

Biogeographic structure of fungal communities in seagrass *Halophilia ovalis* across the Malay Peninsula

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Originality-Significance statement

Seagrasses are critical marine ecosystems that play important roles in helping to mitigate climate change by sequestering carbon. Annual rates of carbon sequestration are up to 10 times higher in seagrass meadows than mature tropical rainforest. Despite their ecological significance, seagrasses in the global epicentre of their biodiversity—Southeast Asia—are being destroyed at an alarming rate. Traditionally, mycobiome-plant interactions have been acknowledged to play integral roles in plant health, yet data on the seagrass mycobiome remain severely limited. This study is the first to examine the mycobiome of the important ecosystem engineer, *Halophilia ovalis*, in Southeast Asia. The results from this study may be used to guide future conservation and ecosystem restoration efforts in this biodiversity hotspot.

Summary

Distributed across both the tropical Atlantic and Pacific oceans, the seagrass *Halophila ovalis* stabilises coastal sediment, thereby preventing shoreline erosion, and is also an important food source for megaherbivores such as dugongs. However, seagrass meadows globally are under severe duress due to both climate change and anthropogenic activities. We characterised the mycobiome of *Halophila ovalis* at seven sites in the Malay Peninsula using ITS1 rDNA amplicon sequences, and investigated differences in fungal community structure. We found that geographic location was a significant factor shaping fungal communities, and that marine sediment harboured a significantly richer diversity when compared to *H. ovalis* leaves, roots and rhizomes. Taken together, it is likely that locality rather than specific plant structure determines fungal community structure in *H. ovalis*. Because the plant mycobiome is known to exert a strong effect on plant health, to maximise the success of future seagrass transplantation and restoration work we propose that these efforts consider the importance of seagrass mycobiomes at all stages.

Introduction

As primary producers, seagrass meadows are a critical ecosystem in the marine environment. They serve multiple ecosystem functions, providing food for marine herbivores (Aragones and Marsh, 1999; Alcoverro and Mariani, 2004), act as nurseries (Verweij *et al.*, 2008), and provide coastal protection (Christianen *et al.*, 2013). Furthermore, seagrass meadows contribute to global fisheries productivity (Unsworth *et al.*, 2019a), mitigate climate change by blue carbon sequestration (Duarte and Krause-Jensen, 2017), and potentially aid in reducing levels of bacterial pathogens from the environment (Lamb *et al.*, 2017). Despite their acknowledged importance, seagrass meadows are being lost at an alarming rate, exacerbated by anthropogenic factors (Dunic *et al.* 2021). Consequently, there is a pressing need to design comprehensive and effective seagrass restoration and conservation policies (Unsworth *et al.* 2019b).

In terrestrial plants, host-fungal interactions are crucial in maintaining plant fitness, and the importance of considering fungal communities is widely acknowledged in terrestrial restoration programmes (Berruti *et al.*, 2015; Asmelash *et al.*, 2016). In contrast, studies on their marine angiosperm counterparts have only recently started gaining traction, with one of the largest efforts characterising the fungal communities within the well-studied *Zostera marina* over its global distribution (Ettinger *et al.*, 2021). The importance of the mycobiome in seagrasses cannot be overstated, particularly when viewed through the lens of conservation management and restoration (Ugarelli *et al.* 2017; Duarte *et al.* 2018; Conte *et al.* 2021).

Found in both the tropical Atlantic and Pacific bioregions with high long-distance dispersal potential (Short *et al.* 2010; Wu *et al.* 2016), *Halophila ovalis* is a fast-growing seagrass that is a preferred food source for the charismatic and threatened megaherbivore *Dugong dugon* (Preen, 1995; Nakaoka and Aioi, 1999). Considering the critical roles played

by seagrasses, the role the mycobiome has in promoting host health, and the sparsity of studies examining seagrass-associated fungi, particularly in Southeast Asia, we investigated the fungal communities associated with *H. ovalis*—across three different plant structures, leaf, rhizome and root—collected from seven sites throughout the Malay Peninsula based on ITS1 rDNA sequencing. Furthermore, we analysed sediment samples in close proximity to each collected plant. The ITS region is the generally accepted fungal barcoding marker (Schoch et al. 2012) and is used in the majority of fungal metabarcoding studies (Lücking et al 2020), this ubiquity allows comparisons between regions, species and ecosystems. However, the currently used primers do not amplify 100% of all known fungal taxa (Lücking et al 2020) which likely leads to an underestimation of fungal diversity.

Based on previous seagrass mycobiome studies (Ettinger and Eisen, 2019; Ettinger *et al.*, 2021; Wainwright *et al.*, 2019b), we hypothesised that fungal communities will be distinct based on the following: (1) between sediment and plant; (2) between plant structures; and (3) between sampling sites.

Results and discussion

Alpha diversity and composition of fungal communities

Ten samples of *H. ovalis* (separated by structure) and sediment were obtained from seven sites across the Malay Peninsula, sediment samples were collected within 1m of each plant, a core sample extending down to approximately 4cm below the surface was taken with a sterile syringe that had the end removed. In total 40 samples were collected from each site and 0.25g of tissue and sediment was used in DNA extractions (Fig. 1; Table S1). Sequencing protocol, read filtering, and identification of amplicon sequence variants (ASVs) followed Wainwright et al. (2019b). Taxonomy was assigned using the UNITE database v8.2 (Abarenkov *et al.*, 2020), with any non-fungal taxa removed from further downstream analyses. Raw sequences were deposited under BioProject ID: PRJNA736417, and the number of reads recovered per sample are indicated in Table S1. Samples were not rarified

(McMurdie & Holmes 2014), inspection of rarefaction curves indicates that sequencing depth was sufficient (Fig. S1). To generate Bray-Curtis distances data was transformed to proportions and all subsequent analyses were conducted using R v4.0.4 (R Core Team, 2021), using phyloseq v1.34.0 (McMurdie and Holmes, 2013) unless otherwise stated.

Following Ettinger and Eisen (2019), we calculated statistical differences in both the number of observed ASVs and Shannon diversity using Kruskal-Wallis tests with 9,999 permutations followed by *post hoc* Dunn tests (Table S2–S3). We detected significant differences between sediment and plant structures but not between plant structures, mirroring findings of Ettinger and Eisen (2019). Similar to this work (Fig. 2A), recent studies on seagrasses also demonstrated higher alpha diversity in sediment-associated fungi compared to plant structures (Hurtado-McCormick *et al.*, 2019; Wainwright *et al.* 2019a & 2019b; Ettinger *et al.*, 2021). The number of shared fungal taxa between different plant structures and sediment ranged from 52 to 92, whereas the number of ASVs shared between only plant structures stood between 6 and 13, with fewer unique ASVs found within each plant structure in comparison to the sediment (Fig. 2B).

Extraction of the top 20 ASVs with the highest relative abundance corroborated high taxonomic overlap between sediment and *H. ovalis* samples (Fig. 3, S3). Unlike *H. ovalis* tissues, sediment samples were typically dominated by either *Penicillium citrinum* or *Hortea werneckii*, whereas all tissues showed higher evenness in relative abundances of fungal taxa. *Penicillium citrinum* is a common marine fungus reported to have antibacterial properties and promote plant growth (Khan *et al.*, 2008; Sabdaningsih *et al.*, 2020). Similarly, Abdel-Kareem *et al.* (2021) showed that the marine fungus *Candida orthopsilosis*, also found here, can enhance growth and disease resistance. Finally, we found two species of *Aspergillus* that were abundant in seagrasses: *A. sydowii* and *A. penicilloides*. The former is thought to be a pathogen of gorgonian seafans in the Caribbean (Alker *et al.*, 2001; but see Toledo-Hernández *et al.*, 2008), whereas the latter is able to efficiently remove heavy metal contaminants from the aquatic environment (Paria *et al.*, 2018).

Within *H. ovalis* structures (excluding sediment), we identified five fungal taxa that were highly abundant (Fig. 3, S3). Notably, *Rigidoporus microporus* was found most abundant in the

rhizome of collections made from Merambong Shoal, it is a known pathogen of rubber and other agricultural or commercially important plants (e.g., coconut trees) (Farid *et al.*, 2009; Watling and Seaward, 2004). While the pathogenic effects of *R. microporus* in seagrasses remain undocumented, long-term studies on the Merambong Shoal population may yield insights into the effects of *R. microporus* on the health of seagrass meadows and how it enters the marine environment. The characterisation of fungal communities can provide insights into host health, fungal symbiotic relationships and the suitability of hosts for transplantation into new environments (Zahn and Amend, 2017; Wainwright *et al.*, 2019b; Lee *et al.* 2019; Lee *et al.* 2020 Egan *et al.* 2021). Furthermore, some of these fungi (e.g. *Papiliotrema mangalensis*, *Sporormiella minima*) have previously been identified in mangrove plants (Kumaresan and Suryanarayanan, 2001; Hagler *et al.* 2017), suggesting connectivity of fungal communities between mangroves and seagrass habitats, or a broad ecological niche. The importance of ecological connectivity between the mangrove-seagrass-coral reef continuum has been documented and recognised in previous studies (Berkström *et al.*, 2013; Du *et al.*, 2020), but research examining the fungal communities in the marine microbiome is sorely lacking (Trevathan-Tackey *et al.* 2019). With a growing body of data, the biogeography and ecological importance of fungal communities along this continuum may be elucidated in the near future (e.g., Wainwright *et al.* 2018).

Beta diversity of fungal communities

Principal coordinate analyses (PCoA) were conducted using Bray-Curtis distances (Fig. 4, S2). The effects of two factors, plant structure and location, on fungal community composition were tested with permutational multivariate analysis of variance (PERMANOVA) using the *adonis* function in vegan v2.5-7 package (Oksanen *et al.* 2020). Due to the disproportionately high alpha diversity in sediment samples (Fig. 2), we further computed the amount of variance for both factors limited to only *H. ovalis* structures in downstream analyses. Analyses showed that fungal communities in sediment samples were clearly distinct from those in the plant structures. Correspondingly, we obtained significant differences for structure, location and structure:location with the inclusion of sediment

samples, but only location and structure:location was significant within *H. ovalis* when sediment was excluded (Table S4).

To determine the contribution of each variable, we performed pairwise PERMANOVA implemented in the EcoUtils v0.1 package (Salazar, 2021) to characterise specific community differences according to location and structure (Table S5). Given the proximity of roots to sediment, we expected the communities of *H. ovalis* roots to be similar to that of sediment, however, we found significant differences in the fungal communities found in roots and the sediment ($p=0.002$) (Table S5). We further tested dispersion between the different sample types to ensure our results were not an artefact of significant differences in dispersion using betadisper, followed by permutest with 9,999 permutations (Table S6) implemented by vegan v2.5-7 and found no significant difference ($p=0.1119$; Table S6). The morphology of *H. ovalis* (Singh *et al.*, 2019)—having a short rhizome (5–20 mm) and leaf (6–25mm)—could contribute to the homogenous fungal community across the seagrass. Plant morphology may be a critical factor in providing an additional dimension for comparison in future meta-analyses comparing differences in mycobiomes across seagrasses.

Corroborating the effects of location on fungal community structure, all pairwise comparisons resulted in significant differences between sites when sediment samples were included, and the exclusion of sediment found similar results with the exception of Cyrene Reef vs. Sentosa, Pulau Semakau vs. Sentosa (Figs. 1, 3; Table S5). The absence of fungal community structure over comparatively small spatial scales when only the host is considered is consistent with work examining the coral mycobiome in Singapore, and suggests a reasonably high degree of connectivity between locations separated by <10km (Rabbani *et al.*, 2021). Furthermore, distance decay of similarity in fungal communities assessed by Mantel test (*mantel* in vegan v2.5-7) for both with and without sediment samples revealed a significant pattern (Table S7). Consistent with previous studies (Ettinger and Eisen, 2019; Wainwright *et al.*, 2019b), location appears to be the main driver of fungal community differences between *H. ovalis* samples. Given these findings, we suggest that any seagrass transplants are taken from, or grown as close as possible to, the recipient site; failure to do so may result in transplants that are maladapted to their new environment.

Conclusion

Consistent with other studies, marine sediment contains high diversity of fungi, significantly more so than fungal communities associated with *H. ovalis*. Nevertheless, location is the main driver shaping fungal communities within *H. ovalis*. We suggest that transplantation of seagrasses in the future might benefit from the consideration of fungal communities, especially given the differences in fungal community structure with location. Finally, this is, to our knowledge, the first characterisation of the mycobiome of a non-tape seagrass, which future studies analysing the effects of plant morphology on mycobiomes may integrate and build upon.

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Ethical approval

All applicable permits, international, national, and/or institutional guidelines required to perform the work were followed. Collections from Malaysia were made under permit JTLM 630-7Jld. 9(9) and from Singapore under permit numbers NP/RP 18-035 & NP/RP 18-035a.

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Figures

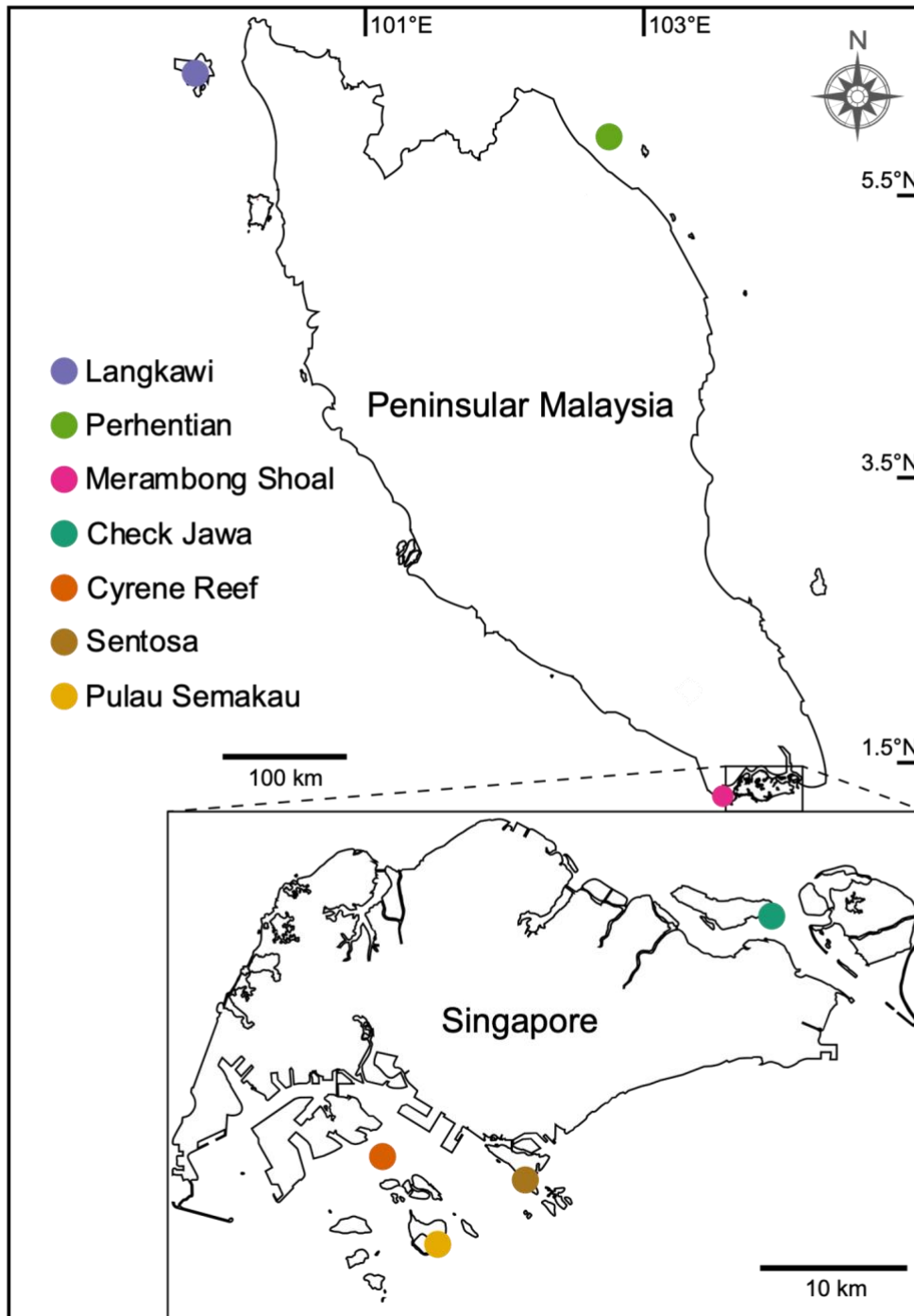


Figure 1. Map of sampling sites across Peninsula Malaysia

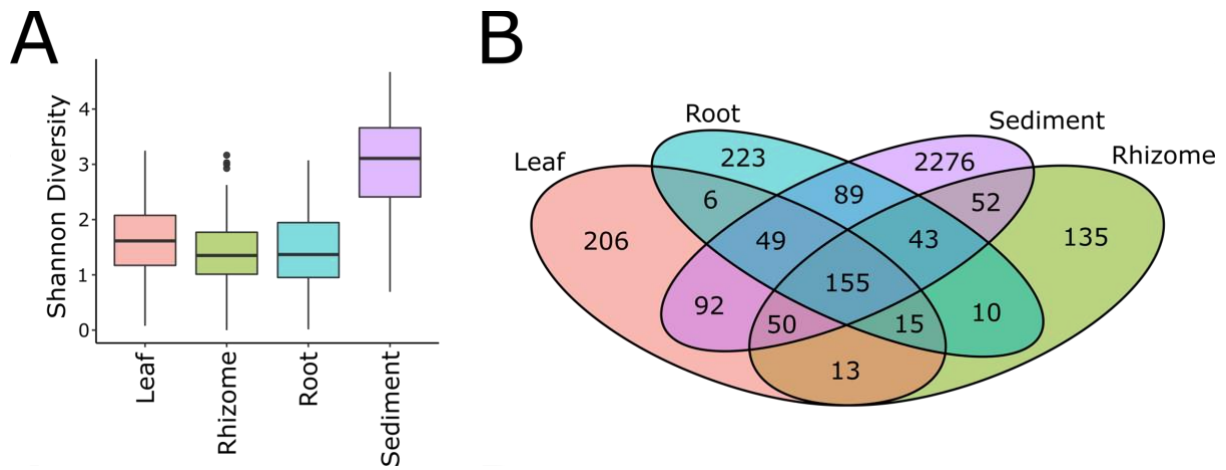


Figure 2. Diversity and richness of fungal taxa recovered across different *Halophilia ovalis* structures and substrate based on ITS1 sequences. (A) Shannon diversity metric; (B) ASV richness and shared taxa

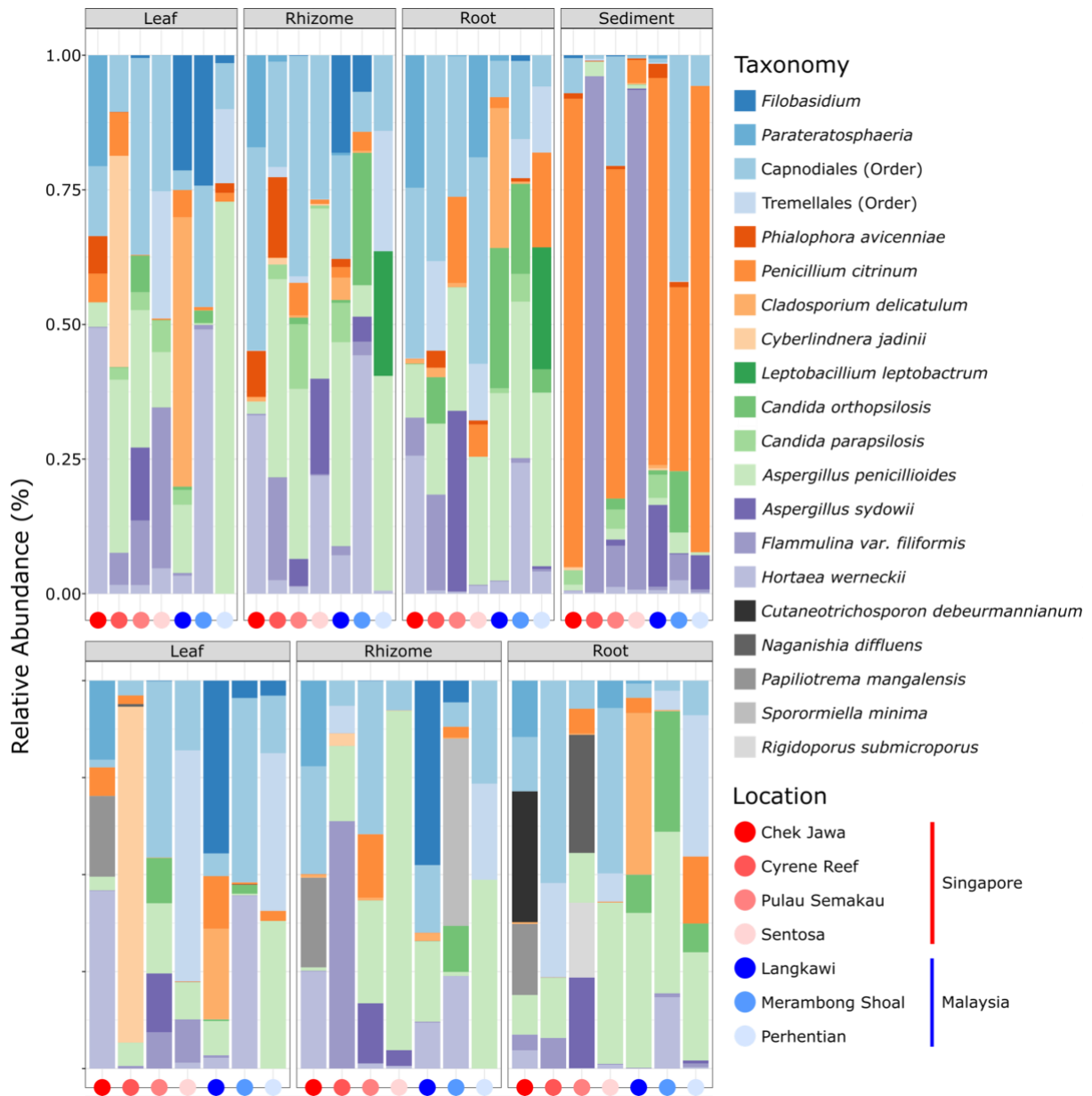


Figure 3. Top 20 ASVs recovered by highest relative abundances based on ITS1 sequences with the inclusion of sediment samples (top), and limited to *Halophila ovalis* tissues only (bottom). Each bar represents the mean relative abundance of the 10 replicates collected for a particular sample.

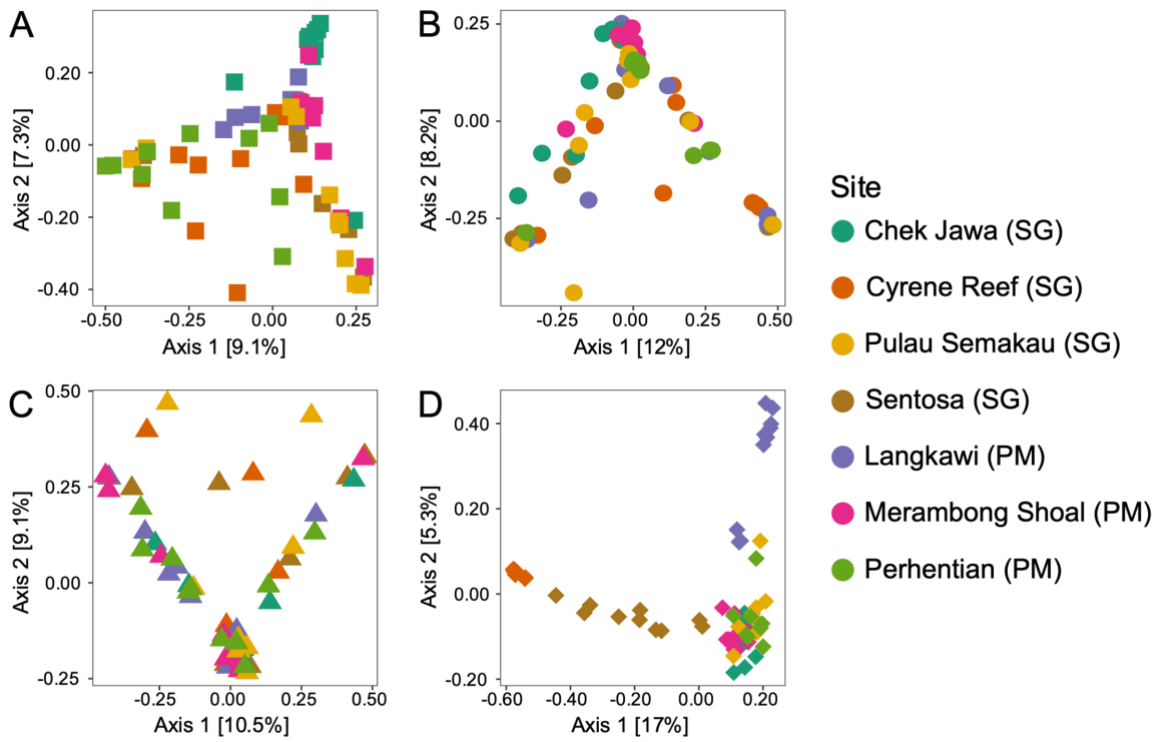


Figure 4. Principal coordinate analysis of ITS1 fungal communities based on Bray-Curtis distances, partitioned by structure. (A) Leaf; (B) Rhizome; (C) Root; (D) Sediment.