

1 **Bacterial but not protist gut microbiota align with**
2 **ecological specialization in a set of lower termite species**

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17

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33 The authors declare no conflict of interest.

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56 **Abstract**

57 The role of microbes in adaptation of higher organisms to the environment is
58 becoming increasingly evident, but remains poorly understood. Protist and
59 bacterial microbes facilitate that lower termites thrive on wood and are directly
60 involved in substrate break down. During the course of evolution lower termites
61 adapted to different diets and lifestyles. In order to test whether there are
62 changes of the termite gut microbiota that co-occur and hence could be related
63 to diet and lifestyle adaptation, we assessed the bacterial and protist
64 communities in a multispecies framework profiling three wood-dwelling and two
65 foraging lower termite species using 16S and 18S rRNA gene amplicon
66 sequencing. Termites were kept under controlled conditions on the same diet to
67 minimize environmental effects on their gut microbiota. We found that protist
68 communities group according to host phylogeny while bacterial communities
69 group according to lifestyle. The change from the ancestral wood-dwelling to a
70 foraging lifestyle coincides with exposure to more diverse and higher
71 concentrations of pathogens as well as a more diverse diet. Accordingly, we
72 identified bacteria that are associated with foraging termites of the genus
73 *Reticulitermes* and could function as probiotics or be metabolically important on
74 a more diverse diet. Furthermore, protist and bacterial diversity are correlated,
75 suggesting not only that many termite gut bacteria are associated with protists,
76 but also suggesting a role of protist diversity in the evolution of bacterial diversity
77 in the termite gut or vice versa.

78

79 **Introduction**

80 The importance of microbes in the evolution of higher organisms is starting to be
81 realized (Feldhaar, 2011; McFall-Ngai et al., 2013; Wang et al., 2015). Metazoan
82 evolution is not only driven by pathogenic microbes. Instead, microbes often are
83 important facilitators of adaptation of higher organisms to the environment

84 (McFall-Ngai et al., 2012; Douglas, 2015). The evolution of organisms is often so
85 strongly intertwined with that of their associated microbes that Zilber-Rosenberg
86 and Rosenberg (2008) suggested the host organism and its associated microbes
87 should be treated as an evolutionary entity, the holobiont. However, most studies
88 that aim at shedding light on the mechanisms of adaptation still focus on the host
89 organisms all but ignoring its associated microbial communities.

90 The gut microbial communities of wood-feeding roaches and termites
91 facilitated their ability to thrive on a wood diet. Wood is difficult to digest and
92 poor in nitrogen. The ability to thrive on this resource has intrigued scientists for
93 decades (e.g. Cleveland, 1923, 1925, Breznak et al. 1973). This interest was
94 further spurred by the perspective to leverage insights into the process of
95 lignocellulose break down in termites for the generation of biofuel (Scharf, 2015).
96 Today, we can rely on extensive insights into the processes of lignocellulose
97 break down and nitrogen fixation in the termite gut (Breznak et al., 1973;
98 Warnecke et al., 2007; Hongoh et al., 2008b, 2008a; Brune and Dietrich, 2015;
99 Ohkuma et al., 2015). For the breakdown of lignocellulose, lower termites depend
100 on protists in their gut (Cleveland, 1923, 1925). These protists belong to the
101 order Oxymonadida (phylum Preaxostyla), which are specific to termite and
102 wood-feeding roach guts, and the phylum Parabasalia. The protists are
103 transmitted between colony members and from parent to offspring via proctodeal
104 trophallaxis. This vertical transmission contributes to patterns of cospeciation
105 between protist and termite host (Noda et al., 2007; Desai et al., 2010). In return,
106 most of the protists live in symbioses with ecto- and endosymbiotic bacteria. The
107 bacterial termite gut microbiome consists of these protist-associated symbionts
108 as well as bacteria freely living in the gut (Bauer et al., 1999; Breznak, 2002).
109 Although vertical transmission of bacteria between termites is not as strict as for
110 the mostly anaerobic protists (Noda et al., 2009), cospeciation can occur (Noda et
111 al., 2007; Ohkuma, 2008; Ikeda-Ohtsubo and Brune, 2009; Desai et al., 2010).

112 The microbial communities of termites share a common origin with the
113 microbial communities of cockroaches (Ohkuma et al., 2009; Schauer et al.,
114 2012; Dietrich et al., 2014). Since the evolutionary split of termites from
115 cockroaches, lower termites have diversified and adapted to a variety of
116 environments (Eggleton and Tayasu, 2001), lifestyles (Abe, 1987), and diets
117 (Donovan et al., 2001). Given the direct involvement of termite gut microbes in
118 food digestion, the microbiota could play a role in adaptation to new diets (Zhang
119 and Leadbetter, 2012). Prokaryotes that are beneficial for the termite host on a
120 new diet can be acquired from the environment. The presence of such newly
121 acquired microbes may indeed be reflected by the many taxon specific gut
122 bacteria found in termites (Hongoh et al., 2005; Dietrich et al., 2014; Rahman et
123 al., 2015; Tai et al., 2015). As a consequence of the acquisition of new bacteria
124 that are beneficial under new dietary conditions, the termite associated microbial
125 communities would change according to host diet. In fact, it has been shown that
126 both, diet and evolutionary history, shape the gut microbiota of termites (Boucias
127 et al., 2013; Dietrich et al., 2014; Rahman et al., 2015; Tai et al., 2015), with a
128 larger impact of diet in higher termites (He et al., 2013; Mikaelyan et al., 2015a;
129 Rossmassler et al., 2015). While the phylogenetic imprint on microbial
130 communities is evident, it is not completely clear from these studies what leads
131 to the diet related differences in microbial communities. These differences could
132 be driven by environmental microbes that are ingested and passage the gut.
133 Such passengers do not necessarily form any evolutionary relationship with
134 termites. However, such a relationship would be expected for microbes that play
135 a role in diet adaptation. In order to effectively disentangle passaging microbes
136 from microbes that persist in the termite gut, potentially forming a stable
137 relationship, it is necessary to control the environment of the termite host
138 including the diet.

139 Furthermore, termites that change their lifestyle from wood-dwelling
140 (single piece nesting according to Abe 1987) to foraging outside the nest
141 (separate and intermediate nesting type according to Abe 1987), are expected to
142 encounter more diverse and a higher load of microbes. Most foraging termite
143 species are exposed to microbe rich soil (4×10^6 colony forming units, Vieira and
144 Nahas, 2005), carrying on average 5000 times more microbes than even the
145 nests of damp wood-dwelling termites (800 colony forming units, Rosengaus et
146 al. 2003). As a consequence microbial diversity should be higher in foraging
147 species. Moreover, an increase in bacterial community diversity should be more
148 pronounced than an increase in protist diversity because bacteria are more
149 readily acquired from the environment when compared to anaerobic protists. The
150 foraging lifestyle also opens the termite colony to invasion by fungal and
151 bacterial pathogens (Korb et al., 2012, 2015) These pathogens exert selection
152 pressures on the hosts that have led to a variety of defensive strategies
153 (Rosengaus et al., 2011). However, the possibility that microbes themselves play
154 a role in the defense of social insect colonies as probiotics or defensive symbionts
155 has largely been neglected. This is surprising because defensive symbionts are
156 known from many other insects (e.g. Kaltenpoth et al., 2005).

157 In this study we sought to test whether an evolutionary switch in host
158 lifestyle and diet co-occurs with a change in microbial communities of lower
159 termites. Furthermore, we intended to identify microbes that correlate in
160 presence or abundance with this evolutionary switch, and hence could be
161 functionally related to evolutionary change. The third objective was to test
162 whether the foraging termites in our study carry an increased microbial diversity,
163 specifically bacterial diversity. Therefore, we analyzed the microbial communities
164 of five termite species that were kept under common conditions. These species
165 comprised two species of wood-dwelling kalotermitids (*Cryptotermes secundus*,
166 *Cryptotermes domesticus*), a wood-dwelling rhinotermitid (*Prorethra*

167 *simplex*), and two species of rhinotermitids that switched from the ancestral
168 wood-dwelling lifestyle to foraging (*Reticulitermes flavipes*, *Reticulitermes*
169 *grassei*, Figure 1). Because there can be substantial variation of microbial
170 communities between different colonies (Hongoh et al., 2005, 2006; Benjamino
171 and Graf, 2016), we analyzed four to eight replicate colonies per species.

172

173 **Results**

174 We used the Illumina MiSeq platform to sequence ~250 base pairs (bp) of the
175 16S rRNA gene to cover the v4 region for bacterial community profiling of the gut
176 microbiota. Protist communities were profiled using a set of 18S rRNA gene
177 specific primers of our own design that targeted the parabasalid protists(see
178 Materials and Methods). Oxymonads were excluded from the analysis because
179 they are difficult to target (e.g. Heiss and Keeling, 2006) and escaped reliable
180 illumina sequencing compatible amplification (data not shown). All termites were
181 kept on *Pinus radiata* wood from the same source. After quality-filtering, our
182 bacterial and parabasalid datasets contained 1 676 707 and 2 747 988 gene
183 sequences respectively (Table S1).

184

185 Diversity of microbial communities

186 OTU (Operational Taxonomic Unit) based analysis of these sequences revealed
187 that bacterial as well as parabasalid diversity was higher in the foraging species
188 that we analyzed (Wilcoxon test on Shannon diversity: $P = 0.005$, and $P = 0.03$
189 respectively, Table 1). Rarefaction curves representing the microbial diversity in
190 each termite species can be found in Supplementary Figure S1. Higher microbial
191 diversity is expected in foraging species if we assume that species frequently
192 leaving their nests to forage have a higher probability of encountering and
193 acquiring new microorganisms.

194 Furthermore, the diversity of parabasalid protists and bacteria was
195 correlated across termite species (Pearson's product - moment correlation
196 coefficient: $P = 0.003$, $r^2 = 0.52$, Figure 2). This correlation supports the notion
197 that many bacteria present in the gut of lower termites are directly associated
198 with protists. However, the bacterial diversity found in samples from the foraging
199 *Reticulitermes* was higher than predicted by the regression from protist diversity
200 of wood-dwelling species (ANOVA: $P = 0.005$, see Table S2 for linear models and
201 tests performed). This suggests that a larger proportion of the bacterial diversity
202 in *Reticulitermes* guts is either associated with oxymonads or does not live
203 associated with protists when compared to wood-dwellers.

204 *Reticulitermes grassei* samples Rg2 and Rg4 as well as *P. simplex* sample
205 Ps9 showed unusual bacterial diversity and community profiles (see discussion).
206 We performed all diversity comparisons with and without these samples and
207 found no qualitative difference in the results. Excluding these samples would
208 increase average *R. grassei* bacterial community diversity, decrease *P. simplex*
209 community diversity and lead to lower p-values in all tests shown in Table 1.

210

211 Composition of microbial communities

212 The correlation of bacterial and protist community diversity that we found
213 suggests that many of the bacteria are associated with protists. Indeed,
214 taxonomic classification of 16S rRNA gene sequences revealed many protist
215 associated bacterial taxa (Figure 3A, B). The four most common bacterial phyla in
216 our samples were *Bacteroidetes* (40.6%), *Spirochaetes* (21.9%), *Proteobacteria*
217 (12.4%), and *Elusimicrobia* (9.1%). Of these phyla the *Bacteroidetes*,
218 *Spirochaetes*, and *Elusimicrobia* contain members known to be important ecto- or
219 endosymbionts of lower termite associated protists (Noda et al., 2005, 2006;
220 Ohkuma, 2008; Strassert et al. 2010).

221 The phylum *Bacteroidetes*, contains the genus *Candidatus* *Armantifilum*,
222 which was common in all *Cryptotermes* colonies (14.7 – 53.7%). *Candidatus*
223 *Armantifilum* *devescovinae* is a filamentous bacterium that can be found
224 attached to the surface of *Devescovina* (Lophomonadidae) flagellates and
225 cospeciates with them in kalotermitids (Desai et al., 2010). Close relatives of
226 *Candidatus* *Armantifilum* are associated with *Snyderella* *tabogae*
227 (Lophomonadidae) in *Cryptotermes* *longicollis* (Desai and Brune, 2012).
228 Accordingly, we found lophomonadids in all *Cryptotermes* colonies (Figure 3C).

229 *Elusimicrobia* are well known endosymbionts of many termite-gut
230 flagellates (Ikeda-Ohtsubo et al., 2007; Brune, 2012). Sequences from a
231 prominent member of the *Elusimicrobia*, *Endomicrobium* were detected in all
232 samples (0.3 – 27.8%). *Endomicrobia* are endosymbionts of *Trichonympha* (Ikeda-
233 Ohtsubo and Brune, 2009) which we found in *Reticulitermes* (8.7 – 51.3%). Other
234 *Endomicrobia* are symbionts of the *Cristamonadida* (Desai et al., 2010), of which
235 we found *Devescovina* (0.2 – 76.2%), *Stephanonympha* (0 – 75.0%) and *Foainia*-
236 species (0.6 – 54.2%) in *Cryptotermes*. Interestingly, a small proportion of
237 sequences from the *Endomicrobiaceae* were found in the colonies of *P. simplex*
238 (0.4 – 3.0%). A protist host for *Endomicrobia* has not yet been described in *P.*
239 *simplex* and Tai et al. (2015) found no evidence for *Endomicrobia* in *P. simplex*.
240 However, our sampling of 16S rRNA gene sequences was 8-10 times deeper than
241 that of Tai et al. (2015) providing more power to detect rare microbes. 2 724 of 3
242 459 (86%) of the sequences from *Endomicrobia* found in *P. simplex* clustered into
243 three OTUs (OTU 90, OTU 252, and OTU 316) that were specific to *P. simplex*.
244 Hence, they are unlikely to represent contamination from non-*P. simplex* samples.
245 These three OTUs are closely related and cluster phylogenetically with sequences
246 from defaunated *R. santonensis* as well as a sequence from a *Zootermopsis*
247 *nevadensis* flagellate suspension (Figure S2).

248 *Spirochaetes*, which are characteristic of termite guts, where they can be
249 free living or live as ectosymbionts of protists (Breznak, 2002; Wenzel et al.,
250 2003; Noda et al., 2003), were found in all samples (9.1 – 50.5%) except the
251 abnormal sample Rg2.

252 Sequences from the spirochaete *Treponema la* fell into several distinct
253 OTUs that were termite taxon specific (e.g. OTUs 2, 3, and 33, see S5). Many
254 other bacterial genera contained termite taxon specific OTUs. For example,
255 sequences from *Endomicrobium* fell into several taxon specific OTUs (7, 12, and
256 20). *Candidatus* *Armantifilum* (OTUs 1, 31, and 48) separated into termite genus
257 specific OTUs as well. The prevalence of host specific OTUs suggests that the
258 microbial communities of the termite species analyzed here are distinct. At the
259 same time, the relatedness of these OTUs from different termite taxa, as
260 reflected by their common classification into a single genus, supports the notion
261 that the members of these communities often share a phylogenetic history
262 (Schauer et al., 2012; Dietrich et al., 2014).

263

264 Analysis of microbial community differences and similarities

265 The phylogenetic relationship between bacteria from different termite species
266 can be incorporated into community distances by analyzing environment specific
267 phylogenetic branch lengths of all community members (Lozupone and Knight,
268 2005). We were especially interested in the question whether similarities in
269 microbial community composition reflect host diet and lifestyle. Assuming that
270 microbes that are ecologically relevant for the host should not be rare, we used
271 the weighted Unifrac metric (Lozupone et al., 2007) for our analysis.

272 The bacterial communities of the wood-dwelling species *C. domesticus*, *C.*
273 *secundus* and *P. simplex* clustered separately from the bacterial communities of
274 the foraging *Reticulitermes* (Figure 4A) and together with the bacterial
275 communities of the wood-dwelling cockroach *Cryptocercus* (Figure S5), which is a

276 sister taxon of termites and also wood-dwelling. In contrast, the parabasalid
277 protist communities clustered strictly according to termite host family (Figure 4B).
278 Interestingly, the parabasalid communities of *R. flavipes* and *R. grassei* were
279 distinct (Approximately Unbiased support = 93%), while there was no consistent
280 difference between the protist communities of *C. secundus* and *C. domesticus*.
281 This might in part be due to the high variability of protist communities within *C.*
282 *domesticus*. The relative sequence abundance of the five most common
283 protists in *C. domesticus* varied substantially (*Devescovina*: 0.4% -
284 77.1%, *Stephanonympha*: 0.3% - 75.0%, *Foaina*: 0.6% - 39.0%,
285 unclassified lophomonadid: 0.07% - 28.3%, unclassified parabasalid:
286 1.4% - 23.3%). Samples Cd2 and Cd6 had similar protist communities that
287 were somewhat distinct from the other *Cryptotermes*. These samples shared a
288 very low relative abundance (1.7% and 1.4%) of an unclassified genus of the
289 Gf10 symbiont group that is common in the other *Cryptotermes* samples (red in
290 Figure 3C). They also share a high prevalence of *Stephanonympha* (62.8% and
291 75.0%).

292

293 Bacteria specifically associated with foraging *Reticulitermes*

294 The clustering of foraging *Reticulitermes* bacterial communities suggests that
295 there are bacteria specifically associated with *Reticulitermes*. In order to detect
296 these bacteria, we used an Indicator Species Analysis (Dufrêne and Legendre,
297 1997). Foraging *Reticulitermes* and wood-dwelling termites were treated as two
298 different habitats and bacterial 97% identity OTUs as species. A full list of
299 Indicator OTUs is available in Table S10.

300 *Reticulitermes* indicator OTUs that are more closely related to
301 environmental bacteria or bacteria found in non-dictyopteran hosts than to OTUs
302 found in *P. simplex* could be recently acquired by *Reticulitermes*. In order to shed
303 light on which bacteria are closely related to *Reticulitermes* indicator OTUs, we

304 constructed a phylogenetic tree. This tree included representative sequences
305 from the indicator OTUs, all OTUs for which we found at least 100 sequences in *P.*
306 *simplex* or *Cryptotermes*, and all sequences in DictDB (Mikaelyan et al., 2015b)
307 (Figure S4). The Newick formatted phylogeny can be found in S8.

308 Table 2 contains a list of *Reticulitermes* indicator OTUs that do not have *P.*
309 *simplex* OTUs nor bacteria from other wood-dwellers amongst their closest
310 relatives and contain more than 1 000 sequences. Close relatives of all of these
311 indicator OTUs were found in association with *Reticulitermes* in previous studies,
312 corroborating our results. Close relatives of several indicator OTUs came from the
313 environment, non-dictyopteran hosts or other foraging termites.

314

315 **Discussion**

316 Profiling bacterial and parabasalid protist communities, we found (i) that protist
317 and bacterial diversity correlate, (ii) evidence for changes in community
318 composition and diversity that correlate with an evolutionary switch from wood-
319 dwelling to foraging, (iii) candidate microbes that are specific to foraging
320 *Reticulitermes*. Furthermore, we found several samples that showed unusual
321 community profiles that require discussion.

322

323 Unusual community profiles and intraspecific variation in microbial communities

324 Three samples showed unusual community profiles (Ps9, Rg2, and Rg4). More
325 than half of the sequences from Rg4 are comprised by OTU 16. OTU 16 was
326 classified as a member of the Enterobacteriaceae. Many enterobacteria are insect
327 pathogens (Grimont and Grimont, 2006; Galac and Lazzaro, 2011; Jurat-Fuentes
328 and Jackson, 2012). Potential insect pathogens can often be found at low titers in
329 natural host populations where they probably live as commensals (Staubach et
330 al., 2013). This is also true for OTU 16 that comprises no more than 8% of the
331 sequences in the other samples. However, these bacteria can reach high titers

332 when entering the hemolymph (Galac and Lazzaro, 2011) causing a systemic
333 infection. This renders it plausible that Rg4 contained a termite that was
334 systemically infected with OTU 16.

335 The bacterial communities of Rg2 and Ps9 contained a smaller fraction of
336 sequences from presumably protist associated bacteria (*Endomicrobium* and
337 *Candidatus Azobacteroides*) than their conspecifics (1.2% instead of 10.1 -
338 27.4%, and 0.01% instead of 19.2% - 49.3%, respectively). This suggests that
339 these samples might have had a lower protist titer. A low protist titer in termites
340 is commonly caused by loss of protists from the hindgut during molts (Honigberg,
341 1970). We selected individuals that had filled guts and carefully excluded
342 individuals that were close to molting indicated by their whitish appearance.
343 However, these samples might have contained termites that have not fully
344 reestablished their protist communities after molting. Because we carefully
345 selected the termites for our study, it appears unlikely that the overall high
346 variability in the protist communities of *C. domesticus* is purely a result of
347 undetected molts. The variance in community composition between conspecific
348 termite colonies that we and others (Hongoh et al., 2005, 2006; Benjamino and
349 Graf, 2016) observed suggests that the use of replicate colonies is important.

350

351 Bacterial and protist diversity

352 We presented, to our knowledge, the first report of a correlation between
353 bacterial and protist community alpha diversity. Studies that analyze both, protist
354 and bacterial communities in parallel, across termite species are still rare. In the
355 two other studies, we are aware of, neither environmental nor dietary conditions
356 were controlled (Tai et al., 2015; Rahman et al., 2015). As a consequence, the
357 diversity patterns of termite associated microbial communities might have been
358 affected by microbes merely passing through the gut. These passengers could
359 have obscured the correlation of protist and bacterial diversity that we observed.

360 A correlation between protist and bacterial diversity does not only suggest
361 that many bacteria are protist associated in the termite gut (Brugerolle and
362 Radek, 2006). This correlation also implies a potential mechanism, how the high
363 bacterial diversity in the termite gut (Colman et al., 2012; Jones et al., 2013) has
364 evolved. A higher protist diversity offers bacteria a larger number of independent
365 bacterial habitats that can be colonized. Because there is good evidence that the
366 gut protists vertically transmit their bacterial symbionts (Noda et al., 2007; Ikeda-
367 Ohtsubo and Brune, 2009; Desai et al., 2010) and can carry a single isolated
368 strain of their symbionts (Ikeda-Ohtsubo et al., 2007; Zheng et al., 2015), these
369 bacteria experience reduced gene flow that allows for the accumulation of
370 genetic differences. Furthermore, the different environmental conditions, each
371 protist species offers, exert selection pressures on their associated bacteria
372 further promoting divergence via natural selection. In fact, the protists could
373 serve as 'speciation reactors' for bacterial symbionts and facilitate the evolution
374 of high bacterial diversity in termite guts. On the other hand, coadaptation
375 between protists and bacteria could promote protist speciation (Dolan, 2001).

376 The bacterial diversity in the gut of *Reticulitermes* was higher than
377 expected given parabasalid protist diversity of the other species. This implies
378 that *Reticulitermes* carries either a higher proportion of oxymonad protist
379 associated bacteria or more bacteria that live independently of protists. Because
380 the proportion of oxymonad to parabasalid diversity is identical in *R. flavipes*
381 (synonym *R. santonensis*) and *C. domesticus* (1:2, according to Yamin, (1979),
382 non-protist associated bacteria are likely to contribute to the additional bacterial
383 diversity in *Reticulitermes*.

384 Our bacterial diversity estimates for *Reticulitermes* are in concordance with
385 estimates from Benjamino and Graf (2016) who sequenced the same fragment of
386 the bacterial 16S rRNA gene. Their estimates of Shannon diversity range from
387 3.54 to 4.48 for different colonies and fit our estimate of 4.3 ± 0.25 well. A

388 previous study that reached a similar sequencing depth of an overlapping region
389 of the 16S rRNA gene also estimated similar Shannon diversities for *R. flavipes* of
390 3.92 (Dietrich et al., 2014). This is within one or two standard deviations of the
391 diversity we calculated for *R. flavipes* and *R. grassei* (4.3+-0.25 and 3.72+-0.87,
392 respectively). Rahman et al. (2015) estimated Shannon diversity of
393 *Reticulitermes* to be somewhat higher (4.91-5.54). However, these authors
394 included protists in their diversity estimates. In contrast, the bacterial diversity
395 estimate in Tai et al. (2015) was lower with Shannon diversity for *R. flavipes* at
396 3.66 versus 3.92 in our study, as well as 0.81 versus 3.14 for *P. simplex*. This
397 difference could result from our ~10X increased sampling depth. Shannon
398 diversity is known to correlate with sampling depth (Preheim et al., 2013).
399 Furthermore, Tai et al. (2015) used a masking approach to remove variable
400 regions from their alignment. This masking can significantly reduce diversity and
401 resolution (Schloss, 2010). Because we used SILVA (Pruesse et al., 2007) based
402 DictDB (Mikaelyan et al., 2015b) for our alignments instead of Greengenes
403 (DeSantis et al., 2006) as in Tai et al. (2015), this masking is not necessary
404 (Schloss, 2010). The same arguments can explain the lower parabasalid protist
405 diversity in Tai et al. (2015) (0.5 for *R. flavipes* compared to 1.63 in our study and
406 0.29 for *P. simplex* compared to 0.7 in our study). That our protist diversity
407 estimates were higher than those obtained by visual inspection (Yamin, 1979) is
408 not surprising since cryptic variation has been repeatedly documented from
409 termite protists (Stingl and Brune 2003; Brugerolle and Bordereau, 2006; Zheng
410 et al., 2015). Especially smaller protists often escape visual methods (Radek et
411 al., 2014).

412

413 Bacterial communities cluster according to lifestyle while protist communities
414 cluster according to host phylogeny

415 As a consequence of the switch from the ancestral wood-dwelling lifestyle to
416 foraging, *Reticulitermes* are not only exposed to more diverse microbial
417 communities from outside the nest, but also experienced a change to a more
418 diverse diet. *Reticulitermes* probably originated in inland habitats (Dedeine et al.,
419 2016) where they feed on a variety of substrates (Waller, 1991). Their natural
420 substrates include decaying wood (Amburgey, 1979), horse dung (Rahman et al.,
421 2015), and they are even able to acquire nutrients from soil (Janzow and Judd,
422 2015). In contrast, *C. domesticus*, *C. secundus* and *P. simplex* are largely
423 restricted to islands and coastal regions where they dwell on dead wood
424 (Eggleton, 2000). These changes in lifestyle and diet align with a change of
425 bacterial communities, but not protist communities. Therefore, microbes that are
426 related to this change in lifestyle are more likely to be found amongst the
427 bacteria. This result adds to the notion that bacterial communities are more
428 variable over evolutionary time than protist communities (Noda et al., 2009; Tai
429 et al., 2015).

430 A higher propensity of random gain and loss of bacteria in *Reticulitermes*
431 that is fueled by exposure of foragers to more diverse microbes could leave the
432 bacterial community of *Reticulitermes* distinct from that of wood-dwellers.
433 However, *Prorhinotermes* have probably reverted back from foraging to wood-
434 dwelling (Legendre et al. 2008, Bourgignon et al. 2015) leaving its ancestors
435 exposed to similarly diverse microbes, and *Prorhinotermes* with a similar
436 propensity for random change of microbial communities. Given similar potential
437 for random change, invoking lifestyle related natural selection either driving the
438 divergence of the *Reticulitermes* microbiota, or the convergence of the
439 *Prorhinotermes* microbiota back to that of wood-dwelling species, or both,
440 appears plausible to explain the distinct bacterial communities of *Reticulitermes*.
441 Candidate bacteria that correlate with the change in lifestyle

442 DictDB (Mikaelyan et al., 2015b) allowed us to classify the majority (75.5%) of our
443 bacterial sequences to the genus level and identify bacteria that correlate with
444 the evolutionary switch in lifestyle from wood-dwelling to foraging in
445 *Reticulitermes*. These bacteria come from diverse families. OTU 120 is a
446 spirochaete from the genus *Treponema* I. Spirochaetes including *Treponema* I
447 strongly correlate with diet in higher termites (Mikaelyan et al., 2015a;
448 Rossmassler et al., 2015). Indicator OTU 307 was classified as *Stenoxybacter*.
449 *Stenoxybacter* colonizes the hind gut wall of *R. flavipes* and contributes to
450 acetate metabolism as well as oxygen consumption (Wertz and Breznak, 2007).
451 Another interesting candidate is OTU 58 (*Lactococcus*). The genus *Lactococcus*
452 has, amongst termites, so far only been reported from *Reticulitermes* and higher
453 termites (Bauer et al., 1999; König et al., 2006; Mathew et al., 2012; Boucias et
454 al., 2013; Yang et al., 2015). Lactococci are powerful probiotics in mammals
455 (Ballal et al., 2015), fish (Heo et al., 2013) and arthropods (Maeda et al., 2013).
456 The acquisition of probiotics would be an obvious evolutionary response to the
457 increased pathogen exposure that is connected to the foraging lifestyle.
458 Furthermore, *Lactococcus* might be metabolically important in *R. flavipes* as it
459 directly or indirectly contributes to acetate production in termite guts (Bauer et
460 al., 1999).

461

462 **Conclusion**

463 We provide evidence that, even after removal of environmental noise through
464 keeping different lower termite species under common conditions, an ecological
465 imprint on their associate bacterial communities remains. Studies that control the
466 termite environment include more species and span several evolutionary
467 switches in lifestyle are necessary to generalize this pattern.

468

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474

475 **Materials and Methods**

476 Termite samples

477 All termites used in the experiments were kept under constant conditions (27°C,
478 70% humidity) and acclimated to the same food substrate (*Pinus radiata* wood) in
479 our lab for at least six weeks prior to the experiment. This period is considered
480 sufficient for termites and their gut microbes to adjust to a new diet (Huang et
481 al., 2013) and lies far beyond the 24h passage time of food through the termite
482 gut (Breznak, 1984). *C. domesticus* and *C. secundus* were collected in Darwin (in
483 2003, 2007 and 2011), Australia. *P. simplex* was collected in Soroa, Cuba (in
484 1964), and colonies of *R. flavipes* as well as *R. grassei* were collected on the Île d'
485 Oléron, France (in 2014). Morphological species identification was confirmed by
486 sequencing of the mitochondrial cytochrome c oxidase subunit II (Figure S6 and
487 supplementary methods) for most of the samples. The foraging species *R.*
488 *flavipes* and *R. grassei* were additionally provided with sand to build their nests.
489 In order to exclude a contribution of microbes or microbial DNA in the sand to the
490 *Reticulitermes* associated microbiota, we applied the same DNA extraction,
491 amplification, and sequencing procedure as for the termite guts to the sand.
492 However, we failed to amplify the targeted fragments from the sand samples.
493 This led us to conclude that the sand did not contribute to the bacterial nor
494 protist community profiles.

495 In order to test whether the time since collection from the wild played a
496 role in diversity and composition of the microbial communities we analyzed the
497 microbial communities of *Cryptotermes* that have been in the lab for different

498 periods of time (2004 - 2012). We could not detect any effect of the time under
499 lab conditions on microbial community alpha diversity (bacteria: $P = 0.99$, $r =$
500 0.001 ; protists: $P = 0.13$, $r^2 = 0.21$) nor composition and beta diversity
501 (Supplementary Figure S3).

502

503 DNA extraction, primer design and library preparation

504 DNA was extracted from the guts of three workers per colony using bead beating
505 and chloroform precipitation (also see supplementary material and methods). We
506 amplified the v4 region of the 16S rRNA gene with the bacteria specific primerset
507 515f (5'-GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-
508 GGACTACHVGGGTWTCTAAT-3') developed by Caporaso *et al.* (2012). Specific
509 barcode combinations were added to the primer sequences following Kozich *et al.*
510 (2013). In order to amplify a region of the 18S rRNA gene of parabasalid protists,
511 we developed custom designed primers. Primer development was based on
512 parabasalid sequences from SILVA SSU Ref Nr 123 that was extended by 18S
513 rRNA gene sequences from protist taxa that we expected to occur in the termite
514 species we analyzed according to Yamin (1979). These additional sequences were
515 downloaded from ncbi and aligned using ClustalW (Larkin *et al.*, 2007) in the
516 BioEdit Sequence Alignment Editor version 7.2.5 (Hall, 1999). The resulting
517 alignment was manually curated and searched for potential forward and reverse
518 primers that would match as many protist sequences as possible but not termite
519 18S rRNA genes. The 18S rRNA gene alignment as well as the corresponding
520 taxonomy reference - file are provided in the supplement (S7). The resulting
521 primerset Par18SF: 5'-AAACTGCGAATAGCTC-3'; Par18SR: 5'-
522 CTCTCAGGCGCCTTCTCCGGCAGT-3', amplified ~250 bp. A detailed PCR protocol
523 can be found in the supplementary material and methods section. Libraries were
524 sequenced on an Illumina MiSeq reading 2x 250bp.

525

526 Analysis

527 Data analysis was performed using MOTHUR version 1.33.3 (Schloss et al., 2009),
528 following the MiSeq SOP. Our detailed MOTHUR script can be found in S9. For
529 taxonomic classification of the bacterial 16S OTUs a representative sequence of
530 each OTU was aligned to a termite specific database DictDb_v3 (Mikaelyan et al.,
531 2015b), while 18S protist OTUs were aligned and classified using all parabasalid
532 sequences from the SILVA SSU Ref Nr 123 database that was extended by our
533 custom made alignment and taxonomy reference file (S7). In order to avoid
534 coverage bias, bacterial and protist datasets were rarefied resulting in 32 824
535 and 22 285 sequences per sample, respectively. Statistical tests and data
536 visualization were performed in R version 3.1.2 (2012). Phylogenetic trees
537 necessary to apply UniFrac (Lozupone et al., 2007) as well as the trees including
538 OTU representative sequences were generated with FastTree version 2.1.7 (Price
539 et al., 2010). The resulting trees were visualized with Dendroscope 3.3.4 (Huson
540 and Scornavacca, 2012). Trees based on Unifrac were generated using the
541 UPGMA algorithm as implemented in the R PvClust package version 2.0.0 (Suzuki
542 and Shimodaira, 2015) and bootstrapped 10 000 times with standard settings.
543 Indicator species were obtained following the method by (Dufrêne and Legendre,
544 1997), as included in MOTHUR.

545

546 **Data availability**

547 Sequence data is available at MGRAST (<http://metagenomics.anl.gov/>) under the
548 IDs 4711469.3 - 4711607.3. Supplementary information is available at the
549 journal website.

550

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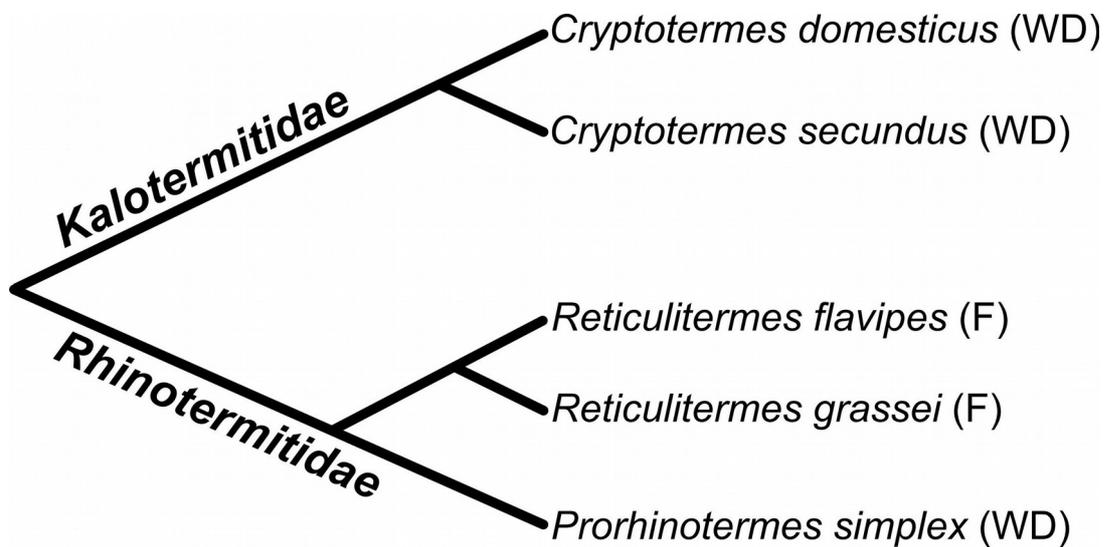
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565 **Figures and tables**



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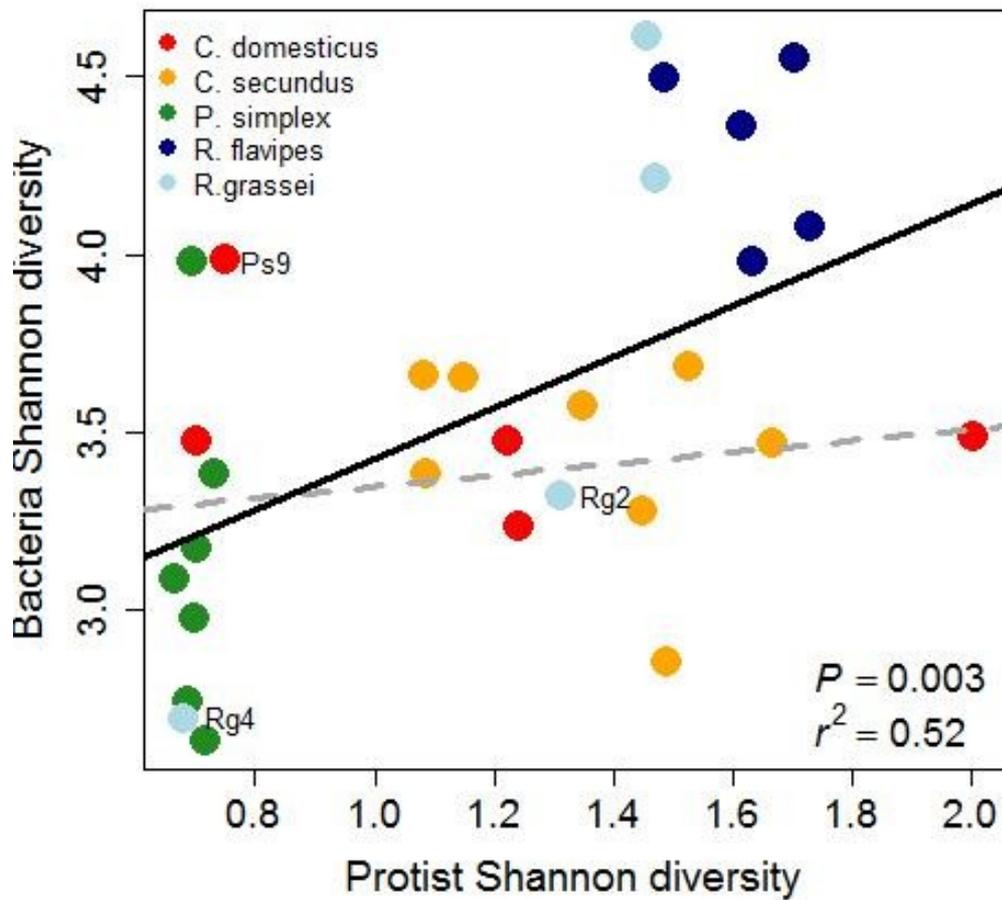
567 **Figure 1: Schematic phylogeny of the five lower termite species**

568 **analyzed in this study.** Letter in () describes host lifestyle. Branch lengths not

569 drawn to scale. WD = wood-dweller; F = forager. See also Figure S6 for a

570 cytochrome c oxidase sequence based tree.

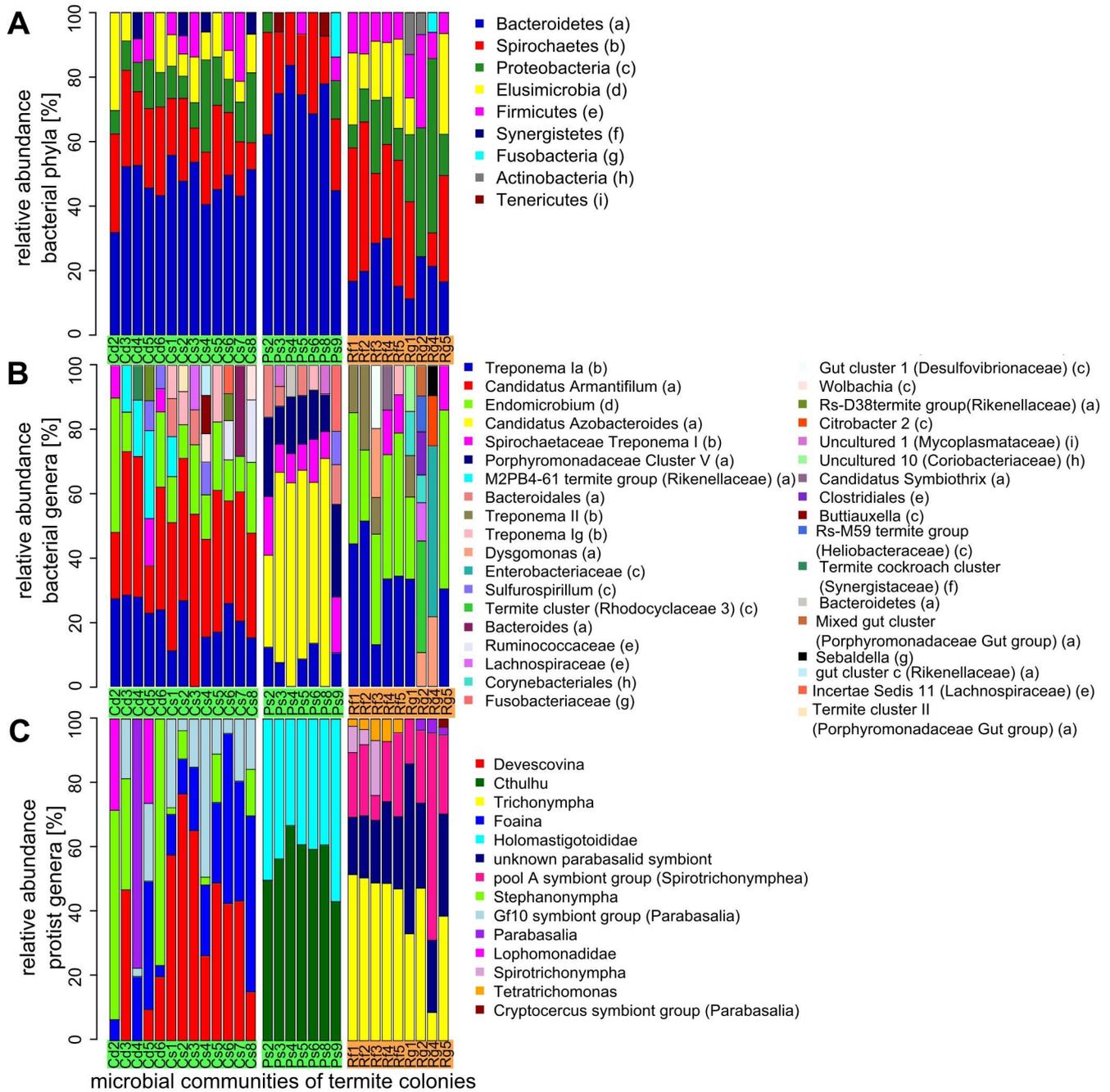
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573 **Figure 2: The Shannon diversities of protists and bacteria are**
574 **correlated across species.** Each dot represents a termite colony. The solid
575 regression line is based on all samples, while the dashed line is based on wood-
576 dwellers only. Colonies with unusual diversity patterns were marked (Ps9, Rg2,
577 and Rg4, see discussion).

578



579

580 **Figure 3: Relative abundance of bacterial and protist taxa as assessed**

581 **by taxonomic profiling of 16S- and 18S rRNA gene sequences.** Legends

582 are sorted by abundance beginning with the most abundant taxa (A) Relative

583 abundance of bacterial phyla. Phyla with a relative abundance <5% were

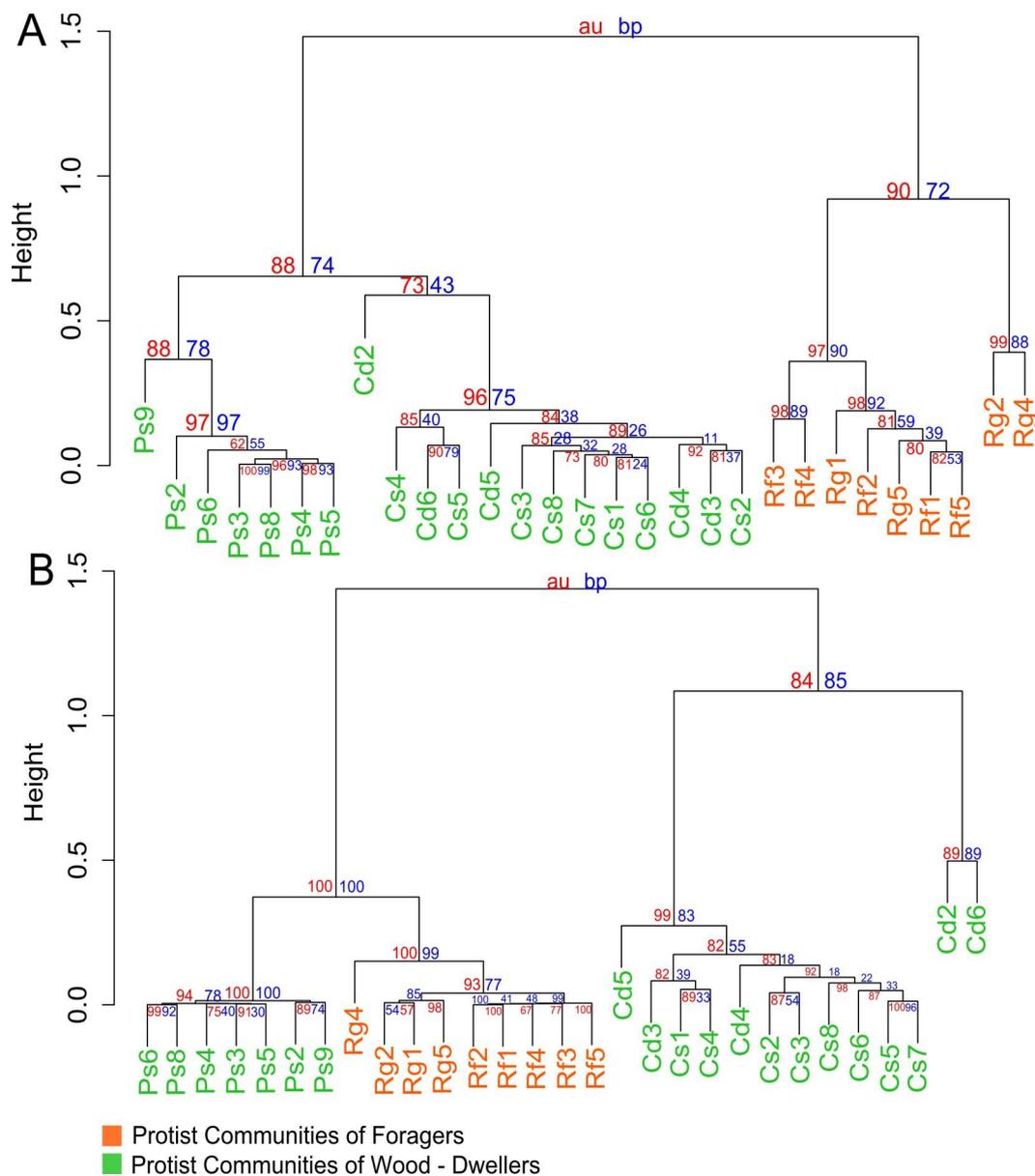
584 removed for clarity. (B) Relative abundance of bacterial genera. Sequences that

585 could not be classified to genus level with sufficient bootstrap support were

586 labeled with the next higher taxonomic rank that could be assigned with

587 sufficient support. Genus level classifications with unconventional names (e.g.

588 “Gut cluster 1”) are followed by family classification in parentheses. Letters in
 589 parentheses correspond to phyla in A. Genera of abundance <5% have been
 590 removed for clarity. (C) Relative abundance of protist genera. See B for non genus
 591 level classifications. Genera with an abundance <2% have been removed. Cd =
 592 colonies of *C. domesticus*, Cs = *C. secundus*, Ps = *P. simplex*, Rf = *R. flavipes*, Rg
 593 = *R. grassei*.
 594



596 **Figure 4: Cluster dendrograms based on weighted UniFrac community**
 597 **distances.** (A) Cluster dendrogram based on bacterial community distances. (B)
 598 Cluster dendrogram based on protist community distance. Cd = colonies of *C.*

599 *domesticus*, Cs = *C. secundus*, Ps = *P. simplex*, Rf = *R. flavipes*, Rg = *R. grassei*.
 600 blue numbers = bootstrap probability; red numbers = approximate unbiased
 601 probability.

602

603 **Table 1: Average Shannon - diversity indices of bacterial and protist**
 604 **communities based on 97% sequence similarity OTUs.** n = number of
 605 replicate colonies for each species; H = Average Shannon-Diversity; SD =
 606 Standard deviation; P = p-values of Wilcoxon test on Shannon-Diversity wood-
 607 dweller vs. forager.

	C. domesticu s	C. secundu s	P. simple x	R. flavipe s	R. grasse i	wood- dweller	forager	P
n	5	8	7	5	4	20	9	
Bacterial community								
OTUs	450	509	490	697	559	481	636	
H	3.56	3.42	3.14	4.3	3.72	3.37	4.04	0.005
SD	0.25	0.3	0.45	0.25	0.87	0.37	0.64	
Parabasalid community								
OTUs	31	21	21	36	36	23	36	
H	1.15	1.37	0.7	1.63	1.23	1.08	1.45	0.03
SD	0.52	0.2	0.02	0.1	0.37	0.4	0.32	

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609

610 **Table 2: Bacterial Indicator OTUs of *Reticulitermes*.** Shown are indicator
 611 OTUs with an indicator value larger than 99.9 and $P < 0.001$ that comprise at
 612 least 1,000 sequences and have no known close relatives in wood-dwellers.
 613 IndVal = Indicator value; DictDb ID refers to the label set in DictDb_v3.

OTU	#sequences	Taxonomy	IndVal	P
Otu00007	23425	<i>Endomicrobium</i>	99.98	<0.001
Otu00020	12960	<i>Endomicrobium</i>	99.98	<0.001
Otu00052	12461	<i>Termite Cluster (Rhodocyclaceae 3)</i>	99.99	<0.001
Otu00003	12282	<i>Treponema la</i>	99.96	<0.001

Otu00049	7539	<i>Treponema ll</i>	100	<0.001
Otu00019	6690	<i>Treponema la</i>	99.97	<0.001
Otu00056	5936	<i>Gut Cluster 1</i> (<i>Desulfovibrionaceae</i>)	99.95	<0.001
Otu00050	3414	<i>Treponema la</i>	99.99	<0.001
Otu00077	2726	<i>Incertae Sedis 34</i> (<i>Lachnospiraceae</i>)	99.98	<0.001
Otu00058	2505	<i>Lactococcus 3</i>	99.98	<0.001
