v.1.5 (Rambaut & Drummond, 2007) after removal of an initial burn-in proportion of 10% of the trees. The tree was calibrated using a secondary constraint from Barrabé *et al.* (2014) for the clade (*Psychotria* clade IV + *Psychotria* Pacific clade (including Hydnophytinae)) of 22 ± 7 million yr ago (Ma), with a normal prior and a standard deviation corresponding to the 95% confidence interval (CI).

Ancestral state reconstructions of nectary types

The floral nectary types of 55 ingroup (including the nine Fiji species) and 22 outgroup taxa were coded '0' for non-ant-rewarding and '1' for ant-rewarding based on published and unpublished observations (Huxley, 1981; M. P. H. Jebb and C. Huxley-Lambrick, pers. comm. to G.C., February 2015 and November 2015; G.C. own observations on Fiji). We used stochastic character mapping to infer possible histories of floral nectary types, using the function 'MAKE.SIMMAP' in the PHYTOOLS package v.04-60 (Revell, 2012), which implements the stochastic character mapping approach developed by Bollback (2006). We estimated ancestral states using a symmetric rate model, and then simulated 1000 character histories on the maximum clade credibility trees from BEAST. We summarized the 1000 simulated character histories using the function DENSITYMAP (also in PHYTOOLS).

Correlated evolution of concealed sugar rewards and symbiosis specialization

To test whether concealed sugar rewards evolved with symbiosis specialization, we used BAYESTRAITS v.2 (Pagel & Meade, 2014), which allows the detection of correlated evolution between pairs of discrete binary traits. Absence of concealed sugar reward was coded as '0' and presence as '1'. Based on observations by C. R. Huxley, M. P. H. Jebb and M. Janda, gathered over the last 35 yr in Papua New Guinea, and by G. Chomicki in Fiji in September–October 2014 and March–April 2015, we distinguished two main mutualism types: facultative, when species were inhabited by several (often unrelated) generalist ant species, and specialized, when species were either obligately inhabited by *P. nagasau* (*Squamellaria grayi*, *S. huxleyana*, *S. imberbis*, *S. major*, *S. thekii*, *S. wilsonii*) or inhabited by one or two specialized plant-ants (from the genera *Philidris* or *Anonychomyrma*, all *Myrmecodia* species, and a few *Hydnophytum*). For all nine Fijian species, we quantified occupancy rates and ant partner types by examining the ants present in at least 20 specimens per species. *Squamellaria jebbiana*, *S. tenuiflora* and *S. wilkinsonii* were inhabited by various generalist ant species (several species of *Pheidole*, *Camponotus chloroticus*, *Colobopsis polynesica* (*Camponotus polynesicus*)). Furthermore, 30–45% of the individuals of this species were not inhabited by ants. Sarnat (2009) reported further ant species inhabiting *S. tenuiflorum*. Altogether, this indicates that *S. jebbiana*, *S. tenuiflora* and *S. wilkinsonii* form only facultative symbioses with ants. By contrast, the six other Fijian *Squamellaria* species (*S. grayi*, *S. huxleyana*, *S. imberbis*, *S. major*, *S. thekii*, *S. wilsonii*) were all inhabited by *P. nagasau* (> 300 mature individuals observed, all were inhabited), indicating an obligate symbiosis with *P. nagasau*. Moreover, *P. nagasau* has never been found outside of *Squamellaria* (Sarnat & Economo, 2012; this study), suggesting that the symbiosis is obligate for both partners. We used two proxies for symbiosis specialization: the number of ant partners per plant species, with species scored as '0' if occupied by ants from two or more genera and as '1' if occupied by ≤ 2 ant species from the same genus; and the level of domatium specialization, with species scored as '0' if their domatia have entrance holes > 0.5 cm in diameter and reticulated, unlinked cavities (indicative of facultative symbioses) or if their domatia have entrance holes > 1 cm and bulbous cavities (forming no symbioses with ants) and as '1' if their domatia have entrance holes < 0.5 cm in diameter and highly reticulated, linked cavities. Although the number of ant partners might directly reflect the presence or absence of concealed sugar rewards, tuber traits are an independent measure of symbiosis specialization. We used the maximum clade credibility (MCC) tree from BEAST, but pruned the 22 outgroups and first ran a model of independent trait evolution estimating the four transition rate parameters α1, α2, β1, β2, wherein double transitions from state 0,0 to 1,1 or from 0,1 to 1,0 are set to zero. We then ran a model of dependent trait evolution with eight parameters (q12, q13, q21, q24, q31, q34, q42, q43). To compare these non-nested models, we calculated the Bayes Factor score.

X-ray micro-computed tomography (micro-CT)

Flowers were fixed in formalin–acetic acid–alcohol (FAA) in the field. For X-ray micro-CT, all samples were treated with a solution of 1% (w/v) phosphotungstic acid in FAA for at least 1 wk, changing the solution every other day following the protocol of Staedler *et al.* (2013). The flowers were imaged at 2–33.7 μm voxel size with a microXCT-200 X-ray tomography system from Zeiss Microscopy (Jena, Germany). This system uses a 90-kV microfocus X-ray source (L9421-02 from Hamamatsu, Hamamatsu City, Japan), a cooled 2k 2k CCD camera, and switchable scintillator objective lens units. The scanning settings are summarized in Table S4. XMRECONSTRUCTOR 8.1.6599 software (Zeiss Microscopy) was used to perform the three-dimensional reconstruction from the scanning data. For samples that were scanned in several steps, XMECONTROLLER 8.1.6599 software was used to stitch together the resulting scan data. TXM3D VIEWER software (Xradia Inc., Concord, CA, USA) was used to acquire images of the samples.

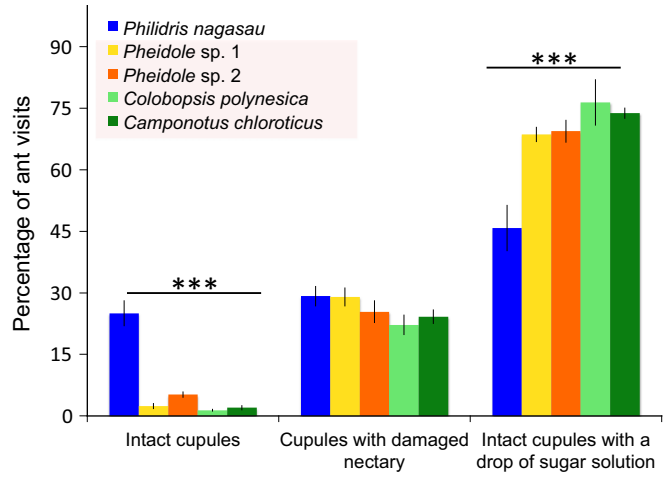

Fig. 2 *Squamellaria* conceals nectar as an exclusive reward. The transparent red square highlights opportunistic ant species (*Camponotus chloroticus*, *Colobopsis polynesica* (*Camponotus polynesicus*), *Pheidole* sp. 1, *Pheidole* sp. 2), which are compared with the specialist plant-ant *Philidris nagasau*. Error bars, ± SE. ***, *P* values significant at the $P < 0.001$ level of a *post-hoc* Tukey's test.

Results

Only a specialized symbiont exploits the concealed sugar reward produced for *c.* 10 d in its hosts' post-anthetic flowers

Two types of symbioses are found in the nine Fijian ant-plant species in the genus *Squamellaria*: facultative symbioses with several generalist ants in *S. jebbiana*, *S. wilkinsonii* and *S. tenuiflora*, and obligate symbioses with a single ant mutualist, the dolichoderine ant *P. nagasau*, in the six remaining species (*S. grayi*, *S. huxleyana*, *S. imberbis*, *S. major*, *S. thekii*, *S. wilsonii*) (Fig. 1a,b). In five of the latter species, old flowers in which the corolla has already been lost stay on the plants unchanged, instead of falling off or beginning to develop into fruits. Each of these old flowers has a conspicuous nectary disc that is not exposed whilst the flowers still have their petals (Fig. 1c). Only *P. nagasau* actively forage on these cup-shaped post-anthetic nectaries by biting into the epidermis with their mandibles (Fig. 1c,d; Movie S1). The absence of any nectar as liquid or as crystals on the nectary disc made post-anthetic flowers

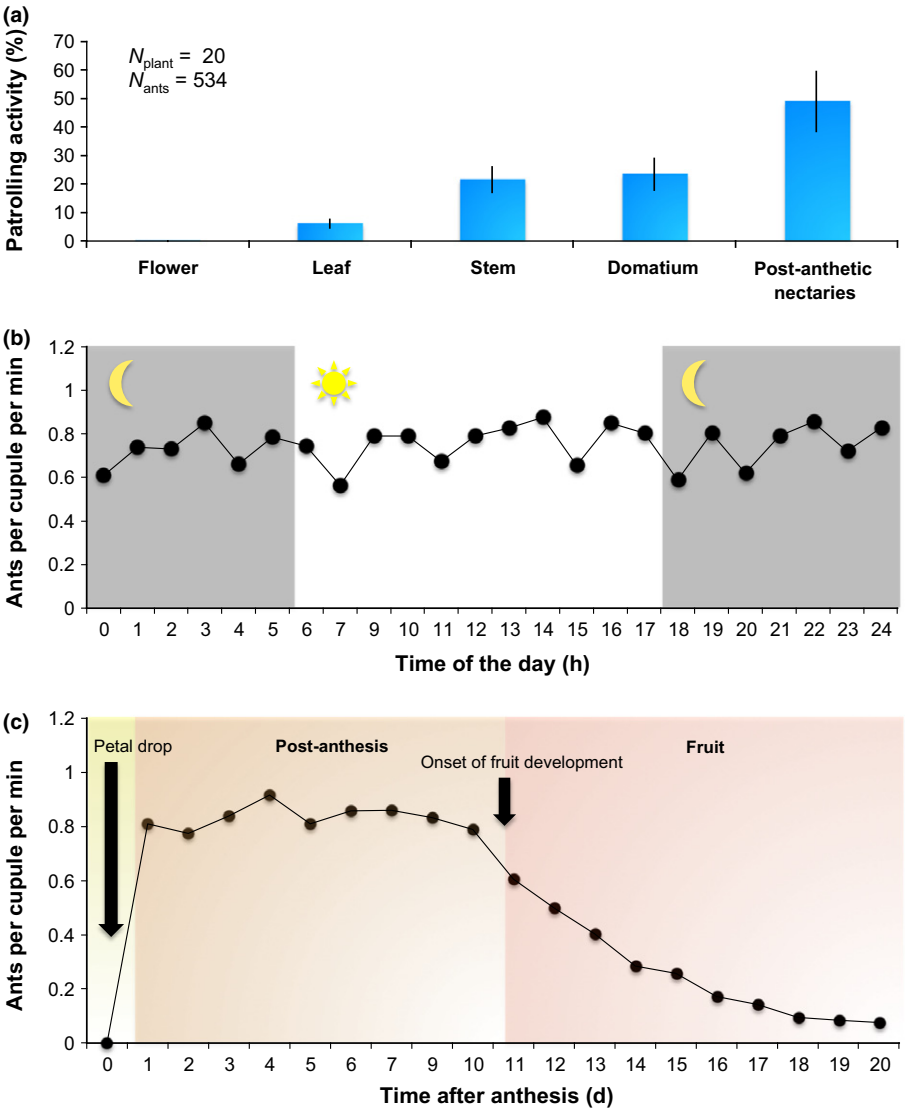

Fig. 3 *Philidris nagasau* foraging behaviour on *Squamellaria* nectaries. (a) *Philidris nagasau* patrolling activity on different *Squamellaria* organs. (b) Ant foraging activity throughout the day. (c) Ant foraging activity on 53 individual nectaries followed after anthesis. Error bars, ± SE.

unattractive to opportunists who appeared unable to perceive or exploit the concealed sugar reward as confirmed by cafeteria experiments (Fig. 2; ANOVA, $P < 0.001$; *post-hoc* Tukey's test, $P < 0.001$). Monitoring of ants also showed that *P. nagasau* hardly patrolled anthetic flowers (Fig. 3a), but visited post-anthetic flowers with their concealed sugar reward more or less constantly during the day and night (Fig. 3b) for *c.* 10 d, after which visitation dropped as fruit development started (Fig. 3c).

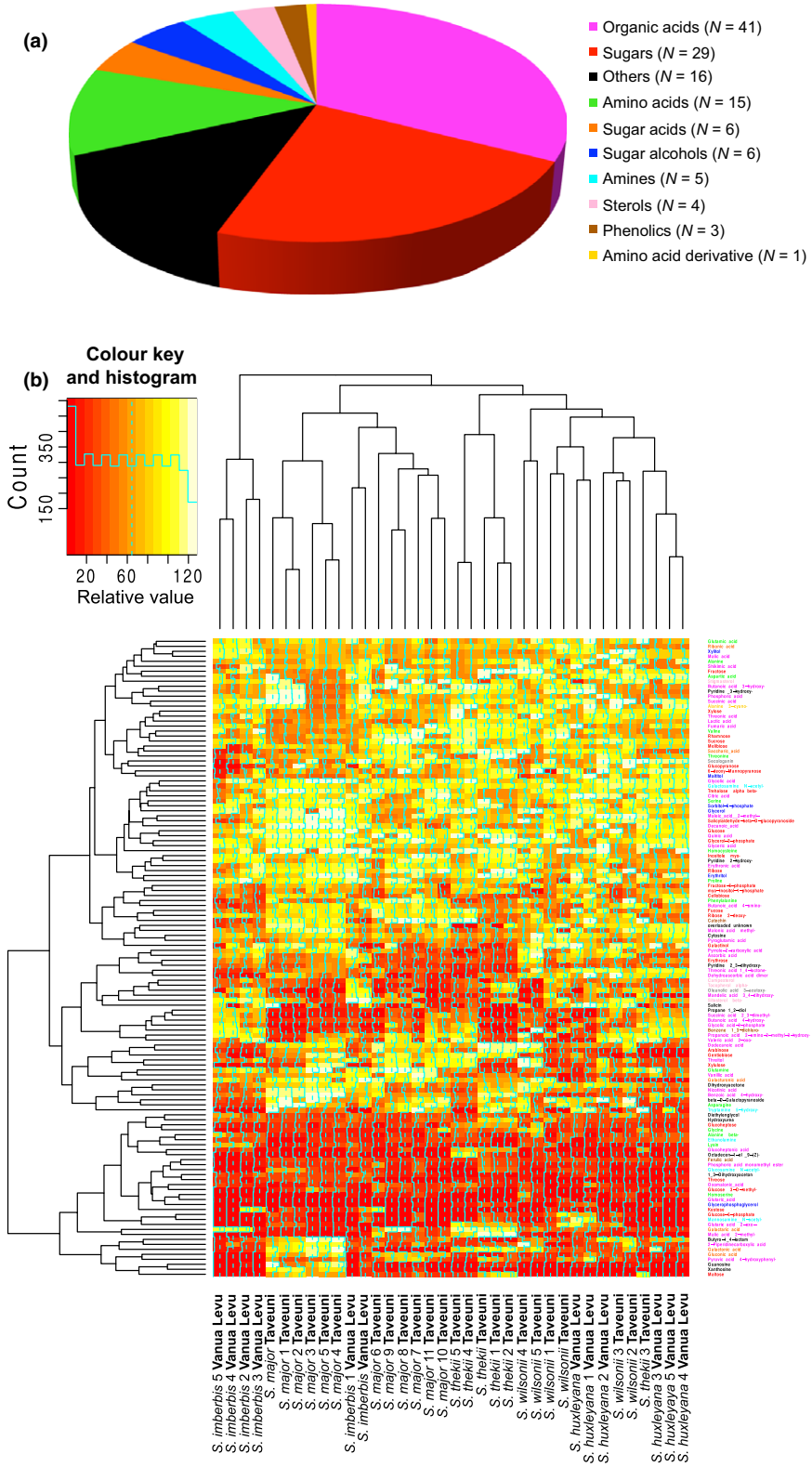

Fig. 4 Metabolomics of *Squamellaria* exclusive post-anthetic sugar rewards. (a) Pie chart showing the main categories for the 128 metabolites common to all five rewarding *Squamellaria* species (*S. huxleyana*, *S. imberbis*, *S. major*, *S. thekii*, *S. wilsonii*). (b) Heatmap showing the relative quantities of all 128 metabolites across all five species and samples. Metabolite names on the right are colour coded as in (a).

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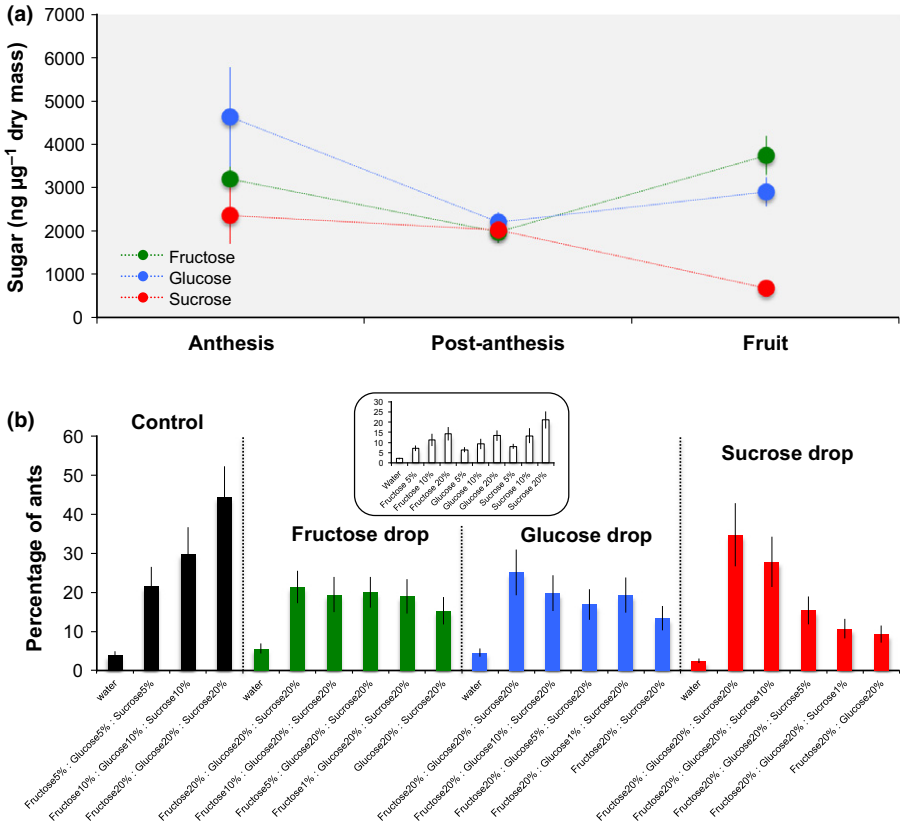

Fig. 5 Sucrose drop during early fruit development regulates *Philidris nagasau* foraging. (a) Sugar concentration in *Squamellaria wilsonii* nectaries at different stages. Post-anthesis nectaries collected on days 2–6; fruit nectaries sampled on immature fruits on days 14–16. (b) *Philidris nagasau* sugar preferences evaluated from cafeteria experiments (see the Materials and Methods section). Error bars, ± SE.

Metabolomic composition of *Squamellaria* post-anthetic concealed sugar reward

Metabolomic analysis of nectary tissue at anthesis, post-anthesis and during early fruit development (see the Materials and Methods section) revealed 128 metabolites, most notably 29 sugars and 15 amino acids (Fig. 4a,b; Table S1), indicating that post-anthetic rewards are very nutritious. At anthesis, the sugary sap is richer in glucose and fructose than in sucrose, but, after anthesis, the concentration of the first two sugars drops to reach *c.* 2400 ng μg^{-1} dry mass, whereas the sucrose level is maintained (Fig. 5a). After the onset of fruit development (i.e. ovary bulging), the sucrose level drops, resulting in correspondingly higher glucose and fructose levels (Fig. 5a).

The association of a drop in sucrose concentration and lower ant visitation suggests that the sucrose level controls *P. nagasau* foraging. To test this, we carried out a second series of cafeteria experiments in which we fed ants with different sugar solutions to test whether *P. nagasau* was sensitive to changes in one of the three sugars (Fig. 5b). This turned out to be true for all three, either separately or together at the same stoichiometry (Fig. 5b and inset). To specifically test how a lower level of one of the three sugars affects *P. nagasau* preferences, we performed three more series of cafeteria experiments in which only one of the three sugars was presented at different concentrations, whilst the two others were kept constant (see the Materials and Methods section). Varying glucose or fructose levels (whilst keeping sucrose constant) resulted in only small decreases in ant attendance (Fig. 5b). By contrast, when sucrose was offered in different concentrations (whilst the levels of glucose and fructose remained unchanged), ant attendance decreased dramatically, tracking the sucrose decrease (Fig. 5b). This confirmed that sucrose levels control *P. nagasau* foraging behaviour.

Evolution of the concealed sugar reward: increase in nectary volume and delayed onset of fruit development

Sugar rewards form early (Fig. 6a), and ant-addressed nectaries have a volume *c.* 25-fold larger than non-ant-addressed nectaries in unspecialized *Squamellaria* or the secondarily reduced glands of the rewardless *S. grayi* (8–10 mm^3 vs 0.3–0.4 mm^3; Fig. S1). In the ant-addressed nectaries, the vascular network is prominent with a high bundle density (Fig. S2).

In addition to the conspicuous difference in gland size, the onset of fruit development in the species of *Squamellaria* with ant-addressed nectaries is phenologically delayed compared with that in related species that do not offer post-anthetic sugar rewards to their symbiotic ants. This delay causes the accumulation of old (post-anthetic) flowers (Figs 1c,d, 6a). Assessment of the timing of fruit development (ovary bulging) in all nine species of Fijian *Squamellaria* by measuring the calyx diameter for 20 d after anthesis revealed that, in the five species with concealed sugar rewards, fruit development started *c.* 10 d after anthesis, whereas in the other (non-ant-rewarding) species, ovary enlargement was noticeable after 48–72 h (Fig. 6b). This delay retards sucrose hydrolysis (previous section), which begins during

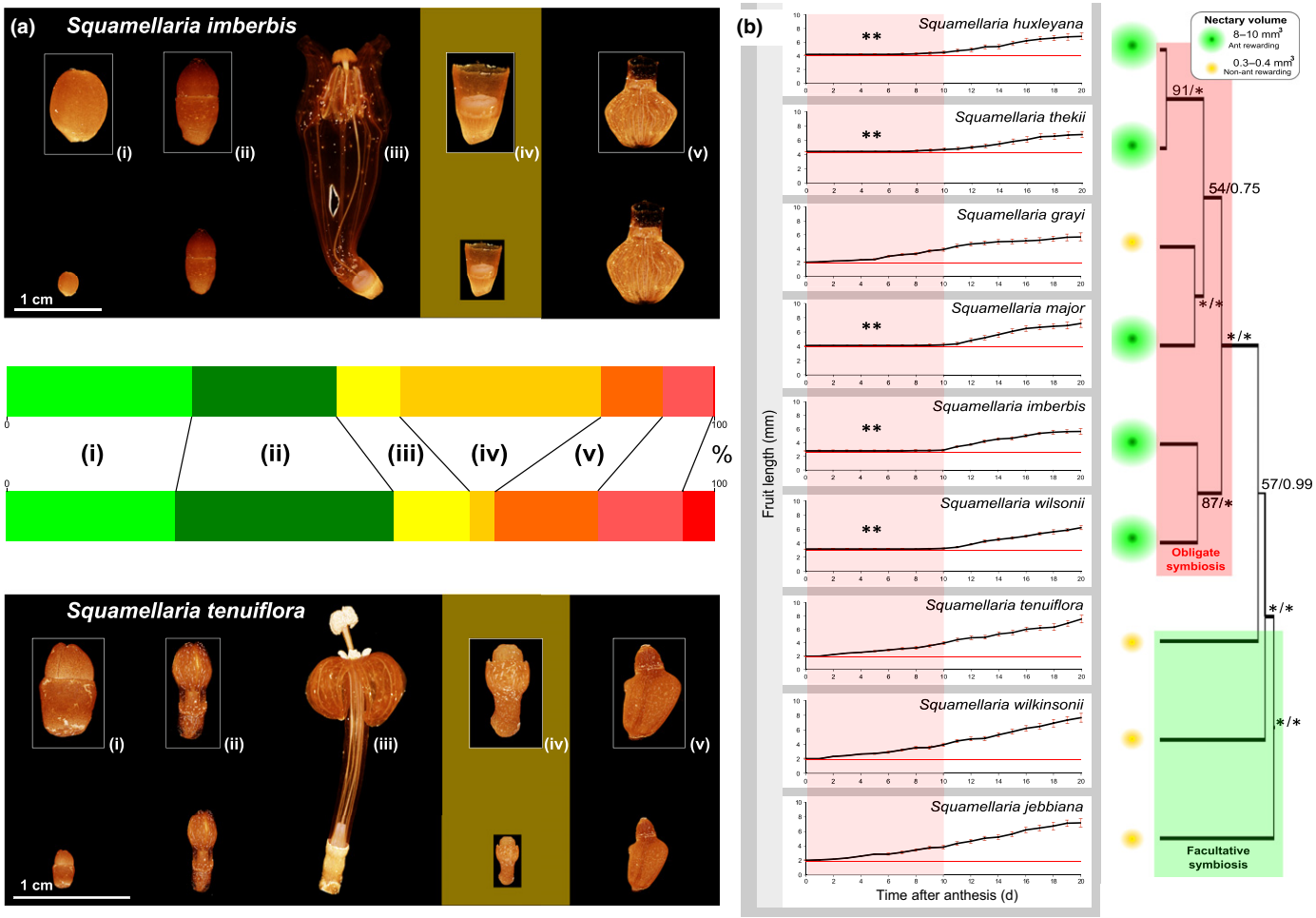

Fig. 6 *Squamellaria* post-anthetic sugar rewards evolved via heterochronic fruit development and nectary enlargement. (a) Micro-computed tomography (μCT) scanning images showing floral developmental stages of the ant-addressed nectaries of *Squamellaria imberbis* and the non-ant-addressed nectaries of *S. tenuiflora*. The middle chart shows the proportion of each of the developmental stages, recorded as a percentage, in 20 inflorescences for each species. (b) Fruit developmental timing in rewarding and non-rewarding Fijian ant-epiphytes. Fruit length (ovary length) is over the time shown, and linked to phylogenetic relationships. Numbers at the branches show the maximum likelihood (ML) bootstrap support and the posterior probabilities. * indicates maximal support. Error bars, ± SE. **, *P* values of *t*-tests significant at the $P < 0.01$ level.

fruit development and results in the accumulation of ant-rewarding post-anthetic flowers. During the months of September/October and March/April when we studied *Squamellaria*, the symbiotic ant colonies were constantly provided with sugar rewards, and observations of *Squamellaria* herbarium specimens (K, FHO, SUVA, L, NSW, US) confirmed that flowers are produced year-round. *Squamellaria* flowering phenology thus ensures that rewards are produced year-round.

Concealed sugar rewards evolved with mutualism specialization

To understand the evolution of concealed sugar rewards produced after anthesis and accessible only to visitors capable of chewing (not pollinators), we investigated nectary ontogeny in all Fijian ant-plant Rubiaceae species. All nine species have floral nectary discs, but *P. nagasau* ants forage only on five of the six *Squamellaria* species it inhabits (*S. huxleyana*, *S. imberbis*, *S. major*, *S. thekii*, *S. wilsonii*, *S. grayi*). To study the evolution of gland structure and volume, we inferred a molecular clock-dated phylogeny based on up to 10 nuclear and plastid DNA markers obtained for 55 species of Hydnophytinae (*c.* 50% of all species in the clade; Chomicki & Renner, 2016). Large ant-addressed nectaries that are sugar-rich post-anthesis evolved in the most recent common ancestor (MRCA) of *Squamellaria*, *c.* 2.1 ± 1 Ma (Fig. S3), and were secondarily lost in *S. grayi*, which has small glands similar to those of unspecialized *Squamellaria* (Figs 6b, 7, S2).

Mapping the evolution of concealed sugar rewards on a large Hydnophytinae phylogeny revealed an apparent correlation with specialized symbiosis (Fig. 7). The BayesTrait test (see the Materials and Methods section) for correlated evolution of ant symbiont specialization and domatium specialization showed that models of correlated trait evolution were strongly favoured over models that assumed independent trait change (Bayes Factor = 55.8 and 43.1, respectively), confirming the concurrent evolution of the cheater exclusion mechanism 'concealed sugar rewards' jointly with increasing symbiosis specialization

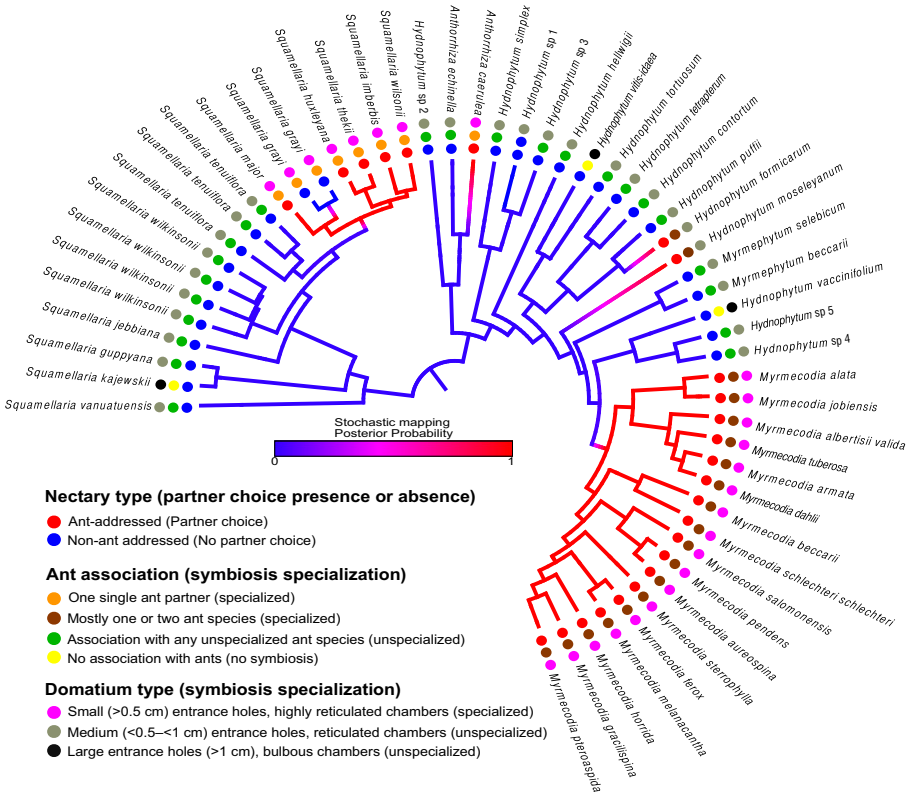

Fig. 7 Evolution of exclusive rewards in the Hydnophytinae and correlated evolution with mutualism specialization. Stochastic mapping reconstruction of nectary type performed on the BEAST maximum credibility tree, and correlated evolution of concealed rewards and symbiosis specialization, as evaluated via two proxies (ant inhabitants and domatium type).

(measured by proxies, namely ant partner number and the domatium traits 'entrance hole diameter' and 'type of cavity').

Discussion

Concealed nectary rewards compared with other partner choice mechanisms in ant–plant symbioses

The concealed sugar reward in these rubiaceous ant-plants (genus *Squamellaria*) filters out opportunistic nectar foragers (Fig. 2). However, the post-anthetic nectar rewards are unlikely to be the main asset that ties *P. nagasau* to *Squamellaria*, given that one species, *S. grayi*, secondarily lacks the sugary rewards (and hence the partner choice mechanism) and still retains its obligate symbiosis with *P. nagasau*. Selective access to food rewards has evolved as a partner choice mechanism in several other ant–plant systems. In Central American *Vachellia*, post-secretory hydrolysis of sucrose by invertase renders the EFN unattractive to opportunistic ants (Heil *et al.*, 2005; Kautz *et al.*, 2009), whereas the mutualist species *Pseudomyrmex ferrugineus* is manipulated by its host (*Vachellia*), which inhibits the digestive ability via chitinase that blocks invertase activity (Heil *et al.*, 2014). *Vachellia* thus filters out opportunistic foragers, but also manipulates its partner to restrict it from exploiting other food sources. Such partner restriction can theoretically stabilize mutualisms (Wyatt *et al.*, 2016). In this *Vachellia–Pseudomyrmex* system, the plant hosts produce food bodies (Beltian bodies) that are protein- and lipid-rich, and that are protected from exploitation by a protease inhibitor that prevents leaf beetles and opportunistic ants from digesting them (Orona-Tamayo *et al.*, 2013). *Squamellaria* concealed sugar rewards differ from these systems in that filtering is physical, not chemical. In South-East Asian domatium-bearing *Macaranga*, about half of the species have slippery waxy stems that limit stem exploitation by opportunists, whereas mutualists possess biomechanical adaptations enabling them to adhere to these waxy surfaces (Federle *et al.*, 1997, 2000). Domatium-bearing *Macaranga* species without waxy surfaces have Beltian bodies hidden under stipules and almost no EFN, whereas waxy *Macaranga* secrete abundant EFN (Federle & Rheindt, 2005), showing that wax-covered stems are also a partner choice mechanism. Yet another type of physical partner choice occurs in one species of the African Fabaceae *Leonardoxa*, in which ant and plant have coevolved to produce a prostoma matching the ant mutualist's size and shape (Brouat *et al.*, 2001).

When is partner choice needed in ant–plant symbioses?

It is currently debated whether partner fidelity feedback alone can maintain mutualism (West *et al.*, 2002; Kiers *et al.*, 2003; Weyl *et al.*, 2010; Kiers *et al.*, 2011; Frederickson, 2013). Frederickson (2013) argued that 'sanction' mechanisms in fig–wasp, yucca–moth and legume–rhizobia mutualisms are a misinterpretation of host pre-adaptations and are instead best understood as partner fidelity feedbacks (Weyl *et al.*, 2010). In *Cordia nodosa*, young shoots that suffer heavy herbivory are shed, which has been interpreted as a 'host sanction' that evolved in response to selection from cheaters (Edwards *et al.*, 2006). This seems unlikely as organ abscission following biotic or abiotic damage is frequent in

plants (e.g. Addicott, 1982), and thus this is likely to be a pre-adaptation, best understood within the partner fidelity feedback framework (Weyl *et al.*, 2010). A potential example of a sanction induced by cheater selection is found in *Hirtella myrmecophila* (Chrysobalanaceae), the leaf pouch domatia of which are inhabited by *Allomerus octoarticulatus*, an ant that protects *Hirtella* against herbivores, but castrates its host. *Hirtella* shed the domatia in older leaves (on shoots that will flower), which mitigates the effect of castration (Izzo & Vasconcelos, 2002). If *Allomerus* is the principal partner of *Hirtella*, this would be a case in which partner fidelity feedback alone cannot efficiently maintain mutualism.

More generally, where ant–plant symbioses involve specialized food rewards, there seems to be selection for reducing the attraction of opportunists (parasites of mutualisms), whereas cheating by the plant's own symbionts appears to be too rare to have induced the evolution of sanctions (Frederickson, 2013). EFNs provide a good example. In over 457 plant lineages and > 3900 species (Weber & Keeler, 2013), EFNs attract a wide range of ants and parasitoid wasp species that forage for nectar and deter herbivores (Heil & McKey, 2003). Of the 158 lineages of vascular plants (685 species) with ant domatia, only 14 have EFNs (Chomicki & Renner, 2015), and almost all of these form facultative symbioses because their nectaries can be exploited by numerous ant species without partner filtering (e.g. *Barteria nigritana* (Passifloraceae), Djiéto-Lordon *et al.*, 2004; *Humboldtia brunonis* (Fabaceae), Gaume *et al.*, 2005). Specialized ant–plant symbioses involving EFN rewards, however, limit opportunistic foraging through partner choice (Heil *et al.*, 2005; Federle & Rheindt, 2005; D. McKey, pers. comm. to G.C., May 2015).

Partner choice evolved with mutualism specialization

Our finding of the striking correlation between partner choice (concealed sugary rewards) and symbiosis specialization provides a strong argument of when partner choice is needed to stabilize a mutualism. It suggests that partner choice is necessary in specialized, coevolved mutualisms when costly trophic rewards are offered, and indirectly shows the strength of food competition from opportunists. In ant–plant symbioses, partner choice mechanisms (reviewed above) are always present in highly specialized mutualisms, all involving costly food rewards. The abundance and ubiquity of opportunists are thus unlikely to be balanced by mere partner fidelity feedback, requiring the evolution of a partner choice mechanism during the transition from facultative to obligate mutualisms.

Conclusion

Our study illustrates a novel partner choice mechanism that consists of post-anthetic sugar rewards and that evolved via a developmental shift in fruit development and nectary enlargement. The concealed sugar rewards appear to have played a central role in the transition from facultative to obligate mutualisms by increasing benefit trading whilst preventing partner exploitation. Both our experimental and comparative data for the nine Fijian species of *Squamellaria*, and our larger scale phylogenetic analysis of the Hydnophytinae, imply the correlated evolution of partner choice and mutualism specialization. Our study highlights that partner choice may be necessary to maintain mutualisms from exploitation by opportunists when mutualisms involve the trading of highly valuable 'goods' between the partners. This suggests that, in such specialized (coevolved) mutualisms, the selection pressure exerted by opportunists exceeds that exerted by cheaters.

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Author contributions

G.C. designed and planned the research; G.C. and Y.S. performed the research and analysed the data; G.C. and S.S.R. wrote the manuscript; all authors read and agreed the manuscript. S.S.R., G.C. and J.S. provided reagents.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1 Computed tomography (CT) scanning images showing the developmental stages of *Squamellaria grayi* nectary.

Fig. S2 Vascular system of nectaries visualized with computed tomography (CT) scanning data.

Fig. S3 Dated phylogeny of the Hydnophytinae.

Table S1 Full list of metabolites of *Squamellaria* nectaries from metabolomic analyses (see the Materials and Methods section)

Table S2 Primers used in this study

Table S3 Plant material included in this study with authors of species names, vouchers and their geographical origin and GenBank accession numbers for all sequences. Herbarium acronyms follow the *Index Herbariorum* (http://sci-web.nybg.org/science2/IndexHerbariorum.asp)

Table S4 Scanning conditions for micro-computed tomography

Movie S1 *Philidris nagasau* foraging on post-anthetic nectaries of *Squamellaria wilsonii*, Taveuni, DesVoeux peak track.

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