

1 **Ecological specificity of the metagenome in a set of lower termite**
2 **species supports contribution of the microbiome to adaptation of the**
3 **host**

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29
30 **Running title**

31 lower termite metagenomes

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51 **Abstract**

52 **Background**

53 Elucidating the interplay between hosts and their microbiomes in ecological adaptation has
54 become a central theme in evolutionary biology. A textbook example of microbiome-
55 mediated adaptation is the adaptation of lower termites to a wood-based diet, as they
56 depend on their gut microbiome to digest wood. Lower termites have further adapted to
57 different life types. Termites of the wood-dwelling life type never leave their nests and feed
58 on a uniform diet. Termites of the foraging life type forage for food outside the nest and
59 have access to other nutrients. Here we sought to investigate whether the microbiome that
60 is involved in food substrate breakdown and nutrient acquisition might contribute to
61 adaptation to these ecological differences. We reasoned that this should leave ecological
62 imprints on the microbiome.

63

64 **Results**

65 We investigated the protist and bacterial microbiomes of a total of 29 replicate colonies
66 from five termite species, covering both life types, using metagenomic shotgun
67 sequencing. The microbiome of wood-dwelling species with a uniform wood diet was
68 enriched for genes involved in lignocellulose degradation. Furthermore, metagenomic
69 patterns suggest that the microbiome of wood-dwelling species relied primarily on direct
70 fixation of atmospheric nitrogen, while the microbiome of foraging species entailed the
71 necessary pathways to utilize nitrogen in the form of nitrate for example from soil.

72

73 **Conclusion**

74 Our findings are consistent with the notion that the microbiome of wood-dwelling species
75 bears an imprint of its specialization on degrading a uniform wood diet, while the
76 microbiome of the foraging species might reflect its adaption to access growth limiting

77 nutrients from more diverse sources. This supports the idea that specific subsets of
78 functions encoded by the microbiome can contribute to host adaptation.

79

80 **Keywords**

81 lower termites, metagenomics, ecology, adaptation

82

83 **Background**

84 The importance of microbes for the evolution of higher organisms is starting to be realized
85 [1, 2]. Metazoan evolution is not only driven by pathogenic microbes, as reflected by fast
86 evolution of immune genes [3]. Rather, microbes often are facilitators of metabolic and
87 environmental adaptations [2, 4, 5]. For instance, the gut microbial communities of wood-
88 feeding roaches and termites facilitate thriving on a wood diet that is difficult to digest and
89 poor in nitrogen. Nitrogen fixation and the digestion of wood depend on the termite gut
90 microbiome [2, 6, 7]. In lower termites, lignocellulose degradation was initially mainly
91 attributed to unicellular eukaryotes (protists) in the gut [8]. Recently, it has become evident
92 that lignocellulose degradation is a synergistic effort of the termite, its associated protists,
93 and bacteria [9–11]. In addition to their role in lignocellulose degradation, bacteria are also
94 essential for the assimilation of nitrogen taken up from the environment. Nitrogen can be
95 acquired from the environment either via fixation from the atmosphere [12, 13], or via
96 nitrate reduction [14]. Also, nitrogen can be recycled from the metabolic waste product uric
97 acid [15, 16]. By using genome sequencing and pathway reconstruction, these processes
98 have been assigned to four major bacterial phyla in the termite gut: Proteobacteria
99 (*Desulfovibrio* [17]), Spirochetes (*Treponema* [18, 19]), Bacteroidetes (*Azobacteroides*)
100 [16], and Elusimicrobia (*Endomicrobium* [20, 21]).

101 Many bacteria in the termite gut live in tight association with protists, where they sit
102 on the surface [22, 23], in invaginations of the cell membrane [17], or even inside the
103 protist cells [24]. Such tight associations lead to frequent vertical transmission of bacteria

104 between protist generations. In return, protists and bacteria are vertically transmitted
105 between termite generations via proctodeal trophallaxis during colony foundation [25].
106 Vertical transmission has led to co-speciation between bacteria and their protist hosts, and
107 sometimes even the termite hosts [26–29]. Evidence for horizontal transfer of protists
108 between termite species, so called transfaunations, is limited to a few exceptions [30].
109 Hence, the termite host species association is rather strict, leading to strong phylogenetic
110 imprints on protist community structure [31–33]. In comparison, the bacterial microbiome is
111 more flexible, frequently transferred between termite host species [34], and affected by
112 diet [33, 35–41].

113 There is evidence that the gut microbiome of termites has contributed to the
114 adaptation of different termite species to their specific ecologies [33, 36, 42–44]. There are
115 pronounced ecological differences between the so-called termite life types [45, 46]. Termite
116 species of the wood-dwelling life type never leave their nest except for the mating flight.
117 They feed on a relatively uniform bonanza resource, that is the piece of wood they built
118 their nest in [47, 48]. On the other hand, foraging species leave their nest to forage for
119 food and have access to additional nutrients [47, 49]. This likely imposes different selection
120 pressures on the termite holobiont, in particular with regard to nutrient uptake. Because
121 the microbiome is directly involved in nutrient uptake, it seems reasonable to hypothesize
122 that it may also play a role in adaptation to life type related ecological differences. In this
123 scenario, one would expect the life types to leave an imprint on microbiome structure and
124 function. As such, searching for microbial imprints of a given life type can possibly provide
125 us with a lead for microbiome-mediated adaptation.

126 One potential pitfall of such an endeavor is that microbiomes may bear imprints
127 from transient microbes that were ingested from the environment. Transient microbes
128 rarely form evolutionary relevant relationships with the host [50, 51]. Instead, they reflect
129 short-term associations with microbes from the local environment the termites were

130 collected from. When the local environment correlates with other ecological differences,
131 these imprints could be falsely interpreted as potentially adaptive changes of the
132 microbiome. Therefore, it is essential to reduce the impact of these microbes on the
133 analysis. In order to explore potential ecological imprints on the microbiome, we focused
134 on an evolutionary switch between wood-dwelling and foraging life types in the
135 Rhinotermitidae (Figure 1). *Reticulitermes* species are of the foraging life type, while
136 *Prorethitermes simplex* is wood-dwelling. If the microbiome was affected by life type
137 specific ecology, we would expect that the microbiome of *Prorethitermes simplex* was
138 similar to that of the other wood-dwelling species (*Cryptotermes*) although these are from
139 a different family (Kalotermitidae). At the same time, the microbiome of the foraging
140 *Reticulitermes* species should bear distinct features. Alternatively, if there was no
141 ecological imprint, we would expect the microbiome to follow a phylogenetic pattern, with
142 the Rhinotermitidae *Prorethitermes* and *Reticulitermes* forming a cluster and the
143 *Cryptotermes* species (Kalotermitidae) forming a second cluster. Using this experimental
144 setup, we recently showed that protist community composition aligned with phylogeny, but
145 bacterial communities aligned more strongly with wood-dwelling and foraging life types
146 [33].

147 To further explore this, we investigated whether changes in microbiome composition
148 are also reflected by changes in microbiome function, as would be expected if the
149 microbiome played a role in adaptation. For instance, we would expect dietary adaptations
150 to be reflected by changes to pathways involved in substrate breakdown and effective
151 provisioning of limiting nutrients such as nitrogen. In order to test whether and which
152 changes in the functional repertoire align with life type and could be involved in potential
153 adaptation to different ecologies, we characterized the metagenome of two foraging
154 species; *Reticulitermes flavipes* and *Reticulitermes grassei*. We compared their functional
155 repertoire to that of three wood-dwelling species *Prorethitermes simplex*, *Cryptotermes*

156 *secundus*, and *Cryptotermes domesticus*. Because there can be substantial variation in
157 microbial communities between colonies [52–55], we analyzed five *C. domesticus*, eight
158 *C. secundus*, seven *P. simplex*, five *R. flavipes*, and four *R. grassei* replicate colonies. We
159 focused on the persistent, long-term differences between microbiomes by controlling short-
160 term effects caused by the influx of transient microbes. This was achieved by feeding a
161 common diet of sterile *Pinus* wood for several weeks prior to sample collection.

162

163 **Results**

164 We analyzed a total of ~440 million metagenomic shotgun sequences. Between 974,176
165 and 8,949,734 sequences per sample were of microbial origin (Table S1). Sequences were
166 subsampled (rarefied) to 1,386,882 bacterial and 2,781 protist annotated sequences per
167 sample. For annotation, sequences were aligned to a reference database of clusters of
168 orthologous groups of genes (COGs) with known function. These COGs represent the
169 lowest level of the eggNOG hierarchical annotation. At the next higher level, the COGs are
170 grouped into pathways (Figure S1, Figure S9), and at the third and highest level, the
171 pathways are grouped into three categories; “information storage and processing”, “cellular
172 process and signaling”, and “metabolism”. We adhere to this definition of the eggNOG
173 hierarchical terms throughout the study.

174

175 “information storage and processing” differentiates the protist metagenomes of wood-
176 dwelling and foraging lower termite species

177 In our previous study [33] on the identical samples, the protist communities of the
178 Rhinotermitidae *Prorhinotermes* and *Reticulitermes* clustered together, supporting a
179 phylogenetic imprint on community composition. Here, we tested whether this pattern was
180 also reflected by the functions encoded by the protist metagenome. Therefore, we
181 annotated metagenome encoded functions in the shot gun sequences and compared the

182 functional metagenome profiles across host species, using Bray-Curtis-Dissimilarity [56].
183 This index considers abundance of functional categories, thus avoiding arbitrary coverage
184 cutoffs.

185 The protist functional repertoire clustered according to host family and genus
186 (Figure 2A), thus showing a dominant phylogenetic imprint. Family wise clustering was
187 supported by Redundancy Analysis (RDA): the model including host family explained more
188 variance in the functional repertoire and produced lower AICs than the model based on life
189 type (Table 1). For a more detailed view, we analyzed the three categories at the highest
190 level in the eggNOG hierarchical annotation (Figure S1) separately. Cluster analysis of the
191 categories “cellular process and signaling” and “metabolism” supported the notion that
192 phylogenetic relatedness is an important factor for functional similarity (Supplement
193 Figures S4B and D). In contrast, the portion of the metagenome assigned to “information
194 storage and processing” (Figure 2B) clustered primarily by life type. The stronger effect of
195 life type than phylogeny on this functional category was also supported by higher
196 explanatory power and lower AICs in RDA (Table 1).

197 Identification of the functions that differentiate the protist metagenomes of the
198 wood-dwelling and foraging species can hold clues with regard to the nature of potentially
199 adaptive phenotypes in the protist metagenome. In order to do so, we performed a linear
200 discriminant analysis (LEfSe: [57]). This analysis identified 22 over-represented COGs in
201 foraging and 14 in wood-dwelling species (Figure 3A, Table S3, $p < 0.05$, $q < 0.05$, LDA
202 score > 2 , Figure S6).

203 The pathway “replication, recombination, and repair” was over-represented in the
204 foraging species (Figure 3A, Table S3, $p = 0.0001$, $q = 0.002$). The over-represented
205 COGs in this pathway included a DNA dependent DNA-polymerase (COG0470) and five
206 helicases (COG0514, COG0553, COG1199, COG1204, ENOG410XNUT, see Figure 3B
207 for grouped analysis and Table S3 for individual COG p- and q-values). In wood-dwelling

208 species, the pathway “transcription” was over-represented ($p = 0.0004$, $q = 0.003$). The
209 over-represented COGs in this pathway contained DNA binding domains and were
210 supposedly involved in transcriptional regulation (COG5147, ENOG4111SAB).

211

212 The bacterial metabolic metagenome aligns with host ecology

213 In our previous study [33], the bacterial community composition of termite hosts clustered
214 primarily by life type, which is consistent with ecology related differences between
215 microbiomes. Following the rationale above, we tested whether this pattern was also
216 reflected by the functions encoded by the metagenome.

217 Against the expectation from our previous study, the functional bacterial profiles
218 showed no life type, but a phylogenetic imprint, which is in line with the protist functional
219 profiles. Most samples clustered according to host family (Figure 2C). Analyzing the three
220 high-level eggNOG functional categories separately provided more detailed insight. The
221 categories “cellular process and signaling” and “information storage and processing”
222 supported the notion of strong phylogenetic effects on metagenome function (Supplement
223 Figures S5B and C). In contrast, the metabolic metagenomes (Figure 2D) clustered
224 primarily according to host life type. Host life type was also a better predictor for metabolic
225 functions than host family in RDA (Table 1).

226 Aside from these general patterns, several samples stood out. Samples Rg2 and
227 Rg4 of *R. grassei* were on long branches in the dendrograms (Figure 2 and Supplement
228 Figure S5), suggesting unusual functional profiles. Notably, these samples already stood
229 out in our previous study [33] because of their unusual abundance of microbial taxa
230 potentially due to infection with pathogens. This unusual composition was confirmed by
231 taxonomic annotation in this study (see Supplement Figure S3). Sample Cs7 (*C.*
232 *secundus*) also clustered separately from the other samples. This was mainly driven by
233 abundant transposases in this sample (53.1% of sequences) (for example COG1662,

234 COG3385, or ENOG410XT1T, see Table S2), accompanied by an increase in the
235 frequency of *Bacteroides* (Figure S3) that are rich in conjugative transposons [58, 59]. We
236 performed all analyses with and without these samples and found no qualitative
237 differences (data not shown).

238 Bacterial metabolic functions that differentiated wood-dwelling from foraging
239 species were identified using linear discriminant analysis (LEfSe). 105 metabolic COGs
240 were over-represented in the wood-dwelling species, while 151 were over-represented in
241 the foraging species (Table S4, $p < 0.05$, $q < 0.05$, LDA score > 2 , Figure S7). All COGs
242 described as over-represented or enriched in the following were subject to these p-value,
243 q-value and LDA cutoffs. Because of their specialized diet, genes involved in nitrogen
244 metabolism and lignocellulose break down like glycoside hydrolases (GH) are of particular
245 interest, when focusing on metabolic differences among gut microbiomes in wood-feeding
246 termites with different ecologies. In fact, among the genes involved in 'carbohydrate
247 transport and metabolism' that were enriched in the microbiome of wood-dwelling termites,
248 GHs were over-represented (43.3% of enriched genes versus 12% expected, exact
249 binomial test: $p = 2.124e-05$, Table S4, S5). In the foraging termite species, only one gene
250 with putative lignocellulolytic activity was over-represented (COG3858), suggesting that
251 the wood-dwelling species have a higher potential for complex carbohydrate degradation.
252 To further investigate differences in GH abundance between the microbiomes of wood-
253 dwelling and foraging species, we performed a detailed pathway analysis using the CAZy
254 database ([60], Figure 4). All GHs acting in hemicellulose break-down were more abundant
255 in the wood-dwelling species (Figure 4B). Among the cellulolytic enzymes, β -glucosidases
256 were significantly more abundant in the wood-dwelling species. The other two enzymes
257 involved (cellulase (endo- β -1.4-glucanase), cellobiohydrolase) showed a trend into the
258 same direction. All of the genes with cellulolytic or hemicellulolytic activity were affiliated
259 with Bacteroidetes (mostly members of the genus *Bacteroides*) or the genus *Treponema*.

260 Additional support for the increased importance of hemicellulose utilization in the wood-
261 dwelling species, was provided by the over-representation of twelve COGs annotated as
262 TonB-dependent receptors (ENOG410XNNV, ENOG410XNPQ or COG4206, see Table
263 S4). Apart from other substrates, these receptors are important for the uptake of plant-
264 derived hemicellulose [61, 62]. All functions annotated as TonB-dependent receptors (or
265 TonB-dependent associated receptor plugs) were affiliated with the genus *Bacteroides*
266 (see Table S4).

267 Because wood is poor in nitrogen, termites depend on an efficient system for
268 conserving and upgrading nitrogen [6]. In the wood-dwelling species, a potential
269 nitrogenase ((*nifH*) COG1348) was over-represented (Figure 4C, Table S4). Nitrogenases
270 are key enzymes in the fixation of atmospheric nitrogen and downstream ammonia
271 synthesis. Nitrogenase activity was mainly affiliated with members of the genus
272 *Treponema* (Figure 4C). In contrast, in the foraging species, COGs involved in
273 dissimilatory nitrate reduction (COG1251, COG5013, COG2181, COG0243, Figure 4C,
274 Table S4) were over-represented. They were affiliated with a variety of different genera
275 ranging from *Desulfovibrio* and *Gordonibacter* to *Stenoxybacter*, *Enterobacter* and
276 *Serratia*. *Serratia* and *Enterobacter* are potential insect pathogens and contributed to the
277 prevalence of one of the three nitrate reductases, narG (COG5013). Closer inspection of
278 the source of these bacteria revealed that they mainly stemmed from the abnormal
279 samples Rg2 and Rg4 that we suspected to carry a potential pathogenic infection. When
280 we remove these samples from the analysis the increase of narG in foragers remains
281 significant ($p = 0.034$).

282 For living on a nitrogen poor substrate, it can also be adaptive to effectively recycle
283 nitrogen from the main waste product of the host's amino acid metabolism, uric acid. Uric
284 acid can be recycled through anaerobic ammonia production and downstream glutamate
285 synthesis [6, 15, 20, 63]. In the wood-dwelling species a putative glutamate

286 dehydrogenase (COG0334), involved in glutamate synthesis by ammonia assimilation,
287 was over-represented. This glutamate dehydrogenase gene was mainly affiliated with
288 members of the genera *Bacteroides*, *Treponema* and *Desulfovibrio*. In the foraging
289 species, COGs with putative glutamine (COG0174) and glutamate synthase (COG0067,
290 COG0069) function, were enriched (Figure 4D). These COGs were affiliated with
291 *Desulfovibrio*, *Treponema*, *Pseudomonas* and *Acetobacterium*.

292

293 **Discussion**

294 In this study, we assessed functional differences of termite metagenomes that underwent
295 an evolutionary switch from wood-dwelling to foraging to identify putative contributions of
296 the microbiome to ecological niche adaptation. To do this, we chose a set of five termite
297 species (two foraging, three wood-dwelling species) and determined whether the
298 functional profiles of the termite gut microbiome followed phylogeny of the host or aligned
299 with host ecology. We hypothesized that alignment of microbiome function with termite life
300 type is consistent with a contribution of the microbiome to termite holobiont adaptation to
301 different ecologies. By comparing the functional content of microbiomes of different host
302 species we focused on long-term evolutionary processes.

303 A potential pitfall of such an approach is that an alignment of the termite microbiome
304 with life type-related ecology could also be caused by short-term differences between
305 microbiomes that are merely transient. For example, microbes in the environment might
306 differ between collection sites for the different host species. Further, ingestion of
307 environmental microbes might lead to an association between microbiome and ecology.
308 Similarly, differences in local food supply can lead to transient, short-term effects on the
309 termite microbiome [55]. Consequently, such short-term differences reflect environmental
310 differences at termite collection sites, rather than potentially adaptive, evolved differences
311 between host-species-specific microbiomes.

312 For this reason, we chose to follow an approach where we control for environmental
313 and dietary differences by acclimating all termites on the same (sterile) food source and to
314 identical environmental conditions. We consider metagenomic patterns that persist under
315 such highly controlled experimental conditions as robust and indicative of long-term,
316 evolutionary acquired differences, rather than short-term imprints originating from
317 differences in the environment or food source. It should be noted that the experimental
318 setup poses a restriction to the number of sampled host species [33].

319
320 Increased potential for replication in the protists of foraging termite species

321 In the protist metagenome of foraging species, genes involved in replication were more
322 abundant. High replication rates are expected to be more frequently under positive
323 selection during recolonization of the gut with protists, when the gut environment has not
324 yet reached carrying capacity [64]. Therefore, we would like to speculate that this
325 difference is related to the fact that *Reticulitermes* guts have to be recolonized more
326 frequently because they molt more frequently; the intermolt periods in *Reticulitermes* are
327 about two weeks long [49], while they average almost two months in *Cryptotermes* [48].
328 During molting the protists are lost and the guts have to be recolonized through proctodeal
329 trophallaxis from nest mates [65]. However, we are aware that differences in the relative
330 abundance of housekeeping genes like those required for replication between protist
331 microbiomes can not be clearly disentangled from differences in average protist genome
332 size and therefore should be interpreted with caution.

333
334 Enrichment of genes for lignocellulose degradation in the microbiome of wood-dwelling
335 termite species

336 While genes involved in replication differentiated the protist metagenomes of wood-
337 dwelling and foraging species in our study, metabolic genes differentiated the bacterial

338 metagenomes. Consistent with differences in their respective diets, the metagenomes of
339 foraging and wood-dwelling species in our study differed by their potential for cellulose and
340 hemicellulose utilization. Several GHs that have cellulolytic and hemicellulolytic function
341 were over-represented in the metagenomes of wood-dwelling species (GH families 2, 3,
342 16, 43, mannosidases, xylosidases, glucanases, xylanases, Figure 4B, Table S4). A more
343 detailed pathway analysis confirmed that hemicellulases are more abundant in the wood-
344 dwelling species. This suggests a more pronounced role for lignocellulose degradation in
345 the metabolism of the wood-dwelling species in our study. Accordingly, TonB dependent
346 transporters were enriched in the microbiome of wood-dwellers. These transporters can
347 shuttle hemicellulose and its building-blocks, in particular xylans and xylose through
348 bacterial membranes [66, 67]. A large fraction of cellulases, hemicellulases, and putative
349 TonB transporters were attributed to the genus *Bacteroides*. In *Bacteroides*, TonB
350 dependent transporters are often co-localized and co-regulated with enzymes for
351 polysaccharide degradation like hemicellulases [59, 68]. This suggests a partnership of
352 enzymes and transporters in polysaccharide degradation. *Bacteroides* species from the
353 human gut are also hemicellulose degraders [69], suggesting a distinctive role for the
354 genus in hemicellulose degradation in termites as well.

355 The above-identified differences in functional potential between the wood-
356 dwelling and foraging species in our study are suggestive of adaptations to utilize diets
357 that differ in hemicellulose content. Hemicellulose content differs between wood species
358 [70, 71]. The wood-dwelling *Cryptotermes* species in our study are mostly found in
359 hardwood mangroves [72] where they can thrive on a bonanza food resource. The other
360 wood-dwelling genus in our study, *Prorhinotermes*, lives in similar coastal habitats with a
361 similar arboreal flora [73]. Hardwood is richer in hemicelluloses and the potential to use
362 hemicelluloses is larger in the microbiome of species living on hardwood. On the other
363 hand, *Reticulitermes* species originated in inland habitats [74], prefer soft woods like pine

364 [75, 76] with lower hemicellulose levels, and accordingly, hemicellulolytic pathways are
365 depleted.

366

367 Termites with different life-types rely on different forms of nitrogen uptake and recycling

368 Nitrogen is scarce in a wood-based diet. As a consequence, termites need to acquire
369 additional nitrogen from the environment. The microbiome is essential for this process. In
370 the microbiome of wood-welling species, which feed on a uniform lignocellulose diet, a
371 potential nitrogenase gene was enriched (nifH, COG1348). Nitrogenases are the key
372 enzymes in the fixation of atmospheric nitrogen and downstream ammonia synthesis. This
373 nifH was mainly affiliated with treponemes that have been shown to play an important role
374 in nitrogen fixation before [12, 18, 19]. In contrast, the microbiome of the foraging species
375 in our study has a higher potential to provide nitrogen to the termite holobiont by
376 dissimilatory reduction of nitrate (Figure 4C). Nitrogen in the form of nitrate naturally
377 occurs in soil. *R. flavipes* has been shown to acquire micro nutrients from soil [77] and to
378 actively balance mineral uptake by food choice [78]. Therefore, it seems reasonable to
379 assume that the microbiome of *Reticulitermes* relies on nitrogen from soil in the form of
380 nitrate to balance the low nitrogen content of wood. The necessary nitrate reductases were
381 found primarily in *Desulfovibrio*, *Gordonibacter* and *Stenoxybacter* that were found in
382 association with *Reticulitermes* before and are shared between a wide range of termites
383 [33, 79, 80].

384 Aside from obtaining nitrogen from the environment (atmosphere, soil), bacteria can
385 also recycle uric acid nitrogen. All of these processes result in ammonia synthesis, the
386 central metabolite of nitrogen metabolism. Ammonia is then further assimilated to
387 glutamate. In the wood-dwelling species a glutamate dehydrogenase (COG0334) was
388 over-represented. It was mainly affiliated with members of the *Bacteroides*, *Desulfovibrio*
389 and treponemes. The foraging species seem to rely on another glutamate synthesis

390 pathway, including glutamine (COG0174) and glutamate synthases (COG0067,
391 COG0069). Accordingly, they were associated with a different set of bacteria including
392 *Pseudomonas*, *Acetobacterium*, *Desulfovibrio*, and treponemes (Figure 4D).

393

394 Phylogeny and ecology align with metagenome-encoded functions

395 Differences in the propensity for nitrogen uptake and recycling are likely to reflect
396 differences in diet of the termite host species. Given differences in diet between the
397 species that represent the different life types, it seems also reasonable to suggest that the
398 changes in the repertoire of hemicellulases reflects adaptations of the microbiome to diets
399 with different hemicellulose content. The finding that this manifested specifically in the
400 metabolic functional repertoire, may suggest that potential selection acts in particular on
401 metabolic functions.

402 Metabolic microbiome mediated adaptation to different diets can happen in two
403 ways. First, acquisition of new microbes with adaptive functions could lead to adaptive
404 changes of the microbiome. Second, genome evolution of microbes that are already
405 associated with the host could lead to adaptation. Microbes that were already present
406 before the onset of lineage specific adaptation are likely to be shared among host species.
407 By contrast, newly acquired microbes are expected to be host lineage specific. We found
408 that the bacterial groups that contributed most to the differentiation of metabolic functions
409 are shared among all five host species (*Treponema*, *Bacteroides*, *Desulfovibrio*,
410 *Dysgomonas*, *Gordonibacter*, *Pseudomonas*, Table S4, Figure S3). This supports that
411 genome evolution of microbes that were already associated with the host contributed to
412 potential adaptation in our model system.

413

414 **Conclusion**

415 We applied metagenomic sequencing of gut microbiomes from a controlled experimental
416 setup to assess a putative contribution of the microbiome to host ecological adaptation that
417 accompanies the evolutionary switch from wood dwelling to foraging life types. We found
418 that the overall pattern of microbiome variation reflected a phylogenetic signal.
419 Interestingly, however, specific functions of the microbiome aligned with the underlying
420 host ecology. The specific ecology related differences in microbiome function led us to
421 hypothesize that the microbiome contributed to dietary adaptations, namely different
422 hemicellulose and nitrogen contents. This hypothesis can now be tested, assessing host
423 fitness under different dietary conditions. Such experiments will be crucial to disentangle
424 adaptive functional changes from selectively neutral functional turnover or side effects of
425 other adaptations.

426

427 **Experimental Procedures**

428 Termite samples

429 All termites were collected from typical natural habitats (see [33]). They were kept under
430 constant conditions (27°C, 70% humidity) on autoclaved *Pinus radiata* wood from the
431 same source for at least six weeks prior to the experiment. The feeding of *Pinus*
432 represents a natural or near natural treatment; *Pinus* is a natural food source of *P. simplex*
433 and *Reticulitermes*. *Cryptotermes* growth and behavior on *Pinus* recapitulates that on
434 natural substrate [72]. The time of the acclimation period was chosen to lie well beyond the
435 gut passage time of 24 h in lower termites [81, 82] and following Huang et al. [83], who
436 showed that six weeks are sufficient for the microbiota to adjust to a new diet. That way, all
437 excretable material like remaining food, transient microbes taken up from the environment
438 that have no mechanisms to persist in the gut, and microbial DNA taken up before the
439 experiment was made sure to be excreted. The samples were identical to those analyzed

440 in our previous study, [33] where detailed information about animal collection, keeping, and
441 cytochrome oxidase II based species identification and a phylogeny can be found.

442

443 DNA extraction and Shotgun sequencing

444 DNA was extracted from a pool of three worker guts per colony using bead beating,
445 chloroform extraction and isopropanol precipitation (see Supplementary material and
446 methods file S7). Each of the 29 colony samples went through independent metagenomic
447 shotgun library preparation and sequencing on an Illumina HiSeq platform (150 bp paired
448 end reads).

449

450 Analysis

451 We employed a double filtering strategy to remove host DNA from our analysis. First,
452 sequences were removed that mapped to available host genomes from *C. secundus* [84]
453 and transcriptomes from *P. simplex* [85] and *R. flavipes*, provided by the 1KITE consortium
454 (www.1kite.org, BioSample SAMN04005235) using BMap [86] (for detailed workflow and
455 more detailed information about used genomes and transcriptomes see Supplementary
456 Figure S2 and file S8). Of note, the sequences were not assembled, but individual reads
457 were directly annotated. In a second step we used taxonomic and functional annotations
458 with Megan6 [87] to retrieve only sequences that could be unambiguously assigned to
459 either bacteria or protists. In order to compare the bacterial and protist data sets of all
460 samples, they were rarefied to the number of sequences in the sample with lowest
461 coverage, resulting in 1,386,882 and 2,781 sequences per sample, respectively. Sample
462 Cs4 was excluded from the analysis for insufficient sequence coverage (974,176
463 sequences), so was Cs5 from the protist data. Sample Ps5 did not pass the analysis
464 pipeline and was also excluded.

465 Functional annotation with the eggNOG database resulted in the highest number of
466 annotated sequences (21,215,480 annotated sequences in total) and was chosen for
467 further functional analysis. Bray-Curtis distances of functional abundances were clustered
468 with the pvClust package in R [88]. Multivariate modeling was performed via RDA
469 (Redundancy Analysis) and AICs as well as values for the proportion of variance explained
470 were derived with the model selection tool ordistep and ordiR2step, as implemented in the
471 R vegan package [89]. Models were compared to the null-model via ANOVA. To identify
472 over-represented functions associated with the two termite life types, a Linear Discriminant
473 Analysis (LDR) was performed using LEfSe [57] and visualized using graphlan [90].
474 Pathway analysis of CAZy GHs was performed by blasting bacterial reads of all samples
475 against the full CAZy protein database, using Diamond [91]. GH abundance was estimated
476 by counting reads with matches on proteins with cellulolytic and hemicellulolytic functions
477 [92]. Pathway analysis of the nitrogen metabolism was performed by searching COG IDs
478 corresponding to the KEGG IDs among the over-represented COGs from the LEfSe
479 analysis. A detailed workflow for full reproducibility can be found in Supplementary Figure
480 S2 and file S10 and S11.

481

482 **Declarations**

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498 *flavipes*.

499

500 Ethics approval and consent to participate

501 Not applicable.

502

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508 German Research Foundation (DFG) through grant no INST 37/935-1 FUGG.

509

510 Availability of data and material

511 The raw data has been uploaded to the ncbi short-read archive (BioProject ID
512 PRJNA509211, Accession: SAMN10573992 – SAMN10574019). Supporting information
513 and analysis workflows are included in the supplementary files in this article.

514

515 Author contributions

516 FS, JK, LW designed the experiment. JK, FD provided study organisms. LW performed the
517 experiments, CV generated the sequence data, LW and FS analyzed the data. LW, FS, JK,
518 CV, and FD wrote the manuscript. All authors read and approved the final manuscript.

519

520 Competing interests

521 The authors declare no competing interests.

522

523 Consent for publication

524 Not applicable.

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534 **Figures and Tables**

535

536 **Table 1: Models of the effects of life type and host family (phylogeny) on functional**

537 **community profiles.** Effects were analyzed with Redundancy analysis using the

538 functional abundance table as response variable. Host family was the best explanatory

539 variable for the combination of all protist functions, however life type explained more

540 variance (R^2) and produced a lower AIC for the category “information storage and

541 processing” than host family. For all bacterial functions taken together, host family again

542 explained a larger proportion of the variance, while life type was the best explanatory

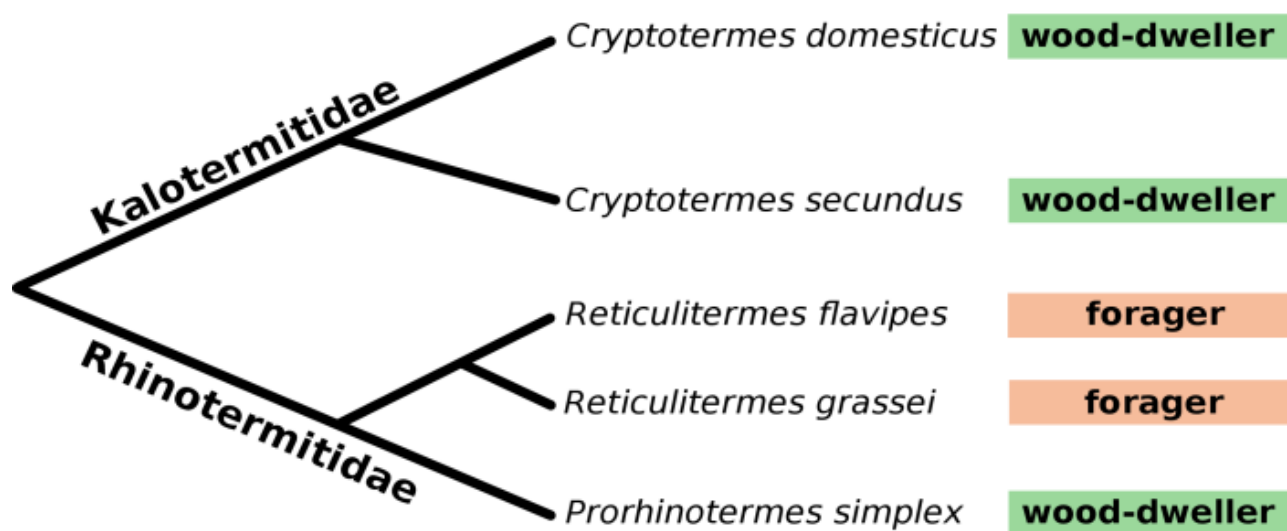
543 variable in the category “metabolism”.

		model	AIC	R²	p
protists	all functions	null	-41.8		
		host family	-49.0	0.27	0.001
		host life type	-45.4	0.16	0.001
	information storage and processing	null	-36.8		
		host life type	-39.0	0.11	0.001
		host family	-38.4	0.09	0.001
bacteria	all functions	null	-85.0		
		host family	-92.7	0.27	0.001
		host life type	-90.1	0.20	0.001
	metabolism	null	-92.4		
		host life type	-98.1	0.22	0.001
		host family	-96.8	0.18	0.001

545

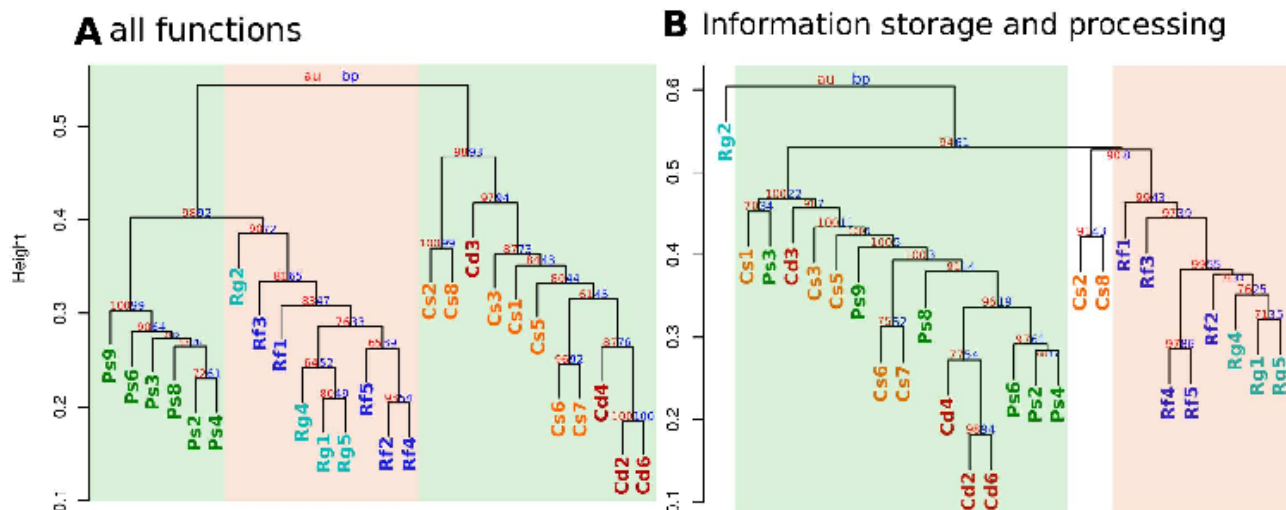
546

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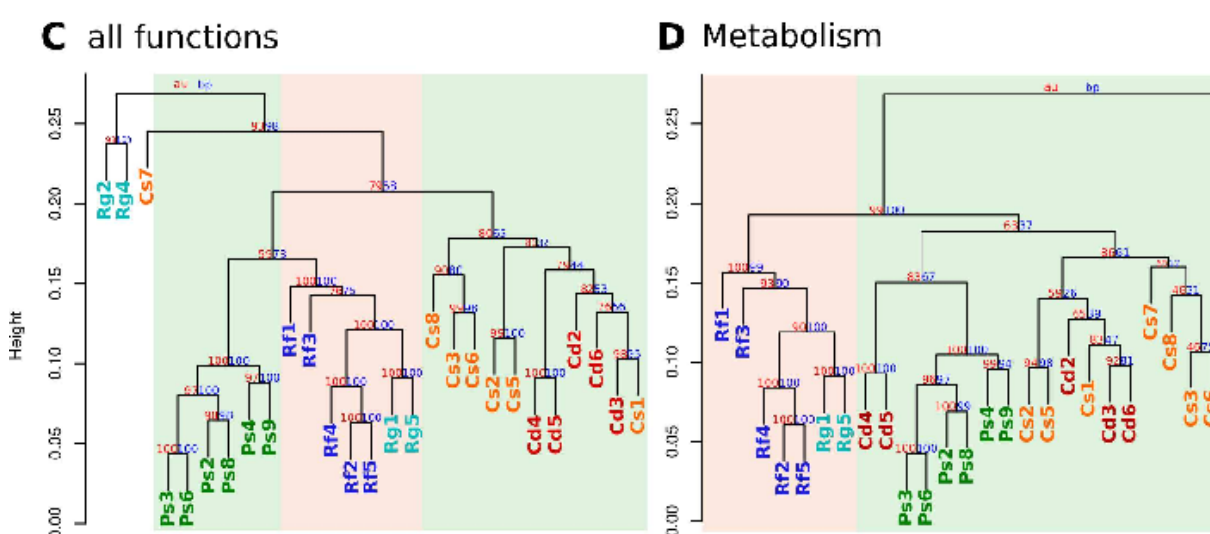


549 **Figure 1: Schematic phylogeny of the five lower termite species used in this study**
550 **from [33].** Branch length not drawn to scale. Colored boxes indicate the life type.
551

Protist functions



Bacterial functions



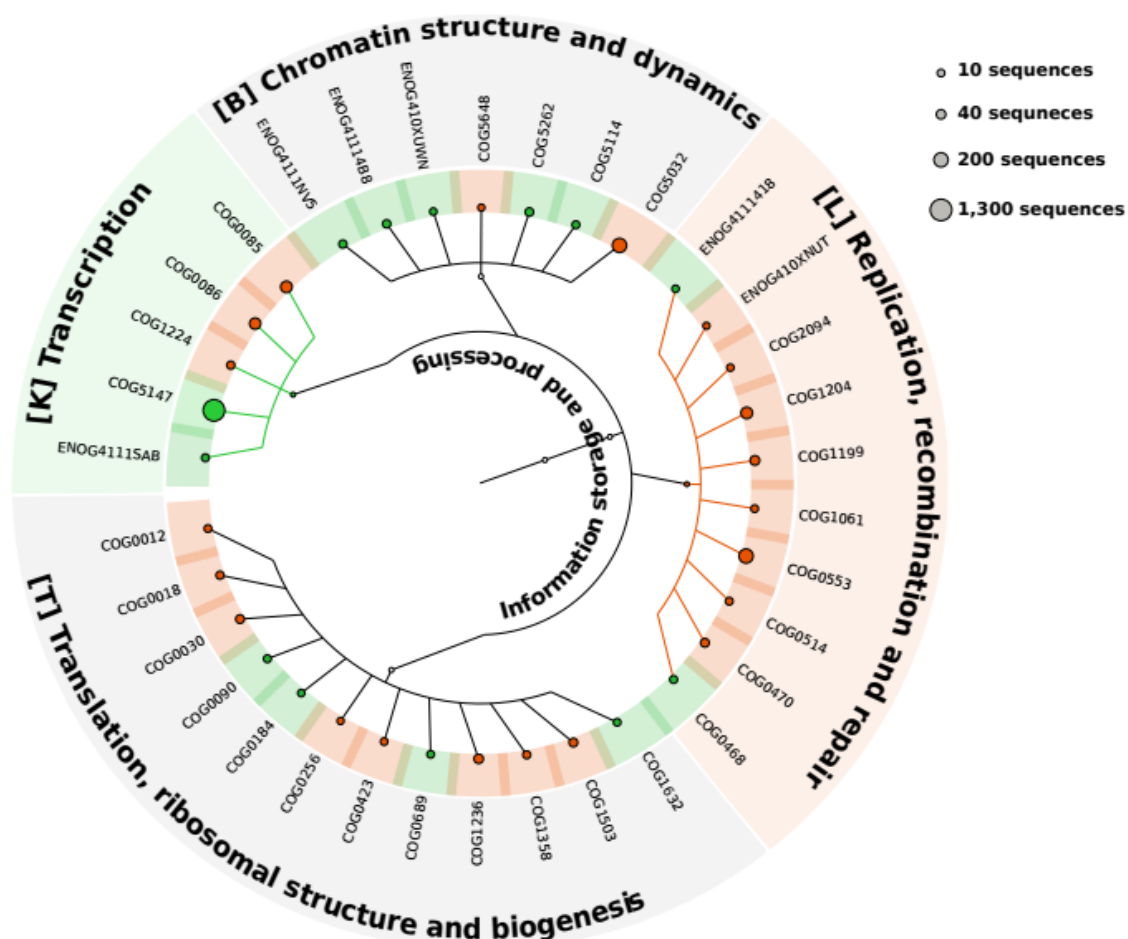
552 **Figure 2: Cluster dendrograms of the functional profiles of the protist and bacterial**
 553 **community.** Community distances are based on Bray-Curtis Dissimilarities of A) all
 554 functions of the protist community (25,795 sequences), B) The category “information
 555 storage and processing” of the protist community (4,527 sequences), C) all functions of the
 556 bacterial community (21,215,480 sequences), and D) the category “metabolism” of the
 557 bacterial community (10,586,058 sequences). Cd (red) = *C. domesticus* colonies; Cs
 558 (orange) = *C. secundus* colonies; Ps (green) = *P. simplex* colonies; Rf (blue) = *R. flavipes*
 559 colonies; Rg (lightblue) = *R. grassei* colonies. Green background = wood-dwelling life
 560 types; orange background = foraging life type. For protist functions involved in “information
 561 storage and processing” in the protist community, samples clustered according to life type.

562 Similarly, the bacterial metabolic metagenomes clustered according to life type. Cluster
 563 dendrograms of all functional categories of protist and bacterial communities can be found
 564 in Supplementary Figure S4 and S5.

565

566

A



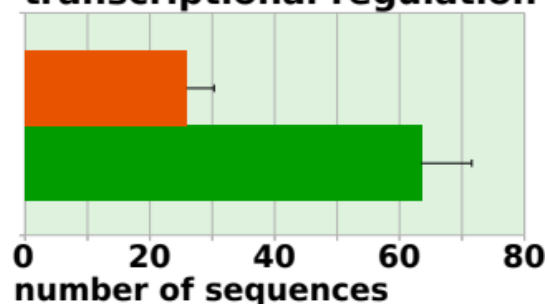
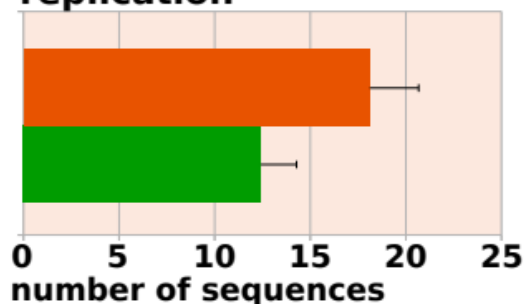
B

[L] Replication, recombination and repair

[K] Transcription

replication

transcriptional regulation



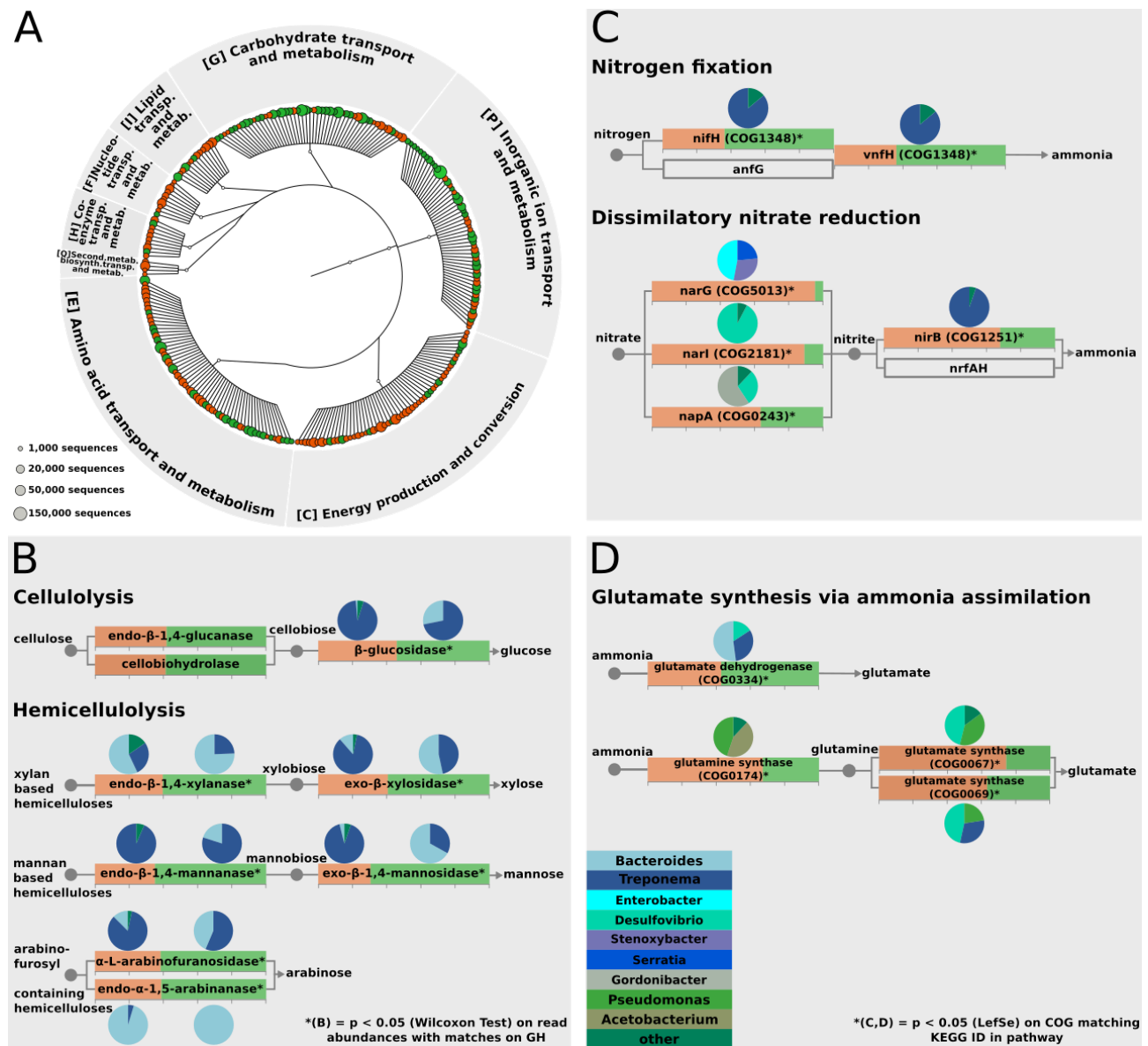
568 **Figure 3: Differences in the functional content of the protist metagenomes of wood-**
569 **dwelling and foraging species.** A) Circular dendrogram/hierarchy of all over-represented
570 COGs in the category “information storage and processing” in wood-dwelling species
571 (green) or foraging species (orange). Circle size at the edges scales with abundance of the
572 COG. Colored branches indicate over-represented pathways. Over-representation was
573 detected with LEfSe [57] ($p < 0.05$, $q < 0.05$, $LDA > 2$). A Venn diagram visualizing the total
574 number, and the differentially abundant number of functions in each of the five pathways
575 that constitute the category “information storage and processing” can be found in
576 Supplementary Figure S6. B) Sequence coverage of wood-dwelling (green) and foraging
577 (orange) species of examples of over-represented COGs mentioned in the text. Error bars
578 represent 95% confidence intervals across replicate colonies.

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583 **Figure 4: Differences in the functional content of the bacterial metagenomes of**
 584 **wood-dwelling and foraging species.** A) Circular dendrogram/hierarchy of all COGs in
 585 the category “metabolism” over-represented in wood-dwelling species (green) or foraging
 586 species (orange). Circle size at the leafs scales with the abundance of the COG. Over-
 587 representation was detected with LefSe [57] ($p < 0.05$, $q < 0.05$, $LDA > 2$). A Venn diagram
 588 visualizing the total number, and the differentially abundant number of functions in each of
 589 the five pathways that constitute the category “metabolism” can be found in Supplementary
 590 Figure S7. B) Pathway analysis of cellulose and hemicellulose degradation. Colored boxes
 591 of cellulolytic or hemicellulolytic genes indicate proportion of relative abundance of
 592 sequences affiliated with wood-dwelling (green) or foraging (orange) species. C) Pathway

593 analysis of nitrogen metabolism. Boxes for genes with functions in nitrogen metabolism
594 indicate relative abundance in the two life types. D) Pathway analysis of glutamate
595 synthesis. Boxes in C) and D) show relative abundance in the two life types of genes with
596 functions in nitrogen/glutamate metabolism. Pie charts show taxonomic association of the
597 gene. All hemicellulolytic genes were over-represented in the wood-dwelling species. Also,
598 a nitrogenase was enriched in the wood-dwelling species, while in the foraging species,
599 genes involved in dissimilatory nitrate reduction were over-represented.

600

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