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Dispersal Limitation Governs Bacterial Community Assembly in the Northern Pitcher Plant (*Sarracenia purpurea*) at the Continental Scale

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ABSTRACT

Aim: Ecological theory suggests that dispersal limitation and selection by climatic factors influence bacterial community assembly at a continental scale, yet the conditions governing the relative importance of each process remains unclear. The carnivorous pitcher plant *Sarracenia purpurea* provides a model aquatic microecosystem to assess bacterial communities across the host plant's north–south range in North America. This study determined the relative influences of dispersal limitation and environmental selection on the assembly of bacterial communities inhabiting *S. purpurea* pitchers at the continental scale.

Location: Eastern United States and Canada.

Time Period: 2016.

Major Taxa Studied: Bacteria inhabiting *S. purpurea* pitchers.

Methods: Pitcher morphology, fluid, inquilines and prey were measured, and pitcher fluid underwent DNA sequencing for bacterial community analysis. Null modelling of β-diversity provided estimates for the contributions of selection and dispersal limitation to community assembly, complemented by an examination of spatial clustering of individuals. Phylogenetic and ecological associations of co-occurrence network module bacteria was determined by assessing the phylogenetic diversity and habitat preferences of member taxa.

Results: Dispersal limitation was evident from between-site variation and spatial aggregation of individual bacterial taxa in the *S. purpurea* pitcher system. Selection pressure was weak across the geographic range, yet network module analysis indicated environmental selection within subgroups. A group of aquatic bacteria held traits under selection in warmer, wetter climates, and

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midge abundance was associated with selection for traits held by a group of saprotrophs. Processes that increased pitcher fluid volume weakened selection in one module, possibly by supporting greater bacterial dispersal.

Conclusion: Dispersal limitation governed bacterial community assembly in *S. purpurea* pitchers at a continental scale (74% of between-site comparisons) and was significantly greater than selection across the range. Network modules showed evidence for selection, demonstrating that multiple processes acted concurrently in bacterial community assembly at the continental scale.

1 | Introduction

The processes that shape the distribution of species assemblages including dispersal, niche selection, speciation, and ecological drift (Vellend 2010), are a central focus of community ecology. However, estimating the relative influence of these different processes on the assembly of microbial communities, particularly at continental spatial scales, remains a challenge. Assembly of free-living bacterial communities distributed across large spatial scales ($> 10^3$ km) is hypothesised to be governed by dispersal limitation and environmental selection by climatic drivers (Martiny et al. 2006; Langenheder and Lindström 2019). While evidence from a variety of systems clearly supports the role of climatic conditions in governing microbial communities at the continental scale (e.g., Sunagawa et al. 2015; Averill et al. 2021), further work is needed to determine the conditions under which dispersal contributes to microbial community structure (Zhou and Ning 2017; Louca 2022).

Evaluating how β-diversity, the variation or turnover in the composition and phylogenetic diversity of communities, compares to null expectation is one approach to infer the relative influence of dispersal and selection processes on community assembly. In niche assembly theory, the phylogenetic distance between species can be used to represent their evolved ecological differences, with the assumption that species that are closely related are more ecologically similar than those that are distantly related (Webb et al. 2002; Kraft et al. 2007; Cavender-Bares et al. 2009). The evolutionary distance (also called phylogenetic diversity) of species in one community can be compared with that of other communities to determine the extent to which the variation between the communities deviates from a null model (Graham and Fine 2008). If it can be assumed that closely related species are more ecologically similar than distant relatives, then this deviation from the null expectation provides a measure of selection on ecological niches (Fine and Kembel 2011; Kraft et al. 2011). Pairs of communities that have greater than expected evolutionary distance compared to the null expectation show evidence of heterogenous selection (i.e., selection by different factors or levels of a given factor), while pairs that have lower than expected evolutionary distance are indicative of homogenous selection pressure (i.e., the same factors are exerting selection pressure on both communities; Stegen et al. 2012, 2013). When pairwise evolutionary distance does not differ from the null expectation, selection is not a dominant force in shaping communities.

Similarly, the degree of compositional variation between communities can be compared to that expected if community assembly was governed primarily by ecological drift (Chase et al. 2011). Dispersal limitation makes communities more susceptible to species loss via stochastic events (drift), resulting in higher turnover than expected under free species exchange models (Hubbell 2001). Conversely, high dispersal rates can reduce variation between communities by homogenising species abundances (Mouquet and Loreau 2002, 2003). After excluding selection-associated turnover, compositional turnover that is significantly higher or lower than the null expectation is consistent with dispersal limitation or homogenising dispersal, respectively (Stegen et al. 2013, 2015), while compositional turnover that does not differ from the null expectation is consistent with ecological drift. In this way, the relative importance of dispersal and selection in generating site-to-site variation in species composition (β-diversity) along ecological gradients can be quantified (Chase and Myers 2011).

Since dispersal and selection act on individuals, species-level analyses can complement the community-level approach described above (Vellend 2010). However, the application of some species-specific approaches (e.g., species distribution modelling) is limited in microbial community studies by the low prevalence of most species (Manel, Williams, and Ormerod 2001). In light of this challenge, co-occurrence network analysis can be used to identify the joint spatial effects of environmental conditions and recruitment (Freilich et al. 2018). In microbial studies that rely on co-occurrence data, biological background knowledge and phylogenetic analyses can be used to contextualise network modules, highly connected subgroups or 'modules' of species (Girvan and Newman 2002), aiding in the identification of macro-scale patterns of individual microbial groups and their drivers (Deng et al. 2012; Goberna and Verdú 2022). Additionally, environmentally constrained null models (Peres-Neto, Olden, and Jackson 2001) can also be used to detect the spatial aggregation of individual taxa in microbial community studies. These individual-level analyses can support the inferences derived from β-diversity as well as elucidate the possible roles of biotic and abiotic factors shaping species distributions.

An additional challenge to investigating the processes that shape bacterial community assembly on a continental scale is that significant habitat variations can obscure the effects of dispersal limitation (Hanson et al. 2012). For this reason, the relatively homogenous internal environment within the pitcher of the carnivorous plant *Sarracenia purpurea* provides an ideal experimental system for such studies (Bittleston et al. 2021). The aquatic ecosystem that develops inside rain-filled *S. purpurea* pitchers (typically around 15–30 mL) has served as a model system for investigating processes in community ecology owing to the plant's continental-scale distribution, from Florida, USA to Newfoundland and Labrador, Canada, as well as its tractable nature (Addicott 1974; Kneitel and Miller 2002, 2003; Buckley et al. 2003; Baiser et al. 2011, 2013; Gray et al. 2012, 2016; Freedman et al. 2021). Furthermore, pitchers are sterile upon opening (Peterson et al. 2008), allowing for the observation of community assembly at known points along a primary successional trajectory. In this study we minimised the habitat variation that is often present at large spatial scales by using the pitcher plant system to identify patterns in bacterial community assembly.

The bacterial community inhabiting *S. purpurea* pitchers appears to assemble by both deterministic and stochastic processes. At the site level, bacterial community composition inside pitchers differs from the surrounding wetland habitat as well as from identically treated artificial pitchers (Bittleston et al. 2018; Ellison et al. 2021; Grothjan and Young 2022), demonstrating environmental selection on the bacterial assemblage by the plant host. The effect of environmental selection is also evident at the continental scale where warmer temperatures are associated with greater top-down trophic regulation and larger pitcher size, resulting in a latitudinal gradient of bacterial richness (Baiser et al. 2011; Freedman et al. 2021; Gray et al. 2016). Lastly, historical contingency can also alter pitcher bacterial community assembly, as the initial bacterial composition in a pitcher influences the composition of the mature community (Bittleston et al. 2020).

The present study aimed to disentangle how dispersal limitation and environmental selection might jointly govern bacterial community assembly in *S. purpurea* pitchers. We hypothesised that between-site variability in bacterial communities across the > 2000 km north–south range of *S. purpurea* was mainly governed by dispersal limitation, and selection imposed by climate-associated conditions was less important (H1). We further hypothesised that the strength of habitat filtering on community assembly was greatest in the higher latitudes (H2) due to greater seasonality in climate, resulting in convergence on similar trait composition (Weiher and Keddy 1995). If dispersal limitation was an important determinant of community structure, then we expected that between-site taxonomic variability (after factoring out significant phylogenetic turnover) would be greater than expected from drift acting alone. Furthermore, we expected that a substantial portion of the bacteria to be more spatially aggregated than expected by chance when weighting for site suitability. If environmental selection was important for community structure, then we expected that groups of co-occurring bacteria would be more closely related than expected by chance and have common resource requirements or habitats. A graphical representation of the analyses and associated hypotheses is provided in Figure 1.

2 | Methods

2.1 | Data Collection and Sequence Analysis

We collected pitcher plant (*S. purpurea*) fluid samples from 36 sites from Florida, USA to Quebec, Canada spanning 2518 km (30.2°–53.7°) of the plant's north–south range in North America (Figure 2a) between June and July 2016. The collection, DNA extraction and sequencing of these samples are fully described in Freedman et al. (2021). Collection at each site occurred 4–6 weeks after pitchers opened to allow sufficient time for the full food web to develop (Buckley et al. 2010). This time window provided the necessary flexibility to sample the large study area, but potentially introduced variation in communities resulting from up to two-week difference in development. We expect the impact of this difference on DNA-based bacterial community composition which was primary target of this study would have been minimal because 'relic DNA' from dead and dormant cells may represent 50% of the total DNA pool (Lennon et al. 2018), and a previous study in *S. purpurea* pitchers found no difference in bacterial community composition between three and five weeks after opening (Korn 2021). At each site, three *S. purpurea* plants were randomly selected along a 120 m transect established through areas with high plant density. Fluid was collected from the largest new pitcher of each selected plant using a sterile pipette to first homogenise the fluid by pipetting up and down and then transfer it to a sterile Falcon tube (Corning Inc., Corning, USA). From each sample, the fluid volume was recorded, the pH was measured using indicator strips, and counts of total prey, flesh flies, midges, and mosquitos were made by visual inspection. Pitcher chlorophyll was measured using optical absorbance with a SPAD-502Plus handheld meter (Konica Minolta Inc., Tokyo, Japan) just below the mouth of the pitcher to the side of the keel (Figure 2b). The pitcher was then collected, measurements of rosette diameter and pitcher length and width were made with a cloth measuring tape, and keel width, mouth diameter, and lip width measurements were made using a digital calliper (Ellison and Gotelli 2002). DNA for bacterial community analysis was extracted in triplicate from 300 μL of homogenised pitcher plant fluid with a PowerSoil DNA isolation kit (MoBIO Laboratories, Carlsbad, CA), pooled, and the V5–V7 region of the 16S rRNA gene was amplified using PCR primers 799f/1193r (Chelius and Triplett 2001). Purified PCR products were sequenced on an Illumina MiSeq using 2 × 300 bp using V3 chemistry by MRDNA (Shallowater, TX, USA). All parameters are listed in Figure 2b.

The pitcher morphology measurements were combined using Principal Components Analysis (PCA) on the scaled and centred data using the correlation matrix. A composite variable consisting of the first PC axis (66% of variance) was used to represent pitcher morphology for further analyses. Average maximum temperature and precipitation values for the sampling period (June–July 2016) were obtained for each site from the WorldClim database (Fick and Hijmans 2017). One sample with a pH value of 1.0 was deemed to be erroneous and discarded. These eight measurements (pitcher fluid volume, pH, total prey, flesh flies, midges, mosquitos, chlorophyll, and pitcher morphology PC1), and the two climatic variables (maximum temperature and precipitation) are collectively referred to as 'environmental variables'.

The 16S amplicon sequence data were processed using qiime2 software (version 2022.2; Bolyen et al. 2019) using 'dada2' (Callahan et al. 2016) for quality control and denoising, resulting in 4,808,433 sequences after quality filtering. Amplicon sequence variants (ASVs) unidentified at the kingdom level, singletons, and any sequences assigned to chloroplast or mitochondria were removed, resulting in 5189 total ASVs. Details on sequence processing are provided in the Supporting Information S.1.1. All further analyses were conducted in R (version 4.2.1; R Core

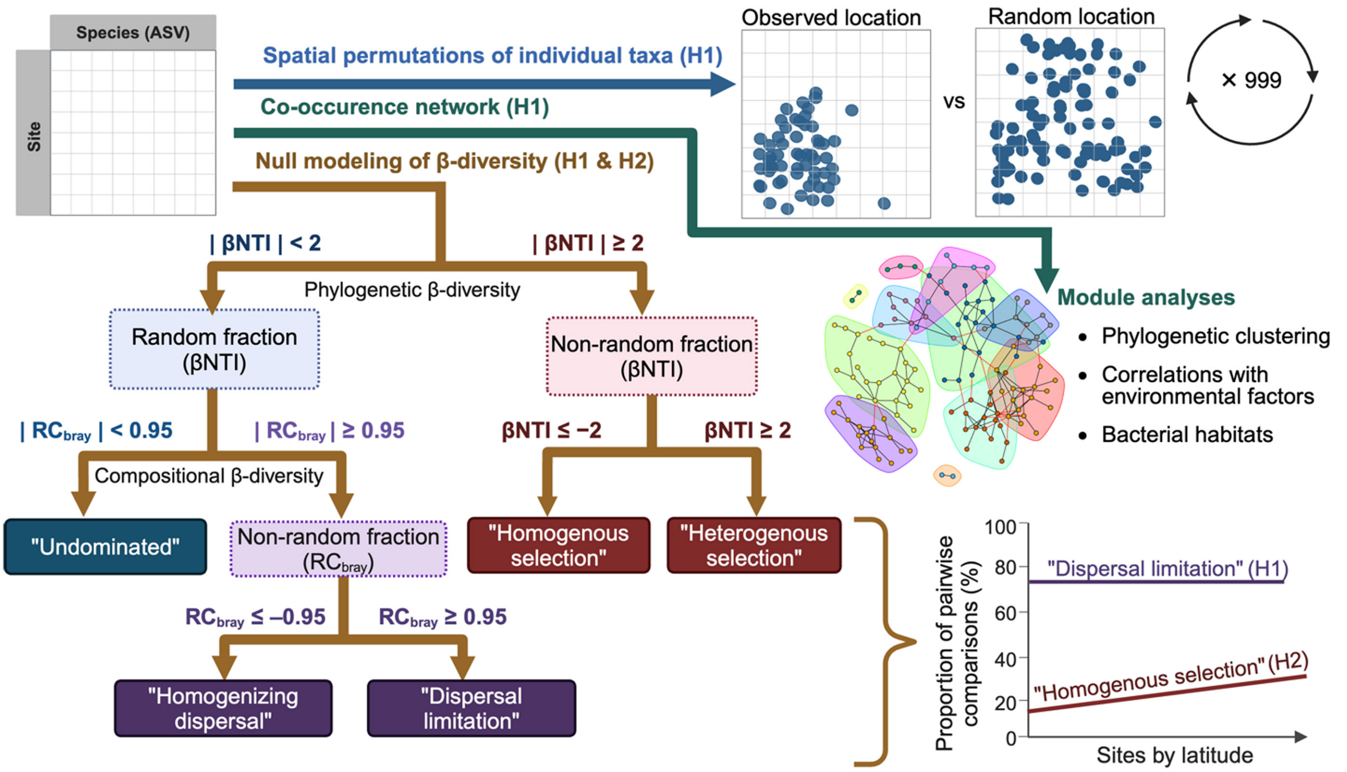

FIGURE 1 | A graphical representation of the analyses and the associated hypotheses: (H1) that between-site variability in bacterial communities across the > 2000 km north–south range of Sarracenia purpurea was mainly governed by dispersal limitation, and selection imposed by climate-associated conditions was less important; (H2) that the strength of environmental selection ('homogenous selection') was greater at higher latitudes than lower latitudes. Taxon-specific dispersal limitation was evaluated using an environmentally constrained null model to test for spatial clustering. Dispersal patterns and habitat preferences of co-occurring subgroups were investigated using module analyses. Deviation in phylogenetic β-diversity from a null model (βNTI) was used to quantify differences in selection between communities. Where βNTI was not significantly different from random, deviation in compositional β-diversity from a null model (RC_{bray}) was used to quantify dispersal. Dispersal limitation was defined as greater-than-expected compositional β-diversity in the absence of significant phylogenetic β-diversity. See Table 1 for additional definitions.

Team 2021); plotting was performed using 'ggplot2' (version 3.4.4; Wickham 2016).

2.2 | Null Modelling of β-Diversity and the Spatial Aggregation of Individuals

Inferring ecological processes from phylogenetic β-diversity assumes that closely related bacteria share habitat preferences to a greater degree than distant relatives (Kraft et al. 2007; Cavender-Bares et al. 2009; Fine and Kembel 2011). We evaluated this assumption by regressing between-ASV phylogenetic distance against between-ASV niche difference as described in Stegen et al. (2012, 2013). First, we identified the environmental variables representing important bacterial niches using distance-based redundancy analysis (dbRDA) of phylogenetic dissimilarity based on the UniFrac index and a forward selection procedure (variables retained at $p < 0.01$; 999 permutations). For this model selection procedure only, 11 missing measurements (midge abundance: 3, volume: 3, pH: 5) were interpolated from random forest analysis as described by Stekhoven and Buehlmann (2012) using 'missForest' (Stekhoven 2022). Then an optimal niche value for each ASV was estimated as the median of the variable across samples where the ASV was present. Between-ASV phylogenetic distance and optimal niche distance were regressed using Mantel correlograms (Oden and Sokal 1986) and correlations were tested for significance with 999 permutations using 'vegan' (version 2.6-4; Oksanen et al. 2020).

We estimated the relative influence of community assembly processes using the null modelling of β-diversity framework described by Stegen et al. (2013, 2015). First, phylogenetic β-diversity was calculated as the between-community mean nearest taxon distance (βMNTD; Fine and Kembel 2011). Then a standard effect size for βMNTD, the β Nearest Taxon Index (βNTI) value, was calculated by taking the difference between the observed βMNTD and the mean of a null distribution generated by randomly shuffling species and recalculating βMNTD 999 times, in units of standard deviations. Next, the Raup-Crick index of Bray–Curtis dissimilarity was calculated by probabilistically reassembling local communities 999 times and taking the Bray–Curtis dissimilarity between each re-assembled pair (RC_{bray}; Chase et al. 2011; Stegen et al. 2013). Differences in diversity among sites were controlled by maintaining the observed richness and number of individuals, and the probability of observing an individual ASV was based on the number of samples it occupied and its relative abundance across all samples (Stegen et al. 2013). This provided a null distribution to which the observed Bray–Curtis dissimilarity was compared, and the resulting RC_{bray} value was scaled from −1 to 1. Calculations were carried out using 'picante' (version

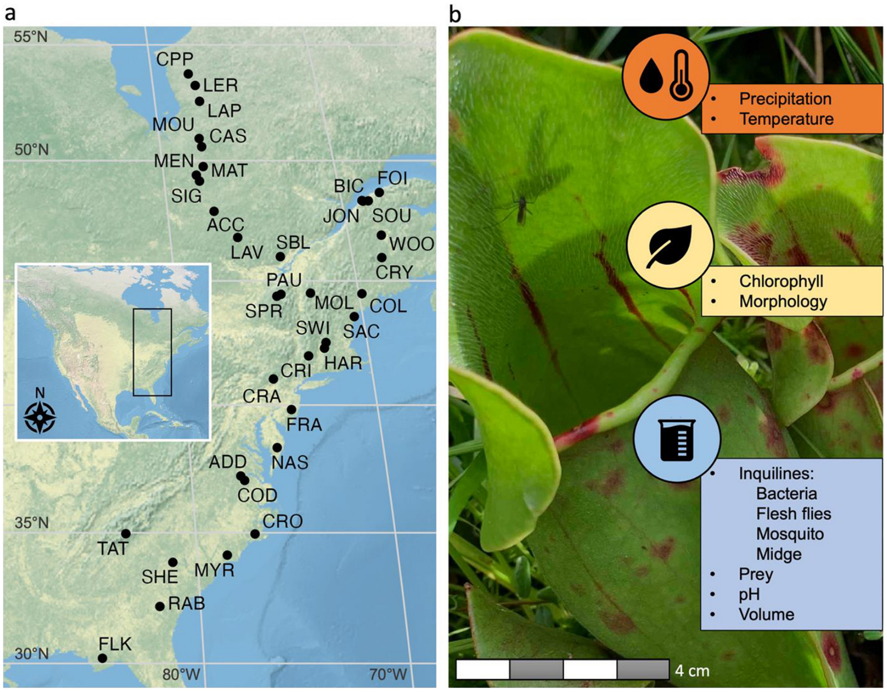

FIGURE 2 | (a) The locations of the sampling sites. The base map was obtained from Natural Earth (naturalearthdata.com) and is in Mollweide projection. (b) A carnivorous purple pitcher plant (*Sarracenia purpurea*) and the associated parameters ('environmental variables') which were measured in this study. Average precipitation and maximum daily temperature data during the sampling period (June–July 2016) were obtained from WorldClim (worldclim.org).

1.8.2; Kembel et al. 2010) and a parallel implementation code developed by Richter-Heitmann et al. (2020) was applied to increase computational efficiency.

The response variables resulting from the null model analysis of β-diversity were the pairwise βNTI and RC_{bray} matrices. The values represent the extent to which phylogenetic (βNTI) and compositional (RC_{bray}) turnover between two communities deviated from the null expectation. Significant deviations from null values were interpreted as described in Stegen et al. (2013, 2015) as follows. Values of $|\beta\text{NTI}| \geq 2$ were designated significantly different from chance and interpreted to indicate a role of environmental selection on community assembly. Lower-than-chance phylogenetic turnover ($\beta\text{NTI} \leq -2$) was termed 'homogenous selection' and greater-than-chance phylogenetic turnover ($\beta\text{NTI} \geq 2$) was termed 'heterogenous selection'. Values of $|RC_{bray}| \geq 0.95$ were designated significantly different from chance. To factor out turnover resulting from environmental similarity, RC_{bray} was only interpreted where βNTI values were not significant (e.g., $|\beta\text{NTI}| < 2$). Then, in the absence of significant βNTI, lower-than-chance compositional turnover ($RC_{bray} \leq -0.95$) was termed 'homogenising dispersal' and greater-than-chance compositional turnover ($RC_{bray} \geq 0.95$) was termed 'dispersal limitation'. The scenario in which both βNTI and RC_{bray} did not differ from the null expectation (e.g., $|\beta\text{NTI}| < 2$ and $|RC_{bray}| < 0.95$) indicated that community turnover was consistent with weak dispersal, weak selection, diversification, or drift, and was referred to as 'undominated' (Stegen et al. 2015). The definitions are provided in Table 1.

The influence of each process was estimated for each sample by counting its between-site pairwise comparisons fitting each of the above definitions. That is, each sample was compared against samples from other sites, and each pairwise comparison was assigned a community assembly classification based on the interpretation scheme in Table 1. The hypothesis that bacterial communities across the north–south range of *S. purpurea* were characterised by dispersal limitation (H1) was evaluated using a Chi-square test to determine if the counts of dispersal limitation were greater than the counts not consistent with dispersal limitation. The hypothesis that the strength of environmental selection was greater at high latitudes (H2) was tested by fitting a generalised linear mixed model (GLMM) with a Poisson distribution for the counts of homogenous selection as a function of latitude. Site was treated as a random effect to account for the grouping structure of observations. The GLMM was fitted and evaluated using 'lme4' (version 1.1-35.1; Bates et al. 2015) and 'lme4Test' (version 3.1-3; Kuznetsova, Brockhoff, and Christensen 2017).

The independent and shared effects of environmental and spatial factors on βNTI and RC_{bray} were examined using variance partitioning and distance-based redundancy analysis (dbRDA; Borcard, Legendre, and Drapeau 1992). Spatial factors were modelled with spatial eigenvector analysis, which quantifies spatial relationships among communities across a range of spatial scales (Borcard and Legendre 2002). Spatial eigenvectors were identified using the distance between sites as input to the function 'pcnm'. Significant spatial eigenvectors for both βNTI and RC_{bray} were determined using forward model selection (Blanchet, Legendre, and Borcard 2008) with stepwise testing of independent variable significance ($\alpha = 0.05$, 1000 permutations), and variable ordering based on improvement in the model's adjusted R^2 using the 'ordiR2step' function in 'vegan'. βNTI and RC_{bray} were scaled to vary between 0 and 1 and evaluated in

TABLE 1 | The framework used for interpreting community assembly processes from the null modelling of β-diversity from Stegen et al. (2013, 2015).

Classification	Equation
Homogenous selection	$\beta\mathrm{NTI} \leq -2$
Heterogenous selection	$\beta\mathrm{NTI} \geq 2$
Homogenising dispersal	$\lvert\beta\mathrm{NTI}\rvert < 2$ and $\mathrm{RC}_{\mathrm{bray}} \leq -0.95$
Dispersal limitation	$\lvert\beta\mathrm{NTI}\rvert < 2$ and $\mathrm{RC}_{\mathrm{bray}} \geq 0.95$
Undominated	$\lvert\beta\mathrm{NTI}\rvert < 2$ and $\lvert\mathrm{RC}_{\mathrm{bray}}\rvert < 0.95$

separate dbRDAs. The variance partitioning analyses included the significant spatial eigenvectors for βNTI and $\mathrm{RC}_{\mathrm{bray}}$, respectively, and the 10 environmental variables described above. Variance partitioning analysis was carried out using the functions 'dbdra' and 'Condition' in 'vegan' as described by Legendre and Legendre (2012).

To evaluate the role of dispersal limitation (H1) at the level of individual ASVs, we conducted an environmentally constrained null model analysis of spatial clustering. The environmental constraints consisted of the variables identified by the dbRDA procedure described above, summarised as the first axis of a PCA. An environmentally constrained null distribution was then created by shuffling the occurrences of ASVs among sites 1000 times weighting by the site-specific probability for each ASV (Peres-Neto, Olden, and Jackson 2001) and calculating the sum of the variances of the x and y spatial coordinates. The observed spatial variance was compared to the mean of the null, and the proportion of ASVs that were more spatially clustered than expected at an alpha of 0.05 was determined. If dispersal limitation was an important process for community assembly (H1), then we expected a large majority of the ASVs to be more spatially clustered than expected by chance.

2.3 | Co-Occurrence Network Construction and Module Analysis

To determine if bacterial co-occurrence patterns were associated with dispersal and habitat preferences, we first identified significant co-occurrences using network analysis. To facilitate the co-occurrence network analysis of highly similar taxa, ASVs were merged at a cophenetic distance of 0.05 using the 'tip_glom' function in the 'phyloseq' package (version 1.42.0; McMurdie and Holmes 2013), resulting in 1498 ASVs. Of these, the ASVs present in at least six samples and with a relative abundance of at least 1% in those samples were retained. The sequencing depth was normalised across samples by rarefying sample counts to the minimum number of sequences per sample (13,574). A single network, where nodes represent ASVs and edges (e.g., links) represent significant co-occurrences, was constructed using all samples and an ensemble approach with two methods: SparCC (Friedman and Alm 2012) and SPIECEASI (Kurtz et al. 2015) using the 'SpiecEasi' package; network construction parameters are provided in the Supporting Information S.1.2. The two networks were then combined by retaining only edges supported by both methods. This consensus co-occurrence network method can reduce bias in applications with high-throughput DNA sequencing datasets (Faust et al. 2012). Three samples from Florida (site FLK) tended to have fewer shared taxa and were omitted to facilitate network construction. The *p*-values for SparCC correlations were calculated by computing 999 bootstrapped correlation coefficients and comparing the observed value to the null distribution and were then adjusted using the false discovery rate method (Benjamini and Hochberg 1995). Edges with an adjusted p-value < 0.01 and a correlation value above the threshold of the mean plus one standard deviation were retained in the network. We focused the network analysis on positive associations to capture bacterial co-occurrences rather than exclusions. The resulting modules are subsets of bacteria with high incidences of co-occurrence, which were identified using the leading eigenvector method (Newman 2006). All network calculations were done using the 'igraph' package (version 1.5.1; Csardi and Nepusz 2006).

The resulting network was evaluated to determine if it was more structured than expected by chance. The observed modularity and clustering coefficient were compared to a null distribution obtained from 999 randomly generated Erdős–Rényi graphs (Erdős and Rényi 1960) that contained the same number of edges and vertices as the observed graph using a one-sample *t*-test. The scale-free property, a characteristic of modular, non-random networks, was evaluated by fitting a power law curve to the degree distribution (Barabási and Albert 1999).

The degree of phylogenetic clustering within each module was calculated to determine if environmental selection was evident as phylogenetic relatedness within modules using complimentary metrics. Standard effect sizes of the mean pairwise distance (MPD), mean nearest taxon distance (MNTD; Webb et al. 2002), and Faith's phylogenetic diversity (PD; Faith 1992) were calculated from 9999 null assemblages generated by randomly shuffling taxa using the functions 'ses.mpd', 'ses.mntd', and 'ses.pd', respectively, in 'picante' (version 1.8.2; Kembel et al. 2010). Modules containing at least 10 nodes were considered in the module analysis with environmental variables.

To determine associations between the modules and environmental variables we conducted singular value decomposition on the expression matrix of each module and used the first principal component, known as the module 'eigengene' to represent the module (Langfelder and Horvath 2007; Zhou et al. 2011; Deng et al. 2012). A significant relationship between an environmental factor and a module was defined as a significant correlation between the module eigengene and the environmental factor in terms of the Pearson correlation coefficient (r) after Holm correction (Holm 1979; Horvath and Dong 2008). Similarly, the extent to which an ASV was associated with a module was defined by its module membership, the correlation between the module eigengene and the ASV's relative abundance (Langfelder and Horvath 2007; Zhou et al. 2011). Module eigengenes were calculated from the ASV table used for network construction with Hellinger-transformed abundance counts using 'WGCNA' (version 1.72-1; Langfelder and Horvath 2008).

To better interpret the module relationships with environmental conditions, we compiled habitat data for the bacterial taxa with high and significant module membership (Holm-adjusted $p < 0.01$ and $r > 0.5$). A high module membership indicated high congruence between the module eigengene and the ASV (Horvath and Dong 2008; Zhou et al. 2011) and we refer to these ASVs as 'representatives' of the respective module. To obtain ecological information on the representative taxa, genetic matches were identified by a BLAST search (Camacho et al. 2009) of the NCBI 16S RefSeq database (downloaded 18-3-2023; O'Leary et al. 2016) using 'rBLAST' (version 0.99.2; Hahsler and Nagar 2019). Information on the isolation sources of taxa was parsed from records and publications associated with the top five matches greater than 99% percent identity from the BLAST search.

To investigate the community assembly processes acting on a module that positively correlated with pitcher fluid volume and exhibited high phylogenetic diversity, we tested for a habitat-amount effect, whereby species richness increases with the amount of surrounding habitat (Fahrig 2013). The cumulative habitat amount (pitcher fluid volume, range 0.1–60 mL) was plotted against the cumulative number of ASVs when aggregating samples from small to large and from large to small (SLOSS analysis). If larger habitats had greater species richness, then the cumulative number of ASVs was expected to accumulate more quickly when aggregated from large to small samples (Quinn and Harrison 1988).

3 | Results

The analysis included samples from 108 pitchers across 36 sites; the maximum geographic distance between sites was 2518 km (Figure 2a). The average daily maximum temperatures during the sampling period ranged from 18°C to 32°C. Approximately half of the total bacterial relative abundance across the range was accounted for by three orders: Burkholderiales (24% ± 0.05%; mean relative abundance ± standard deviation), Flavobacteriales (15% ± 0.09%), and Sphingomonadales (10% ± 0.05%). The most abundant ASV was a *Chryseobacterium* sp. (8.4% ± 18%) followed by a *Pseudomonas* sp. (6.9% ± 12%).

Prior to inferring selection from the extent that phylogenetic β-diversity deviated from a null model, we tested the assumption that closely related bacteria shared habitat preferences by regressing between-ASV phylogenetic distance against between-ASV niche difference. The environmental variables significantly associated with bacterial community composition included daily maximum temperature, pitcher fluid volume, and pitcher leaf chlorophyll (Table S1). Mantel tests of between-ASV phylogenetic distance indicated that phylogenetic distance was positively correlated with niche difference for these variables across short phylogenetic distances (Figures S1 and S2), supporting the use of to βNTI to evaluate the influence of selection.

3.1 | Dispersal Limitation Dominates Community Assembly in *S. purpurea* at the Continental Scale

The hypothesis that community assembly was heavily influenced by dispersal limitation (H1) was evaluated using null modelling of β-diversity and spatial clustering of individual ASVs. Dispersal limitation, defined as greater-than-expected compositional turnover in the absence of significant phylogenetic turnover, was the predominant community assembly process across the range and represented 74% of pairwise comparisons $X^2(1, N = 108) = 2959$, $p < 0.01$ (Figure 3a). Through variance partitioning analysis, spatial factors explained a significant portion of the variation in $\mathrm{RC}_{\mathrm{bray}}$ when controlling for environmental factors ($F_{6,91} = 1.51$, $p < 0.01$, $R^2 = 0.11$). However, spatial factors did not significantly explain variation in βNTI when controlling for environmental factors ($F_{2,95} = 1.52$, $p = 0.12$, $R^2 = 0.03$), as most of the explained variation was shared between environmental and spatial factors ($R^2 = 0.07$). In contrast, little variation in $\mathrm{RC}_{\mathrm{bray}}$ was shared between environmental and spatial factors ($R^2 = 0.01$). The full results of the variance partitioning analysis are provided in Table S2. Analysis of the spatial clustering of individual ASVs compared with an environmentally constrained null model indicated 21% of ASVs were more spatially clustered than expected from random placement ($p < 0.05$; Figure 3b).

The hypothesis that the strength of environmental selection varied across the range (H2) was evaluated using a GLMM to test if selection (i.e., $|\beta\mathrm{NTI}| \geq 2$; see Table 1) was related to latitude. There was no significant relationship between selection and latitude GLMM $\chi^2(1, N = 108) = 0.8$, $p = 1$.

3.2 | Evidence for Selection Within Some Co-Occurrence Network Modules

A total of 170 ASVs met the filtering criteria for inclusion in the co-occurrence network (Figure 4a), which ultimately consisted of 126 nodes (representing ASVs) and 187 edges (representing significant co-occurrences) after removing isolated nodes. The ASVs included in the network constituted 86% of the total abundance. The network was scale-free, as indicated by fitting the degree distribution to a power law ($R^2 = 0.79$; $p < 0.01$). Modularity was significantly greater than the null model (modularity = 0.70; $p < 0.01$), indicating the presence of modules in the network. A scale-free state and modular structure are properties of non-random networks (Barabási and Albert 1999; Girvan and Newman 2002). Ten modules were identified within the network, and five fit our size criterion for further investigation.

Evaluation of the modules' phylogenetic relatedness and relationships with the environmental variables revealed several in which the relatedness of bacteria within modules was significantly greater than that of the overall network and the module eigengenes significantly correlated with environmental

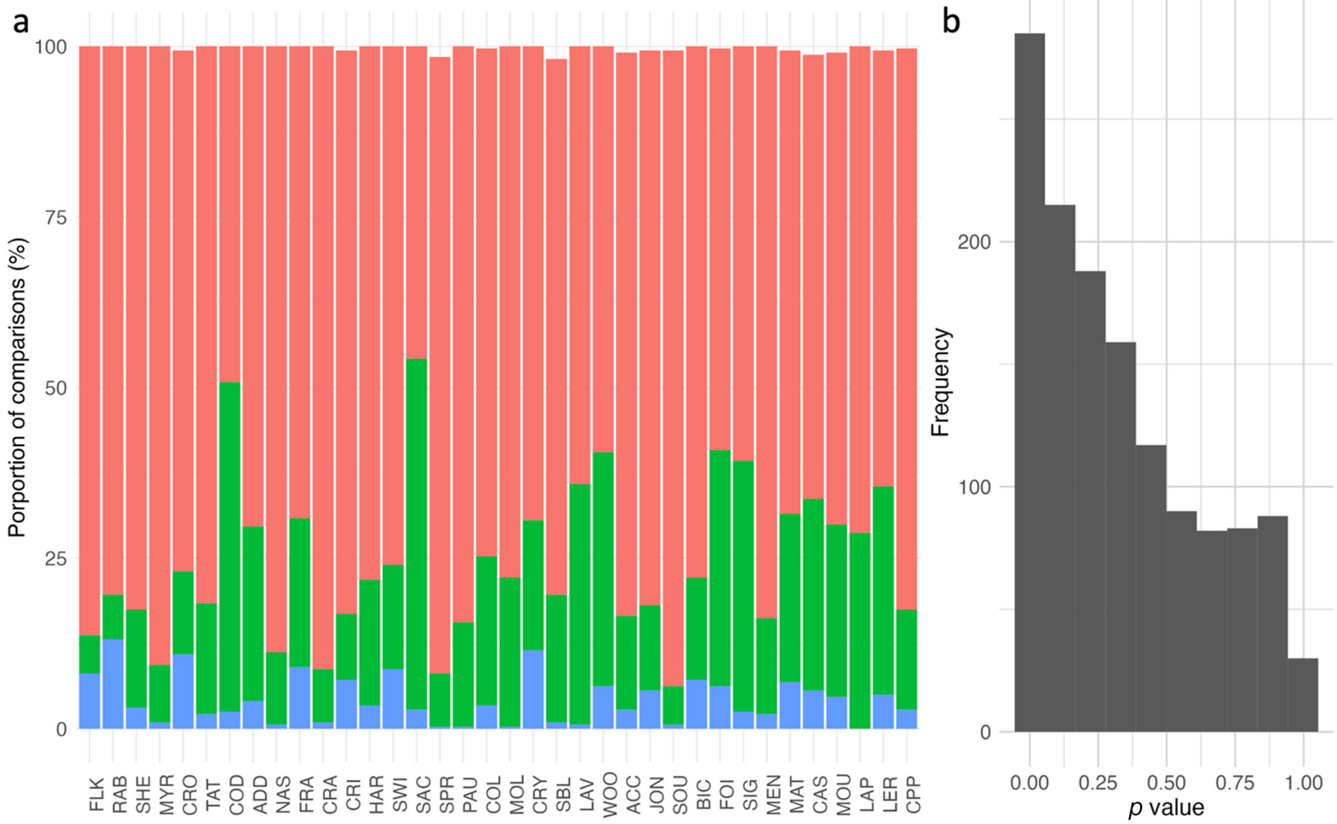

FIGURE 3 | (a) Bacterial communities inhabiting *S. purpurea* were dispersal-limited at the continental scale. The contributions of dispersal limitation (red), homogenous selection (blue) and 'undominated processes' (green) are represented by the count of the between-site pairwise comparisons fitting the definition of each process (see Table 1 for quantitative definitions). The mean of three samples collected at each site is shown for each site. The sites are arranged by increasing latitude from Florida (FLK) to northern Quebec (CPP); locations are shown in Figure 2a. The minor contributions of homogenising dispersal and heterogenous selection are evident where sites do not total to 100%. (b) The frequency distribution of *p*-values from the environmentally constrained null model analysis of spatial aggregation. A large proportion of bacterial amplicon sequence variants were more clustered in space than expected from random placement when weighting for site suitability (21% at $\alpha = 0.05$).

variables. Module 1 (M1) was the only module which exhibited significant phylogenetic relatedness based on MPD (Table 2). Phylogenetic relatedness based on Faith's PD was significantly lower than expected in M1, module 2 (M2), and module 4 (M4), than expected by chance; the MNTD results followed a similar trend as Faith's PD (Table 2). Three of the five modules exhibited significant correlations with the environmental variables (Figure 4b). The strongest observed relationship was a negative correlation between M1 and average maximum daily temperature during the sampling period ($r = 0.57$; $p < 0.01$). M2 was most strongly correlated with the abundance of midges present in the pitcher fluid ($r = 0.40$; $p < 0.01$), and module 3 (M3) was most strongly positively associated with pitcher fluid volume ($r = 0.39$; $p < 0.01$). We considered if the trend of increasing diversity with volume in M3 could be due to a habitat-amount effect. The SLOSS analysis showed that smaller pitchers had greater ASV richness than larger pitchers (Figure S3). Hence, the species accumulation curves did not support the idea that increasing bacterial diversity with pitcher fluid volume was due to a habitat-amount effect.

Investigating the primary habitat of the representative taxa (e.g., module membership $r > 0.5$ with matches available in the NCBI database) to gain a better understanding of the environmental associations of the module bacteria revealed habitat differences among modules. Of the seven representative taxa in M1, five belonged to the order Burkholderiales and two were members of the Sphingomonadales (Figure S4; Table S4). Investigation of publication records and NCBI isolation source data revealed that the top five bacteria in M1 matched with aquatic taxa. The taxon with the highest M1 module membership score was *Aquincola amicola* (Family Commonadaceae) isolated from a freshwater river (Chen et al. 2018). The next highest module membership score was a taxon from the genus *Undibacterium* (Family Oxalobacteraceae) with matches to *U. curvum*, isolated from a tropical stream (Lu et al. 2021), and *U. crateris*, isolated from a crater lake (Phurbu et al. 2021). In contrast, M3 representitive taxa were bacteria from soil and plant roots (Laranjo, Alexandre, and Oliveira 2014; Weon et al. 2008; Table S4). Taxa in M4 included several plant leaf-associated bacteria and seed endophytes including *Staphylococcus epidermis* (Chaudhry and Patil 2016), *Stenotrophomonas maltophilia* (Hardoim et al. 2012) and *Pseudomonas putida* (Berg et al. 2005; Table S4). The taxa in M5 were a diverse and cosmopolitan group of bacteria, including reported plant-associates (*Sphingomonas faeni*, *Pantoea agglomerans*, *Pantoea brenneri*; Bhardwaj, Jain, and Kumar 2022; Walterson and Stavrinides 2015), as well as two taxa with isolates from

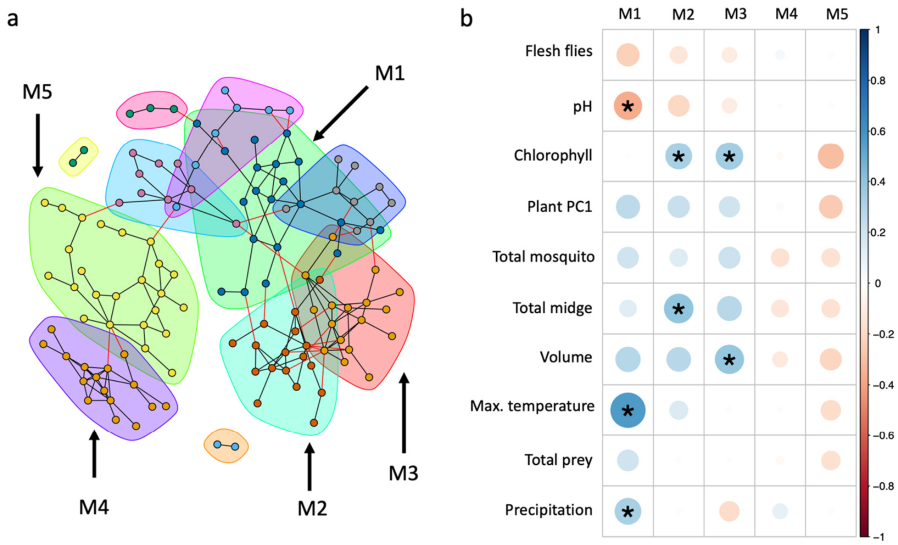

FIGURE 4 | (a) A co-occurrence network representing the bacterial inhabitants of *Sarracenia purpurea* pitchers across the host's north–south range in North America exhibits a modular property. Bacterial taxa (amplicon sequence variants or 'ASVs') are the nodes and significant co-occurrences are the connections. Modules, regions of greater interconnectedness within a network, are indicated by the different coloured hulls underlaying the nodes. Red lines mark intramodule connections and black lines show intermodule connections. The network's modularity and clustering coefficient were significantly greater than a null distribution generated from randomly assembled networks ($p < 0.01$). Five large modules (> 10 ASVs) named M1–M5 were considered in further analyses; five smaller modules that were below the threshold size for analysis are also shown. (b) Correlation analyses between network modules M1–M5 and the environmental variables revealed significant relationships for M1, M2, and M3. The size of the points indicates the value of the Pearson correlation coefficient between the module eigengene and the variable. The colour represents the direction of the relationship, where blue is positive and red is negative, as shown by the guide. Those marked by an asterisk were statistically significant ($p < 0.05$) after adjusting for multiple comparisons. The plot data are provided in Table S3.

TABLE 2 | Standardised effect sizes (Z) and permutation quantile (p-value) of phylogenetic diversity within modules based on null model analysis.

		MPD		**MNTD**		**Faith's PD**	
Module	**Num. taxa**	Z	p	Z	p	Z	p
M1	20	−2.68	< 0.01	−1.53	0.06	−1.96	0.02
M2	17	−0.32	0.38	−2.18	0.01	−1.92	0.02
M3	17	0.79	0.78	−0.68	0.26	0.20	0.59
M4	24	−0.44	0.34	−1.47	0.07	−2.07	0.01
M5	16	0.10	0.55	0.05	0.53	0.04	0.54

glaciers (*Sphingomonas glacialis*), and Arctic and Antarctic terrain (*Massilia* sp.). Nine of the 14 taxa with high module membership in M2 did not have matches in the NCBI reference database, indicating that have not yet been isolated in culture. The taxon with the highest module membership score for which a match was available in M2 was *Roseiarcus fermentans*, which was isolated from a methanotrophic culture established from an acidic peat soil (Kulichevskaya et al. 2014).

4 | Discussion

We tested the hypothesis that dispersal limitation and selection by climatic drivers together structured bacterial communities in the pitcher plant *Sarracenia purpurea* across a range of 2518 km four to six weeks after pitcher opening. We used quantitative definitions of these community assembly processes from a well-established framework based on null modelling of β-diversity (Stegen et al. 2013, 2015). Supporting hypothesis H1, we found that dispersal limitation, defined as greater-than-expected compositional turnover in the absence of significant phylogenetic turnover, was predominant across the range. Biogeographical separation was associated with stochastic community assembly and not with selection as evidenced by a significant pure spatial effect for RC_{bray} but not for βNTI. A null model analysis of individual ASVs supported the conclusion that a substantial portion were more spatially aggregated than expected by chance. The relative importance of environmental selection, defined as lower-than-chance phylogenetic β-diversity, was not associated with high latitudes, providing no support for H2. Network inference allowed for a deeper analysis of additional phylogenetic and ecological information on modules of co-occurring bacteria. We identified that a module of aquatic bacteria was under selection by conditions associated with warmer temperatures and a module of saprotrophs under selection by conditions that corresponded with greater midge abundance, whereas larger volume pitchers appeared to weaken the effect of selection. Overall, our results supported the hypothesis that the bacterial inhabitants of *S. purpurea* pitchers were dispersal-limited across the host plant's north–south range in North America and suggested that heterogenous environmental selection pressures acted on different groups of bacteria.

The conditions under which dispersal limitation contributes to bacterial community structure is a topic of current investigation that has not been fully resolved (Louca 2022). For example, a study of Antarctic soils deglaciated for millennia reported that dispersal limitation was the predominant microbial community assembly process at the scale of 10^2 km (Lemoine et al. 2023). The results of that study and ours beg the question if these patterns were the result of contemporary dispersal limitation. Alternatively, density-dependent mechanisms such as competition could have maintained patterns that originated during post-glacial recolonisation such as is hypothesised for some macro-organisms (Waters, Fraser, and Hewitt 2013). For example, competitive exclusion by some species of *Streptomyces* has been shown to result in their spatial aggregation at the regional scale (Choudoir, Doroghazi, and Buckley 2016). While our study identified a pattern in turnover consistent with dispersal limitation, further work is needed to determine if this pattern was the result of contemporary dispersal limitation, competitive exclusion, or historical contingencies.

Our analysis of network modules allowed us to disentangle community characteristics associated with dispersal and environmental selection by generating information on groups of bacteria that were associated with specific abiotic conditions (Goberna and Verdú 2022). Module M1 was positively correlated with maximum temperature and precipitation, and the representative taxa were from within the Families Comamonadaceae and Oxalobacteraceae (Order Burkholderiales) and Sphingomonadaceae (Order Sphingomonadales) that are common in freshwater lakes (Newton et al. 2011). Accordingly, M1 may represent a module within the *S. purpurea* microbiome that is similar to those found in aquatic systems (Sirota et al. 2013; Northrop et al. 2017). The traits conferring fitness in this module appear to be deeply conserved based on the significant MPD, a metric which is sensitive to phylogenetic clustering of distant relatives (Webb et al. 2002; Mazel et al. 2016), and under a selective pressure associated directly or indirectly with temperature (Hall, Neuhauser, and Cotner 2008; Berggren et al. 2010; Young, Sielicki, and Grothjan 2018; Grothjan and Young 2019; Zhang et al. 2020).

In contrast, M3 exhibited the lowest degree of phylogenetic relatedness among taxa of all the modules. M3 was also positively correlated with pitcher water volume and the representative taxa were plant- or soil-associated bacteria, which may suggest that these taxa enter the *S. purpurea* pitcher aquatic system through dispersal from contrasting ecosystems. As water volume is highly related to pitcher size, larger volume pitchers with wider openings may simply be more likely to capture exogenous material (e.g., soil particles and plant material) and associated microbial taxa than smaller pitchers. Air-borne taxa are also likely an important recruitment source (Grothjan and Young 2022), and larger pitchers may collect more bacteria from the air. We considered if the higher phylogenetic diversity observed in this module could be due to increased habitat amount, but this was not supported by a SLOSS analysis. Thus, the more parsimonious explanation is that larger pitcher volume represents increased target for dispersal. We posit that the co-occurrence pattern in M3 of diverse plant- and soil-associated bacteria suggests that dispersal governs the community assembly of this module. This would represent a mass effects scenario, in which the selective force of the environment is masked by the dispersal of species from locations outside the pitcher.

As another example of how the habitat associations of taxa within a network module and their phylogenetic relationships provided insights into community assembly, M2 positively correlated with the abundance of the midge (*Metriocnemus knabi*) which shreds detritus, increasing the availability of organic matter available for bacterial decomposition (Butler, Gotelli, and Ellison 2008, Baiser et al. 2011). M2 had significant phylogenetic diversity based on MNTD and Faith's PD, but not MPD, which together suggest that the processes generating modularity in M2 operated on traits that evolved more recently rather than traits conserved from a more distant ancestor (Mazel et al. 2016). It is plausible that the bacteria in M2 were highly responsive to an increase in organic C substrate availability from midge activity such as has been shown with *Wyeomyia smithii* (Arellano, Young, and Coon 2024). Thus, we would expect to observe a pattern of midge effects on bacterial communities at the tips of the phylogenetic tree because the simple C substrate acquisition traits are shallowly conserved in bacteria (Martiny et al. 2015). Deeper investigation into the representative taxa in M2 showed they included saprotrophs (Weon et al. 2009; Tran and Dahl 2016), methanotrophs (Bogan et al. 2003; Dunfield et al. 2003), and syntrophic partners in methanogenic consortia (Weon et al. 2009; Kulichevskaya et al. 2014; Tran and Dahl 2016). Thus, the co-occurrence of bacteria in M2 appears to be consistent with selection based on substrate availability or possibly metabolic collaboration. The low identification rate of taxa in this module suggests that future work utilising alternative methods like shotgun metagenomics would be useful to improve understanding of bacterial responses to variation in midge abundance.

Putative habitat preference or dispersal routes of the bacteria comprising M4 and M5 were not apparent because these modules were not significantly associated with the environmental variables. Modularity in M4 and M5 could be governed by an unmeasured variable such as oxygen or nitrogen, or historical contingency (Bittleston et al. 2020), implying a role of an additional selective force or dispersal pattern not identified here, or an important priority effect or interaction in the assembly of the *S. purpurea* microbiome.

There are several limitations to the present study that warrant further testing in future studies. First, the null modelling framework we applied is unable to distinguish between true dispersal limitation and other effects that increase compositional turnover, such as competitive exclusion (Choudoir and Buckley 2018). As with all DNA-based analyses, it is not possible to determine whether these bacteria were metabolically active at the time of sampling (Carini et al. 2016). Further inquiry directed towards identifying the dispersal and persistence of bacteria in *S. purpurea* pitchers is warranted to support the results reported here. Specifically, further study to disentangle possible spatial autocorrelation resulting from dispersal limitation that can lead to spurious environmental correlations with ASVs should be considered (Viana, Keil, and Jeliazkov 2022). Additionally, whether the community dynamics observed here have functional consequences is untested in this study and metagenomics, transcriptomics or direct functional measurements are needed to further interrogate the biogeography of community function in the pitcher plant system. Our study offers only a single snapshot, and time series analysis would further improve understanding of community assembly processes in *S. purpurea* pitchers. Despite the limitations, these results contribute to our understanding of large-scale bacterial community assembly by showing that dispersal limitation occurs in a model system at the continental scale.

5 | Conclusions

This study quantified the relative influence of dispersal and selection on bacterial community assembly in *S. purpurea* pitchers across the plant's latitudinal range. Our results revealed that a substantial portion of bacterial communities as well as individual taxa were spatially clustered while controlling for environmental variation, consistent with the hypothesis that community assembly at the continental scale is governed to a greater extent by dispersal limitation than by selection (Martiny et al. 2006; Langenheder and Lindström 2019). Network inference based on bacterial co-occurrences revealed several modules with significant phylogenetic relatedness and correlation to environmental variables, which suggests that selection may have acted on specific groups of bacteria within the community. Additionally, the network module analyses indicated that pitcher volume weakened the effect of selection, possibly through a dispersal-mediated increase in bacterial diversity. These results suggest that dispersal limitation and selection occur concomitantly at the community level. Further study of dispersal patterns and selective pressures at the level of individual taxa or co-occurring groups could provide further insight into bacterial community assembly mechanisms in this model system and beyond.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw sequence reads for the pitcher plant microbiome are available from the National Center for Biotechnology Information (NCBI) under accession no. PRJNA641293. The environmental variables and rounded spatial coordinates are provided in Table S5 as well as on Dryad doi:10.5061/dryad.18931zd4q. Code used in to run the analyses is publicly available from the sources cited in the text.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.