

Microdiverse bacterial clades prevail across Antarctic wetlands

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Abstract

Antarctica's extreme environmental conditions impose selection pressures on microbial communities. Indeed, a previous study revealed that bacterial assemblages at the Cierva Point Wetland Complex (CPWC) are shaped by strong homogeneous selection. Yet which bacterial phylogenetic clades are shaped by selection processes and their ecological strategies to thrive in such extreme conditions remain unknown. Here, we applied the phylscore and feature-level β NTI indexes coupled with phylofactorization to successfully detect bacterial monophyletic clades subjected to homogeneous (HoS) and heterogeneous (HeS) selection. Remarkably, only the HoS clades showed high relative abundance across all samples and signs of putative microdiversity. The majority of the amplicon sequence variants (ASVs) within each HoS clade clustered into a unique 97% sequence similarity operational taxonomic unit (OTU) and inhabited a specific environment (lotic, lentic or terrestrial). Our findings suggest the existence of microdiversification leading to sub-taxa niche differentiation, with putative distinct ecotypes (consisting of groups of ASVs) adapted to a specific environment. We hypothesize that HoS clades thriving in the CPWC have phylogenetically conserved traits that accelerate their rate of evolution, enabling them to adapt to strong spatio-temporally variable selection pressures. Variable selection appears to operate within clades to cause very rapid microdiversification without losing key traits that lead to high abundance. Variable and homogeneous selection, therefore, operate simultaneously but on different aspects of organismal ecology. The result is an overall signal of homogeneous selection due to rapid within-clade microdiversification caused by variable selection. It is unknown whether other systems experience this dynamic, and we encourage future work evaluating the transferability of our results.

KEYWORDS

Antarctica, homogeneous selection, microdiversity, null models

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1 | INTRODUCTION

Antarctic ecosystems display extreme environmental conditions (Hughes et al., 2015) that drive bacterial selection pressures (Cavicchioli, 2015; Logares et al., 2018). In particular, the Cierva Point Wetland Complex (CPWC), located on the north-west coast of the Antarctic Peninsula, represents a macrobiodiversity hotspot assigned as an Antarctic specially protected area (Antarctic Treaty Secretariat, 2013; Wilhelm et al., 2016). This complex is completely covered by snowpacks that can exceed 1 m depth during winter (April–December) and is mostly snow free during the short austral summer (Wilhelm et al., 2016). Its bacterial metacommunity consists of a set of local communities, inhabiting lentic (ponds, lakes), lotic (streams, seepages, wet rocks) and terrestrial (soils, mosses, snow) environments, spatially connected by species dispersal (Leibold et al., 2004). A previous study revealed that bacterial assemblages at CPWC are shaped by strong homogeneous selection, which together with dispersal limitation acting in concert with drift and microdiversification, appear to be leading to different but very closely related bacterial amplicon sequence variants (ASVs, 100% sequence similarity operational taxonomic units; Callahan et al., 2016) across lentic, lotic and terrestrial environments (Quiroga et al., 2022). However, we still do not know which bacterial taxa or phylogenetic clades are shaped by selection processes in the CPWC nor their ecological strategies to thrive in such extreme conditions.

Here, we implemented both phyloscore (Fodelianakis et al., 2022) and feature-level β NTI (β NTI_{feat}; Danczak et al., 2022) analyses to detect bacterial phylogenetic clades subjected to selection processes. These approaches take theoretical concepts from community-level methods to quantify the ecological processes underlying community assembly (i.e. community-wide phylogenetic turnover; Dini-Andreote et al., 2015; Fodelianakis et al., 2022; Stegen et al., 2013, 2015). This allows evaluation of each taxon within the metacommunity in terms of the assembly processes it experiences (i.e. per-taxon phylogenetic turnover). Selection generates deterministic changes in community structure due to among-individual fitness differences (Vellend, 2010). Selection pressures are dictated by the spatial variation of environmental conditions. Spatially homogeneous environmental filters will consistently select close relatives with similar fitness leading to more similar among-communities structures, referred to as 'homogeneous selection'; while spatially variable (i.e. heterogeneous) environmental filters will select more distant relatives with fitness differences leading to more dissimilar among-communities structures, referred to as 'heterogeneous selection' (Dini-Andreote et al., 2015; Stegen et al., 2015; Zhou & Ning, 2017). These are ecological selection processes that do not require evolution and they occur at the community level. The type of ecological selection is identified based on the observed phylogenetic turnover between communities (i.e. β MNTD), which can be lower than, higher than or equal to the null model expectation (i.e. when selection does not influence community turnover; Dini-Andreote et al., 2015; Stegen et al., 2013, 2015). Lower than expected phylogenetic turnover indicates homogeneous selection,

while higher than expected phylogenetic turnover indicates heterogeneous selection. In line with this approach, the phyloscore (Fodelianakis et al., 2022) and β NTI_{feat} (Danczak et al., 2022) analyses identify bacterial taxa with lower or higher than expected phylogenetic turnover across communities. Thus, phylogenetic clades composed of bacterial taxa with lower than expected phylogenetic turnover are identified as subjected to homogeneous selection (HoS clades), while clades encompassing taxa with higher than expected phylogenetic turnover are identified as being shaped by heterogeneous selection (HeS clades) (Fodelianakis et al., 2022). Clades with phylogenetic turnover that does not deviate from null model expectations are identified as being primarily governed by stochasticity (not shaped by selection, non-S clades).

Considering that community-wide lower than expected phylogenetic turnover dominates the bacterial metacommunity within the CPWC (Quiroga et al., 2022), we expect to find numerous HoS clades with high relative abundances. Furthermore, we hypothesize that these HoS clades are ubiquitously distributed across multiple habitats. Ubiquitous and abundant (i.e. ecologically successful) bacterial clades can show high microdiversity (Fodelianakis et al., 2022; García-García et al., 2019; Needham et al., 2017; Props & Deneff, 2020). Microdiverse taxa contain genetically similar subtaxa, or ecotypes (Cohan, 2001), that can partition niche space within the environment. Microdiversity patterns then can be identified by studying the different spatial or temporal abundance distribution of distinct ecotypes across environmental gradients (García-García et al., 2019; Larkin et al., 2016; Needham et al., 2017; Thompson et al., 2021). Therefore, we further examine microdiversity within the bacterial HoS clades identified in the CPWC by means of phylogenetic indexes and relative abundance patterns. Our ultimate objective is to understand the interplay among different eco-evolutionary processes operating at different scales (i.e. ecological selection at the community and clade levels, and microdiversification within clades), and how they jointly influence bacterial community assembly.

2 | MATERIALS AND METHODS

2.1 | Samples and data processing

We analysed a set of 64 samples (i.e. 64 communities) from the CPWC surveyed over the early 2018 Antarctic summer (Quiroga et al., 2022). Each sample was collected and processed individually as previously described (Quiroga et al., 2022). Briefly, the 16S rRNA genes (V3–V4 regions) were sequenced using an Illumina MiSeq platform (2 × 300 paired-end reads). ASVs were determined using DADA2 v1.16.0 (Callahan et al., 2016) and classified using *assignTaxonomy* with the SILVA database v138 (Quast et al., 2013). The ASV count table was normalized to an equal sampling depth of 6284 reads per sample, and a total of 402,176 total reads and 3960 ASVs were retained for further analysis. A phylogenetic tree was constructed using qiime2 (Bolyen et al., 2019) with the

q2-phylogeny plugin (align-to-tree-mafft-fasttree pipeline). This pipeline starts by creating a sequence alignment using MAFFT, after which any alignment columns that are phylogenetically uninformative or ambiguously aligned are removed (masked). The resulting masked alignment is used to infer a phylogenetic tree and then subsequently rooted at its midpoint. The phylogenetic tree is constructed using fastTree (Price et al., 2010), which infers approximately maximum-likelihood phylogenetic trees. FastTree uses the Jukes–Cantor or generalized time-reversible (GTR) models of nucleotide evolution. To account for the varying rates of evolution across sites, FastTree makes use of a single rate for each site (the 'CAT' approximation). To quickly estimate the reliability of each split in the tree, FastTree computes local support values with the Shimodaira–Hasegawa test.

Sequences were aligned using *align.seqs* with the SILVA v138 reference file *silva.seed_v138.align* (Quast et al., 2013) in Mothur v.1.48 (Schloss et al., 2009), filtered using *filter.seqs* and pairwise nucleotide dissimilarities were calculated with *dist.seqs*. Additionally, bacterial ASVs were clustered into 97% sequence similarity operational taxonomic units (OTU97) using the Opticlust algorithm (Westcott & Schloss, 2017) following García-García et al. (2019). The sequence data are publicly available at NCBI BioProject database (ID PRJNA719989, 64 sequence data links, <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA719989>).

2.2 | Statistical analyses

2.2.1 | Identification of phylogenetic clades under selection processes

Phylogenetic clades were considered to be subjected to selection processes only when they were consistently identified with both phyloscore and $\beta\text{NTI}_{\text{feat}}$ indexes. Phyloscore for each ASV was calculated following the R code available at https://github.com/sfodel/phylo_z_scores (Fodelianakis et al., 2022). Among the 10 phylogenetic clades identified with this approach, seven were consistently identified using the sum, average and median phyloscore metrics. Thus, we report the average phyloscore values, referred hereafter as phyloscores (Supporting Methods, Figure S1). $\beta\text{NTI}_{\text{feat}}$ for each ASV was estimated modifying the script available at <https://github.com/danczakre/betaNTI-feature> (Danczak et al., 2022) to account for the low phylogenetic turnover in our system (see Supporting Methods, Figures S2 and S3 for details). Briefly, first we removed all comparisons among community pairs where the focal ASV was present in both communities. The original code considered as equivalents the minimum phylogenetic distance between a focal ASV in one community and its nearest ASV in another community when the latter was itself or a closely related ASV (i.e. both zero phylogenetic distances), although they represent distinct phylogenetic scenarios (Larkin & Martiny, 2017; Sriswasdi et al., 2017). Not removing comparisons among community pairs where the focal ASV was present in

both communities could diminish the ability of the original $\beta\text{NTI}_{\text{feat}}$ code to detect HoS clades by decreasing the $\beta\text{MNTD}_{\text{feat}}$ values (i.e. observed $\beta\text{MNTD}_{\text{feat}}^{\text{obs}}$, average value of the null model distribution $\langle \beta\text{MNTD}_{\text{feat}}^{\text{null}} \rangle$ and standard deviation of the null model distribution $\beta\text{MNTD}_{\text{feat}}^{\text{sd}}$; see equation 2 in Supporting Methods). Secondly, a natural logarithm transformation (Legendre & Legendre, 1998) was applied to the $\beta\text{MNTD}_{\text{feat}}$ indexes (i.e. $\beta\text{MNTD}_{\text{feat}}^{\text{obs}}$, $\langle \beta\text{MNTD}_{\text{feat}}^{\text{null}} \rangle$ and $\beta\text{MNTD}_{\text{feat}}^{\text{sd}}$). Working with a natural logarithm scale (related to proportional change) instead of a linear scale (related to absolute change) improved the capability of $\beta\text{NTI}_{\text{feat}}$ to detect homogeneous selection in our system. The reason is that ln-transformation increases the quantitative spread across small values, thereby increasing the ability of $\beta\text{NTI}_{\text{feat}}$ to resolve small phylogenetic distances associated with HoS. $\beta\text{NTI}_{\text{feat}}$ modified code is available at GitHub (<https://github.com/mvquiroya/NullModels>).

The ASVs phyloscore and $\beta\text{NTI}_{\text{feat}}$ values were used for phylofactorization with phylofactor R package (Washburne et al., 2019). Phylofactorization iteratively partitions the phylogeny, cutting at the edge that maximizes a contrast between two groups (on either side of the edge) at each iteration, allowing the identification of clades with distinct ASVs phyloscore or $\beta\text{NTI}_{\text{feat}}$ values. We implemented 10 iterations (i.e. factors). As $\beta\text{NTI}_{\text{feat}}$ showed a normal distribution, the default two-sample contrast method of phylofactorization was applied, whereas the non-normal distribution of the phyloscore required a Wilcoxon test for phylofactorization. All identified monophyletic clades (i.e. group 1 of each factor) were significantly different (i.e. contrast test $p < .001$) from their complementary group (i.e. group 2 of the corresponding factor). Each identified monophyletic clade was assigned as subjected to homogenous or heterogeneous selection (i.e. HoS or HeS clades, respectively) based on their ASVs phyloscore or $\beta\text{NTI}_{\text{feat}}$ values (i.e. negative or positive mean values, respectively; Table S1). These assignments are done without considering a significance threshold beyond the phylofactorization analysis (see Discussion for further details). The remaining complementary groups were considered together as the outgroup and assigned as not being under selection (non-S clades). To check for biases due to inadequate sequencing depth, individual rarefaction curves were generated for each identified clade and the outgroup with *rarecurve* (*vegan*; Oksanen et al., 2019). Data visualization and graphics were performed with *ggplot2* (Wickham, 2016) and *ggtree* (Yu, 2022).

2.2.2 | Putative microdiversity evaluation

We assessed microdiversity within each phylogenetic clade under selection and the outgroup (i.e. non-S clades) by comparing the distribution of their ASVs' nearest taxon distance (NTD) and nucleotide similarity of β -nearest ASVs (Fodelianakis et al., 2022). Statistical differences between clades (i.e. HeS clade, five HoS clades and the outgroup) were evaluated independently for NTD and nucleotide similarity using global Kruskal–Wallis tests, and further Mann–Whitney post hoc pairwise comparisons applying Bonferroni

correction for testing multiple clade pairs. The NTD and nucleotide similarity of β -nearest ASVs indexes measure the phylogenetic distance and the nucleotide similarity, respectively, between a given ASV and its closest relative in each community where the focal ASV is not present. The value of each index per ASV was estimated by averaging the values across all community pairs. Both indexes were derived from the nearest phylogenetic distance ($d_{i,j,k}$) in the phyloscore formula (see Supporting [Methods](#)). The codes are available on GitHub (<https://github.com/mvquiroga/NullModels>). Within a clade, microdiversification should shift the distribution of NTD towards lower values (i.e. lower phylogenetic distance) compared to the outgroup; whereas fine-scale ecological differentiation should shift the distribution of nucleotide similarity among β -nearest ASVs towards values $>97\%$ 16S rRNA gene identity, indicating spatial substitution among subtaxa (Fodelianakis et al., 2022). In this context, microdiversification refers to very small sequence divergences within clades; whereas fine-scale ecological differentiation refers to subtaxa that have greater than 97% 16S rRNA gene similarity and distinct niche space. The distinction is important because microdiverse sequence divergence can have, or not, adaptive implications. By definition, NTD and nucleotide similarity of β -nearest ASVs examine the shortest phylogenetic distances, and therefore, they should not be affected by the clade breadth. As we observed a significant positive relationship between the mean NTD and the phylogenetic depth (i.e. ASV distance from the root), we calculated a linear regression as a detrending method to further work with the NTD residuals ([Figure S4](#)). This NTD detrending is a prerequisite to compare clades with unequal phylogenetic depth (Fodelianakis et al., 2022).

To further identify distinct habitat associations of potential ecotypes across Cierva Point environments, we inspected the abundance patterns of the ASVs clustering into unique OTU97 within each HoS clade via Venn diagrams with *microeco* (Liu et al., 2021) and heatmaps with *complexHeatmap* (Gu, 2022). According to their origin, samples were distributed into three environment types: lentic (ponds and lakes, $n=22$), lotic (streams, seepages and wet rocks, $n=18$) and terrestrial (soils, mosses and snow, $n=24$).

3 | RESULTS

3.1 | Phylogenetic clades consistently identified with phyloscore and $\beta\text{NTI}_{\text{feat}}$ indexes

Seven phylogenetic clades were consistently identified with phylofactorization using both indexes and were therefore retained for further analysis ([Figure 1](#)). Individual rarefaction curves, based on normalized sample counts, for these selected phylogenetic clades showed that the number of ASVs in most of the samples reached a plateau ([Figure S5](#)), suggesting there was no bias due to inadequate sequencing depth.

We focus on the five clades, with 93–309 ASVs each ([Figure 1](#), [Table S1](#)), that showed low phylogenetic turnover (i.e. mean negative phyloscore and $\beta\text{NTI}_{\text{feat}}$ values), indicating that they might be

subjected to homogeneous selection (HoS clades). The consensus taxonomy of these clades affiliated to class Actinobacteria (309 ASVs), order Xanthomonadales (102 ASVs) and families *Comamonadaceae* (178 ASVs), *Flavobacteriaceae* (116 ASVs) and *Sphingobacteriaceae* (93 ASVs). Remarkably, although the metacommunity was shaped by homogeneous selection (Quiroga et al., 2022), we identified one HeS clade (i.e. showing high phylogenetic turnover, mean positive phyloscore and $\beta\text{NTI}_{\text{feat}}$ values) composed of 1311 ASVs affiliated to 27 different phyla. This clade hosted a subclade of 157 ASVs affiliated to the phylum Firmicutes ([Figure 1](#), [Table S1](#)). This subclade was not analysed further.

The HeS clade (1311 ASVs), the five HoS clades (798 ASVs) and the non-S clades (1851 ASVs) accounted for, on average, 24%, 30% and 46% of the total number of ASVs per sample respectively ([Figure 2a](#)). The five HoS clades dominated the communities representing, on average, 46% of the total number of reads per sample ([Figure 2b](#)), while the HeS clade showed a low relative abundance per sample (average 12%). The non-S clades accounted for, on average, 42% of the relative abundance per sample. The number of reads in clades per sample normalized by the number of ASVs in clades per sample were, on average, 27, 55 and 97 reads per ASV in HeS clade, non-S clades and HoS clades respectively.

3.2 | Abundant HoS clades showed signs of microdiversity

We examined the distribution of the NTD and nucleotide similarity among β -nearest ASVs within each phylogenetic clade and the outgroup (non-S clades) to assess putative microdiversity patterns. Interestingly, the outgroup showed an NTD residual close to zero (average 0.08), which is significantly lower than that of the HeS clade (average 0.35, $p < .001$) and significantly higher than those of the five HoS clades (average from -0.93 to -0.35 , all $p < .001$, [Figure 3a](#)). In concordance, the distribution of nucleotide similarity among β -nearest ASVs significantly shifted towards values $>97\%$ in HoS clades, with 82%–100% of these replacements occurring among subtaxa ([Figure 3b](#)). The opposite trend was observed for the HeS clade, with only 6% of the replacements occurring among ASVs that showed $>97\%$ similarity. Within the outgroup, 48% of the replacements occurred among subtaxa.

Four monophyletic HoS clades were composed mainly of putative subtaxa (groups of ASVs sharing $>97\%$ nucleotide identity) ([Figure 3c](#)). Those clades, affiliated to families *Sphingobacteriaceae*, *Flavobacteriaceae*, *Comamonadaceae* and order Xanthomonadales, had 100%, 95%, 82% and 93% of their ASV pairwise comparisons, respectively, showing $>97\%$ nucleotide identity. In the other HoS clade, affiliated to class Actinobacteria, 40% of the ASV pairwise comparisons were among subtaxa. In contrast, in the HeS clade and the outgroup (non-S clades) only 2% and 7% of the ASV pairwise comparisons, respectively, were between subtaxa ([Figure 3c](#)). As the HeS clade and non-S clades did not show signs of microdiversity they were not analysed further.

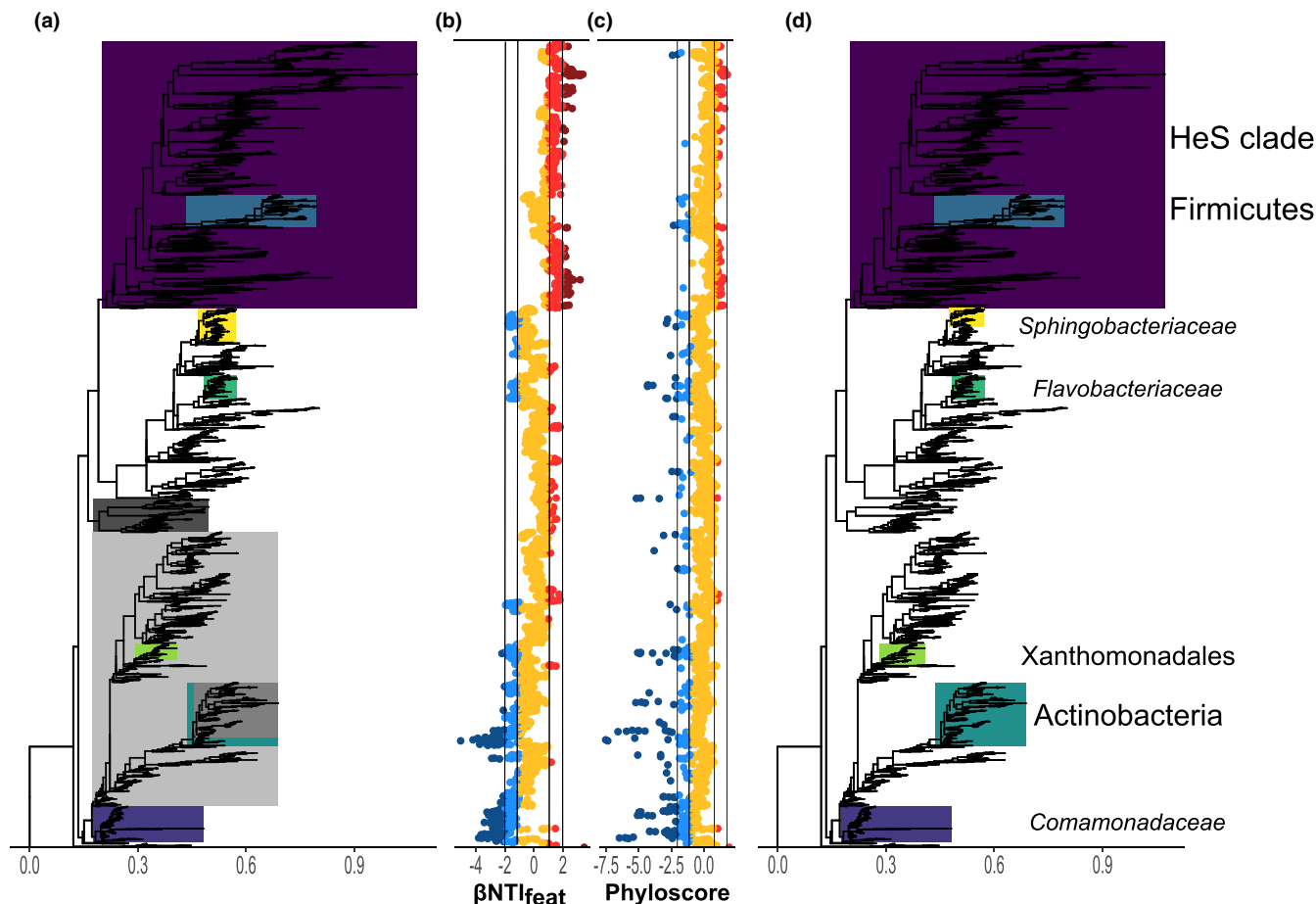


FIGURE 1 Identification of phylogenetic clades under selection processes. (b) $\beta\text{NTI}_{\text{feat}}$ and (c) phyloscore values for each ASV. (a), (d) Phylogenetic clades identified with phylofactorization for each index respectively. The consensus taxonomy of the HoS (homogeneous selection) clades consistently identified with both indexes is indicated on the right with font size proportional to taxonomic depth. The HeS (heterogeneous selection) clade is composed by 27 phyla, with a Firmicutes subclade. (b), (c) Dot colour and vertical lines indicate significance thresholds (i.e. -2, -1, 1, 2) suggested by Danczak et al. (2022).

3.3 | OTU97 composition of HoS clades and their ASVs distribution patterns

Those HoS clades affiliated to families *Spingobacteriaceae*, *Flavobacteriaceae*, *Comamonadaceae* and order Xanthomonadales displayed the 100% (93/93), 97% (113/116), 90% (161/178) and 96% (98/102) of their ASVs clustering into a unique OTU97 (genus *Pedobacter*, *Flavobacterium*, *Polaromonas* and *Rhodanobacter* respectively) (Figure 4). In contrast, the ASVs within the HoS clade affiliated to class Actinobacteria clustered into three OTU97 (genus *Glaciihabitans*, *Humibacillus* and *Nakamurella*; Figure 4). It is noteworthy that the five HoS clades contained 7 of the 10 most abundant OTU97 across the CPWC.

For each of the five HoS clades, we selected the ASVs that clustered into specific OTU97 and analysed their distribution patterns. Those unique OTU97 in HoS clades affiliated to *Spingobacteriaceae*, *Flavobacteriaceae*, *Comamonadaceae* and Xanthomonadales, and the three OTU97 affiliated to Actinobacteria are shown in Figure 5. Relatively few ASVs (4–21% of ASVs accounting for 22–66% of the total reads per OTU97) were shared between the three

environments (Figure 5). Conversely, most of the ASVs (57%–83% of ASVs comprising 15%–48% of the total reads per OTU97) were only present in a specific environment. In addition, 44 ASVs from genus *Rhodanobacter* (HoS clade Xanthomonadales) were specific to terrestrial environments, showing higher average sample reads (Figure S6) and representing 38% of the total reads for this OTU97 (Figure 5).

4 | DISCUSSION

4.1 | Putative microdiverse HoS clades prevail across the Antarctic wetland complex

In line with the high contribution of ecological homogeneous selection in the bacterial community assembly at CPWC (Quiroga et al., 2022), we consistently identified five bacterial HoS clades driving these assemblages. As expected, the HoS clades showed high relative abundance across all samples, resembling ecologically successful clades that thrive in the extreme CPWC. Furthermore,

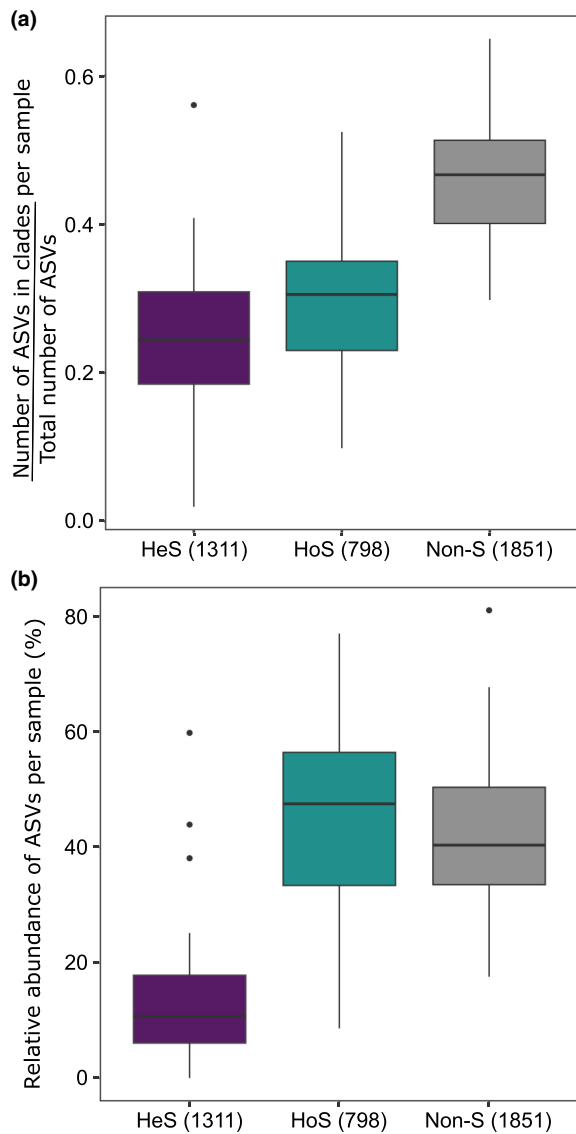


FIGURE 2 HoS clades contained approximately a third of the ASV richness and half of the relative abundance per sample. (a) Ratio of the number of ASVs in clades (HeS) or group of clades (HoS, non-S) over the total number of ASVs per sample. (b) Relative abundance of ASVs per sample. HeS: heterogeneous selection clade, HoS: all five homogeneous selection clades, Non-S: non-selection clades (outgroup). Points outside the whiskers represent outliers. The number of ASVs per clade or group of clades is shown between brackets.

the ASVs within HoS clades displayed, on average, 1.8 and 3.6 times higher number of reads per sample than those from non-S and HeS clades respectively. In addition, the identified HeS clade showed distinctly low relative abundance per sample, potentially explaining the overall strong homogeneous selection detected at the community level (Quiroga et al., 2022). Signs of strong homogeneous selection were previously reported for bacterial communities from other extreme ecosystems, such as Antarctic lakes (Logares et al., 2018), glacier-fed streams (Fodelianakis et al., 2022), proglacial floodplain streams (Brandani et al., 2023) and the oligotrophic South Pacific Gyre (Allen et al., 2020). In all, the present results are consistent with

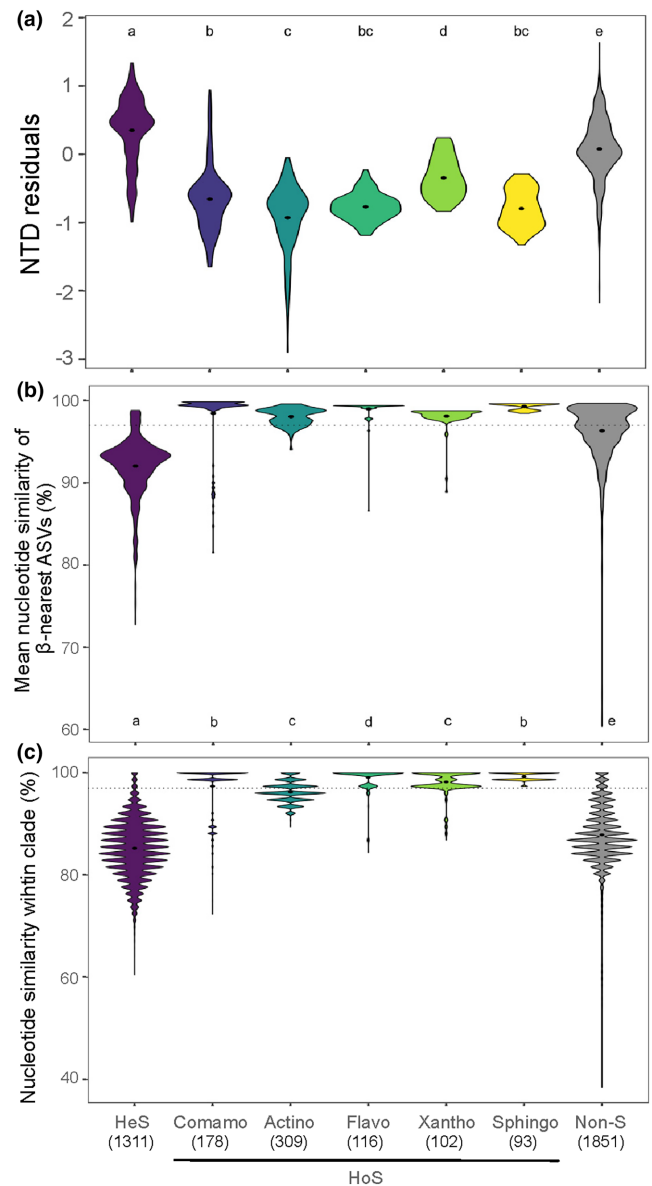


FIGURE 3 HoS clades showed signs of microdiversity. Violin plots of (a) NTD residuals and (b) mean nucleotide similarity of β -nearest ASVs across phylogenetic clades. (c) Nucleotide similarity within each clade. Different letters indicate significant differences between group means (Mann-Whitney post hoc pairwise comparisons, $p < .001$). HeS: heterogeneous selection clade, HoS: homogeneous selection clades, Comamo: *Comamonadaceae*, Actino: Actinobacteria, Flavo: *Flavobacteriaceae*, Xantho: Xanthomonadales, Sphingo: *Sphingobacteriaceae*, Non-S: non-selection clades (outgroup). The number of ASVs per clade is shown between brackets. Points represent means per clade, dotted lines represent 97% nucleotide similarity.

our working hypothesis that HoS clades are highly abundant and ubiquitously distributed across the different environments within the CPWC.

Strikingly, only the HoS clades showed signs of putative microdiversity (Fodelianakis et al., 2022). In other words, ASVs within HoS clades had more similar closest relatives compared to ASVs within the HeS and non-S clades, and most of these replacements occurred

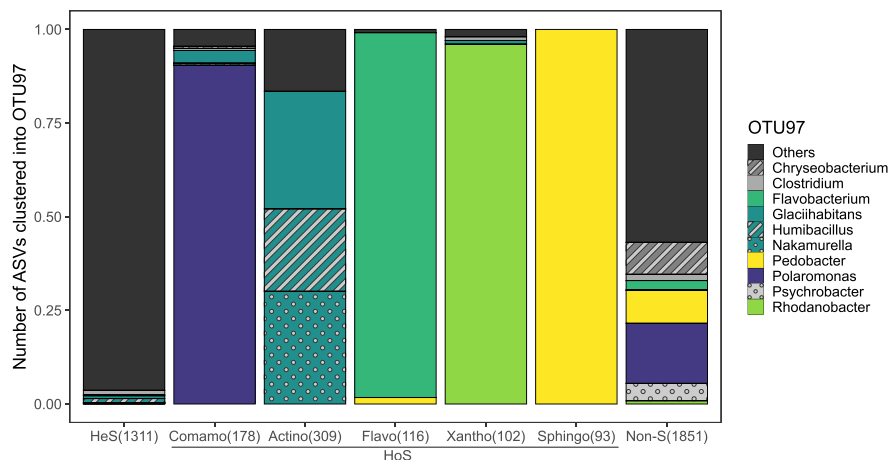


FIGURE 4 HoS clades were mainly composed by ASVs that clustered into a unique 97% sequence similarity operational taxonomic unit (OTU97), except for class Actinobacteria (Actino). The taxonomic classification (genus) of the top 10 most abundant OTU97 are shown. HeS: heterogeneous selection clade, HoS: homogeneous selection clades, Comamo: *Comamonadaceae*, Actino: Actinobacteria, Flavo: *Flavobacteriaceae*, Xantho: Xanthomonadales, Sphingo: *Sphingobacteriaceae*, Non-S: non-selection clades (outgroup). The number of ASVs per clade is shown between brackets. The group 'Others' refers to less abundant OTUs.

among subtaxa sharing >97% nucleotide identity. In the CPWC, microdiversification of HoS clades appears to occur within local communities potentially isolated by the snow-covered landscape during most of the year. Certainly, literature highlights that microdiversification appears to be especially important within communities with extremely low or zero dispersal rates (Georgiades & Raoult, 2011; Leibold et al., 2004; Stegen et al., 2013).

This work points to an eco-evolutionary scenario of ecological selection-dictated (i.e. non-random) microdiversification, as postulated by Larkin and Martiny (2017), Nemergut et al. (2013) and Zhou and Ning (2017). As microdiverse bacterial taxa are usually composed by ecotypes, or subtaxa (Cohan, 2001), that can partition niche space within the environment, the microdiverse HoS clades identified in the CPWC should display microdiversification-dictated ecological versatility that enables them to occupy different ecological niches. These HoS clades were composed by versatile bacterial phylotypes that are common, and abundant, inhabitants of Antarctic environments (Doytchinov & Dimov, 2022; Soto et al., 2022). For instance, members of the genus *Polaromonas* (HoS clade *Comamonadaceae*), generally found on glacier surfaces, can contain a high level of metabolic diversity (Smith et al., 2014), which facilitate their adaptation to specific environmental conditions (Ciok et al., 2018). Indeed, Gawor et al. (2016) found evidence of niche differentiation and microdiversification within this genus. *Flavobacterium* spp. (HoS clade *Flavobacteriaceae*) inhabit different Antarctic habitats, such as lakes, ponds, microbial mats, glaciers and snow (Doytchinov & Dimov, 2022; Soto et al., 2022), and some display a remarkably diverse metabolic potential to utilize different carbon sources (Gangwar et al., 2011). *Polaromonas* spp. co-occur with *Pedobacter* (HoS clade *Sphingobacteriaceae*) and *Flavobacterium* spp. in snow algae blooms in maritime Antarctica, apparently competing for resources (Soto et al., 2022). In agreement with this, the enzymatic activity at 4°C of *Pedobacter* and *Polaromonas* isolates indicates their capacity to metabolize complex organic compounds as lipids, carbohydrates and lignin, even at low temperatures (Antony et al., 2016). The

draft genome of a *Humibacillus* strain (HoS clade Actinobacteria), isolated from maritime Antarctic soil, revealed the presence of cold-shock proteins and potential biosynthesis of cryoprotectant substances, suggesting their capability to resist extremely cold temperatures and withstand prolonged desiccation periods (Thomas et al., 2018). The strictly aerobic *Nakamurella antarctica* (strain S14-144) and *Glacihabitans arcticus* (strain RP-3-7) (HoS clade Actinobacteria) were reported to grow at 4–28°C in a wide pH range (5.0–8.0 and 6.0–8.5, respectively), showing diverse physiological and biochemical characteristics (Da et al., 2019; Dahal & Kim, 2019). Furthermore, the denitrifying bacterial genus *Rhodanobacter* (HoS clade Xanthomonadales) was previously reported as abundant in penguin-impacted Antarctic soils (Aislabie et al., 2009; Ramírez-Fernández et al., 2019, 2021). *Rhodanobacter* also shows a versatile metabolism, including facultative anaerobiosis (Prakash et al., 2012) and use of amino acids rather than sugars for growth (Lee et al., 2007). In summary, numerous taxa found in HoS clades have previously been shown to have significant ecological and metabolic flexibility, which we propose is key to the dominance of HoS clades across the CPWC.

Based on the ASV distribution of each HoS OTU97, the ecological success of the HoS clades appears to also be facilitated by a combination of relatively few 'generalist' ASVs ubiquitously distributed across the wetland complex and a larger group of 'specialist' ASVs adapted to specific (i.e. lentic, lotic, terrestrial) environments within the landscape. It is important to highlight that we are not discussing the generalist versus specialist concept at the species level (Sriswasdi et al., 2017; Székely & Langenheder, 2014), but rather at the intraspecific level. In this context, 'specialist' ASVs can represent ecotypes shaped by variable selection processes in the CPWC. We postulate that this environment-dictated ASV distribution reflects ecologically distinct groups of 'specialist' ASVs within each OTU97, which appear to be shaped by eco-evolutionary processes leading to subtaxa niche differentiation. Such differential spatial distributions across environmental gradients have been previously observed for

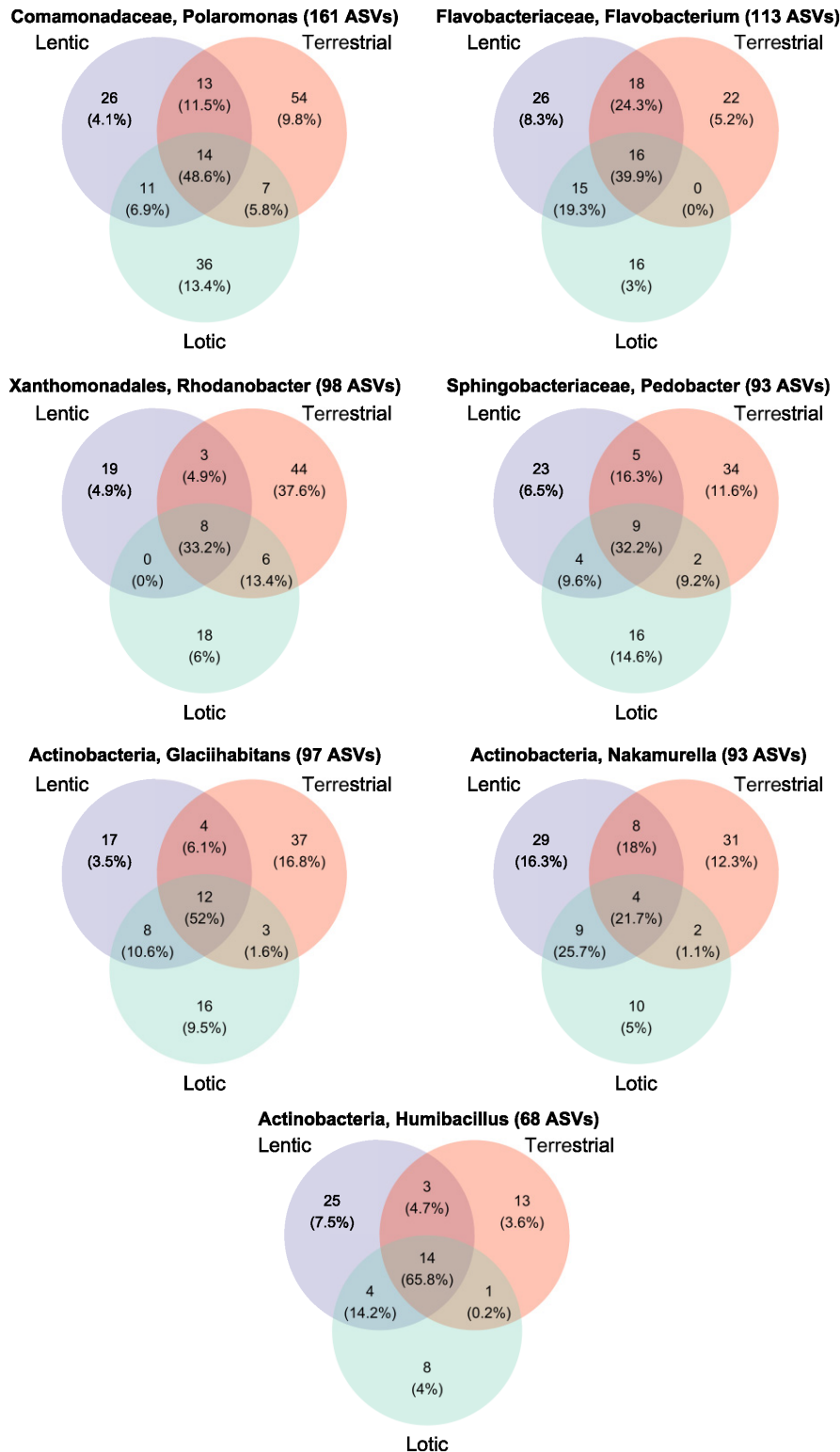


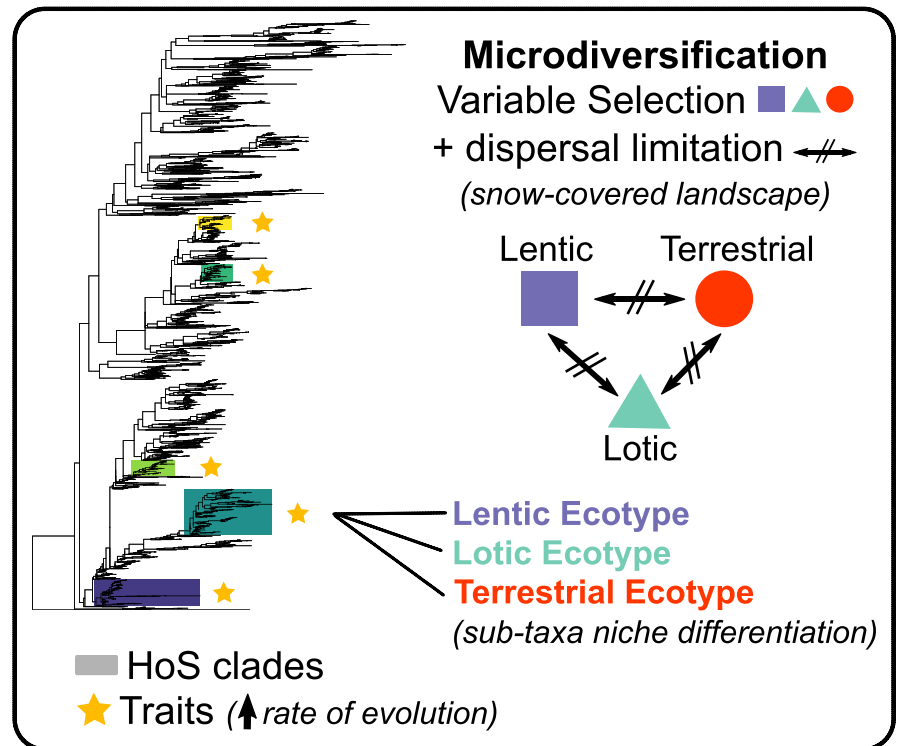
FIGURE 5 Environmental distribution of ASVs clustering into unique OTU97 within each HoS (homogeneous selection) clade. Subtitles indicate the consensus taxonomy of the clade (class, order or family), the genus of the OTU97 and the number of ASVs per OTU97 between brackets. Venn diagrams show the number of unique and shared ASVs. The percentages of sequences associated with those ASVs are shown between brackets. Samples were grouped as proceeding from lentic, lotic and terrestrial environments.

distinct ecotypes (García-García et al., 2019; Larkin et al., 2016; Nagarkar et al., 2021; Needham et al., 2017; Thompson et al., 2021). Considering that ecotypes consist of populations of cells adapted to a given ecological niche, this might suggest that there are lentic, lotic and terrestrial ecotypes, consisting of groups of 'specialist' ASVs for each HoS OTU97. Furthermore, we hypothesized that the 'generalist' ASVs ubiquitously distributed across the landscape represent

the microdiverse pool that through evolutionary adaptation will result in 'specialist' ASVs, similar to the generalist-specialist evolutionary cycle proposed by Sriswasdi et al. (2017).

Taking into account these results, we propose a conceptual model to explain the eco-evolutionary processes shaping the bacterial HoS clades of this metacommunity (Figure 6). Briefly, in the CPWC, the microdiversification of bacterial clades is driven by strong selection

FIGURE 6 A conceptual model of the eco-evolutionary processes shaping HoS bacterial clades within the CPWC. We hypothesize that these ecologically successful HoS bacterial clades have phylogenetically conserved traits that accelerate their rate of evolution, facilitating the microdiversification processes, which are enhanced by dispersal limitation. This variable selection process appears to be sufficiently fast, on an evolutionary timescale, to lead to subtaxa niche differentiation (i.e. different ecotypes) and an overall signal of homogeneous selection.



pressures that vary in both space and time. This variable selection process appears to be sufficiently fast, on an evolutionary timescale, to lead to niche differentiation among different ecotypes and an overall signal of homogeneous selection both at the community (Quiroga et al., 2022) and phylogenetic clade levels. The detection of a strong selection signal points towards a low-dispersal scenario, as high-dispersal rates should erode the signal of selection (Dini-Andreote et al., 2015; Stegen et al., 2013, 2015). More specifically, the heavily restricted dispersal imposed by the snow-covered CPWC landscape appears to facilitate the microdiversification processes (Nemergut et al., 2013; Stegen et al., 2013; Zhou & Ning, 2017). We also hypothesize that microdiverse bacterial clades thriving in this system have phylogenetically conserved traits that accelerate their rate of evolution, enabling them to rapidly adapt to strong and spatio-temporally variable selection pressures within the CPWC environments, resembling trade-offs related to trait evolutionary responses (Schmid et al., 2022). Nevertheless, as variation in highly conserved marker genes (e.g. 16S rRNA) represents distant evolutionary divergences (Martiny et al., 2023), experimental work in conjunction with shotgun metagenomics and genome sequences of closely related strains is needed to evaluate the suggested eco-evolutionary processes.

4.2 | Methodologies to detect bacterial phylogenetic clades shaped by selection processes

The phyloscore (Fodelianakis et al., 2022) and $\beta\text{NTI}_{\text{feat}}$ (Danczak et al., 2022) indexes coupled with phylofactorization (Washburne et al., 2019) applied in this work detected bacterial phylogenetic clades

composed of ASVs subjected to homogeneous and heterogeneous selection. The original $\beta\text{NTI}_{\text{feat}}$ code (Danczak et al., 2022) detected a weak homogeneous selection signal within our data set, which can be attributed to (1) the inclusion of uninformative pairwise comparisons in which both communities contain the focal ASV, and (2) a highly positive skewed distribution of $\beta\text{NTI}_{\text{feat}}$ values. Therefore, considering that both community-wide phylogenetic turnover (i.e. βNTI ; Quiroga et al., 2022) and per-taxon phylogenetic turnover (i.e. phyloscore) detected clear signals of homogeneous selection in our data set, we developed and applied a modified version of the $\beta\text{NTI}_{\text{feat}}$ code that improved its capability to detect ASVs with lower than expected phylogenetic turnover in the CPWC. This was achieved by removing all comparisons among community pairs where the focal ASV was present in both communities and transforming the linear scale of $\beta\text{NTI}_{\text{feat}}$ values to a natural logarithmic one to achieve a less skewed distribution (i.e. more balance tails) (Legendre & Legendre, 1998) (see Materials and Methods and Supporting Methods sections for details). We propose that this modified $\beta\text{NTI}_{\text{feat}}$ code may be useful to identify ASVs with low phylogenetic turnover in other ecosystems displaying a strong signal of homogeneous selection at the community level.

It is important to highlight the fact that we restricted the phylofactorization analysis (Washburne et al., 2019) to identify only the first 10 factors, and we acknowledge that undetected HoS clades could remain within the outgroup (non-S clades). We suspect that several ASVs with negative phyloscore and $\beta\text{NTI}_{\text{feat}}$ values, NTD residuals <0 (Figure 3a) and showing replacements among subtaxa (Figure 3b) within the non-S clades, correspond to one or several HoS clades (Fodelianakis et al., 2022) not detected with our phylofactorization pipeline.

4.3 | The path forward

A significant challenge to studying selection processes shaping individual taxa within communities and metacommunities is the lack of standard methodologies. Both phyloscore and $\beta\text{NTI}_{\text{feat}}$ indexes were published quite recently (Danczak et al., 2022; Fodelianakis et al., 2022) and although they intend to measure the same ecological processes, they tackle the question through slightly different approaches. In addition, establishing a significance level for these indices is a controversial step, especially when working with operational taxonomic units at the subtaxon-level like ASVs. For example, if two different ASVs displaying zero phylogenetic distance (i.e. zero-length tree branch) show negative phyloscore or $\beta\text{NTI}_{\text{feat}}$ values of -2.1 and -1.9 , respectively, we question whether only the first ASV should be considered as 'significantly' (i.e. <-2) subjected to homogeneous selection. On the contrary, if a shallow monophyletic clade contains several ASVs with index values close to zero (i.e. not subjected to selection) plus one ASV with a highly significant negative value (i.e. -5), does this represent an ecologically important trend or it is just an outlier or methodological noise? Indeed, both analytical frameworks (Danczak et al., 2022; Fodelianakis et al., 2022) are generally applied to studies that use, as in our case, short amplicon sequencing of the 16S rRNA gene, which might not properly resolve the topology near the tips of the phylogeny (Chase et al., 2017). This scenario becomes even more complex taking into account taxonomy annotation and tree errors in the 16S rRNA databases (Edgar, 2018) and the many challenges to building reliable phylogenetic trees (Kapli et al., 2020).

Although both phyloscore and $\beta\text{NTI}_{\text{feat}}$ indices are continuous variables, their quantitative information is lost when the individual ASVs are simply classified as not subjected to selection (non-S) or subjected to homogeneous (HoS) or heterogenous (HeS) selection with the traditional significance thresholds (i.e. $\text{HoS} < -2$, $\text{non-S} = |2|$, $\text{HeS} > 2$; Danczak et al., 2022). Phylofactorization (Washburne et al., 2019) uses these indexes as continuous variables and identifies monophyletic clades significantly different from their complementary group based on two-sample contrast test that assesses relative differences between groups as opposed to comparing each group against the static predefined traditional thresholds for indexes values. This allows the definition of clades across the homogeneous selection–non-selection–heterogenous selection continuum. In the present work, the phylofactorization identified bacterial clades likely subjected to selection processes showing mean index values significantly different than those of the non-selection clades (i.e. HoS: negative values, HeS: positive values), without using the traditional significance thresholds (i.e. $\text{HoS} < -2$, $\text{non-S} = |2|$, $\text{HeS} > 2$). Future work may find it useful to classify the identified clades along the homogeneous to heterogeneous selection continuum based on clade metrics (e.g. phyloscore/ $\beta\text{NTI}_{\text{feat}}$ sum, mean, median, mode), and look for associations between these metrics and explanatory variables (e.g. alpha diversity, biological processes, environmental features).

In addition to experimental work (e.g. competition experiments), simulation modelling could also be used to test the hypothesis proposed in the conceptual model (Figure 6), as previously implemented by Dini-Andreote et al. (2015) and Stegen et al. (2015). Simulation models should be set up to study the response of different bacterial phylogenetic clades with contrasting microdiversification timescales through different metacommunity assembly-process scenarios. This *in silico* experimentation could provide further insights into the underlying mechanisms driving metacommunity assembly. In all, to better understand the eco-evolutionary processes shaping bacterial phylogenetic clades, the path forward involves testing these new methodological approaches (Danczak et al., 2022; Fodelianakis et al., 2022) coupled with phylofactorization (Washburne et al., 2019) and experimental and simulation data to robustly generate and test detailed hypotheses.

AUTHOR CONTRIBUTIONS

GM designed research. GM and DC obtained funding. PHL performed lab work. MVQ and AV performed bioinformatics and statistical analyses. MVQ, AV and JCS wrote the manuscript. All authors commented on the manuscript and approved the final manuscript for submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The sequence data are publicly available at NCBI BioProject database (ID PRJNA719989, 64 sequence data links, <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA719989>). R code for nearest taxon distance (NTD) and nucleotide similarity of β -nearest ASV indexes,

and the modified version of feature-level β NTI index are available at GitHub (<https://github.com/mvquiroya/NullModels>).

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our sequence data and R code on public database and repository, respectively, as described above.

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SUPPORTING INFORMATION

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