Supplementary materials

Characteristics of *H. physophora* and *Allomerus* populations

The *H. physophora* populations studied differed in the ratio of their associated ant species, ranging from 100% of the plants inhabited by *A. octoarticulatus* in Camp Patawa, 60% in Saül, 20% in Fourgassier, and 15% in the Montagne des Singes whereas 100% of the plants in Petit Saut were inhabited by *A. decemarticulatus*. These ratios were obtained from a non-destructive census of the ant species inhabiting the *H. physophora* in the above-mentioned areas, regardless of plant size and ant colony developmental stage, except for founding queens and incipient colonies.

Molecular phylogeny of *Allomerus* ants

Methods

Total DNA was extracted from ant workers. Each ant worker was incubated overnight at 55°C in 10 µL of Proteinase K solution (Qiagen) and 150 µL of 10% Chelex solution (BioRad, Marnes-la-Coquette, France). We used 2 µL of the solution obtained to amplify fragments of the cytochrome c oxydase subunit I (COI) and the cytochrome *b* (cyt *b*) genes in two different PCR.

We amplified a segment of the COI with primers LCO1490 and HCO2198 [1] and a segment of the cyt *b* gene with primers CB1 and tR^{S} or CB2 [2, 3]. PCR products were sequenced by Millegen (Labège, France). In addition, individuals from four Solenopsidini species were used as outgroups: *Monomorium subopacum* collected in *El Puerto de Santa María*, Cádiz (Spain), and *Diplorhoptrum* sp., *Solenopsis saevissima* and *Wasmannia auropunctata* collected in *Petit Saut* (French Guiana). The sequences are available at GenBank through accession numbers KX585770-KX585863.

The sequences were edited, aligned and concatenated using Geneious v9.1.5 (http://www.geneious.com) [4]. The alignment was performed using MAFFT v1.3.5 [5] as implemented in Geneious. The final alignment consisted of 56 sequences and was 1,594-bases long. Phylogenetic reconstruction was performed by maximum likelihood inference (ML) using the GTRGAMMA substitution model in the RAxML software [6]. The best-scoring ML tree was selected after 50 iterations. Branch support for the topology was assessed by bootstrapping the original data; the autoMRE automatic bootstopping criterion resulted in 850 replicates.

Results

The *Allomerus* clade was monophyletic in the context of the outgroup species provided. We identified four very well supported clades corresponding to four of the five species/subspecies studied; *A. dentatus*, which was represented by only one individual, was basal to these four clades. *Allomerus decemarticulatus* individuals were grouped as a monophyletic clade, basal to *Allomerus septemarticulatus*; *A. octoarticulatus* and *A. octoarticulatus* var. *demerarae* were grouped together. The three *A. septemarticulatus* individuals were in their own monophyletic clade, basal to *A. octoarticulatus sensu lato*. Finally, *A. octoarticulatus* and *A. octoarticulatus* var. *demerarae* were almost perfectly divided into two separate monophyletic sister-clades. Geographical origin and host-plant species did not seem to influence the phylogenetic grouping for *A. octoarticulatus*.

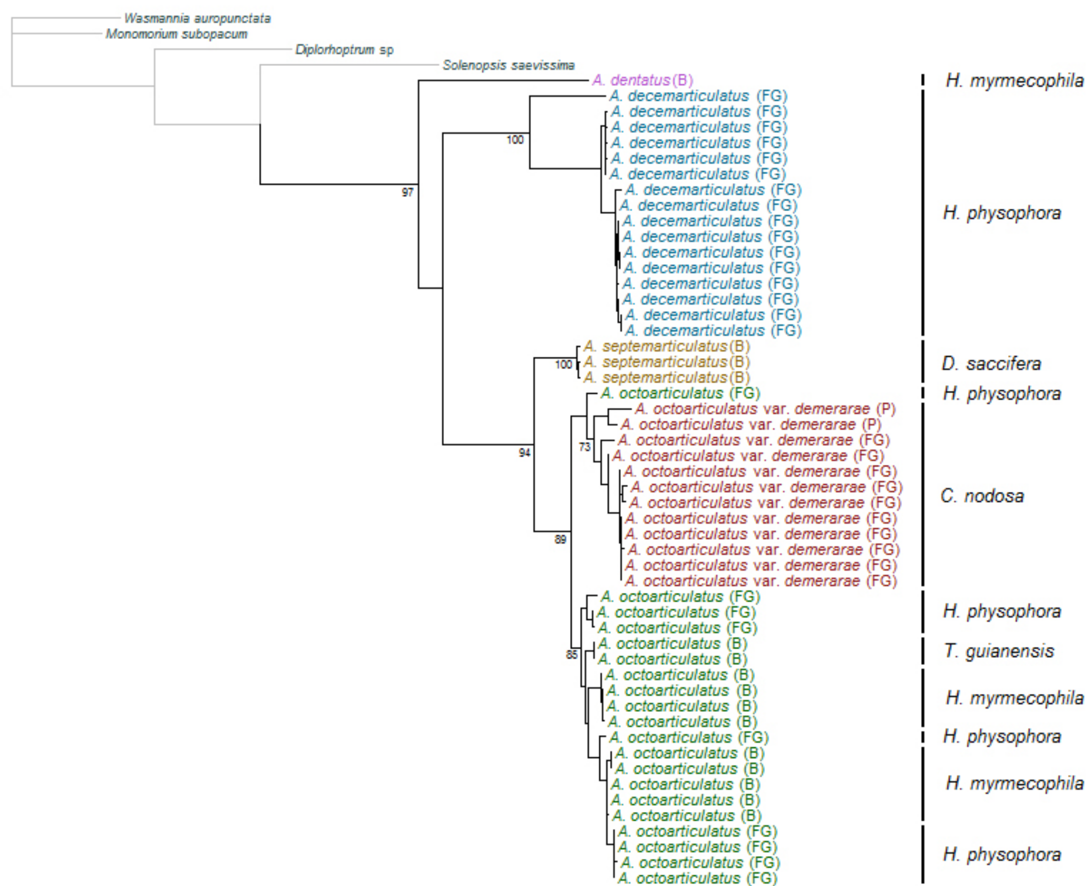


Figure S1. Partial phylogeny of the *Allomerus* genus based on cyt *b* and COI mtDNA sequences (1060 bp). Bootstrap values above 50% are indicated for each branch. Each ant species is highlighted in a different color and followed by its area of origin [Brazil (B), Peru (P), French Guiana (FG)] and the identity of its associated host plant.

53

54 **Chemical basis of biotic protection**

55 Methods

56 We collected *Allomerus* workers from several colonies (not mixed) and *H.*
57 *physophora* leaves from different trees than those sheltering the collected ants in the
58 morning for behavioral experiments to be conducted in the afternoon. Because it has
59 been shown that nurses responded differently to leaf tissue extracts [7, 8], only
60 patrolling workers were collected with a mouth aspirator. Once transported to the
61 laboratory, they were provided with water and honey and kept in small vials (h=70
62 mm; Ø=30 mm) until their use in the afternoon.

63 Four types of leaves were collected according to the stages described in
64 Grangier *et al.* [9]. Juvenile leaves (hereafter, young leaves) were collected whole
65 whereas well-developed leaves (stage 2-4) were cut off leaving the domatia on the
66 tree to avoid collecting ants with the leaf. Leaves were individually placed in plastic
67 bags for transport to the laboratory where the patrolling ants were carefully removed.
68 Less than 1 hour after the leaves were collected in the field, their compounds were
69 extracted by soaking them in a hexane bath for 30 min to obtain volatile surface and
70 primary compounds [10, 11]. Young leaves were soaked in a 4 ml vial (15x45 mm)
71 filled with hexane, only the cut petiole remaining out of the solvent bath. Older leaves
72 (stage 2-4) were soaked in a 22 ml vial (23x84 mm) with 4 ml of hexane with only a
73 part of the tip of the leaves soaking in the solvent to obtain approximately the same
74 surface area of washed leaf as for the young leaves. For each extract, three leaves
75 from three different trees were consecutively soaked in the same hexane bath. After
76 each leaf, hexane was added to ensure a volume of 4 ml because of partial evaporation
77 or absorption by the trichomes. Extracts were kept at -20°C until their use in the
78 afternoon.

79 The isolation of active compounds involved in the induction of the patrolling
80 behavior was performed *via* the fractionation of crude extracts to the compounds'
81 polarity on a silica gel column (2 cm, 70-230 mesh, 60 Å). Elution was carried out
82 successively with 4 ml of hexane, 1 ml of dichloromethane and 4 ml of methanol.
83 Fractions were dried under nitrogen and re-dissolved in 4 ml of hexane for bioassays.

84 The chemical analysis of leaf tissue extracts and fractions was conducted
85 through Gas Chromatography-Mass Spectrometry (GC-MS) with a Varian 450-GC

86 coupled to a MS240 ion-trap mass spectrometer. The entire system was operated by
87 using the Varian MS workstation data system, version 6.9.2. MS spectra were
88 recorded over a mass range of 50-650 mass units with two scans per second. An
89 aliquot of 1 µl was injected in splitless mode with an injector temperature of 250°C, a
90 detector temperature of 300°C and a manifold temperature of 300°C. An apolar
91 Varian factorFourVF-5MS capillary column (30m, 0.25mm i.d., 0.25 µm film
92 thickness, 5% diphenyl and 95% dimethylpolysiloxane) was used. Elution was carried
93 out with helium at 1 ml/min. The oven temperature was programmed as follows: 60°C
94 for 2 min, 60 to 280°C at 15°C/min, 280 to 320°C at 10°C/min and left at 320°C for
95 10 min. Identifications were processed with spectral databases and with a series of
96 standard linear alkanes. Standards of compounds of interest were injected for control.

97 Behavioral tests were performed in a neutral arena (Petri dish; 90mm in
98 diameter and 10mm deep) whose walls were coated with Fluon® to prevent the ants
99 from climbing out. Thirty workers from the same colony were placed in the arena 10
100 min before the bioassay for acclimation. 300 µl of extract or control solvent (hexane)
101 were deposited on a 4 cm^2 piece of filter paper and allowed to evaporate during 15
102 seconds before being introduced in the arena. The number of ants in contact with the
103 papers were then scored every minute during 20 min as described in Edwards *et al.*
104 [8]. Ant workers and filter papers were used only once and the arenas were cleaned
105 with ethanol between each experiment.

106 As bioassays on the different fractions identified tocopherols as putative
107 attractive compounds, the same bioassays were performed with synthetic α-tocopherol
108 and β-tocopherol (Sigma-Aldrich). Different combinations were tested: "α- and β-
109 tocopherol", "α- and β-tocopherol+EOL" (EOL = extracts from old leaves), "α-
110 tocopherol+EOL", and "β-tocopherol+EOL". The α- and β-tocopherol were mixed in
111 proportions similar to the ones observed in young leaves (see Table S1).

112 The effect of each treatment on the number of ants attracted was analyzed using
113 a repeated-measures ANOVA comparing the treatments and their respective controls
114 using SPSS 16 software. Time (n=20) was considered as the repetition factor and the
115 number of ants was $\sqrt{(x+0.5)}$ transformed. To account for violations of the sphericity
116 assumption of variances, the Greenhouse–Geisser correction was applied to the
117 degrees of freedom.

118

119 Results

120 The extracts from young leaves induced worker recruitment by
121 A. decemarticulatus in the first few minutes after being presented (i.e., a significant
122 time effect, Table S1). Note that the time effect noted in the other experiments (i.e.,
123 other leaf developmental stages) was related to a loss of interest in the filter papers
124 during the course of the trials.

125 The compounds identified in the leaf extracts were mainly linear hydrocarbons
126 (69.9% and 98.7% in young and old leaves, respectively) (Table S2). Young leaves
127 showed a more diversified profile than did old leaves with some triterpenes (α-
128 amyrin, β-amyrin and α-amyrone), tocopherols (α- and β-tocopherol), and an alcohol.
129 Among the volatile compounds, only methyl salicylate was found in both young and
130 old leaves.

131 The fractionation of the extracts according to the polarity of the compounds
132 showed that only the dichloromethane fraction attracted *A. decemarticulatus* workers
133 (Table S3). The GC-MS analysis of compounds in the different fractions showed that
134 α-tocopherol was the only compound present in all of the active fractions (i.e.,
135 dichloromethane and young leaves) and absent from all of the inactive fractions (i.e.,
136 old leaves, hexane and methanol fractions) (Table S3). β-tocopherol was present in all
137 of the fractions but its proportion was relatively higher in the active dichloromethane
138 fraction. Because of their quite similar molecular structures, both compounds were
139 selected for behavioral tests.

140

141

142 **Table S1.** Repeated-measures ANOVAs examining the ability of extracts
143 from H. physophora leaves to attract *A. decemarticulatus* and *A. octoarticulatus*
144 according to leaf developmental stage (1-4). Probabilities corrected for sphericity are
145 provided using the Greenhouse–Geisser correction (G-G). Significant results are
146 indicated in bold.
147

	A. decemarticulatus				*A. octoarticulatus*			
	df	**F**	***p*-value**	**G-G**	***df***	**F**	***p*-value**	**G-G**
Stage 1 *vs.* hexane								
Treatment	1	44.66	**<0.001**		1	3.61	0.065	
Error	38				38			
Time	19	3.55	**<0.001**	**<0.001**	19	2.84	**<0.001**	**0.001**
Time x Treatment	19	1.66	0.039	0.095	19	0.73	0.787	0.722
Error	722				722			
Stage 2 *vs.* hexane								
Treatment	1	0.71	0.405		1	1.14	0.292	
Error	38				38			
Time	19	3.68	**<0.001**	**<0.001**	19	3.29	**<0.001**	**0.001**
Time x Treatment	19	1.44	0.101	0.155	19	1.12	0.326	0.348
Error	722				722			
Stage 3 *vs.* hexane								
Treatment	1	1.73	0.197		1	1.32	0.258	
Error	38				38			
Time	19	0.90	0.587	0.540	19	2.42	**0.001**	**0.005**
Time x Treatment	19	0.50	0.965	0.900	19	1.50	0.079	0.124
Error	722				722			
Stage 4 *vs.* hexane								
Treatment	1	0.92	0.345		1	1.44	0.237	
Error	38				38			
Time	19	1.10	0.344	0.359	19	4.94	**<0.001**	**<0.001**
Time x Treatment	19	1.13	0.316	0.338	19	0.91	0.574	0.536
Error	722				722			

148

Table S2. Retention index, identification and relative proportion of the compounds in extracts from young and old *H. physophora* leaves and their fractions. tr: traces (<0.1%), Unk: unknown, - : absent. Fractions that attracted *A. decemarticulatus* are in bold.

Peak	Retention index	Identification	Leaves		Fractions (young leaves)			
			Young	Old	**Evaporated**	Hexane	**Dichloromethane**	Methanol
1	1207	Methyl salicylate	**0.2**	0.2	**tr**	tr	**-**	-
2	2300	n-Tricosane (n-C_{23})	**0.2**	tr	**0.1**	0.3	**0.1**	0.3
3	2500	n-Pentacosane (n-C_{25})	**4.1**	0.1	**2.4**	0.6	**0.1**	0.2
4	2600	n-Hexacosane (n-C_{26})	**0.5**	tr	**0.4**	0.8	**tr**	-
5	2641	Unk.	**0.3**	tr	**0.2**	0.2	**0.5**	-
6	2700	n-Heptacosane (n-C_{27})	**4.1**	0.1	**3.4**	6.3	**-**	-
7	2704	Unk. polar	**1.5**	-	**1.1**	-	**5.9**	8.4
8	2800	n-Octacosane (n-C_{28})	**1.4**	-	**0.9**	1.2	**-**	-
9	2805	Unk. polar	**0.7**	-	**0.5**	-	**2.2**	2.7
10	2845	Unk.	**0.6**	0.1	**0.2**	0.1	**1.7**	-
11	2900	n-Nonacosane (n-C_{29})	**19.1**	10.0	**20.1**	19.5	**-**	-
12	2910	Heptacosan-2-ol	**18.2**	-	**14.4**	-	**57.1**	65.1
13	3000	n-Triacontane (n-C_{30})	**2.8**	1.5	**4.1**	5.0	**-**	-
14	3077	β-tocopherol	**4.7**	0.2	**2.0**	0.2	**19.5**	1.6
15	3100	n-Entriacontane (n-C_{31})	**30.2**	56.5	**37.7**	52.4	**-**	-
16	3155	α-tocopherol	**0.8**	-	**0.7**	-	**4.3**	tr
17	3200	n-Dotriacontane(n-C_{32})	**1.5**	4.1	**1.9**	2.7	**tr**	-
18	3300	n-Tritriacontane(n-C_{33})	**5.7**	26.4	**7.7**	10.7	**-**	-
19	3438	β-amyrin	**1.4**	-	**1.1**	-	**3.3**	11
20	3455	α-amyrone	**0.4**	0.7	**-**	-	**2.1**	
21	3484	α-amyrin	**0.8**	-	**0.7**	-	**1.7**	5.6
22	3572	Unk. polar	**0.7**	-	**0.3**	-	**1.4**	5.1

152 **Table S3.** Repeated-measures ANOVAs examining the attractiveness for *A.*
153 *decemarticulatus* of fractions of extracts from young *H. physophora* leaves.
154 Probabilities corrected for sphericity are provided using the Greenhouse–Geisser
155 correction (G-G). Significant results are indicated in bold.
156

	df	F	*p*-value	G-G
Evaporated *vs.* hexane				
Treatment	1	63.85	**<0.001**	
Error	38			
Time	19	0.55	0.941	0.869
Time x Treatment	19	0.77	0.744	0.669
Error	722			
Hexane fraction *vs.* hexane				
Treatment	1	0.22	0.623	
Error	38			
Time	19	1.56	0.061	0.113
Time x Treatment	19	0.95	0.513	0.485
Error	722			
Dichloromethane fraction *vs.* hexane				
Treatment	1	154.32	**<0.001**	
Error	38			
Time	19	0.79	0.725	0.659
Time x Treatment	19	0.70	0.819	0.744
Error	722			
Methanol fraction *vs.* hexane				
Treatment	1	0.96	0.334	
Error	38			
Time	19	1.33	0.153	0.209
Time x Treatment	19	0.58	0.923	0.834
Error	722			

157

158

159 **Table S4.** Repeated-measures ANOVAs examining the ability of synthetic
160 tocopherols and their mixture with extracts from old leaves (EOL) to attract
161 A. decemarticulatus. Probabilities corrected for sphericity are provided using the
162 Greenhouse–Geisser correction (G-G). In bold: significant results.
163

	df	F	*p*-value	G-G
α- and β-tocopherols mix *vs.* hexane				
Treatment	1	2.07	0.158	
Error	38			
Time	19	1.02	0.440	0.427
Time x Treatment	19	0.82	0.682	0.598
Error	722			
α- and β-tocopherols + EOL mix *vs.* hexane				
Treatment	1	43.14	**<0.001**	
Error	38			
Time	19	1.68	**0.035**	0.088
Time x Treatment	19	3.11	**<0.001**	**0.001**
Error	722			
α-tocopherol + EOL mix *vs.* hexane				
Treatment	1	7.07	**0.011**	
Error	38			
Time	19	2.12	**0.004**	**0.041**
Time x Treatment	19	0.67	0.847	0.696
Error	722			
β-tocopherol + EOL mix *vs.* hexane				
Treatment	1	2.48	0.124	
Error	38			
Time	19	4.89	**<0.001**	**<0.001**
Time x Treatment	19	0.60	0.911	0.757
Error	722			

164
165
166 **Nutrient transfer via ^{15}N labeling**

167 Methods

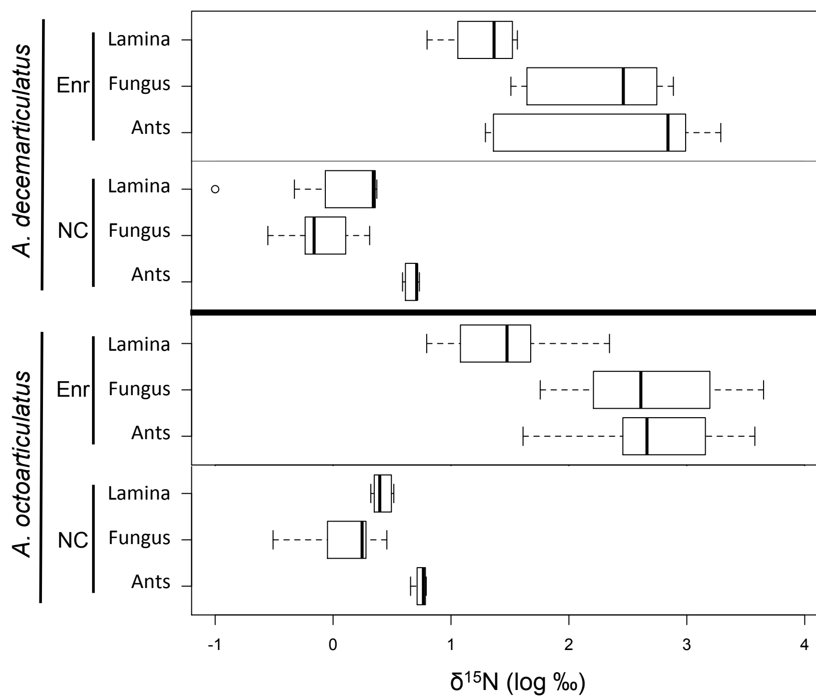
168 We investigated the role of the two mutualistic ant species in myrmecotrophy by
169 providing the ants with food artificially enriched in ^{15}N. This experiment was
170 performed on neighboring plant individuals inhabited by either *A. decemarticulatus* or
171 *A. octoarticulatus* from the MdS population to avoid site-related variations in isotopic
172 signatures. Isotopic labeling was performed using agar discs (ca. 5 mm diameter;
173 mean weight ± SE: 111 ± 17 mg) containing 2% of enriched glycine (99 atom%
174 $^{15}NH_2CH_2COOH$), 3% of non-enriched peptone, 15% of non-enriched fructose and
175 4% of agar. The agar discs were placed in plastic cups wired to the base of the plant,
176 just besides the first basal branching. No direct contact occurred between the ^{15}N-
177 enriched food and the plant or the galleries. The plastic cups were sealed with tapped
178 caps to keep out food-robbing insects and a small hole facing the host plant stem was
179 made to provide access to the ants. The plastic cups were placed 10 days prior to the

180 beginning of experiment to allow the ants to adapt to the setup. The ants were fed
181 with ^{15}N-enriched food each day for 9 consecutive days. Before re-supplying each
182 colony with a new, predefined quantity of agar discs, we removed any remaining food
183 and weighed it in order to determine the total food consumption by the ant colonies.
184 Ant, plant and fungal samples were collected one month after the end of the
185 experiment. The youngest leaf was harvested from each plant and a 4-cm^2 piece of the
186 lamina was dissected although the main nerve was spared. The ants sheltering in the
187 domatia were also collected. Fungal samples were not collected directly from the
188 galleries to avoid any bias as the latter are not constituted of pure fungus and also
189 because the two ant species do not build similar galleries in terms of composition and
190 structure. Pure fungal samples were collected as described in Ruiz-Gonzàlez *et al.*
191 [12]. Briefly, sampling consisted of making a cut of ca. 5mm^2 in the domatia walls of
192 the six youngest, mature leaves and waiting for the ants to fill it with pure fungus.
193 Cuts were made 2 days after the end of the enrichment procedure.
194 To quantify the %N, %C, $\delta^{15}N$ and $\delta^{13}C$ contents of the three partners, all of the
195 samples collected were vacuum-dried and around 5 mg of each were analyzed. Stable
196 isotope analyses were conducted at the Colorado Plateau Stable Isotope Laboratory
197 (Northern Arizona University, USA) using a Thermo-Finnigan Delta^{Plus} Advantage
198 gas isotope-ratio mass spectrophotometer (ThermoFisher Scientific) interfaced with a
199 Costech Analytical ECS4010 elemental analyzer.
200 $\delta^{15}N$ and $\delta^{13}C$ were calculated as follows:

$$\delta X = \left(R_{sample}/R_{standard} - 1\right) \times 1.000$$

201
202 where X is the element of interest, and Rsample and Rstandard are the molecular
203 ratios (i.e., $^{13}C/^{12}C$ or $^{15}N/^{14}N$) of the sample and the standard, respectively [13]. The
204 carbon and nitrogen contents (%N and %C) were quantified in the negative controls
205 for the three partners by calculating the ratio between the mass of carbon or nitrogen
206 atoms and the total mass of the dried sample.
207
208 Results
209 The provisioning of the ants with ^{15}N-enriched food resulted in a significant
210 increase in $\delta^{15}N$ for all three associated partners compared to the negative controls
211 (Fig. S2). The ^{15}N enrichments of the ants and the fungus were of the same magnitude
212 (*A. decemarticulatus vs.* fungus: MW, $U = 37$, $p = 0.645$; *A. octoarticulatus vs.*

213 fungus: MW, $U = 26$, $p = 0.901$). On the contrary, the ^{15}N enrichments of the plants
214 were significantly lower compared to both the ants and the fungus
215 (*A. decemarticulatus*: ant *vs*. plant: MW, $U = 52$, $p < 0.05$, fungus *vs*. plant: MW, $U =$
216 61, $p < 0.01$; *A. octoarticulatus*: ants *vs*. plant: MW, $U = 46$, $p < 0.05$, fungus *vs*.
217 plant: $U = 46$, $p < 0.05$). The comparison of the two systems also showed no
218 significant differences in ^{15}N translocation between the ants, the fungus and the
219 plants, demonstrating a similar functioning in the two associations
220 (*A. decemarticulatus vs. A. octoarticulatus*: MW, $U = 23$, $p = 0.612$;
221 *A. decemarticulatus- vs. A. octoarticulatus*-associated fungus: MW, $U = 16$ $p = 0.189$;
222 *A. decemarticulatus- vs. A. octoarticulatus*-associated plants: MW, $U = 24$, $p =$
223 0.694).



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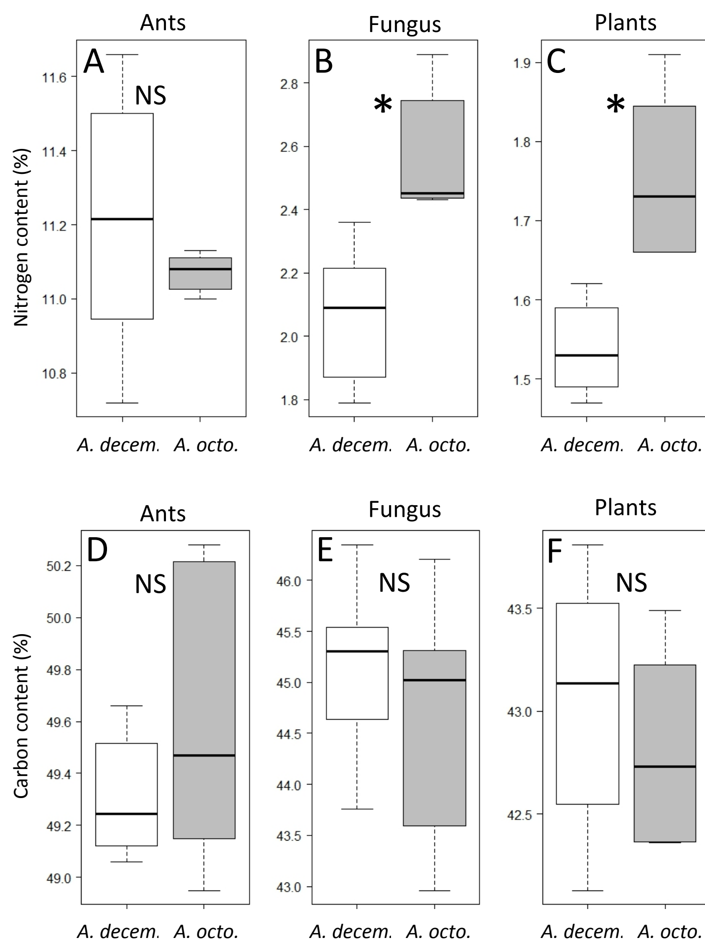
225
226 **Figure S2.** Effect of experimental ^{15}N provisioning to the ants on the three partners in
227 the two model systems. $\delta^{15}N$ (log $^{0}/_{00}$) values of the three partners are shown for the
228 two treatments: Negative control (NC) or ant ^{15}N-enriched food (Enr). The two
229 species of ants, *Allomerus decemarticulatus* and *A. octoarticulatus*, were provisioned
230 during 9 consecutive days with agar discs containing 2% of 99 atom% ^{15}N-glycine.
231 Box and whisker plots represent, top to bottom, the 90th percentile, 75th percentile,
232 median, 25th percentile, and 10th percentile and open circles indicate outliers.

233



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235 **Figure S3.** Nitrogen (**A-C**) and Carbon (**D-F**) contents of the three associated
236 partners. **A, D** are the comparisons between the two ant species,
237 *Allomerus decemarticulatus* and *A. octoarticulatus*; **B**, **E** are for the ant-associated
238 fungus and **C**, **D** for *Hirtella physophora.* Box and whisker plots represent, top to
239 bottom, the 90th percentile, 75th percentile, median, 25th percentile, and 10th percentile
240 and open circles indicate outliers. 'NS' or asterisks indicate the absence or presence of
241 a significant difference between species, respectively.
242

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