# The bacterial community of the European spruce bark beetle in space and time

Short title: The bacterial community of *Ips typographus*

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**Abstract:** The European spruce bark beetle *Ips typographus* is a pest causing severe damages in forests dominated by the Norway spruce in Europe. Microorganisms play an essential role in the host species performance, including nutrition, fitness as well as in overcoming host defenses. Here, we performed high-throughput 16S rRNA metabarcoding of *I. typographus* across different populations in Europe, to assess its bacterial community. We investigated four postglacial refugial areas in Europe and focused specifically on a current bark beetle hot spot in the Dolomites where we compared populations with different epidemiological phases (outbreaking vs. non-outbreaking) and across different seasons (pre-overwintering vs. overwintering). Our results show that the bacterial community structure varied among populations from the refugial areas and between different regions within the Dolomites. We found a significant difference in the bacterial community between pre-overwintering and overwintering individuals, but we did not find differences between epidemic and endemic populations. The prevalence of the genus *Erwinia* which was present in every individual and *Pseudoxanthomonas* in almost every individual across all populations, suggests that these taxa form the core bacterial community of *I. typographus*. Furthermore, several additional bacterial taxa occurred in all populations, but with variable frequencies. This study highlights a complex interaction of *I. typographus* and various bacterial taxa across different regions and ecological phases of *I. typographus* populations and provides new insights into the role of microorganisms in the biology of this important pest species.

**Key words**: Curculionidae, *Ips typographus*, forest pest, bacterial diversity, microbiome, endosymbionts, *Erwinia, Pseudoxanthomonas, Spiroplasma*

## Introduction

The European spruce bark beetle *Ips typographus* (L.) (Coleoptera: Curculionidae, Scolytinae) is one of the most destructive forest pests in Europe, which has caused significant ecological and economic disturbances over the last decades (Schelhaas et al. 2003). In Europe, *I. typographus* has a wide geographic range overlapping with its main host tree species, Norway spruce *Picea abies* (Karsten). Normally *I. typographus* exist as endemic populations were beetles breed and develop within the phloem of weakened and dying trees, especially wind-thrown and drought-stressed trees with impaired defenses. In epidemic phases when population outbreaks occur, this beetle can colonize and kill also apparently healthy trees (Schroeder 2010). In 2018, the storm Vaia destroyed more than 410 $km^2$ of forests, mainly dominated by Norway spruce, in the Southern Alps of North-Eastern Italy and Southern Austria (Udali et al. 2021). Moreover, heavy snow falls affected the Dolomites region in the years 2019 and 2020. These forest disturbance events provided vast amounts of suitable host material for *I. typographus* reproduction and development and resulted in a dramatic bark beetle outbreak in the Southern Alps (Nardi et al. 2022).

Insects are associated with various microorganisms that provide physiological and ecological benefits to their hosts. They play a significant role in various aspects of insect life histories, including nutrition, development, morphogenesis, behavior, and immunity (Douglas 2015). In particular, microorganisms are of high significance for phytophagous insects. For instance, despite having endogenous detoxification systems, insects are often associated with microbes to detoxify protective plant secondary metabolites (Itoh et al. 2018).

Bacterial symbionts also play a significant role in the life histories of numerous bark beetles, such as in the economically important genera *Ips* and *Dendroctonus*, by contributing to the detoxification of plant metabolites, protection of beneficial fungal symbionts, nutrition, detoxification, production of pheromone-like molecules, and protection against pathogens

through the production of antimicrobial compounds (García-Fraile 2018). For instance, the bacterial symbiont *Erwinia typographi* associated with *I. typographus* showed resistance to high concentrations of the spruce monoterpene myrcene, highlighting a potentially important role of this bacterium in the defense against terpenoids and phenolic compounds and thus assisting in overcoming host defense mechanisms (Skrodenytė-Arbačiauskienė et al. 2012). The genus *Pseudomonas* has been isolated from different *I. typographus* ontogenic stages and was shown to play a potential role in nutrient provisioning, protection against pathogens, and degradation of toxic compounds (Peral-Aranega et al. 2020). Therefore, bacteria might assist in the successful colonization of trees and might therefore play an important role in mass outbreakings of *I. typographus.*

Although our understanding of the bacterial community structure of *I. typographus* has increased in recent years (Chakraborty et al. 2020; 2023; Fang et al. 2020; Yu et al. 2022; Veselská et al. 2023), surprisingly little knowledge exists about the taxonomic composition and diversity across different geographic regions, epidemiological and overwintering phases. Here, we first describe the taxonomic composition and core-microbiome of *I. typographus* by characterizing the bacterial communities of different populations from a large area of Europe, including the four major refugial areas, in the Apennines, the Dinaric Alps on the Balkan Peninsula, the Carpathian Mountains and in the Russian plain (Tollefsrud et al. 2009). Subsequently, we focus particularly on the Dolomites (Northeastern Italy and Southern Austria), where currently an outbreak of this beetle occurs. In this region, we compare the microbial composition of endemic and epidemic populations to investigate the role of bacteria in the population dynamics of *I. typographus* and investigate the potential role of bacteria in overwintering individuals by comparing the bacterial communities of populations in different overwintering phases.

## Materials and methods

*Sample collection*

Adults of *Ips typographus* were collected from seven geographic regions. Populations from the Apennines (Abetone – Italy, Abe), the Dinaric Alps in Croatia (Vrhovine, CR), the Carpathian Mountains in Romania (Belis, RO), and the Russian plain (Alexandrov – Russia, RU) were included to assess differences in the bacterial community across a broad geographic scale and evaluate potential effects of historic events during the Pleistocene (Figure 1; Table S1). Moreover, we focused specifically on populations within the Dolomites that were affected by the storm Vaia in 2018 and by the snowfall events in the following years, namely four populations from Eastern Tyrol (Austria; ET1-ET4), three populations from South Tyrol (Italy; ST1-ST3) and six populations from Veneto (Italy; VT1-VT6) (Figure 1). To compare the bacterial communities between epidemic and endemic populations on a small local scale, the four populations in Eastern Tyrol were collected in different epidemiological phases in summer 2020 (two endemic sites, ET2 and ET4, and two epidemic sites, ET1 and ET3). The populations from Veneto were sampled before and during winter of 2019 and 2020, to compare the microbial community from pre-overwintering and overwintering individuals. For each population, 8-12 individuals were collected from infested *Picea abies* trees (Suppl table S1). To avoid the analysis of siblings, single adult beetles were collected from different galleries. Live beetles were immediately transferred to absolute ethanol and stored at -20 °C. Detailed information about the locations is listed in Table S1.

*DNA extraction and sequencing*

DNA was extracted from single *I. typographus* individuals using the Sigma-Aldrich GenElute Mammalian Genomic DNA Miniprep Kit (Saint Louis, Missouri, USA) following the manufacturer's protocol. DNA was quantified with the Invitrogen™ Qubit® dsDNA High

119 Sensitivity (HS) Assay Kit (Life Technologies, USA) and DNA quality was checked with a
120 Nanodrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA). As our aim was to
121 investigate the whole bacterial community associated with *I. typographus*, the collected
122 individuals were not surface-sterilized prior DNA extraction. The bacterial communities of 192
123 individuals were amplified using barcoded primers 515f and 806r which amplify the V4 region
124 of the 16S rRNA gene and sequenced on an Illumina MiSeq platform using 2×250 bp paired-
125 end chemistry by a commercial provider (StarSEQ GmbH, Mainz, Germany). Moreover, four
126 no template controls containing ultrapure $H_2O$ were included as negative controls and a mock
127 community including ten bacterial taxa was included as positive control. Raw reads were
128 deposited at NCBI with the BioProject ID PRJNA1020540.

129 *Data analysis*

130 Raw reads were analyzed using the QIIME2 pipeline (version 2021.4). Due to the poor
131 quality of the reverse reads, only the forward reads (which span almost the entire V4 amplicon)
132 were used for the subsequent analyses. DADA2 was used for quality filtering, denoising, and
133 Amplicon Sequence Variant (ASV) calling using the plug-in q2-dada2 with the denoise-single
134 method. Taxonomic assignment was done against the SILVA database version 138 (Quast et
135 al. 2012). ASVs identified as chloroplasts, mitochondria, archaea, and eukaryotes were filtered
136 out along with rare ASVs (i.e., singletons and ASVs represented by < 10 reads). Moreover, all
137 ASVs present in the negative controls were removed, besides one ASV corresponding to
138 *Erwinia* which was present in low abundance in one of the four controls, but in high abundance
139 across all individuals. The resulting ASVs table (Table S2) was then used for the subsequent
140 analyses.

141 Data analysis was done using R (version 4.1.2) using the 'phyloseq' package
142 (McMurdie & Holmes 2013). Shared and unique ASVs of the different geographic locations

143 were identified, considering only ASVs with 10 or more reads per sample. Visualization of the
144 intersecting and unique ASVs was carried out using the 'UpSetR' package (Conway et al.
145 2017).

146 Bacterial diversity was analyzed using the packages 'phyloseq' (McMurdie & Holmes
147 2013) and 'vegan' (Oksanen et al. 2015), after data normalization using the relative log
148 expression (RLE) method (Robenson & Oshlack 2010). The alpha diversity indices Chao 1 and
149 Shannon's diversity index as well as Good's coverage (to validate the representativeness of the
150 sequencing effort) were determined for each sample. Normal distribution of data was evaluated
151 using the Shapiro-Wilk test. ANOVA and Tukey's Honest Significant Difference (HSD) post-
152 hoc test were used to test for differences between bacterial diversity among populations and
153 localities. Additionally, Welch's t-test was used to test for differences in diversity between
154 endemic and epidemic populations as well as between pre-overwintering and overwintering
155 populations.

156 Beta diversity was analyzed using Canonical Analysis of Principal coordinates based
157 on Bray-Curtis and unweighted UniFrac distance metrics using the 'CAPSCALE' package
158 (Oksanen et al. 2015) to determine differences in community structure among the different
159 geographical locations. Permutation multivariate ANOVA (Adonis) was used to assess
160 differences in community structure among different geographical locations with 1,000
161 permutations followed by a pairwise comparison. Furthermore, a circular pattern hierarchical
162 clustering of samples collected from the Dolomite region was performed based on both
163 unweighted UniFrac distance metrics and Bray-Curtis dissimilarity using the 'ggtree' package
164 (Yu et al. 2017).

165 Differentially abundant taxa between pre-overwintering and overwintering populations
166 of Veneto were identified using the linear discriminant analysis effect size analysis (LEfSe;

167 Segata et al. 2011) with default settings using the diff_analysis function in MicrobiotaProcess
168 R package.

169 Metagenome functions were predicted based on the ASVs abundances of the
170 microbiomes using the original python implementation of PICRUSt2 v2.5
171 (https://github.com/picrust/picrust2) and MetaCyc (Caspi et al. 2010). Results were visualized
172 in the Marker Data Profiling module of MicrobiomeAnalyst version 2.0 (Lu et al., 2023) with
173 default parameters (low count filter: 4 counts, 20% prevalence; low variance filter: 10% based
174 on inter-quartile range; centered log ratio transformation). Differences between populations,
175 regions, seasons, and epidemiological phases were tested for using t-test or one-way ANOVA
176 (Benjamini–Hochberg method, FDR $q < 0.05$).

177

178 **Results**

179 *Predominant taxa in the bacterial community of* Ips typographus

180 After quality control and sequence filtering, a total of 6,797,490 reads ranging from 3,738
181 to 65,356 reads per individual were retained (Table S2). These were clustered into 11–390
182 ASVs per individual (mean = 124.24 ± 5.34). These ASVs comprised 31 bacterial phyla, 70
183 classes, 174 orders, 299 families, and 613 genera. The Good's coverage was greater than 99.5%
184 in all 192 samples and all rarefaction curves reached a plateau, indicating that the sequencing
185 coverage was sufficient to capture the bacterial communities of all samples (Figure S1).

186 The bacterial community composition across all *I. typographus* individuals revealed ten
187 major bacterial phyla. The most abundant phyla were Pseudomonadota (formerly
188 Proteobacteria, 25.1%), Bacteroidota (formerly Bacteroidetes, 17.93%), Bacillota (formerly
189 Firmicutes, 11.2%), and Actinobacteria (6.18%), while Myxococcota, Bdellovibrionota,
190 Planctomycetota and Abditibacteriota were present at lower abundance across all regions

191 (Figure 2A). At the genus level, 15 bacterial taxa were present in most of the studied regions,
192 including *Acinetobacter, Allorhizobium-Neorhizobium, Brevundimonas, Chryseobacterium,*
193 *Erwinia, Izhakiella, Mycobacterium, Novosphingobium, Pseudomonas, Pseudoxanthomonas,*
194 *Sphingobacterium, Spiroplasma, Williamsia* and *Wolbachia* (Figure 2B). The genus *Erwinia*
195 was the most represented genus, being present in all individuals. Moreover, the genus
196 *Pseudoxanthomonas* was present in all but three individuals in Russia (98.44% of all
197 individuals) and occurred in all studied regions (Figure S2). Therefore, *Erwinia* and
198 *Pseudoxanthomonas* can be considered as the core bacteria of *I. typographus*.

199

200 *Differences in bacterial communities across geographic regions*

201 Overall, 15 ASVs were shared among all populations, which belonged to the genera
202 *Erwinia, Pseudoxanthomonas, Acinetobacter, Spiroplasma, Pseudomonas, Chryseobacterium,*
203 *Sphingobacterium,* and *Allorhizobium-Neorhizobium*. Additionally, eleven ASVs were
204 common in all populations except Russia (Figure 3) which included the genera *Tyzzerella,*
205 *Taibaiella, Gordonia, Patulibacter* and *Endobacter.* In contrast, the majority of ASVs were
206 exclusively present only in single regions: 312 in the four populations from Eastern Tyrol, 535
207 in the three populations from South Tyrol, 763 in the six populations from Veneto, 227 in
208 Abetone, 93 in Romania and 335 in Croatia and 477 in Russia. Most ASVs were exclusively
209 present in single populations (Figure 3). At the genus level, 25 genera were shared among all
210 regions and populations, which included *Acinetobacter, Brevundimonas, Chryseobacterium,*
211 *Erwinia, Novosphingobium, Prevotella, Pseudoxanthomonas, Pseudomonas, Seratia,*
212 *Sphingobacterium, Sphingobium, Spiroplasma* and *Williamsia.* Most genera were exclusively
213 present only in single populations: 13 in Eastern Tyrol, 27 in South Tyrol, 26 in Veneto, seven
214 in Abetone, two in Romania, ten in Croatia and 44 in Russia (Figure S3).

215 Overall, bacterial species richness differed significantly between regions (ANOVA, F=
216 13.39, df= 6, $p < 0.001$; Figure 4). Pairwise comparison of species richness showed significant
217 difference between several regions (Table S3). Bacterial diversity based on the Shannon index
218 also differed significantly between regions (ANOVA, F= 20.92, df= 6, $p < 0.001$; Table S4).
219 Beetles from Veneto had the highest Shannon index of 3.62 whereas individuals from Eastern
220 Tyrol had the lowest of 1.80 (Figure 4). The bacterial community composition of *I.*
221 *typographus* differed significantly between and within regions. This was confirmed by a
222 distinct segregation and a significant difference between the geographical regions based on
223 unweighted UniFrac distance (PERMANOVA, df= 6, F= 12.733, $R^2$= 0.29227, $p < 0.001$;
224 Figure 5; Table S5). The bacterial species richness was also significant different between the
225 different refugial areas (Chao1: ANOVA, F= 15.07, df= 3, $p < 0.0003$), whereas bacterial
226 diversity was not significantly different among them (Shannon: ANOVA, F= 1.06, df= 3, p <
227 0.377).

228 The specific focus on populations within the Dolomites allowed the comparison of the
229 bacterial communities of different *I. typographus* populations on a smaller geographic scale.
230 Overall, the bacterial species richness was significantly different between locations from the
231 Dolomites (Chao1: ANOVA, F= 17.2, df= 2, $p < 0.001$). Pairwise comparisons showed a
232 significant difference between ET and ST ($p < 0.02$), ST and VT ($p < 0.03$), and populations
233 from ET and VT had a significantly different bacterial diversity ($p < 0.0001$; Figure 4).
234 Likewise, bacterial species diversity was significantly different among the Dolomites regions
235 (Shannon: ANOVA, F= 64.28, df= 2, $p < 0.0001$). Pairwise comparisons showed no significant
236 difference between ET and ST ($p = 0.477$) but a significant difference between ET and VT (p
237 < 0.0001) and VT and ST ($p < 0.0001$). These results were confirmed by a PERMANOVA
238 based on unweighted UniFrac distance metrics, which revealed a significant difference in the
239 bacterial community structure between different Dolomite regions (PERMANOVA, df= 2, F=

240 16.034, $R^2$= 0.18529, $p < 0.001$; Figure 5). However, the pairwise comparison showed that
241 there is no significant difference in the bacterial community structure between locations from
242 Eastern Tyrol and South Tyrol. The hierarchical clustering showed the segregation among the
243 different Dolomites regions which was strongly pronounced in the case of VT populations
244 when compared to ET and ST populations (Figure S4).

245

246 *No difference between the microbial communities of epidemic and endemic populations*

247 To investigate potential differences in the bacterial community composition between
248 populations in different epidemiological phases we compared the microbial communities
249 between endemic (ET2 and ET4) and epidemic populations (ET1 and ET3) from Eastern Tyrol
250 on a small local scale. The bacterial community composition was similar between the epidemic
251 and endemic populations at both phylum and genus levels (Figure 6A; 6B). The bacterial
252 species richness and diversity were similar between groups (Welch's t-test: Chao1, t = 0.51824,
253 df= 39.199 p = 0.6072; Shannon, t = 0.24332, df = 41.578, p = 0.809). Moreover, Canonical
254 Analysis of Principal coordinates based on unweighted Unifrac and Bray-Curtis dissimilarities
255 showed a strong overlap between the endemic and epidemic populations (Figure 6C; 6D). This
256 was confirmed by Adonis multivariate analysis of variance, showing that there was no
257 significant difference between the bacterial communities of endemic and epidemic populations
258 of *I. typographus* (PERMANOVA, df= 1, F= 0.4194, R2= 0.00904, p = 0.7363). These results
259 might indicate that the bacterial community is rather influenced by the geographical region
260 than the epidemic status. However, several taxa had different relative abundances between
261 epidemic and endemic populations. In particular, *Pseudoxanthomonas*, *Spiroplasma* and
262 *Brevimonas* were more abundant in epidemic populations, whereas
263 *Allorhizobium−Neorhizobium* and *Erwinia* were more abundant in endemic populations
264 (Figure 6B).

265

266 *Different bacterial communities between pre-overwintering and overwintering populations*

267 To study the influence of overwintering on the bacterial community composition, we
268 compared the microbial communities between pre-overwintering VT2, VT3 and VT6 (which
269 were sampled in August and September 2020) and the overwintering VT4 and VT5 populations
270 (which were sampled in December 2019 and January 2020). Despite sharing the same bacterial
271 composition on the phylum level between pre-overwintering and overwintering individuals,
272 there were differences in the abundance where *Allorhizobium-Neorhizobium* was more
273 abundant in pre-overwintering and Erwinia, *Pseudomonas,* and *Pseudoxanthomonas* in
274 overwintering populations. Particularly, the relative abundance of the genera *Acinetobacter,*
275 *Serratia,* and *Spiroplasma* was significantly higher in pre-overwintering individuals. In
276 contrast, the genera *Brevendomonas, Gordonia*, *Pajaroellobacter*, *Pedobacter,* and *Williamsia*
277 were significantly more abundant in overwintering individuals (Figure S5). Both permutated
278 multivariate ANOVA (Adonis) and pairwise comparison of PERMANOVA revealed a strong
279 segregation between the pre-overwintering and overwintering individuals (all $p < 0.001$; Figure
280 7A; 7B).

281

282 *Predicted pathways of bacterial communities in I. typographus*

283 Metagenome function predictions highlight differences among samples from different
284 populations, regions, and season. The PICRUSt2 analysis predicted 419 gut microbial MetaCyc
285 functional pathways showing a pattern across the different populations of *I. typographus*
286 (Figure S6). Statistical analysis revealed that 333 (t-test/one-way ANOVA, FDR $q < 0.05$) and
287 59 biochemical pathways were differentially abundant across the different populations and
288 between individuals of different overwintering phases, respectively. However, there was no

statistical difference between the epidemiological phases. There were 26 biochemical pathways significantly enriched within the top 50 pathways divided by regions, 24 by populations, and 6 by season (Figure S6; Table S6). These included pathways related to the biosynthesis and degradation (of lipids, amino acids biosynthesis, as well as pathways mainly related to the "generation of precursor metabolites and energy", and "nucleoside and nucleotide biosynthesis."

The heatmap with the 44 most abundant varying pathways of this dataset is displayed, consistently showing patterns of abundance differences between the regions (Figure S6). The cluster A contains mainly pathways related to the synthesis of amino acids, which are generally more abundant among the samples of Eastern Tyrol, Russia (AI), Veneto (AII), while they are less abundant in South Tyrol (AII). The lipidic pathways enclosed in cluster B, was more abundant in the samples from Veneto. Conversely, they are less abundant in some populations from South Tyrol and Eastern Tyrol. Lastly, most of the pathways of cluster C related to energy and nucleotide biosynthesis are more common in all the regions except for Veneto. Within Veneto populations, biosynthesis of isoleucine (I, II, III), of valine, of branched amino acids, and pyruvate fermentation to isobutanol (engineered) significantly increased during overwintering (FDR $q < 0.05$) Moreover, fatty acids and beta-oxidation were also more common (raw-$p < 0.05$). Conversely, biosynthesis of gondoate, (5Z)-dodec-5-enoate, oleate IV, CDP-diacylglycerol (I, II), phospholipids, (Z)-octadec-11-enoate, palmitoleate, and fatty acid elongation-saturated significantly decreased (raw-$p < 0.05$). Additionally, in Eastern Tyrol lysine, threonine, and isoleucine I biosynthesis, was more present than in the other regions.

## Discussion

Here, we present a comprehensive assessment of different factors influencing the bacterial community composition of the European spruce bark beetle *I. typographus* across a wide geographic range including the four main postglacial refugial areas. Moreover, we specifically investigated possible changes of the bacterial community among populations in different epidemiological phases and beetles before and during overwintering in an area with a current bark beetle mass outbreak within the Dolomites.

While previous studies were based on pooled *I. typographus* guts from one restricted geographic area (Chakraborty et al. 2020; Fang et al. 2020; Yu et al. 2022; Veselská et al. 2023), our approach allowed a fine-scale analysis of several European populations on a single individual level. Our analysis revealed that the genera *Erwinia* and *Pseudoxanthomonas* which were previously described in *I. typographus* in the Czech Republic and in China (Chakraborty et al. 2020; Fang et al. 2020; Yu et al. 2022) can be considered the core bacterial community being present in all individuals (*Erwinia*) and in 98.44% of the individuals (*Pseudoxanthomonas*). The omnipresence of these two genera across most individuals from different populations across Europe suggests that these bacteria might be essential for *I. typographus* to successfully colonize trees and utilize the subcortical material as a food source. Since various other bark beetle species of the genera *Ips* (Chakraborty et al. 2020) and *Dendroctonus* (Adams et al. 2013) also harbored these bacteria, they might play an important role in the life histories of these widespread conifer bark beetles. Strains of *E. typographi* isolated from *I. typographus* were found to be tolerant to high concentrations of the monoterpene myrcene, one of the defensive compounds of Norway spruce (Skrodenytė-Arbačiauskienė et al. 2012), whereas members of the genus *Pseudoxanthomonas* contribute to cellulose and lignocellulose degradation and therefore play a potential role in the breakdown of plant cells in conifers (Kumar et al. 2015).

336 Apart from these two prevalent genera, other bacterial taxa were present in multiple
337 populations, but at different frequencies. Since not all individuals harbor them, they might not
338 be essential for the survival of the host but might play an additional important role for the
339 beetle. For example, *Brevundimonas* and *Pseudomonas,* have been shown to contribute to the
340 detoxification of conifer phytochemicals in *Dendroctonus* (Boone et al. 2013). While
341 *Brevundimonas* is able to reduce diterpene abietic acid levels at low concentrations, two
342 *Pseudomonas* species were shown to reduce concentrations of monoterpenes under controlled
343 conditions (Boone et al. 2013). Since both taxa were present in all investigated populations, we
344 assume that these bacterial taxa might be involved in overcoming host defenses and might
345 therefore be especially important in colonizing healthy trees. Moreover, the genomic
346 characterization of *Pseudomonas* strains isolated from *I. typographus* revealed various
347 important pathways involved in the inhibition of entomopathogenic fungi as well as pathways
348 for the hydrolyzation of cellulose, xylan, starch, and pectin (Peral-Aranega et al. 2020).
349 Additionally, other dominant bacteria common among *Ips* species have been shown to be
350 important for the biology of bark beetles: *Sphingobacterium* contributes to the decomposition
351 of hemicellulose (Zhou et al. 2009) whereas *Acinetobacter* and *Williamsia* to the
352 decomposition of lignin (Bugg et al. 2011). While *Sphingobacterium* and *Williamsia* were
353 present in most individuals from all different regions, *Acinetobacter* was present in lower
354 abundance in fewer individuals from most populations.

355 Glacial refugial areas during the Pleistocene have been shown to be important for the
356 current geographic range and genetic structure of many species (Schmitt 2007). While most
357 studies focused on how the glacial isolation of different populations shaped their population
358 structure of different bark beetles (Bertheau et al. 2013), the potential effect on the microbial
359 community has not been investigated. Moreover, a growing body of research supports the idea
360 that microorganisms exhibit biogeographic patterns (Martiny et al. 2006) and therefore

361 symbionts could be used to reconstruct the evolutionary history of their hosts. Here, we
362 compared the bacterial communities of populations from the main putative refugial areas of *I.*
363 *typographus* from the Apennines (Abetone), the Dinaric Alps (Croatia), the Carpathians
364 (Rumania) and the Russian Plain (Stauffer et al. 1999; Bertheau et al. 2013), and observed
365 significantly different bacterial communities between these regions. While the populations
366 from the Apennines and Dinaric Alps clustered with the populations from Eastern Tyrol and
367 South Tyrol, the populations from the Carpathian Mountains clustered with the populations
368 from Veneto and Russia forming a unique cluster. Although we cannot exclude that ecological
369 differences between the sampling years and sampling seasons had an impact on the outcome,
370 our results indicate a potential influence of the Carpathian refugial area onto the Southern Alps,
371 which is different from the genetic structure of *I. typographus* (Stauffer et al. 1999, Bertheau
372 et al. 2013). Therefore, our study sheds new light on the evolution and post-glacial history of
373 this bark beetle and its associated bacteria.

374 Although we found significant differences between the bacterial communities from
375 different regions, we did not detect major differences between the bacterial communities of
376 populations in different epidemiological phases, neither in terms of taxonomic richness and
377 diversity nor community structure. These results suggest that *I. typographus* outbreaks might
378 not be linked to a major shift in the bacterial community, at least not in the early phase of an
379 outbreak. However, minor changes were observed for specific genera among the most abundant
380 bacteria, such as *Pseudoxanthomonas* and *Spiroplasma* which had a higher relative abundance
381 in the epidemic than in the endemic populations. While *Pseudoxanthomonas* was reported to
382 have a role in the biodegradation of celluloses and lignocelluloses (Kumar et al. 2015) and
383 might therefore play a role in digestion of xylem and phloem in the host (Fang et al. 2020),
384 *Spiroplasma* can have various effects on their hosts, ranging from beneficial (such as increasing
385 tolerance to natural enemies in their host to reproductive parasitism, particularly male-killing

386 (Ballinger & Perlman 2019). It is unclear if and to which extant they influence the
387 epidemiology of *I. typographus*.

388 An additional important factor in the biology of *I. typographus* is its overwintering
389 behavior. *I. typographus* increases its cold tolerance and enters a reproductive diapause at the
390 end of the season to increase the chance of overwinter survival (Annila 1969; Schebeck et al.
391 2017). Seasonal variations and diapause behavior might influence the bacterial community
392 structure and the latter could support the insect in overcoming the harsh winter conditions, for
393 instance by nutrient storage to build up energy reserves (Dittmer & Brucker 2021). A change
394 in the bacterial community structure across seasons and during overwintering was observed in
395 *Dendroctonus* bark beetles (Wang et al. 2017). By comparing populations sampled before and
396 during the overwintering phase, we found a significant difference of the bacterial community.
397 In particular, the genera *Acinetobacter*, *Seratia, Spiroplasma* and *Tyzzerella* were found at
398 higher abundances in pre-overwintering beetles, while *Williamsia*, *Pseudomonas* and
399 *Sphingobacterium* were more abundant in overwintering populations. The last two genera were
400 also enriched during winter in the gut microbiota of *Dendroctonus armandi* larvae (Wang et
401 al. 2017), highlighting a potential contribution to overwintering of bark beetles. It has been
402 hypothesized that bacteria from the family *Sphingobacteriaceae* are associated with insect
403 overwintering and survival at low temperatures (Wang et al. 2017), whereas *Pseudomonas*
404 might be involved in increasing *I. typographus* resistance to fungal pathogens and thus
405 increasing the chance of survival (Peral-Aranega et al. 2020). Additionally, the genus
406 *Novosphingobium* was significantly more abundant in overwintering beetles. Cheng et al.
407 (2018) reported the involvement of *Novosphingobium* isolates in naringenin degradation
408 activity in red turpentine beetle.

409 To predict the functional capabilities of the bacterial community we performed a
410 PICRUSt analysis and found several amino acids such as branched-chain-, aromatic-,

411 isoleucine, lysine, valine, threonine, glycine, and serine that were predicted within the major
412 biochemical pathways, likely supporting the fitness of *I. typographus* (García-Fraile, 2018).
413 Isoleucine is an amino acid that is crucially needed to emit several aliphatic/aromatic alcohols
414 and esters in fungi, including those associated with bark beetles (Lehenberger, 2021). Thus, its
415 importance is ultimately linked to the chemical communication between microbe and bark
416 beetle interactions. Beta oxidation reactions are involved in detoxification mechanisms of pine
417 terpenes by bark beetle-associated fungi (Hammerbacher et al., 2019). Interestingly, bacteria
418 seem to provide several lipid sources, which in the populations in Veneto are more abundant
419 in pre-ovewintering individuals. These could be then used by the beetle as fat reserve for the
420 following months and in preparation for overwintering (Botterweg 1983). Lastly, our data show
421 several similarities with the recent findings on the predicted bacterial pathways of the *I.*
422 *typographus* pathosystem (Chakraborty et al., 2023). This implies that such pathways not only
423 can be common to adult beetles from different regions, but also to the larvae and galleries,
424 suggesting conserved functions of the bacteriome associated with this pest.

425 In conclusion, our study on the bacterial community of *I. typographus* across different
426 geographic regions and epidemiological and seasonal phases highlights the important role of
427 bacteria in this pest species. Especially the presence of *Erwinia* and *Pseudoxanthomonas* in all
428 analyzed individuals suggests their essential role, likely as obligatory symbionts for this bark
429 beetle. Further studies are needed to investigate their influence on the host and transmission
430 route to the offspring. Similarly, the role of several less-frequent genera associated with *I.*
431 *typographus* need to be further investigated to understand their role for its host.

432

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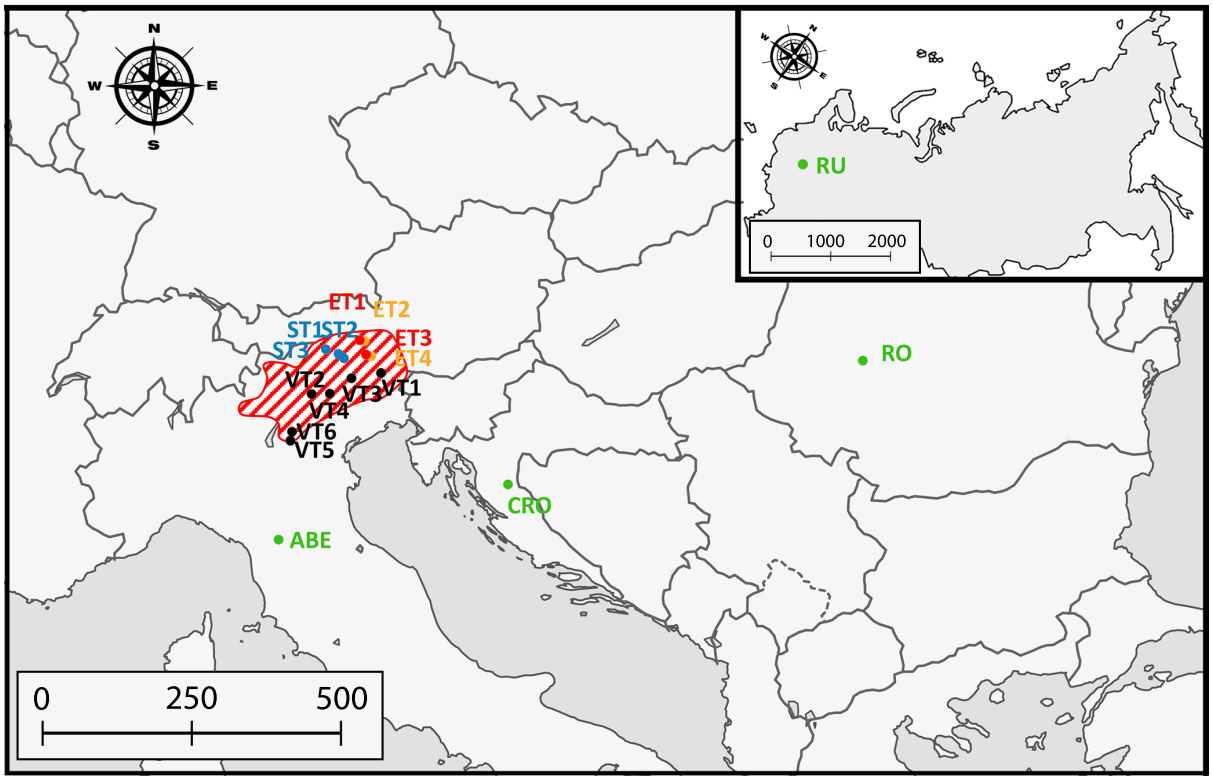
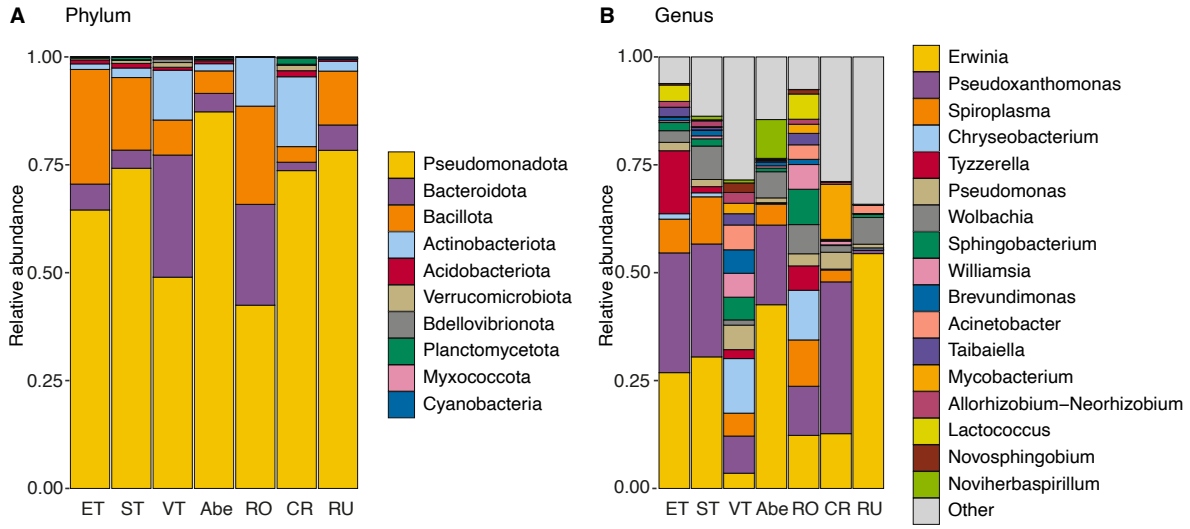
**Figures:**

**Figure 1:** Overview of *Ips typographus* sample locations. ET1-4: Eastern Tyrol; ST1-3: South Tyrol; VT1-6: Veneto; ABE: Abetone; CR: Croatia; RO: Romania and RU: Russia. Populations depicted in orange represent populations in endemic phase, whereas red indicates epidemic phase. Populations in green represent populations sampled before overwintering, whereas populations in blue were sampled during overwintering. The dashed background shows the area affected by the current *I. typographus* outbreak in the Southern Alps. Details of population localities are given in Table S1.



599 **Figure 2:** Microbial composition of *Ips typographus* across different populations at phylum

600 level (A) and genus level (B). In (B) only the 17 most abundant genera were plotted.



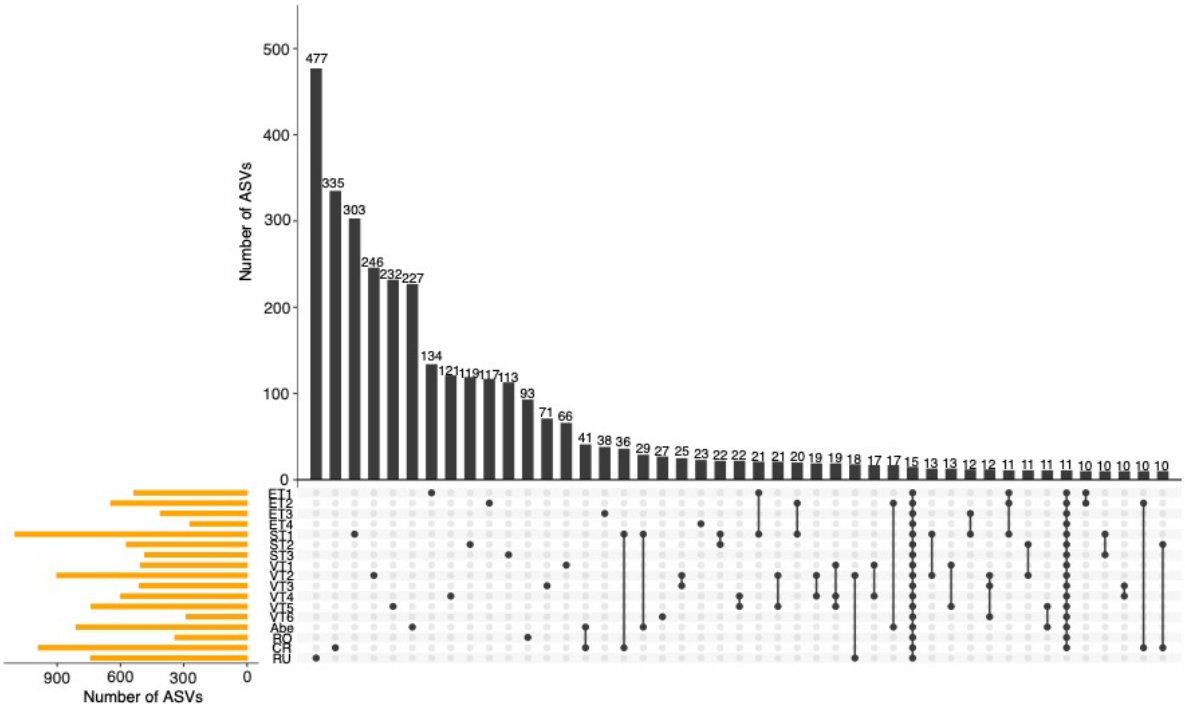

601

602 **Figure 3:** Distribution of ASVs across different populations of *Ips typographus*. Vertical bars

603 (black) and the dot matrix represent the number of shared or unique ASVs, whereas

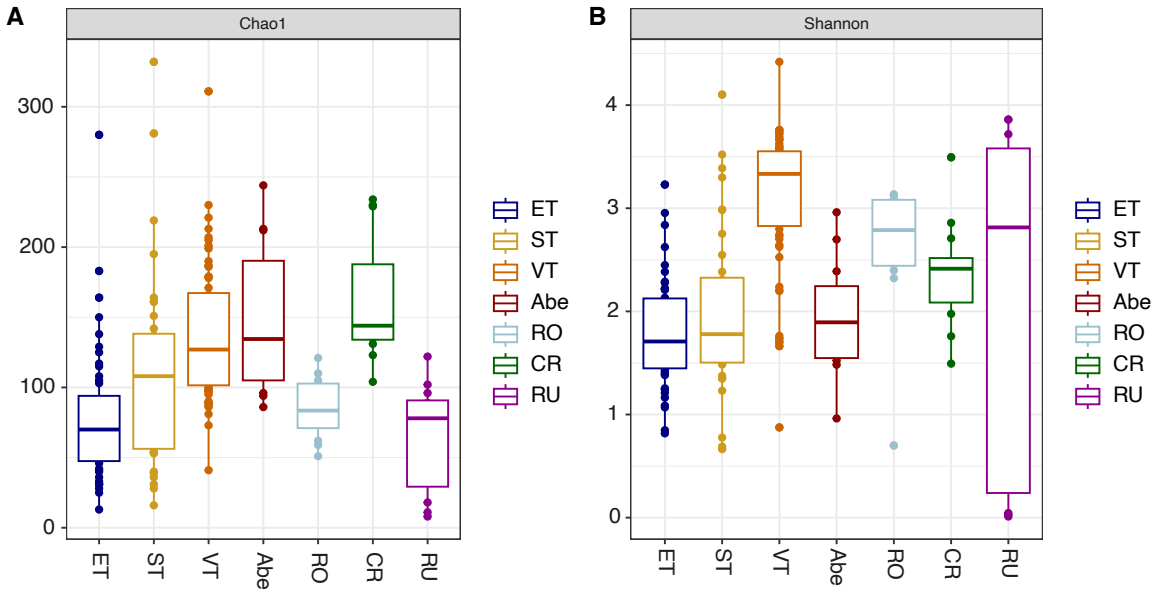
604 horizontal bars (yellow) show the total number of ASVs present in each population. Only the

605 40 most common intersections are shown.



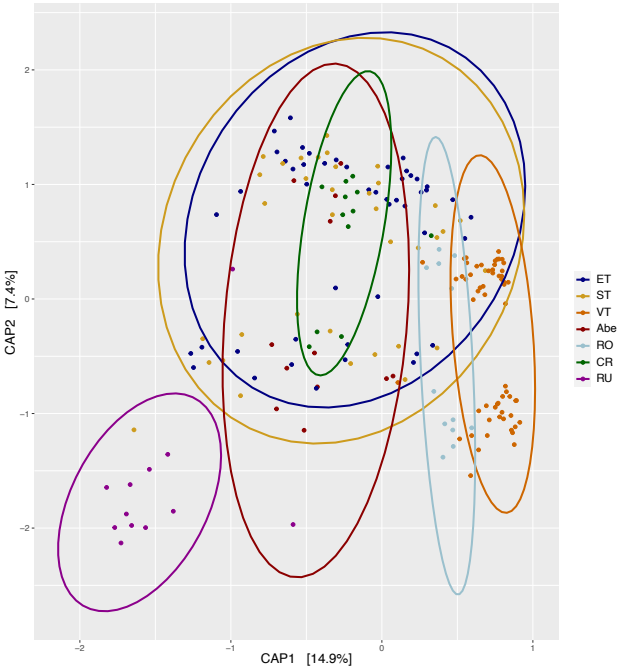

606

607 **Figure 4:** Alpha diversity of the bacterial communities of *Ips typographus* from different
608 geographical regions. Comparison of bacterial species richness (Chao1, A) and bacterial
609 diversity (Shannon index, B).



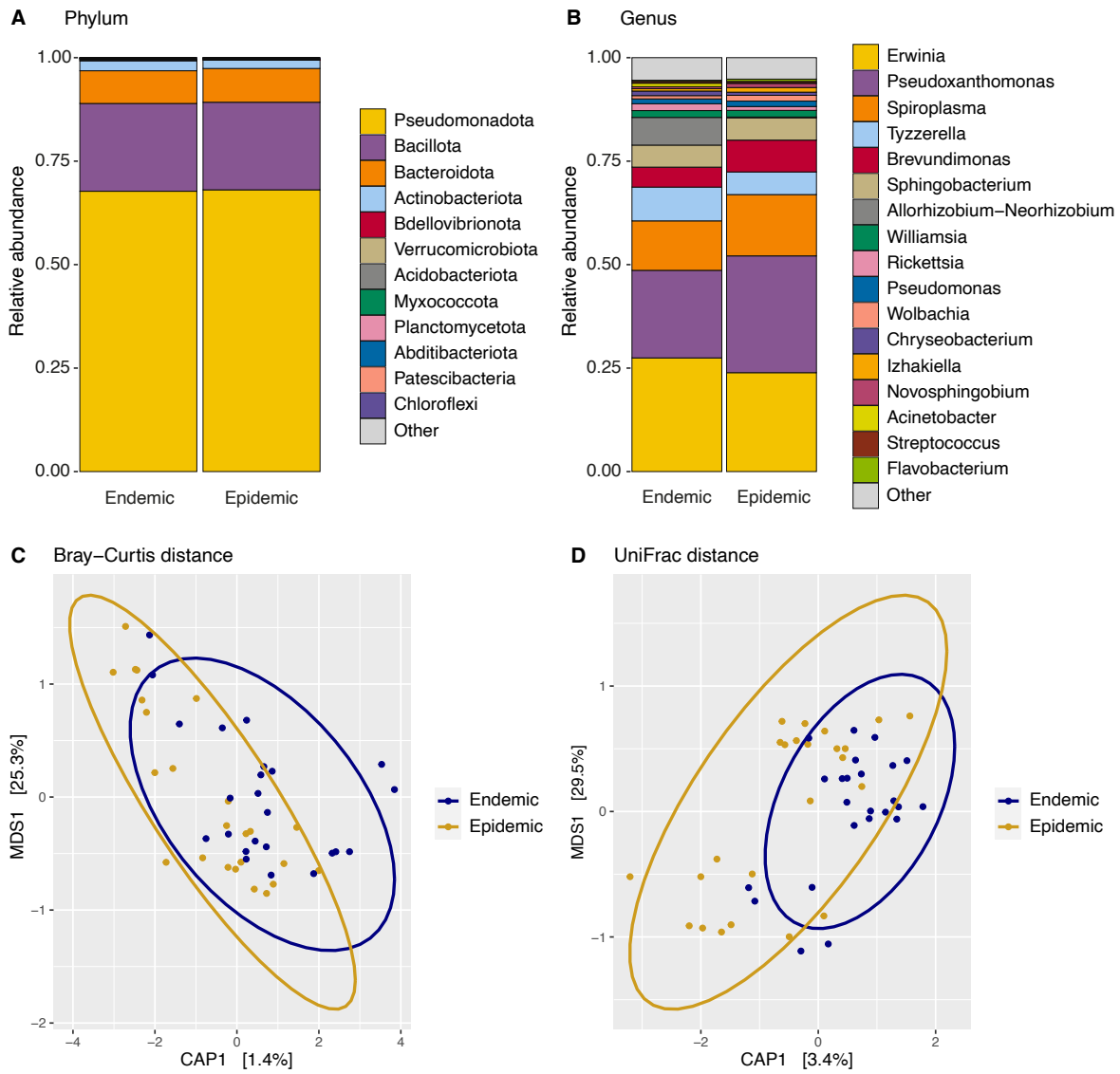

610

611 **Figure 5:** Canonical analysis of principal coordinates (CAP) based on unweighted UniFrac
612 distance showing differences in bacterial community structure of *Ips typographus* among
613 different geographic regions.



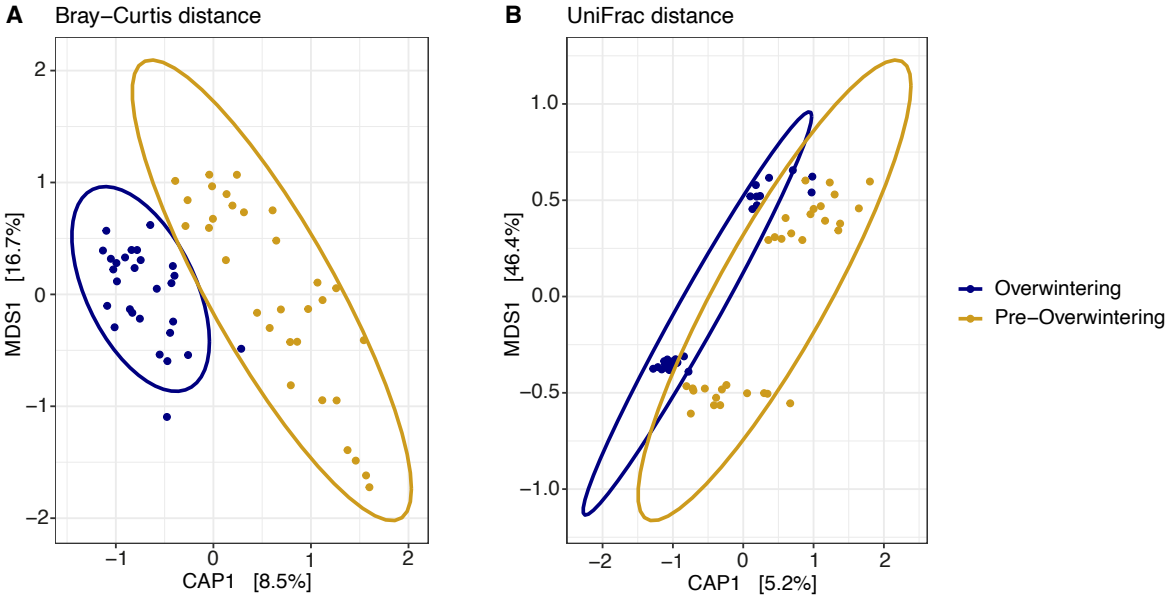

614

615 **Figure 6:** Bacterial community of endemic and epidemic populations of *I. typographus* from
616 Eastern Tyrol at phylum level (A) and genus level (B). In (B) only the 17 most abundant genera
617 were plotted. Canonical analysis of principal coordinates (CAP) based on Bray-Curtis
618 dissimilarity (C) and unweighted UniFrac distance (D) between endemic and epidemic
619 populations.




620

621 **Figure 7:** Canonical analysis of principal coordinates (CAP) based on Bray-Curtis
622 dissimilarity (C) and UniFrac distance (D) between pre-overwintering and overwintering
623 populations of *I. typographus* from Veneto.




624