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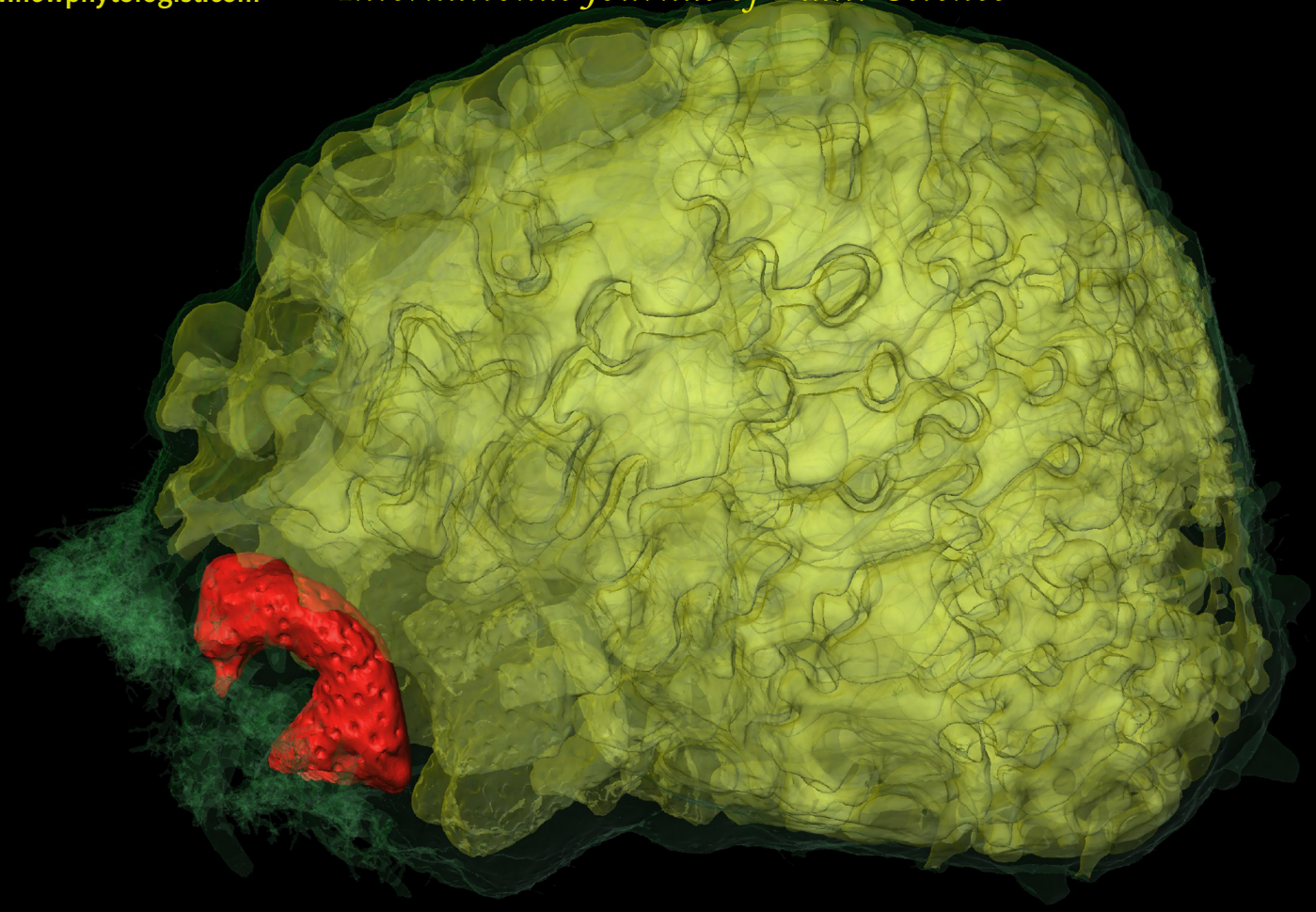

TANSLEY INSIGHT REVIEWS

New Phytologist

Research

Farming by ants remodels nutrient uptake in epiphytes

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Summary

- True agriculture – defined by habitual planting, cultivation, harvesting and dependence of a farmer on a crop – is known from fungi farmed by ants, termites or beetles, and plants farmed by humans or ants. Because farmers supply their crops with nutrients, they have the potential to modify crop nutrition over evolutionary time. Here we test this hypothesis in ant/plant farming symbioses.
- We used field experiments, phylogenetic-comparative analyses and computed-tomography scanning to investigate how the evolution of farming by ants has impacted the nutrition of locally coexisting species in the epiphytic genus *Squamellaria* (Rubiaceae).
- Using isotope-labelled mineral and organic nitrogen, we show that specialised ants actively and exclusively fertilise hyperabsorptive warts on the inner walls of plant-formed structures (domatia) where they nest, sharply contrasting with nitrogen provisioning by ants in nonfarming generalist symbioses. Similar hyperabsorptive warts have evolved repeatedly in lineages colonised by farming ants.
- Our study supports the idea that millions of years of ant agriculture have remodelled plant physiology, shifting from ant-derived nutrients as by-products to active and targeted fertilisation on hyperabsorptive sites. The increased efficiency of ant-derived nutrient provisioning appears to stem from a combination of farming ant behaviour and plant 'crop' traits.

Introduction

Mutualisms that involve the cultivation of one species by another have evolved repeatedly across the tree of life, including in social amoeba and deep-sea crabs that farm bacteria (Brock *et al.*, 2011; Thurber *et al.*, 2011), snails that farm a fungus (Silliman & Newell, 2003), and damselfish and three-toed sloths that farm green algae (Hata & Kato, 2006; Pauli *et al.*, 2014). However, true agriculture – defined by habitual propagation of the cultivated species (the 'crop') by the cultivating species (the 'farmer'); enhancement of the crop's growth by the farmer, through fertilisation or defence; harvest of the crop by the farmer; and dependence of the farmer on the crop – is restricted to ants, termites and beetles that farm fungi (Mueller *et al.*, 2005) and to humans or ants that farm plants (Chomicki & Renner, 2016a). Ants and termites have been farming fungi since at least the Eocene (Mueller *et al.*, 2005), and similar to crop domestication by humans, insect farmers have imposed directional selection on their fungal crops, modifying their traits and nutrient status (Nygaard *et al.*, 2016). In the case of the ant/plant farming mutualism, there is habitual planting of the crop plants' seeds, protection of seedlings from herbivores, fertilisation of the seedlings, harvesting of products from mature crop plants and obligate dependence on both sides (Chomicki & Renner, 2016a; Chomicki *et al.*, 2016). Farmer ants also obligatorily nest inside crop-provided nesting structures (domatia) because they have lost the ability to build carton nests (Chomicki & Renner, 2016a). While ant/plant symbioses relying on domatia have evolved in at least 159 plant genera and 50 families (Chomicki & Renner, 2015), ant/plant farming symbioses have only been reported from epiphytic Rubiaceae subtribe Hydnophytinae, more specifically in Fijian species of *Squamellaria*, in which the evolution of obligate farming has been demonstrated (Chomicki & Renner, 2016a).

Nutrient provisioning by farmers can deeply impact crop physiology. For instance, in fungiculture by attine ants, the fungal crop has lost some genes encoding lignin-degrading enzymes because the ant has modified the nutrition of the fungus, so that the fungus no longer depends on lignin breakdown (Nygaard *et al.*, 2016). The three million-year-old interactions between arboreal farmer ants and their epiphyte *Squamellaria* crops (Chomicki & Renner, 2016a) raise the possibility of similar nutritional remodelling, for example physiological adaptations to the uptake of ant-derived nutrients, but this remains untested. Efficient nutrient uptake by the farmed plants would be beneficial for both partners as it would allow for the coupling of plant and ant colony growth, thereby stabilising the mutualism. Such coupling would be especially important for the ants, because farmed epiphytes produce energy-demanding food rewards for their ant partner (Chomicki *et al.*, 2016) and the ants obligately depend on the plant for nesting (Chomicki & Renner, 2016a). Likewise, it should be beneficial for the plants, because epiphytes

that live in tree canopies and have no connection to the soil are nutrient-limited.

Here we investigate if and how the evolution of plant farming by ants has remodelled nutrition in their crops. We address this question using field experiments on nutrient uptake and then relate physiology-related traits to macroevolution using phylogenetic comparative analyses. Our experimental study system consists of a clade of six species of *Squamellaria* that are obligatorily farmed by the ant species *Philidris nagasau* (Dolichoderinae) and of three closely related species that form nonfarming symbioses with generalist arboreal ant species (Chomicki & Renner, 2016a). *Philidris nagasau* cultivates and manages large *Squamellaria* colonies by collecting and planting the plants' seeds under tree bark and fertilising its crop from the seedling stage onward. The ants feed on sugar- and amino-acid-rich floral food rewards produced by mature *Squamellaria* plants (Chomicki & Renner, 2016a; Chomicki *et al.*, 2016). On Fiji, farmed and nonfarmed species coexist, sometimes even in the same tree canopy, providing an opportunity to directly compare nutrient acquisition in the two types of symbioses.

In addition to our experimental system on Fiji, we used the Hydnophytinae clade to study the evolution of plant nutrition in a macroevolutionary context. The Hydnophytinae comprise *c.* 105 species in South-East Asia and northern Australia that engage in symbioses with ants (Chomicki & Renner, 2015), presenting a diverse range of mutualistic strategies (Chomicki & Renner, 2017). Some species form generalist and facultative symbioses with a range of ant species; others form specialised symbioses with one or two Dolichoderinae species from the genus *Philidris* or more rarely from *Anonychomyrma*. Differences between the various symbiotic strategies involve seed dispersal, which in the farmed species is by ants, but in nonfarmed species by both ants and birds (Chomicki & Renner, 2016a, 2017; Chomicki *et al.*, 2017).

Specifically, we wanted to answer the following questions: How does nitrogen uptake differ between farmed and nonfarmed species of *Squamellaria*? How does fertilisation by ants differ in farmed vs nonfarmed *Squamellaria*? Is nitrogen uptake more efficient in obligate farming symbioses or in facultative nonfarming symbioses with generalist arboreal ants? Does nitrogen uptake capacity parallel the evolution of different types of symbioses in Hydnophytinae at a macroevolutionary scale?

Materials and Methods

Study sites and taxonomic sampling

All fieldwork was conducted jointly with members of the University of the South Pacific, Suva, Fiji (see Acknowledgements) and complied with the relevant regulations. No DNA material was collected for this study.

In September 2014, March 2015, and August 2016, 2017 and 2018 the first author conducted fieldwork on Viti Levu, Vanua Levu and Taveuni. 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 study sites were in 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 study sites were 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). *Squamellaria* taxonomy follows Chomicki & Renner (2016b). Experimental work (below) was conducted on *S. huxleyana* (farmed), *S. wilsonii* (farmed), and *S. wilkinsonii* and *S. jebbiana* (nonfarmed). Computed-tomography scanning was performed on all Fijian *Squamellaria* species (*S. huxleyana*, *S. wilsonii*, *S. imberbis*, *S. major*, *S. thekii*, *S. grayi*, *S. jebbiana*, *S. tenuiflora* and *S. wilkinsonii*). *Squamellaria* were accessed by tree climbing, using a rope secured by a partner on the ground except when they could be reached from the ground.

^{15}N uptake and feeding experiments and $\delta^{15}N$ isotope analyses

We designed two types of experiments to determine the uptake capacity of the cavity types found in farmed and nonfarmed *Squamellaria* (uptake experiments) and to quantify the pattern of fertilisation by ants (feeding experiments). For the uptake experiments, we selected two species: *S. wilkinsonii* (nonfarmed species, inhabited by generalist ant species) and *S. huxleyana* (farmed species, inhabited by *Philidris nagasau*). These species coexist in rainforests of Vanua Levu, Fiji. We tested the uptake capacity of mineral and organic nitrogen using ammonium chloride, $^{15}NH_4Cl$ ($^{15}NH_4^+$; Isotec; Sigma-Aldrich, 98 at.%), as mineral nitrogen source and ^{15}N glycine (Isotec; Sigma-Aldrich, 98 at.%) as organic nitrogen source. We followed the protocol of Gegenbauer *et al.* (2012). To determine the potential for nutrient uptake in the cavities of facultative and obligate hosts, ^{15}N-labelled mineral or organic nitrogen-containing molecules were injected in the domatium cavities with a syringe for an incubation time of 1 h. To prevent the solution from leaking (flowing out) from the many domatium entrance holes, the whole domatium was wrapped in parafilm M, with the plant being intact (shoots attached to domatium; plant freshly collected on the day when the experiments were done).

We used $^{15}NH_4^+$ and ^{15}N glycine at concentrations of 75, 250 and 500 μM with three replicates per nitrogen form and concentration. For each species (farmed: *S. huxleyana*; nonfarmed: *S. wilkinsonii*) three controls were injected with distilled water only. To ensure replicability, we selected similar sized plants with domatia of *c.* 12–15 cm diameter. After 1 h of incubation, apoplastically bound ions were removed by flushing the domatia twice with a 10 mM $CaCl_2$ solution (for 1 min each). Domatia were subsequently cut in half and further washed carefully with distilled water. Samples from warty and smooth chambers (for *S. huxleyana*) and from warty-like and smooth-like chambers (for *S. wilkinsonii*, see Fig. 1a) were then dissected with a scalpel, with a slice thickness of *c.* 1 mm (i.e. removing 1 mm of plant tissue below the surface of the chamber) and immediately microwave-

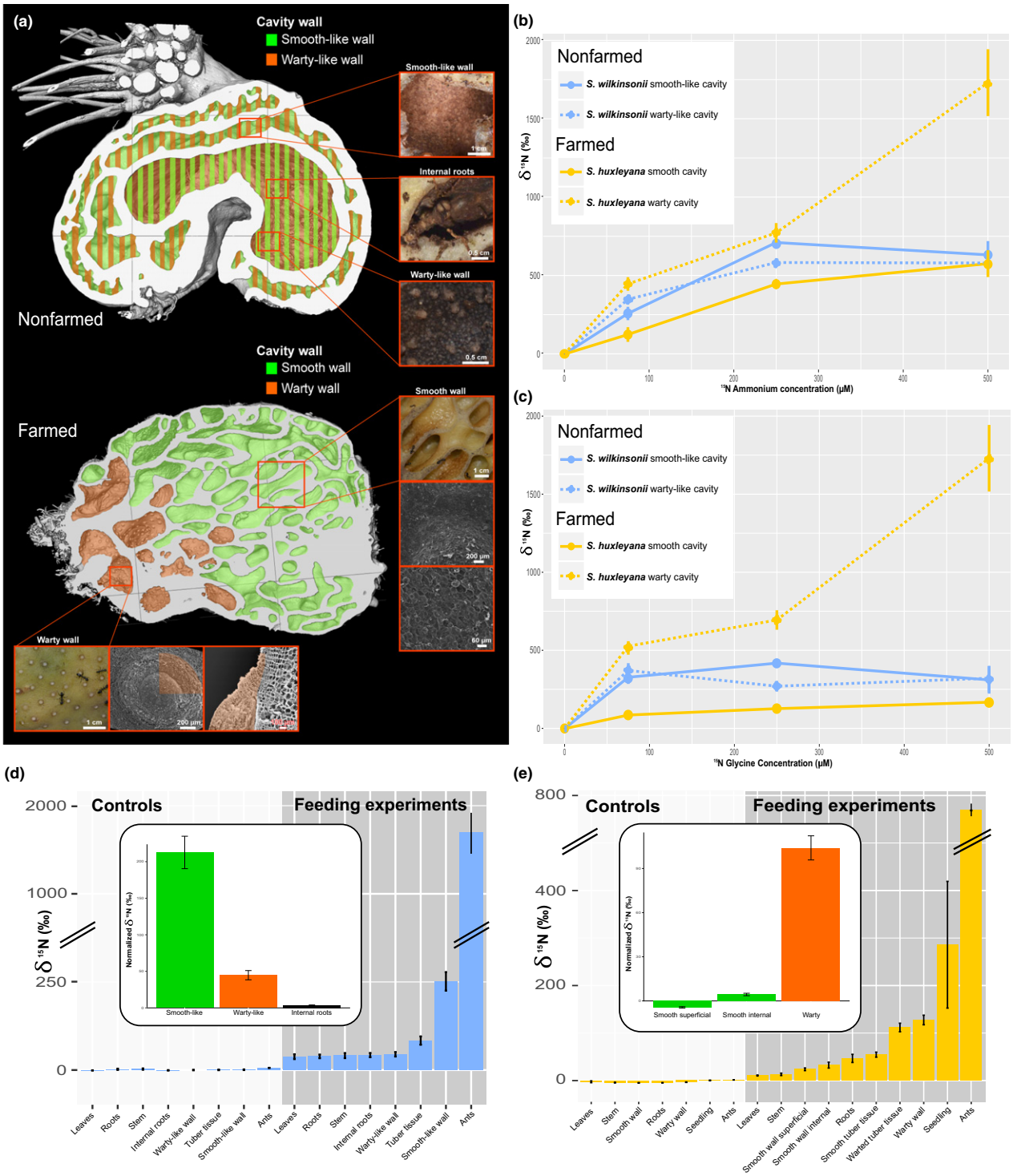

Fig. 1 Targeted nitrogen acquisition in farmed *Squamellaria*. (a) Longitudinal sections from computed-tomography scanning and scanning electron micrographs showing the internal structure of domatia and their wall surface types in nonfarmed (*S. tenuiflora*, upper) and farmed (*S. wilsonii*, lower) *Squamellaria*. In *S. tenuiflora*, the banded pattern of green and orange schematically reflects the intermixing of 'warty-like' and 'smooth-like' areas. (b, c) Uptake experiments that used different concentrations of mineral ($^{15}NH_4Cl$, b) or organic (^{15}N glycine, c) labelled compounds injected into the domatium (see the Materials and Methods section). $\delta^{15}N$ values show the level of absorbed isotopes in each wall type for a nonfarmed (*S. wilkinsonii*, blue) or farmed (*S. huxleyana*, gold) *Squamellaria* species. (d, e) Feeding experiments in which ants were fed with a sugary solution containing ^{15}N glycine for 10 d. (d) Nonfarmed ant-plant *S. wilkinsonii*, inhabited by several generalist ant species. (e) Farmed ant-plant *S. huxleyana*, inhabited only by *Philidris nagasau*. The graphs show the level of isotopes detected in each tissue (grey-shaded background) vs controls (white background). The insets give the normalised $\delta^{15}N$ value resulting from ant defecation (see the Materials and Methods section). The seedling data have been re-plotted from Chomicki & Renner (2016a), for comparison. In all cases, graphs show the means ± SE. See also Supporting Information Fig. S1.

dried (for definitions of domatium tissue type, see the section 'Ancestral state reconstruction of domatium wall differentiation'). Standardising the thickness was essential so that the same amount of wall surface was present per gram of dried sample, and because every sample was precisely weighted before Isotope-ratio MS (IR-MS), this ensured that our results are comparable across samples. Each experiment was replicated three times (for each of the three nitrogen concentrations and the three controls), meaning 12 uptake experiments for each of the two species, and hence 24 uptake experiments, with each plant replicate inhabited by a distinct ant colony. The rate of N uptake was obtained by dividing the $\delta^{15}N$ value minus the baseline value derived from the three controls that were injected with distilled water (equivalent to the value at t0) by the incubation time. For all 24 uptake experiments, the incubation time was 1 h. The values at t0 were close to zero (because they were simply the nonenriched $\delta^{15}N$ values), and the magnitude of change in $\delta^{15}N$ was 200–2000-fold (Fig. 1b,c). Hence, in this case, uptake rate *c.* $\delta^{15}N$ value.

For the ant feeding experiments, we used the same two plant species: *S. wilkinsonii* (nonfarmed) and *S. huxleyana* (farmed). The aim of the experiment was to determine where exactly ant defecation and plant nutrient uptake occur. We selected plants on neighbouring trees with separate ant colonies to achieve a sample of $n = 10$ plants each of the two species, with five control plants and five treated plants. We then placed a solution of 20 mM ^{15}N glycine (enriched at 98 at.%; Isotec) with a 40% (w/v) 1 : 1 : 1 mix of sucrose, glucose and fructose in an Eppendorf tube, itself placed within a falcon tube close to a mature plant (see Fig. S1a,b). A paper wick allowed the ants to reach the solution without drowning in it. The falcon tube was placed under a large branch to prevent rainwater from entering the tube. We added 2 ml of solution to the falcon tube twice a day during the 10 d of the experiment. On the 11th day, we collected the mature plant closest to the nitrogen source. Each plant was cut in half, washed and dried as described above (see also Fig. S1). This experimental design, based on the removal of just a subset of plants from several colonies, was sustainable for the symbiotic colonies of these rare Fijian endemics, and our survey revealed that all colonies recovered in the subsequent year.

The amount of ^{15}N measured in our samples using IR-MS reflects deposition, presumably by defecation, absorption and translocation/transport. The $CaCl_2$ washes ensure that measured ^{15}N reflected only absorbed and transported ^{15}N. To identify the sites of fertilisation, we needed to distinguish ^{15}N arriving by vascular transport from ^{15}N absorption in tissues fertilised by ants. To do so, we generated a baseline, using tissues that the ants do not directly fertilise, namely stems, leaves and roots (which have a structural role and are not visited by ants). ^{15}N fertilisation by ants was quantified as follows: $^{15}N_{fert} \sim$ (measured $\delta^{15}N$tissue − mean ($\delta^{15}N$root, $\delta^{15}N$leaf, $\delta^{15}N$stem)). These values are reported in the insets to Fig. 1(d,e).

For both the uptake and the feeding experiments, samples were homogenised with a ball mill, and *c.* 1–3 mg of dry powder was then weighted in tin capsules. IR-MS analyses were performed at the GeoBiocenter, University of Munich (LMU). Capsules were combusted in an elemental analyser (NC2500; Carlo Erba, Barcelona, Spain) in a continuous helium flow at 1080°C. The combustion gases passed through a reaction tube filled with chromium and silvered cobaltous oxides, a subsequent reduction tube (560°C) filled with copper wires, a water trap filled with magnesium perchlorate and a GC column. The isolated gases N_2 and CO_2 were analysed in an IR mass spectrometer (DeltaPlus, Thermo-Finnigan, Waltham, MA, USA) to determine the isotope ratio of nitrogen ($\delta^{15}N$). $\delta^{15}N$ values are reported as per mil values relative to international standards (air for nitrogen). Total organic carbon (TOC) and total nitrogen (TN) mass percentages were calculated from sample peak areas using the elemental standards atropine, cyclohexanone-2,4-dinitrophenylhydrazone and peptone for calibration.

Ancestral state reconstruction of domatium wall differentiation

The inner walls of Hydnophytinae domatia have different surfaces, which we refer to as smooth, warty, smooth-like and warty-like (for Results, see the images shown in Fig. 1a), and for each of the 81 taxa sampled in our Hydnophytinae phylogeny (Chomicki & Renner, 2017), we coded the type of inner domatium cavity surface. Outgroups (terrestrial shrubs; without domatia) were coded as '0', species with variable cavity surfaces as '1' (warty-like and smooth-like walls, Fig. 1a), species with fully differentiated domatium walls as '2' (clear-cut smooth and warty walls, Fig. 1a), and species that have lost warts and retain only smooth-like cavities as '3'. Some of the last still have cavity walls with a few warts (M. H. P. Jebb, pers. comm. to G. C., March 2015). Each trait was coded from the literature (Jebb, 1985, 1991; Chomicki & Renner, 2016b, 2017; Jebb & Huxley, 2019) and personal observations by G.C. on specimens either in the field, in cultivation or in herbaria.

To trace the evolution of domatium wall specialisation along a phylogeny, we used stochastic character mapping in the function 'make.simmap' in the PHYTOOLS R package v.04-60 (Revell, 2012), which implements the stochastic character mapping approach developed by Bollback (2006). We estimated ancestral states under the equal rates (ER) model (best fitting model as determined with the Akaike information criterion), and then simulated 1000-character histories on the maximum clade credibility (MCC) phylogeny. We summarised the 1000 simulated character histories using the function 'describe simmap'. The density of stochastic changes for each character transition was plotted using the function 'density'.

To account for phylogenetic uncertainty, we used the reverse-jump Markov chain Monte Carlo (MCMC) method implemented in the program BAYESTRAITS v.2 (Pagel & Meade, 2013). We used a sample of 1000 trees from our BEAST analysis (Chomicki & Renner, 2017) to account for phylogenetic uncertainty, a chain of 50 million generations, and rate coefficients and ancestral states were sampled every 1000th generation. We ensured that the acceptance rate was between 20% and 40%, as recommended in the manual, and reconstructed the nodes of interest using the command 'addnode'. We reconstructed all key nodes and report the probabilities above nodes in Fig. 4.

Independent origin test

To infer the minimal number of independent evolutionary gains of domatium wall differentiation while simultaneously accounting for phylogenetic uncertainty, we used the continuous time Markov chain model and Bayes factor test on a set of 1000 phylogenies from our previous molecular clock analysis (Chomicki & Renner, 2017), using the R package INDORIGIN (Minin *et al.*, 2014). The Bayes factors were calculated from the probability of the data conditioned on a null and alternative hypothesis specifying a minimum number of domatium wall differentiation gains occurring on the set of phylogenies.

Testing whether domatium wall differentiation and symbiotic strategies are correlated

To test whether wall differentiation or wart loss correlate with symbiotic strategies, we again used BAYESTRAITS v.2 (Pagel & Meade, 2013), which allows detection of correlated evolution between pairs of discrete binary traits. Specifically, we tested the following two hypotheses: Do fully differentiated domatium walls correlate with the evolution of symbioses with Dolichoderinae (*Philidris* and *Anonychomyrma*) ants? In Fiji, the endemic *Philidris nagasau* is the only Dolichoderinae ant inhabiting Hydnophytinae species (*Squamellaria*), but in New Guinea, several species of *Philidris* and *Anonychomyrma* inhabit Hydnophytinae species (see Chomicki & Renner, 2017). In addition: Does wart loss correlate with ant symbiosis breakdown? To test the first hypothesis, we coded domatium differentiation as '1' fully differentiated and '0' variable or absent, and main ant inhabitants as Dolichoderinae as '1' vs any generalist ant species or no ants as '0'. For hypothesis 2, we coded species with secondary wart losses (as inferred in our previous analyses, shown in Fig. 4) as '1' vs species with variable or fully differentiated walls as '0'. Each trait was coded from the literature (Jebb, 1985, 1991; Chomicki & Renner, 2016a,b, 2017) and personal observations by G.C. on specimens either in the field, in cultivation or in herbaria. We used the MCC tree from our previous BEAST analysis (Chomicki & Renner, 2017) but pruned the outgroups and first ran a model of independent trait evolution and estimated the four transition rate parameters $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$, 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.

Quantifying ant colony size in farmed vs nonfarmed *Squamellaria*

We quantified domatium volume and ant colony size in farmed vs nonfarmed *Squamellaria* by collecting the whole plant with its domatium, immediately placing them in large individual zip bags so that no ants could escape, and then adding 50% ethanol to collect the ants. We selected nine plants of a farmed *Squamellaria* species (*S. imberbis*) and nine similar-sized nonfarmed *Squamellaria* (*S. wilkinsonii*), growing along the cross-island road in Vanua Levu. To count ants, domatia were dissected in 50% ethanol as the majority of ants were hidden in the complex domatium. We quantified domatium volume by water displacement (in the field).

Computed-tomography 3D reconstructions

Domatia were collected in the field and immediately immersed in a 70% EtOH solution until scanned. Computed-tomography scanning was performed on a Nanotom m (Phoenix) X-ray tomography scanner at the Zoologische Staatssammlung in Munich. 3D processing was performed with the software AMIRA (v.6.0.1; TGS Europe SA, Merignac Cedex, France; Mercury Computer Systems Inc., Chelmsford, MA, USA). For slice alignment, the section edges representing the bottom of the block (mould) were used as reference in addition to bringing the specimen structures of neighbouring slices to a maximum congruence. Labelling of structures (AMIRA: segmentation) was done by hand, with the brush (internal structures) and lasso (external surfaces) tools. Initially, every third slice was labelled, with subsequent interpolation of structures on intervening slices, followed by a check of each interpolation and correction if necessary. Before surface rendering, structures were separated from the 'master' LabelField '.am' file into several LabelFields, each containing one specimen component. Specifically, we separated each independent cavity, as well as the surface outline of each tuber (obtained by merging all elements to 'tuber'). This allowed easy visualization of the tuber 3D structures. In addition, most new LabelFields were reduced in resolution by applying the Resample module to enable (fast) surface rendering, mostly using the default settings (binning x and y values by 2). Surface rendering was performed with the SurfaceGen module, leaving all settings at default. This was followed by the smoothening of the reduced surfaces using the SmoothSurface module, with mostly 40 iterations. Surfaces and the volume of each cavity cast and total domatium were quantified using AMIRA. This allowed us to precisely quantify the nesting surface available to ants in all domatia scanned.

Quantifying the types of domatium walls over ontogeny

To determine the relative proportions of smooth and warty walls over ontogeny, we sampled $n = 8$ *S. wilsonii* with domatia ranging from 3 to 28 cm in diameter (Fig. S2). To estimate the relative proportions of smooth and warty walls over the internal surface of the whole domatium, domatia were fully dissected so that their complex 3D arrangement could be placed on a flat surface as peels and photographed. We then used the software IMAGEJ (http://rsb.info.nih.gov/ij) to measure the area of each wall type, specifically relying on the tools 'polygon section' and 'measure' from IMAGEJ.

Comparing the amount of nitrogen gained from ants in farmed and nonfarmed *Squamellaria* species

To determine whether farming by ants leads to a nutritional upgrading in their host plants, we used (1) a 16-month-long

(March 2015–August 2016) exclusion experiment in a large *S. huxleyana* colony inhabited by the farmer ant *P. nagasau*; (2) a likewise 16-month exclusion experiment in *S. jebbiana* inhabited by generalist ants (Fig. S3); and (3) a natural experiment (lasting > 6 months) in which individuals of normally *P. nagasau*-inhabited *S. wilsonii* lacked domatia because their domatia had been eaten, but the plants had survived and regrown.

For experiment 1, we selected a group of *S. huxleyana* individuals growing on a *Macaranga harveyana* tree along the Cross Island road in Vanua Levu that was inhabited by a single *P. nagasau* colony. Removing the *P. nagasau* colony was achieved by removing the plant with the queen-bearing (i.e. largest (Chomicki & Renner, 2016a)) domatium from the tree. This leads to the death of the entire colony, presumably driven by predators, including from other ant species observed to attack *P. nagasau*. Ten days after removal of the queen-bearing domatium, all domatia were empty (but at this stage, still perfectly healthy). We confirmed that *P. nagasau* workers were not hiding inside domatia by dissecting the domatia of three plants scattered on the tree. The experiment thus did not require any insecticide application. To prevent other ants from re-colonising the empty domatia, we applied a sticky coating (Tangle-trap®/Tanglefoot®; The Ortho Group, Marysville, OH, USA) directly around the branches on which these epiphytes grew. The colonies chosen for the exclusion experiments were on the side of roads or tracks isolated from other *P. nagasau* colonies. The controls of experiment 1 consisted of $n = 5$ ant-inhabited *S. huxleyana* plants from five *P. nagasau* colonies.

For experiment 2, we used *S. jebbiana* plants, some of which were naturally occupied by generalist ants, while others were unoccupied. We selected $n = 5$ plants without ants in Taveuni (along the DesVoeux peak track) and applied Tangle-trap® along the trunk of the tree on which they were growing and around the bottom of the domatium, where most ant entrance holes are located. The plants thus grew ant-free for > 16 months. The controls of experiment 2 consisted of five plants inhabited by *Pheidole knowlesi* from distinct colonies. When collecting the plants, we ensured that no ants had re-colonised the plant before collecting the tissue.

For experiment 3, we took advantage of naturally occurring *S. wilsonii* individuals (again along the track leading to the DesVoeux peak) the domatia of which had been eaten, but had survived (Fig. S3f). We selected five such 'domatium-ablated' plants, all having regrown for at least 6 months (based on the shoots present on the remaining base of the domatium (Fig. S3f)) and compared them to five control plants inhabited by *P. nagasau* ants (all from different colonies).

At the end of each of the three experiments, we selected five treatment and five control plants for IR-MS to measure the nitrogen content (% N of dry weight) of different tissues. To do this, plants were cut in half, washed, and sampled for leaf tissue, tuber tissue (domatium tissue without inner walls) and smooth, smooth-like, warty or warty-like wall tissue. Samples were microwave-dried and then ground to fine powder with an MM301 mixer mill (Retsch, Haan, Germany). Stable N isotope values relative to air N_2 were determined from 10 (± 0.5) mg of sample with a MAT253 stable isotope ratio mass spectrometer (Thermo Scientific, Wilmington, DE, USA), a Flash 2000 Elemental Analyzer (Thermo Scientific) and a Conflo IV (Thermo Scientific) at the Institute for Geological Sciences of the University of Mainz, Germany. We used two standards (USGS-40 δ^{15}Nair = −4.5, and USGS-41a δ^{15}Nair = 47.55) and at least one control sample (e.g. IVA Casein) for which we knew the N content to check that the standards were working correctly.

Determining the nitrogen isotopic composition of ants

To determine the trophic level of the farming and nonfarming ants, we measured their ^{15}N level (Davidson *et al.*, 2003). IR-MS was performed in Mainz as described in the preceding section. For each species, we pooled 10–20 workers to have sufficient material for accurate determination of N isotopic composition.

Quantifying herbivory and evaluating leaf chlorosis as a result of ant removal

To determine how farming and nonfarming ants protects obligate vs facultative *Squamellaria* hosts against herbivores, we quantified herbivory in ant-excluded and ant-occupied plants, using the ant-exclusion experiments described above. Leaf herbivory was measured as the percentage of leaves eaten at > 10%:

$$\text{Herbivory} = \frac{\text{Number of leaves eaten at} > 10\%}{\text{Total number of leaves per shoot}} \times 100. \quad \text{Eqn 1}$$

This index is ideal as it easy to visually evaluate which leaves have been eaten at > 10%, and hence a large number of *Squamellaria* could rapidly be assessed nondestructively. Sample sizes match those of the ant-exclusion experiments except for the farmed (*S. huxleyana*) experiments where more plants ($N = 22$) were scored. For each plant, we typically quantified herbivory using three mature shoots.

Nitrogen deprivation typically results in chlorosis, a syndrome where leaves lack Chl and are pale. To evaluate whether the presence of ants impacts chlorosis, we used a colorimetric index with three levels (green, light green/yellow, yellow). The scale used is shown in Fig. S4. Sample sizes were the same as for the herbivory measurements.

Statistical analyses

All statistics were performed in R. Normality was tested by the Shapiro–Wilk test. When normality was not verified, we used a generalised linear model (GLM) using the function 'glm' with a quasiprobability error distribution, implemented in the R package STATS. In each case, our GLMs examined each predictor variable alone as well as all combinations of interactions among predictor variables. The most important statistics are given in the Results section, and all results are also provided in Tables S1–S3. Difference in frequencies was assessed using the Kolmogorov test. Equal variances were assessed by *F*-tests. When a normal distribution was met, differences in means were assessed by one-way and

two-way analyses of variance (ANOVAs) (or using *t*-tests if comparing only two groups with a single variable). Data are represented as the mean ± SE.

Results

Ant nitrogen fertilisation and plant nitrogen uptake varies starkly in farmed and nonfarmed species

To address whether farmed and nonfarmed *Squamellaria* species differed in nitrogen uptake efficiency, we first inspected the inner surface of their domatia. This revealed that domatium walls in farmed species (those exclusively inhabited by the ant species *P. nagasau*) have two kinds of surfaces, one smooth, the other with evenly distributed small protuberances ('warts') (Fig. 1a), similar to those described from *Myrmecodia* (Huxley, 1978). The domatium inner surfaces of nonfarmed species (inhabited by generalist ant species) are poorly differentiated (Fig. 1a). To investigate the physiological function of these distinct surfaces, we performed 'uptake' experiments in which we injected solutions of different concentrations of mineral (ammonium) and organic (glycine) ^{15}N stable isotopes into the domatia of different species (Fig. S1; see the Materials and Methods section). This revealed that the warty-walled surfaces of the farmed species *S. huxleyana* at 500 μM were > three-fold more absorptive for mineral nitrogen and 10-fold more absorptive for organic nitrogen (GLM, tissue type: t-value = 8.89, $P < 0.001$; Table S1) than the smooth-walled surfaces of the same species. They were also five-fold more absorptive (at 500 μM) than the inner domatium surfaces of a nonfarmed species (*S. wilkinsonii*) (Fig. 1b,c, GLM, species × tissue type × concentration: t-value = 6.28, $P < 0.001$; Table S1). In the domatia of the nonfarmed species, the $\delta^{15}N$ value also did not significantly increase further after 75 μM for ^{15}N glycine and 250 μM for ^{15}N ammonium (Fig. 1b,c), suggesting saturation of nitrogen uptake. This indicates that in the domatium walls of nonfarmed *Squamellaria*, smooth-like and warty-like wall areas both have comparable uptake capacity, while the differentiated warts in the farmed species are highly absorptive.

To compare the fertilisation effects of farming ants (*Philidris nagasau*) and nonfarming ants (*Pheidole knowlesi*) in farmed (*S. huxleyana*) and nonfarmed (*S. wilkinsonii*) plants, we performed experiments in which ^{15}N glycine was fed to ants in a sugary solution for 10 d (Fig. S1). The smooth-like and warty-like surfaces of the tested nonfarmed *Squamellaria* species were both ^{15}N-enriched, with the smooth-like wall tissue being the most enriched (Fig. 1d, GLM, tissue type: t-value = 3.43, $P < 0.001$; Table S2), indicating that the generalist *Pheidole* ants do not target their defecation and detritus to particular surfaces and instead deposit them in the same cavities where they raise their brood. By contrast, domatium walls in the *P. nagasau*-farmed *Squamellaria* species showed a high ^{15}N enrichment only in the tissue directly below warty-walled surfaces (Fig. 1e, GLM, tissue type: t-value = 3.43, $P < 0.001$; Table S2). Tissue below smooth walls was not more enriched than were leaves, stems or roots (Fig. 1e, inset), indicating that the amount of ^{15}N found in smooth walls is the result of nitrogen translocation, but not ant deposition.

Domatium wall differentiation and 3D structure modulate crop and farmer needs over ontogeny

For epiphytes, the seedling is a highly critical stage for water and nutrient provisioning, yet the structural costs of domatia – which are formed regardless of the presence of ants – is probably highest in young plants that are not yet ant-occupied (Blatrix *et al.*, 2012; Chomicki & Renner, 2019). This conflict could be reduced if young plants had a high density of absorptive warts on their inner domatium walls. When examining the inner domatium walls throughout ontogeny we indeed found that young walls of farmed species are entirely covered by warts, while smooth-walled surfaces form only later. The domatia of adult plants have *c.* 20% warty walls and 80% smooth walls (Fig. S2). This maximises nutrient provisioning to seedlings, which gain more than twice as much nitrogen from ant defecation compared with mature plants (Fig. 1e). The trade-off between the plant's need for nutrients early on (via warty walls) and the ant's need for nesting space (via smooth walls) is resolved by the multigenerational nature of these farming symbioses, with seedlings (whose domatia lack smooth walls) coexisting in the same 'plantation' in canopy trees with adult plants that reward the ants by offering smooth-walled cavities for nesting (Fig. 2a).

To compare the inner surface area in farmed and nonfarmed *Squamellaria*, we generated 3D models of their domatia using computed-tomography (CT) scanning, which enabled us to precisely quantify surface and volume available for nesting. CT scanning revealed a surface-area-to-volume ratio more than two times larger in farmed than nonfarmed *Squamellaria* (Figs 2b, S5, ANOVA, $F_{1,11} = 23.07$, $P < 0.001$), resulting from a finer reticulation of the internal cavities.

To test if larger surfaces (for the same volume) lead to higher worker numbers per domatium, we compared ant colony size over ontogeny in farmed vs nonfarmed species. We found a tight positive correlation between domatium volume and colony size in farmed ant-plants but not in generalist nonfarmed ant-plants (Fig. 2c; farmed: $R^2 = 0.98$ vs 0.37 in nonfarmed; Pearson's correlation coefficient 0.99 vs 0.63), and overall a much larger number of workers in large plants (Fig. 2c with, for instance, six-fold as many ant workers in farmed as compared to nonfarmed *Squamellaria*, both with domatia of 3.5×10^6 mm^3, corresponding to *c.* 30 cm in length) (Fig. 2c). We estimate that the largest *P. nagasau* colonies contain over 250 000 workers (spanning dozens of *Squamellaria* individuals). The massive difference in ant colony size between farmed and nonfarmed *Squamellaria* suggests that nutrient gain is amplified by larger nesting space (nesting space over volume ratio) allowing larger ant colonies.

Farming increases ant-derived nutrients to plants

To determine to what extent farmed and nonfarmed *Squamellaria* depend on fertilisation by ants, we performed 16-month-long exclusion experiments in which we precluded farming (*P. nagasau*) and nonfarming (generalist) ant species from nesting inside the domatia (Fig. S3; see the Materials and Methods section). All plants gained significantly more nitrogen with ants than without ants (GLM, experiment: t-value = −3.21,

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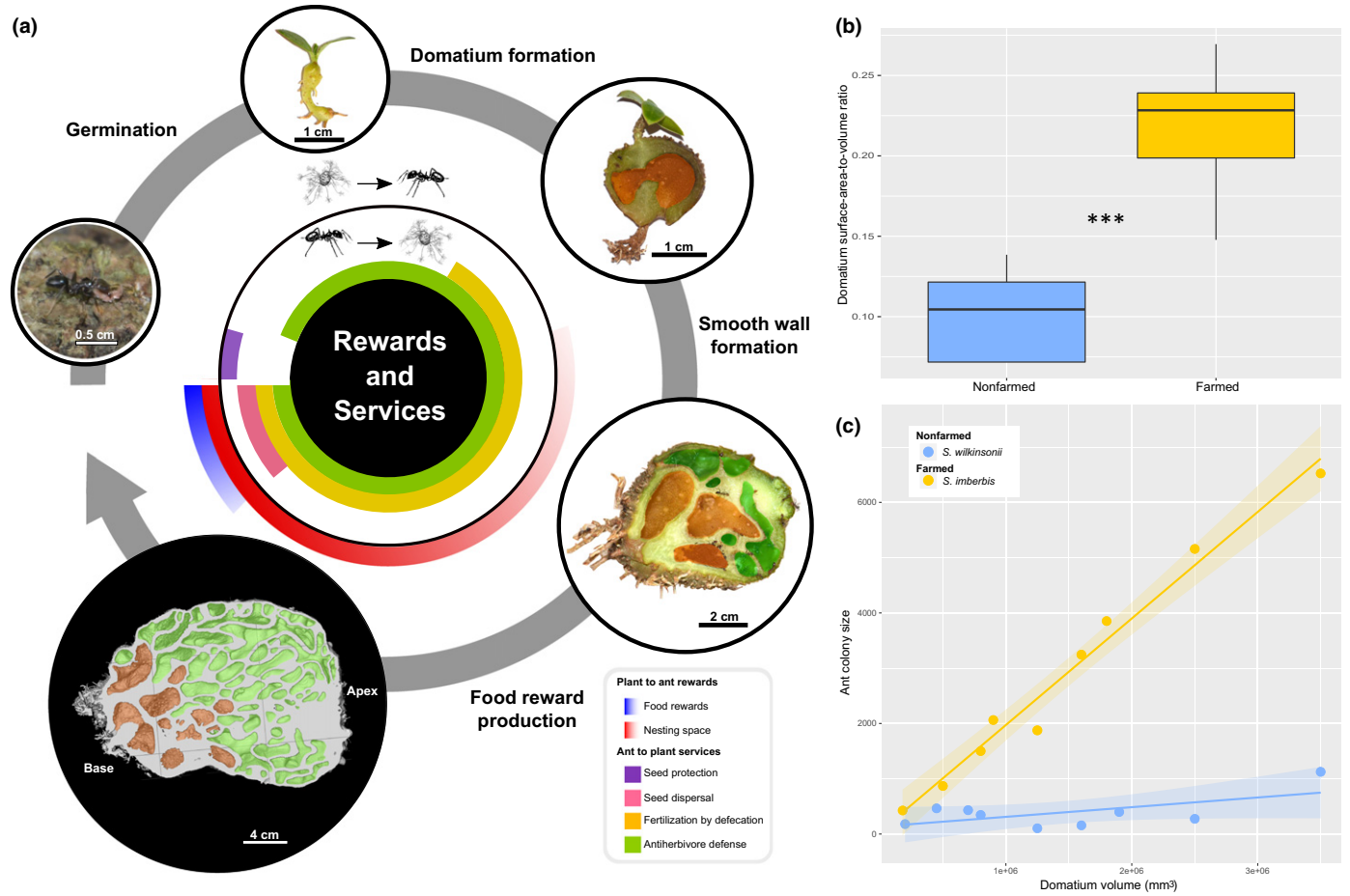

Fig. 2 Domatium wall differentiation and 3D structure modulate crop and farmer needs over ontogeny. (a) Rewards exchanged between *Squamellaria* and *Philidris nagasau* across the plant ontogeny. In the domatium longitudinal section, orange refers to a warty cavity and green to a smooth cavity. (b) Domatium internal surface to volume ratio in farmed vs nonfarmed *Squamellaria*. Measurements relied on CT scanning-based 3D models of all Fijian *Squamellaria* species. Asterisks indicate the level of significance (***, $P < 0.001$) of an ANOVA (details in main text). (c) Relationships between ant colony size and domatium volume in farmed vs nonfarmed *Squamellaria*. Lines show linear regression, with the shading indicating the 95% confidence interval. See also Supporting Information Figs S2 and S5.

$P = 0.002$; Table S3), indicating that in both cases these symbioses are nutritional mutualisms. Farmed *S. huxleyana* gain 43.3% of their nitrogen from farming ants, while nonfarmed *S. jebbiana* inhabited by *Pheidole knowlesi* ants only gained 19.3% from its nonfarming ant (Fig. 3, GLM, ant × tissue type: t-value = −2.73, $P = 0.01$; Table S3). Ablation of the ant-housing domatia (Fig. S3) in another farmed species (*S. wilsonii*) indicated similar nitrogen gains (Fig. 3). The exclusion experiments further revealed that uninhabited *S. huxleyana* plants suffered high mortality rates, especially in seedlings, had chlorotic leaves suggesting nitrogen deprivation, and sustained increased herbivory (ANOVA, $F_{2,20} = 569.7$, $P < 0.01$; post-hoc test, control vs ant exclusion $P < 0.001$; Fig. S4), uncovering a defensive role of *P. nagasau* in the farming nutritional symbiosis. When a generalist ant species (*Pheidole knowlesi*) was excluded from its host (*S. jebbiana*), this affected neither herbivory nor the chlorotic status of the host (Fig. S6). This further highlights the obligate dependence of farmed, but not nonfarmed, *Squamellaria* species on their ant partner.

The high nitrogen gains in farmed *Squamellaria* could be the result of richer faeces or detritus nitrogen content in some ant species than others, for example insect-feeding ants compared to nectar-feeding ants. However, comparison of $\delta^{15}N$ to evaluate the trophic level (Davidson *et al.*, 2003) of *Philidris nagasau* and *Pheidole knowlesi* ants (Fig. S7) suggests that they belong to the same level, and thus probably produce excretions of similar nutrient status. The higher nutrient gain in farmed *Squamellaria* species is therefore most parsimoniously explained by the much larger number of ant workers per plant enabled by the 3D structure of the domatia of farmed species (Fig. 3b,c), and possibly also their differentiated domatium walls with hyperabsorptive warts to which *P. nagasau* workers target their defecation (hence fertilisation).

Evolution and loss of hyperabsorptive tissue mirror mutualism evolution in the Hydnophytinae

The Rubiaceae subtribe Hydnophytinae to which *Squamellaria* and *Myrmecodia* belong includes *c.* 105 species of ant-plants that vary in domatium wall structure (Jebb, 1985, 1991; Chomicki & Renner, 2016b, 2017). Some species have differentiated walls (above), others have walls with wart-free but nonwaxy areas interspersed with 'warty-like' areas (warts of often variable size and

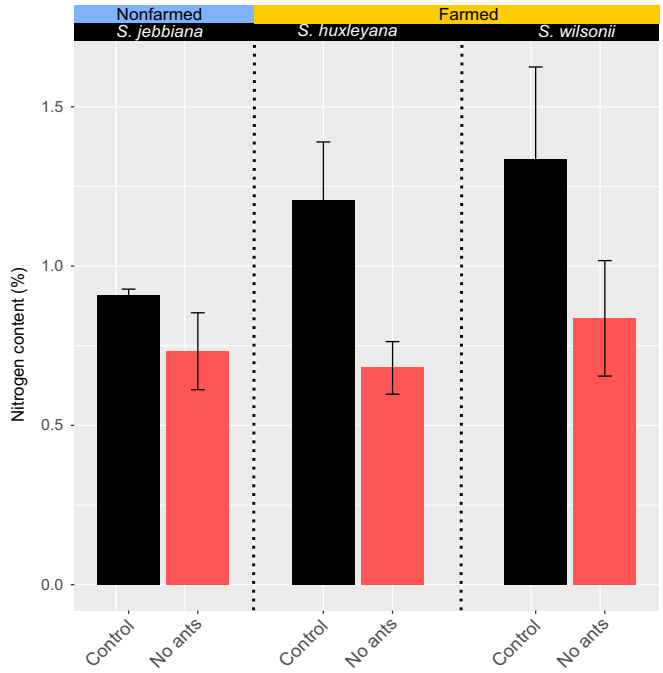

Fig. 3 Ant exclusion experiments reveal differential nitrogen gains in farmed and nonfarmed *Squamellaria* (see also Supporting Information Fig. S4). Graph shows mean ± SE for $n = 5$ plants. Details of statistical analysis are reported in the main text.

spacing), and some species have no warts at all. To investigate the evolution of domatium wall differentiation (mirroring nutrient uptake capacity; previous section) and test if this reflects distinct evolutionary stages of the symbiosis, we used a phylogeny sampling 81 of the *c.* 105 species (Chomicki & Renner, 2017). This revealed that fully differentiated domatium walls evolved four times (Figs 4a,b, S8; Table S4) and that warts were lost about nine times (Figs 4a,b, S8; Table S4). To investigate the ecological context of domatium wall differentiation, we conducted tests of correlated evolution (Fig. S8). These analyses show that domatium differentiation evolved with the colonisation by seed-planting Dolichoderine ants (*Philidris* and *Anonychomyrma*; Bayes factor (BF = 71.12)) and that wart losses coincide with mutualism breakdown (BF = 50.81), usually in ant-poor montane regions where larger arthropods or even small vertebrates, such as tree frogs, inhabit the domatia (Chomicki & Renner, 2017). Species with fully differentiated warts on their inner domatium walls have finely reticulate domatia which large internal areas. Wart-free species without ant symbioses have large and globose domatium cavities, and species forming facultative and generalist ant symbioses have variable wall differentiation and intermediate reticulation (Fig. 4c).

Discussion

Targeted nutrient fertilisation evolved in Hydnophytinae species farmed by Dolichoderine ants

We showed that farmed *Squamellaria* species living in obligate symbiosis with *P. nagasau* farming ants have differentiated domatium walls with specialised hyperabsorptive warts that are the sites of active and exclusive fertilisation by ants. Such differentiated domatium walls apparently evolved from poorly differentiated walls, as are found in nonfarmed *Squamellaria* species. The physiological partitioning of the domatium walls (with smooth and warty surfaces serving different functions) matches the behaviour of *P. nagasau* ants, which exclusively fertilise warty walls. Our ^{15}N feeding experiments suggest strongly that the main fertilisation mechanism is active defecation on the warts, but we could not directly document this because our attempted filming inside the domatium with an endoscope greatly perturbed the ants. Targeted defecation is the only mechanism that can explain the results of our feeding experiments, especially the absence of ant-deposited ^{15}N on smooth walls (Fig. 1b,c), which shows that *P. nagasau* does not to defecate in smooth-walled chambers where the ants raise their brood, probably benefiting colony health. Defecation away from brood chambers is frequent in communally living insects (Weiss, 2006) and might have played a role in the evolution of differentiated domatium walls in *Squamellaria*.

Warts on inner domatium walls were first discovered in *Myrmecodia* in the 1880s and were believed to function either in nutrient absorption (Beccari, 1884–86) or in aeration (Treub, 1888). Miehe (1911) showed that in *Myrmecodia*, warty walls can absorb water and possibly solutes while smooth walls cannot. Experiments by Huxley (1978) using phosphate and sulphur radioisotopes revealed that warts were more enriched in these isotopes than other plant parts, but the possibility of differential uptake of warty and smooth wall types was not tested. Our experiments now demonstrate the physiological differentiation of domatium walls in farmed ant-plant species. The evolution of farming thus remodelled plant nutrition, with domatium walls acquiring hyperabsorptive warty areas and poorly absorptive wax-covered smooth areas.

The occurrence of differentiated domatia with warty and smooth walls in several lineages of the Hydnophytinae is correlated with the nesting of Dolichoderinae ants in their domatia (genera *Philidris* and *Anonychomyrma*; Fig. S8). The extent of coevolution between plants and ants is unclear; the differentiated domatium walls clearly reflect the ant's fertilisation behaviour, but the extent to which defecation behaviour has changed in Dolichoderinae ants that started to farm Hydnophytinae is unknown. The repeated gains and losses of domatium nutrient uptake capacity mirror the evolution of these symbioses at a macroevolutionary scale, and recurrent colonisation by distinct farming Dolichoderinae lineages (Chomicki & Renner, 2016a; Chomicki *et al.*, 2017) may have imposed similar selection regimes on epiphytic Rubiaceae.

Active fertilisation by ants in epiphytes

Vascular epiphytes are well known for their nutrient-scavenging structures, such as phytotelma (water tanks), specialised trichomes, root velamen or litter-trapping leaf arrangements (Benzing, 1990; Zotz & Hietz, 2001). Ants are by far the most common animals associated with epiphytes, and it is clear that they can provide nutrients to epiphytes (Davidson & Epstein,

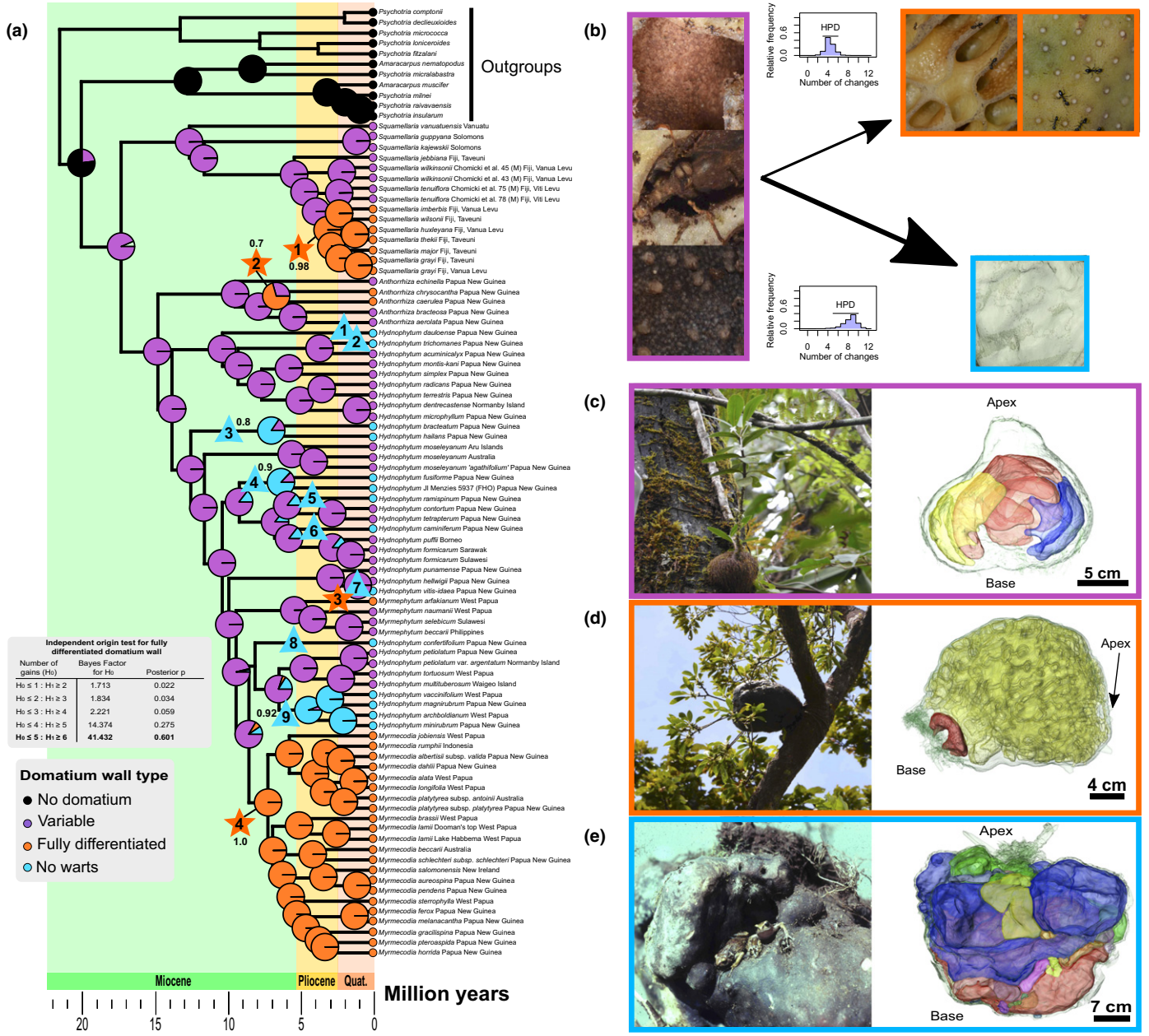

Fig. 4 Evolution and loss of hyperabsorptive warts. (a) Ancestral state estimations performed on a dated phylogeny sampling *c.* 75% of the Hydnophytinae using 1000 simulations of character states (colour pies), a Bayesian reverse-jump MCMC approach using 1000 trees from a Bayesian clock analysis (numbers close to clades that shift in state), and an independent-origin test taking into account differences in evolutionary rates and phylogenetic uncertainty (grey table, see also Supporting Information Table S4). Purple pies (with the state 'variable') refer to domatium walls that have some wart-free but nonwaxy areas ('smooth-like'), others with warts of variable size and spacing ('warty-like'), and occasional internal roots (inset photographs); orange pies refer to fully differentiated warty and smooth areas; blue pies refer to secondary losses of warts, with the inferred losses marked by blue triangles and numbered 1–9. Orange stars (numbered 1–4) show the convergent evolution of fully differentiated hyperabsorptive warts. See also Fig. S8. (b) Summary from the stochastic mapping analysis, illustrating key evolutionary changes in domatium wall structure in the Hydnophytinae. (c–e) Photos of Hydnophytinae (left) and 3D model of their domatia based on CT scanning data (right) with variable (c, *Squamellaria wilkinsonii*), fully differentiated (d, *S. wilsonii*) and no warts (e, *Hydnophytum myrtifolium*). Plant photos in b–d: Guillaume Chomicki; e: Matthew Jebb; CT scans: all G. Chomicki. Statistical support for the phylogeny is shown in Chomicki & Renner (2017: Fig. S1).

1989). However, the scenario so far was that ant-derived nutrients come from the passive uptake of detritus deposited within the domatia (Davidson & Epstein, 1989; Gay, 1993; Treseder *et al.*, 1995; Gegenbauer *et al.*, 2012). Indeed, many ant-plants have adaptations for gaining nutrients from ant waste, such as suberin incrustations in *Piper* (Tepe *et al.*, 2007) and *Caularthron* (Gegenbauer *et al.*, 2012), pitted sclerenchyma with numerous plasmodesmata in *Leonardoxa* (Defossez *et al.*, 2010) and *Humboldtia* (Chanam & Borges, 2017), and internal roots as in *Dischidia* (Treseder *et al.*, 1995) and nonfarmed *Squamellaria*

(this study, Fig. 1a). In nonfarmed *Squamellaria*, nutrient deposition is simply a by-product of the ants' nesting in the plants' domatia, while the active and exclusive fertilisation of warty walls in the farming symbioses is a unique type of nutrient acquisition in epiphytes. Nonfarmed species of *Squamellaria* are bird-dispersed (Chomicki & Renner, 2016a) and are found in typical epiphyte positions (e.g. tree forks) frequently growing with other epiphytes and litter. By contrast, farmed species of *Squamellaria* are planted by the ants high up in the canopy and occur on naked branches. They may thus be more dependent on the active fertilisation by farming ants. Indeed, our experiments suggest that the nitrogen gain from *P. nagasau* ants in farmed *Squamellaria* exceeds that from a less specialised *Philidris* species that inhabits *Dischidia* leaf pouch domatia (N gained from ants: *S. huxleyana*: 43%; *Dischidia*: 29%; this study; Treseder *et al.*, 1995).

The *P. nagasau*/*Squamellaria* farming symbioses differ from both Neotropical (Davidson, 1988) and South-East Asian (Kaufmann & Maschwitz, 2006) ant-gardens in that the former are obligate and specialised while the latter are facultative and generalist systems. Most ant-gardens therefore are not farming mutualisms *sensu stricto* (Mueller *et al.*, 2005; see the Introduction section). Also, the fertilisation of epiphytes in ant-gardens is typically passive while in the *P. nagasau*/*Squamellaria* farming symbioses it is active and targeted to specific structures that result from ant domestication.

Crop domestication in the *Squamellaria*/*P. nagasau* mutualism compared to other insect agriculture mutualisms

The *Squamellaria*/*P. nagasau* farming mutualism shows several convergences with insect fungiculture, such as the monitoring of planted propagules (Chomicki & Renner, 2016a), sustainable harvesting of crop-produced food (Chomicki *et al.*, 2016) and the protection of the crop against consumers (this study). However, it also shows a number of features that differ from insect fungiculture. For example, dependence of the ants on their crop for food is facultative, but dependence for nesting is obligate because *P. nagasau* has lost the ability to form carton nests (Chomicki & Renner, 2016a). The targeted crop fertilisation that we report in this study, however, parallels the targeted crop fertilisation in leafcutter ants in which the inoculum is fertilised by the queen's faeces and mature gardens are fertilised by workers applying faecal droplets to concentrate enzymes (Mueller *et al.*, 2005; De Fine Licht *et al.*, 2013).

An important difference between other insect agriculture mutualisms and the mutualism studied here is that sexual reproduction is the only means for crop propagation in the *Squamellaria*/*P. nagasau* symbiosis, while insect fungiculture involves clonal dispersal of the fungus (Aanen *et al.*, 2002, 2009; Mueller *et al.*, 2005). This leads to the multigenerational *Squamellaria* 'farms', a conspicuous feature of this farming mutualism.

Similar to crop domestication by humans, insect farmers have imposed directional selection on key food rewards, such as the inflated hyphae tips in fungal crops (De Fine Licht *et al.*, 2014) and the postanthetic rewards (nectaries of old flowers providing concealed sugars and amino acids) in *Squamellaria* crops (Chomicki *et al.*, 2016). In fungus-farming ants, a change in fungus crop nutrition followed the transition to herbivory in leafcutter ants, with the loss of some lignin-degrading genes (Nygaard *et al.*, 2016). By contrast, it is the recruitment of a new ant lineage (*Philidris*) with a distinct fertilisation behaviour that drove the evolution of nitrogen uptake by specialised wall outgrowths in Fijian *Squamellaria*.

Farming increases the benefits of mutualism

Mutualisms can be classified according to consumer resource dynamics (Holland & DeAngelis, 2010), types of services (transportation, protection, nutrition), intimacy (symbiotic or non-symbiotic), dependence (facultative or obligate) or specificity (generalist or specialised) (Bronstein, 2015). Comparing the efficiency of these diverse types of mutualism is challenging (Schupp *et al.*, 2017). It has long been predicted that obligate or specialised mutualisms are more efficient than facultative or generalist mutualisms (Herrera & Jordano, 1981; Thøstesen & Olesen, 1996), although efficiency can also be a direct function of the level of investment (Heil *et al.*, 2009). Our experiments show that nitrogen fertilisation is much more efficient in farmed than in nonfarmed symbioses (Figs 1, 3). The farming symbiosis is both obligate (for both ants and plants) and specialised (a single ant partner). The higher efficiency appears to result from both the targeted fertilisation minimising nutrient loss (quality) and the larger domatium surfaces, which allow for more workers per plant (quantity). In the farming symbiosis, efficiency for plants (fertilisation by ants) and ants (food reward and nesting space provisioning) are coupled because the larger inner domatium surface in farmed plants directly increases nesting space and nutrients (and hence the production of food rewards), and similarly the targeted fertilisation increases plant growth (and hence nesting space and food rewards). The lack of a strong positive correlation between worker colony size per plant and domatium volume in nonfarmed symbioses (with generalist ant species) is consistent with a lower efficiency (Fig. 2c), confirmed by our exclusion experiments (Fig. 3).

Conclusion

Our results reveal that the evolution of plant farming by ants has deeply remodelled their crops' nutrition, resulting in an increase in nutrient uptake driven by ants. The starting point appears to have been the recruitment of an ant lineage (*Philidris*) with a distinct social structure (polydomy), loss of carton nest-building ability and the behaviour to defecate inside even seedlings too small to house a colony, which is crucial for the plants' establishment in soil-free bark cracks. The hyperabsorptive warts (on inner domatium walls) appear to be analogous to root hairs, and their extreme mineral and organic absorption rates could hold clues for improved nitrogen uptake. Investigation of the genetic pathways underlying hyperabsorption of nitrogen is the subject of further research and may in the future help to engineer plants with improved nitrogen uptake, analogous to other ongoing crop improvement programmes (Covshoff & Hibberd, 2012; Schroeder *et al.*, 2013).

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Author contributions

GC and SSR conceived the study, GC performed the experiments and analysed the data, GC and SSR wrote the paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Experimental design for the isotope labelling 'feeding' and 'uptake' experiments.

Fig. S2 Change in the proportions of the two cavity wall types during ontogeny in *Squamellaria wilsonii*.

Fig. S3 Experimental design for ant exclusion experiments.

Fig. S4 Analyses of the ant exclusion experiments in *Squamellaria huxleyana*.

Fig. S5 Surface area-to-volume ratio in the Fijian *Squamellaria*, measured from 3D models based on CT scanning data.

Fig. S6 Analyses of the ant exclusion experiments in nonfarmed *Squamellaria wilkinsonii*.

Fig. S7 $\delta^{15}N$ of farming vs nonfarming ants.

Fig. S8 Wart evolution reflects specialisation and breakdown of symbiosis with ants.

Table S1 GLM uptake experiments.

Table S2 GLM feeding experiments.

Table S3 GLM exclusion experiments.

Table S4 Independent origin analysis of fully differentiated domatium walls using the R package INDORIGIN.

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