Variability in the dual mycorrhizal associations of tea tree, Melaleuca alternifolia

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Abstract

Background & aims.

Tea tree (*Melaleuca alternifolia*) is an economically important crop plant with a limited natural distribution in eastern Australia. Coastal and upland tea tree ecotypes have been identified based on unique shoot and root traits. Dual mycorrhization, the ability of plants to associate with both arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi, is particularly frequent among the Australian Myrtaceae, including *Melaleuca* species. However, the mycorrhizal type of tea tree is currently unknown.

Methods.

We investigated tea tree mycorrhizal associations in three coastal and two upland populations using ITS2 metabarcoding and root anatomical observations.

Results.

Our results revealed that tea tree is a dual mycorrhizal plant showing variability in root symbioses among populations. We found that ECM percentage root colonisation was significantly lower in coastal tea tree populations compared to upland populations, despite coastal tea tree populations exhibiting significantly higher levels ECM fungal richness. In contrast, we showed moderate evidence that AM richness was higher in coastal tea tree ecotypes than in upland ecotypes, yet comparable levels of AM root colonisation were observed between ecotypes. Mycorrhizal fungal community composition also differed significantly between coastal and upland plants.

Conclusions.

Our study provides evidence that tea tree is a dual-mycorrhizal species that can host AM and ECM fungi simultaneously within individual plants. Our findings suggest that environmental factors among coastal and upland sites, particularly soil drainage and nitrogen availability, can affect mycorrhizal traits in native tea tree populations.

1. Introduction

Melaleuca alternifolia (Maiden & Betche) Cheel, commonly known as tea tree, is a native Australian Myrtaceous species commercially cultivated for its unique essential oil. The natural range of tea tree extends from the warm-and-wet climate of coastal north-eastern New South Wales to the cool-and-dry temperate climate of the Great Dividing Range (Fig. 1) (Butcher 1994). North-eastern New South Wales is recognised as the hub of tea tree genetic diversity and has been a global centre for the industry since the mid-1900s (Southwell and Lowe 1999). In response to rising global demand and foreign industry

competition, a breeding program was launched to enhance the quality and yield of tea tree oil (Doran et al. 2006). This program evaluated genetic and chemotype variation across native tea tree populations (Rossetto et al. 1999; Homer et al. 2000). Two tea tree ecotypes, coastal and upland, were identified based on adaptive shoot and root traits (Shepherd et al. 2015b). Upland tea tree ecotypes have more deep root systems, likely due to the dry climate and sandy soils of the Great Dividing Range (Shepherd et al. 2015a). In contrast, coastal tea tree ecotypes show enhanced development of aerenchyma and adventitious roots when exposed to waterlogged conditions—a response to the inundation that frequently occurs in coastal tea tree habitats (Shepherd et al. 2015a).

Mycorrhizal fungi are mutualistic root symbionts that support plant nutrient and water uptake, stress tolerance, and pathogen defence while receiving carbon and lipids from their hosts (Smith and Read 2008). In agricultural systems, mycorrhizal fungi have been shown to improve plant performance and crop yield under certain conditions (Lutz et al. 2023) and can enhance essential oil content in medicinal plants (Wicaksono et al. 2018; Azizi et al. 2021). There are two dominant groups of mycorrhizal fungi, namely arbuscular mycorrhizal (AM) fungi and ectomycorrhizal (ECM) fungi that differ in their ability to benefit their host plants (Tedersoo and Bahram 2019). The ability of a host plant to exploit both AM and ECM strategies simultaneously can provide complementary nutritional and non-nutritional benefits (Teste et al. 2020).

While it is commonly assumed that individual plant species exclusively associate with one type of mycorrhizal fungi, dual mycorrhization—where plants simultaneously associate with both AM and ECM fungi—is gaining recognition (Teste et al. 2020). Understanding the factors driving variability in mycorrhizal associations is complex due to the unique evolutionary histories of AM and ECM plants and their fungal partners (Hoeksema et al. 2018). Consequently, dual mycorrhizal plants have emerged as valuable models for exploring the dynamics of mycorrhizal associations within the same host, accounting for underlying host effects (Tedersoo and Bahram 2019; Teste et al. 2020). For example, dual mycorrhizal species have been consistently observed to shift from AM dominant in flood prone habitats with poor soil drainage to ECM dominant in dryer habitat with well-drained soil, due to ECM sensitivity to very wet conditions (Tedersoo and Bahram 2019; Teste et al. 2020). Dual mycorrhization is particularly prevalent among Australian members of the Myrtaceae family, including *Melaleuca* species (Brundrett and Tedersoo 2018). However, the mycorrhizal type and root-associated fungi of tea tree remains unexplored.

We aimed to characterise the mycorrhizal type of tea tree and to evaluate variations in mycorrhizal associations across ecotypes, focusing on three coastal and two upland native populations. To achieve this, we measured mycorrhizal root colonisation and identified root-associated fungi using ITS2 metabarcoding. We hypothesised that (1) tea tree is a dual mycorrhizal plant, and (2) AM fungal colonisation and richness would peak in coastal sites with poor soil drainage and frequent flooding, while ECM colonisation and richness would be higher in upland sites that harbor a drier climate and well-drained soil (Mikryukov et al., 2023).

Distribution of tea tree (*Melaleuca alternifolia*) from native upland and coastal populations. Herbaria records include all tea tree voucher specimens (*n* = 177) on Atlas of Living Australia (2024a). We excluded seven outlier points that occurred up to 400 km south of the map extent. Numbers within site icons indicate tea tree sample sizes in our study.

2. Materials and Methods

2.1. Site descriptions of native tea tree populations

We conducted sampling in three coastal and two upland populations from across the natural tea tree range (Fig. 1). Coastal sites, represented by tall open forests in Candole (CAN 29°42'08"S; 153°13'51"E), Branch Creek (BC; 28°59'20"S, 153°00'36"E) and Devils Pulpit (DP; 29°14'54"S, 153°15'08"S), exhibited medium-heavy to heavy clay soils. Notably, these soils featured an anoxic (grey) layer approximately 5 cm below the surface, a consequence of the frequent flooding typical of coastal tea tree habitats (Shepherd et al. 2015a). These coastal areas were waterlogged due to above-average rainfall at the time of sampling (Supporting Information: Table S1). In contrast, the upland sites—Ballandean (BAL; 28°47'10"S, 151°49'06"E) and Cannon Creek (CC; 28°34'48"S, 151°50'58"E)—had relatively sandy, well-drained soil without an apparent anoxic layer. Coastal sites experience significantly higher mean annual temperature and precipitation yet have a significantly lower soil pH and higher plant available nitrogen (ammonium) than upland sites (Supporting Information: Table S2) (Shepherd et al. 2015b). Coastal sites also had significantly higher soil moisture levels during sampling than upland sites (Supporting Information: Table S2).

Both upland sites (BAL and CC) were open shrublands dominated by tea tree with a sparse (BAL) or dense (CC) grassy ground cover. The vegetation in two of the coastal sites, CAN and BC, was dominated by eucalypts, which typically form ECM associations as mature trees (Teste et al. 2020), with notable occurrences of *Melaleuca* species (*M. alternifolia, M. nodosa, M. quinquenervia, M. sieberi and M. thymifolia*) near water courses. In contrast, the forest type at DP was a *Melaleuca* swamp, dominated by *M. alternifolia*, *M. nodosa*, and *M. quinquenervia*. These species are assumed to be dual-mycorrhizal; that is, previous evidence showed that that *M. quinquenervia* is a dual mycorrhizal species in drained sites but only associates with AM fungi in swamps (Khan 1993).

2.2. Root sampling

In May 2021, we sampled roots from six to eleven well-spaced adult tea tree individuals per population, resulting in 20 samples per ecotype. A minimum of ten metres between plants was maintained to ensure local independence (Tedersoo et al. 2011). Fine roots (< 1 mm diameter) were extracted by tracing roots from the base of each tea tree individual in cardinal directions. Although we did not verify the host identity of root samples using molecular methods, we meticulously sampled each tea tree individual to ensure the roots originated from the targeted individuals. The roots were stored in a portable refrigerator during transport and processed within 48 hours of collection. Undamaged fine roots were thoroughly

washed with tap water, and subsamples were either stored in 70% ethanol for root anatomical observations or frozen at -80 °C for molecular analyses.

2.3. Root mycorrhizal colonisation

We examined the occurrence and percentage of AM and ECM fungal colonisation in tea tree roots using a clear and stain technique optimised for darkly pigmented roots (Brundrett et al. 1996). Fine roots were cut into ca. 1.5 cm fragments and cleared in excess 10% (w/v) KOH solution for 5–8 h (depending on pigmentation) at 90°C. Cleared roots were rinsed with deionised (DI) water and bleached (1 NH_4OH : 1 H_2O_2 : 200 DI H_2O) for 10–40 min, depending on residual pigmentation. Bleached roots were rinsed with DI water and stained with trypan blue at a concentration of 0.05% (w/v) in lactoglycerol (1 lactic acid : 1 glycerol : 1 DI water) for 5 min at 100 °C. Stained roots were rinsed with DI water and de-stained in lactoglycerol for a minimum of 1 week.

We used the magnified gridline intersect method (Mcgonigles et al. 1990) to estimate the percentage of root length colonised by AM fungi. One hundred observations across ca. 60 cm of fine root were recorded at 200 x magnification for each tea tree individual. The presence of AM fungi was recorded if the microscope crosshair intersected an intraradical AM feature (arbuscule, hyphal coil, aseptate hypha, vesicle or spore). We quantified ECM fungal colonisation by systematically assessing all stained root tips (ECM and non-ECM) at 100 x magnification until 100 ECM or 100 non-ECM root tips were counted. Percentage root tips colonised by ECM fungi were then estimated using the equation ECM counts / (ECM counts + non-ECM counts) (Teste et al. 2006).

2.4. Molecular analyses

The Australian Genome Research Facility (Melbourne, Australia) performed DNA extraction, library preparation, and sequencing. DNA was extracted from 0.25g of fine root per tea tree individual using the DNeasy PowerSoil Pro Kit (QIAGEN). Fungal ITS2 barcodes were amplified using the ITS86F (Turenne et al., 1999) and ITS4R (White et al., 1990) primer pair and Platinum SuperFi II mastermix (Life Technologies). PCR products were cleaned using magnetic beads, and samples were visualised on 2% Sybr Egel (Thermo-Fisher). Amplicons were indexed with Platinum SuperFi II mastermix (Life Technologies), cleaned with magnetic beads, quantified by fluorometry (Promega Quantifluor), and normalised at equimolar concentration before pooling. The equimolar pool was cleaned using magnetic beads, and the pooled concentration was measured using a High-Sensitivity D1000 Tape on a 2200 TapeStation (Agilent). The DNA pool was diluted to 5nM and quantified using a Qubit High Sensitivity dsDNA assay (ThermoFisher). Sequencing was conducted on an Illumina MiSeq instrument (San Diego, CA, USA) with a V2, 500 cycle kit (2 x 250 bp paired-end sequencing).

2.5. Bioinformatics

Demultiplexed paired-end reads were assembled using *VSEARCH* v2.21.0 (Rognes et al. 2016) and unpaired reads were discarded. Primers were trimmed using *cutadapt* v4.2 (Martin 2011), and reads without primers were discarded. The IST2 region was extracted using *ITSxpress* (Rivers et al. 2018).

Quality filtering and denoising, as well as de-novo and reference-based chimera removal, were performed in *VSEARCH* using the UNOISE3 and UCHIME3 algorithms, respectively (Edgar, 2016a). Chimera detection was conducted against UNITE + INSDC for all eukaryotes v9.0 (Abarenkov et al. 2023). Reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity.

We assigned taxonomy to OTUs by running BLASTn queries against UNITE + INSDC v9.0 (Abarenkov et al. 2023) using BLAST + v2.12.0 (Camacho et al. 2009). AM fungi were assigned to all OTUs annotated as Glomeromycota using sequence coverage and similarity thresholds of 90% and 85%, respectively (Tedersoo et al., 2022a). We used FungalTraits (Põlme et al. 2020) to assign OTUs to ECM fungi using a sequence coverage threshold of 90% and taxon-specific similarity thresholds ranging from 85–95% (Tedersoo et al., 2022a). We accepted generic-level annotations at 66.67% consensus across the top three BLAST hits. We removed low abundance OTUs sample-wise using an abundance threshold of 0.05% to control for putative contaminants. To address index switching, we performed a library-wise filter by removing OTUs from samples where their relative abundance was < 0.1%.

2.7. Data analyses

Statistical tests were performed in R v4.2.2 (R Core Team 2022), plots were visualised using *ggplot2* v3.4.2 (Wickham 2016), and mapping was conducted in *QGIS* v.3.30.0 (QGIS Development Team 2022).

To test for differences in root mycorrhizal colonisation and alpha diversity between upland and coastal tea tree populations, we fitted linear and generalised linear mixed models using *lme4* v1.1.31 (Bates et al. 2015). Root colonisation measurements (i.e. proportions based on positive mycorrhizal observations over total observations) were fitted on a binomial error distribution, and OTU observed richness with a Poisson error distribution, with samples nested within sites as random effects. Shannon diversity models were fitted on a Gaussian error distribution with sites as random effects. Effects coding was used for categorical variables (i.e. ecotype). Assumptions of residual normality, homogeneity, dispersion, and autocorrelation in the residuals were tested and visualised using *DHARMa* v0.4.6 (Hartig 2021). When spatial autocorrelation was detected, models were refitted to include significant Moran eigenvector maps (MEMs) to account for the spatial dependency. Significant MEMs were estimated using *adespatial* v0.3.20 (Dray et al. 2012). All diversity analyses were performed on non-rarefied data because rarefaction curves indicated that OTU counts were in saturation across all individual root samples (Supporting Information: Figure S1).

The community composition of AM and ECM fungi was assessed on OTUs that occurred in at least two samples. Differences in community composition between upland and coastal tea tree populations were visualised with NMDS plots and tested using PERMANOVA analysis with the 'adonis2' function in *VEGAN* v0.4.6 (Oksanen et al. 2022). Tests were conducted on both Jaccard (presence/absence) and Bray-Curtis (abundance-based) distances. OTU abundances were normalised before estimating Bray-Curtis distances to account for differences in sequencing depth. Differentially abundant fungal genera between ecotypes were evaluated with *ANCOM-BC* v2.0.1 (Lin and Peddada 2020), with *p*-values adjusted using the Benjamini–Hochberg method.

3. Results

3.1. Mycorrhizal root colonisation across ecotypes

The associations of ECM associations were characterised by comparatively short unbranched lateral roots with a thin and poorly developed mantle (Figs. 2a–b). Intracellular AM structures were typified by hyphal coils (Fig. 2d), with relatively few Paris-type arbusculate coils (Fig. 2c) and no Arum-type arbuscules. Simultaneous AM/ECM infection was observed within the individual root segments (Figs. 2e–f) in 36 of the 40 samples examined. All root samples from upland sites (n = 20) exhibited colonisation by both AM and ECM fungi (Fig. 2). In contrast, although AM colonisation was observed in all coastal plants (n = 20), only 80% were simultaneously colonised by ECM fungi. It should be noted, however, that we still observed ECM colonisation in the remaining 20% of roots, albeit at very low levels that escaped our colonisation assessment method (i.e. we observed them in < 1% of fine roots that were not part of the tested roots).

Dual mycorrhizal colonisation on tea tree roots from upland and coastal populations. Arrows showcase characteristic ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) features from tea tree in our study: **a**–**b** poorly developed thin mantle; **c** Paris-type arbusculate coils; **d** hyphal coils and spore; **e**–**f** mantles (ECM) and vesicles (AM) illustrating simultaneous AM/ECM infection within individual root segments. Scale = 100 μm.

We observed a slightly higher percentage of AM colonisation in tea tree roots from coastal populations compared to upland populations, but this difference was not significant (Fig. 3a) ($ß_{Coastal}$ = -0.89, 95% CI [-1.30, -0.48]; $ß_{Upland}$ = -0.25, 95% CI [-0.60, 0.16], p = 0.235). On the contrary, we found strong evidence that the percentage of ECM colonisation was higher in upland populations than in coastal populations, a result that was statistically significant (Fig. 3a) ($ß_{Coastal}$ = -1.74, 95% CI [-1.97, -1.52]; $ß_{Upland}$ = 1.03, 95% CI [0.81, 1.25], p = 5.17e-20).

Differences in arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungal root colonisation and diversity on tea tree roots from coastal and upland populations: **a** root colonisation (%), **b** operational taxonomic unit (OTU) observed richness, **c** fungal community composition based on Bray–Curtis dissimilarity. **a**–**b** Left panels show scatterplots of raw data, and boxes representing marginal means with standard errors and 95% confidence intervals (CIs); right panels show mean differences in effect size around the grand mean (dotted line) with 95% CIs; statistical tests were performed independently for AM and ECM association types. **c** Enlarged points are centroids and ellipses represent 95% CIs. Significance values: ** ≤0.01, *** ≤0.001.

3.2. Taxonomic diversity and composition across ecotypes

The 7,119,114 reads that passed quality filtering clustered into 719 fungal OTUs, ranging from 51,259 to 380,142 reads and 14 to 113 OTUs per sample. Species richness curves indicated that fungal OTU richness was in excess within individual root samples (Supporting Information: Figure S1), affirming that

sequencing depth adequately addressed within-sample diversity levels. However, species accumulation curves revealed that sample size only captured a proportion of the diversity of root-associated fungi across coastal and upland populations (Supporting Information: Figure S2).

The relative abundance of AM and ECM reads were higher in upland than in coastal sites, yet the observed richness of AM and ECM OTUs was higher in costal than in upland sites (Table 1). Prominent non-mycorrhizal guilds included saprotrophs (soil, litter, and wood) and putative plant pathogens, comprising 39.0% (2,786,193) and 9.4% (672,573) of reads, and 38.8% (279) and 17.2% (124) of OTUs, respectively.

Table 1
Relative abundance of read counts and operational taxonomic unit (OTU) richness for arbuscular mycorrhizal (AM) fungi and ectomycorrhizal (ECM) fungi across all samples and subsets from upland and coastal regions.

	All samples		**Upland samples**		**Coastal samples**	
Mycorrhizal type	Relative abundance	OTU richness	Relative abundance	OTU richness	Relative abundance	OTU richness
Arbuscular mycorrhizal	1.46%	72	1.58%	18	1.29%	59
Ectomycorrhizal	43.1%	106	50.1%	49	33.6%	71

We provide moderate evidence showing that AM fungal OTU richness was significantly higher in coastal populations than in upland populations (Figs. 3b) ($ß_{Coastal}$ = 1.81, 95% CI [1.50, 2.13]; $ß_{Upland}$ = -0.40, 95% CI [-0.71, -0.09], p = 0.012). Additionally, we found strong evidence indicating that ECM fungal OTU richness was significantly higher in coastal populations compared to upland populations (Figs. 3b) ($ß_{Coastal}$ = 2.43, 95% CI [2.27, 2.58], $ß_{Upland}$ = -0.26, 95% CI [-0.41, -0.10], p = 0.001). Similar results were observed when estimating alpha-diversity with the Shannon diversity index for AM fungi ($ß_{Coastal}$ = 1.08, 95% CI [0.71, 1.44]; $ß_{Upland}$ = -0.49 (95% CI [-0.86, -0.13], t_{36} = -2.74, p = 0.010) and ECM fungi ($ß_{Coastal}$ = 0.74, 95% CI [0.62, 0.87]; $ß_{Upland}$ = 1 -0.19, 95% CI [-0.32, -0.07], t_{36} = -3.13, p = 0.003). The composition of AM and ECM fungal communities was significantly different between ecotypes based on Bray-Curtis distances (Fig. 3c) (AM, SSE = 13.8, R^2 = 0.09, p = 0.002; ECM, SSE = 12.68, R^2 = 0.05, p = 0.004) and Jaccard distances (AM, SSE = 14.34, R^2 = 0.09, $F_{(1,38)}$ = 3.41, p < 0.001; ECM, SSE = 14.14, R^2 = 0.05, $F_{(1,38)}$ = 1.98, p = 0.003).

The AM fungal community that we detected was composed of members of Glomeraceae, Entrophosporaceae, and Claroideoglomeraceae, with genera *Entrophospora* (Entrophosporaceae) and *Rhizophagus* (Glomeraceae) showing the highest relative abundance in upland and coastal sites, respectively (Fig. 4). Indeed, the relative abundance of *Entrophospora* was significantly higher in upland sites based on Log^2 fold change (Fig. 5), despite being the most OTU-poor AM fungal genus (Fig. 4).

Rhizophagus and *Glomus* (Glomeraceae) emerged as the most OTU-rich AM genera in our dataset (Fig. 4).

Tomentella (Thelephoraceae) and *Ruhlandiella* (Pezizaceae) were the most OTU-rich and relatively abundant ECM genera within the ECM fungal community (Fig. 4), with a notably higher abundance in upland than in coastal sites, although these differences were not statistically significant (Fig. 5). *Sebacina* (Sebacinaceae) was almost exclusively found in coastal samples, resulting in significantly higher abundance in coastal than in upland populations. *Cenococcum* (Gloniaceae) had a comparatively low relative abundance within the ECM community yet was significantly more abundant in coastal than in upland populations (Fig. 5).

Differential abundance of mycorrhizal fungal genera in upland and coastal tea tree populations. Log_2 fold change above the dotted line indicates genera with higher abundance in upland communities, and below the line represents higher abundance in coastal communities. Bars are standard errors, and dotted lines are 95% CIs. Detailed test statistics can be found in Supporting Information: Tables S5–S6. Significance values are Benjamini–Hochberg adjusted: * ≤0.05, ** ≤0.01, *** ≤0.001.

4. Discussion

4.1. Tea tree is a dual mycorrhizal host

Our study provides empirical evidence confirming the dual mycorrhizal status of tea tree, with 90% of the adult individuals studied exhibiting simultaneous root colonisation by AM and ECM fungi (Fig. 2e–f). This finding aligns with 77% of the mycorrhizal observations documented on other *Melaleuca* species (Supporting Information: Table S7).

The AM roots of tea tree displayed a Paris-type arbuscle morphology (Fig. 2c), which is consistent with expectations for woody plants (Dickson 2004). The ECM roots of tea tree in our study exhibited limited mantle development (Fig. 2a–b), a characteristic shared with other *Melaleuca* species, such as *M. preissiana*, *M. uncinata* and *M. systena* (Albornoz et al., 2016a; Bougoure et al., 2009; Teste et al., 2014). Such poor ECM development has raised questions regarding the functionality of ECM roots in *Melaleuca* species (Brundrett 2017), given the demonstrated positive correlation between Hartig net thickness and plant growth in *Eucalyptus grandis* (Burgess et al. 1994). However, a common garden microcosm study on *M. preissiana* later showed that higher levels of ECM colonisation coincided with enhanced growth and shoot nutrition, despite limited ECM development, emphasising the potential benefits of ECM fungi to *Melaleuca* species (Teste et al., 2014).

The most notable ECM fungal genera colonising tea tree roots were *Tomentella*, *Ruhlandiella* and *Sebacina* (Fig. 4). *Tomentella* is frequently detected in soils of temperate and tropical regions worldwide (Tedersoo et al., 2022b) and occurs in most forest types across Australia (Atlas of Living Australia 2024a), including in association with *M. systena* grown on field soil (Albornoz et al. 2021). *Ruhlandiella* is a genus of truffle-like fungi native to the southern hemisphere that frequently form ECM associations

with *Eucalyptus* and *Melaleuca* species in Australia, as well as in plantations worldwide (Kraisitudomsook et al., 2019). We primarily detected *Sebacina* in coastal tea tree roots, which was unexpected considering its affinity to dry rather than wet tropical forests (Corrales et al. 2022). Likewise, the higher abundance of *Cenococcum* in coastal sites was surprising, as *Cenococcum* typically thrives at high latitudes (Obase et al. 2017) and is predicted to prevail in cooler regions with four distinct seasons (Zheng et al. 2023). These unexpected results highlight the gap in knowledge of ECM fungal diversity and ecology in Australia, with paradigms described in the northern hemisphere that may not be well-mirrored in southern latitudes (Albornoz et al. 2021).

Given that *Tomentella, Ruhlandiella*, and *Cenococcum* are early successional taxa known to occur in disturbed habitats (Warcup 1991; Jairus et al. 2011; Sugiyama and Sato 2021), they could represent promising native candidates for tea tree nursery trials. These ECM genera were shown to abundantly colonise *M. systena* seedlings when grown on field soil (Albornoz et al. 2016b). Although developing specific inoculum could be challenging for the cryptic genera *Ruhlandiella* and *Cenococcum*, previous attempts to isolate species of these genera in culture have been successful (Warcup 1991).

The relatively low abundance of AM fungi that we detected in our dataset is likely due to the ITS primers we used that are known to poorly amplify some AM fungi (Lekberg et al., 2018). Subordinate families such as Acaulosporaceae, Gigasporaceae, Ambisporaceae, Archaeosporaceae, and Paraglomeraceae are typically disregarded when using ITS2 metabarcoding, including in our study. On the other hand, families such as Glomeraceae, Claroideoglomeraceae, and Entrophosporaceae are well-detected by ITS primers and were well represented in our dataset. Notably, *Entrophospora* dominated the upland community but was almost absent in coastal plants. These findings align with previous studies that considered *Entrophospora* as indicator species for AM fungi in shrublands, but not in forest soils in the Colorado Plateau, USA (Chaudhary et al., 2018).

4.2. Variation in mycorrhizal associations across tea tree ecotypes

Mycorrhizal switching is the phenomenon whereby dual mycorrhizal plants transition between AM and ECM dominance depending on environmental factors, such as flood frequency, soil drainage and nutrient availability, or temporal factors, namely plant age (Teste et al. 2020). Indeed, flooding is a common disturbance in coastal tea tree plantations and habitats, with the consequence of inhibiting tea tree growth and oil yield (Jing et al. 2009; Shepherd et al. 2015a). In response to flooding, tea tree develops hypertrophied lenticels on submerged stems (Jing et al. 2009) and aerenchyma on roots (Shepherd et al. 2015a) to facilitate gas exchange and provide roots with oxygen under hypoxic conditions (Yamamoto et al. 1995; Visser 2003). Unlike ECM fungi, the endosymbiotic habit of AM fungi enables them to access oxygen in submerged roots (Miller and Sharitz 2000), allowing the carbon-phosphorous exchange to persist on inundated soil, albeit at a decreased rate than on well-drained soil (Bao et al. 2019). In contrast, limited oxygen supply across the ECM interface under waterlogged conditions adversely affects ECM root development (Read and Armstrong 1972). Thus, differences in flood frequency and soil

drainage between coastal and upland habitats were likely the dominant factors contributing to the lower levels of ECM colonisation on coastal tea tree individuals, highlighting the critical roles these factors can play in regulating mycorrhizal switching.

For example, *M. quinquenervia, Casuarina cunninghamiana* and *Salix babylonica* were colonised by ECM fungi on well-drained soil but not in swamps, whereas AM colonisation levels were constant across sites regardless of habitat conditions (Khan 1993), which is reasonably consistent with the findings in our study. By contrast, *Leptospermum scoparium* (Moyersoen and Fitter 1999; Weijtmans et al. 2007) and *Quercus rubra* (Watson et al. 1990) were predominantly colonised by AM fungi on poorly drained lowland soils yet were almost exclusively colonised by ECM fungi in an adjacent upland region characterised by drier conditions and more well drained soil. These findings collectively, albeit indirectly, support the hypothesis that dual mycorrhizal plants tend to preferentially maintain AM associations in wet environments with poorly drained soils, while ECM fungi tend to gain a competitive edge in dryer environments with well-drained soils (Tedersoo and Brundrett 2017; Teste et al. 2020).

Interestingly, soil available N (ammonium) was significantly lower and correlated with a higher percentage of ECM root colonisation in our upland tea tree study sites. The lower N availability in upland habitats would ostensibly enhance ECM root colonisation in upland tea tree ecotypes, as N limitation typically promotes ECM connectivity (Treseder 2004; Plett et al. 2020). Therefore, soil N levels likely contributed to the observed differences in ECM colonization in our study, at least to a certain extent.

Contrary to our second hypothesis, we observed the highest richness of ECM fungi in the subtropical coastal tea tree habitat, despite upland populations having higher ECM root colonisation (Fig. 3). Typically, ECM richness peaks in cool, dry climates of temperate and boreal forests (Tedersoo et al. 2012; Mikryukov et al. 2023). Coastal tea tree populations are located in ECM plant-diverse forests, while upland stands are in open shrublands dominated by tea tree. Thus, the higher ECM fungal richness in coastal tea tree roots could be due to greater ECM plant diversity (Saitta et al. 2018). Similarly, the increased diversity of AM plants in coastal sites correlated with higher AM fungal richness, aligning with expectations that AM fungi thrive in warm, wet climates (Stürmer et al. 2018; Mikryukov et al. 2023). However, our findings on spatial variation in mycorrhizal diversity should be interpreted cautiously due to our small and unbalanced sampling design, which likely detected only a portion of the mycorrhizal fungal diversity. Further studies are needed to adequately capture the mycorrhizal fungal diversity across tea tree habitats.

4.3. Perspective for the use of mycorrhizal fungi to improve tea tree oil quality and yield

The mycorrhizal associations of tea trees may represent an untapped natural resource for the tea tree industry, though their functionality cannot be inferred from our study. While dual mycorrhizal inoculations generally enhance plant growth and nutrient uptake (Teste et al., 2020), their effect on essential oil yield and composition is underexplored. A study on *Myrtus communis* showed that AM fungal inoculations can boost essential oil production, particularly under drought stress (Azizi et al. 2021). Additionally, the

yield and essential oil composition of *Leptospermum scoparium* were found to depend on AM fungal isolate identity (Wicaksono et al., 2018), while inoculations with the AM fungus *Glomus lamellosum* significantly enhanced essential oil production in *Salvia officinalis*, *Lavandula angustifolia*, *Geranium dissectum*, and *Origanum dictamnus* (Karagiannidis et al. 2011).

Future research should focus on evaluating how both AM and ECM fungi influence tea tree growth, oil yield, and quality to determine their potential benefits for the tea tree industry. Moreover, investigating the role of AM fungi in supporting tea tree growth during flooding is essential, especially given the increasing frequency and intensity of flooding observed and projected under future climate scenarios in regions with high tea tree plantation density (Herold et al. 2021). Understanding how AM fungi can enhance the resilience of tea trees to these extreme conditions could provide critical insights for sustaining tea tree oil production in a changing climate.

5. Conclusion

Our study provides empirical evidence showing that tea tree is a dual mycorrhizal plant, capable of forming both AM and ECM associations simultaneously. The observed mycorrhizal variation between coastal and upland ecotypes in our study underscores the potential role of environmental factors—especially soil drainage and nitrogen availability—in shaping tea tree mycorrhizal associations. Our findings position tea tree as a promising model for examining the trade-off and synergies between different mycorrhizal types in relation to both nutritional and non-nutritional benefits, including their impact on essential oil content, content while accounting for host-specific effects.

Declarations

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Competing Interests

All authors declare no conflicts of interest.

Author Contributions

LF and TJR conceived the ideas and designed the methodology. LF collected the data. LF and MTR analysed the data. LF and CT led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data Availability

The data from this study are available on figshare (https://doi.org/10.26181/25241788) in five files: an information file, sample metadata file, OTU sequences in fasta format, OTU-by-sample matrix and taxonomy table annotated with mycorrhizal trait information.

Code Availability

The code used for the bioinformatic and statistical analyses in this study is available at GitHub: https://github.com/LukeLikesDirt/TeaTreeMycorrhizas.

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Figures

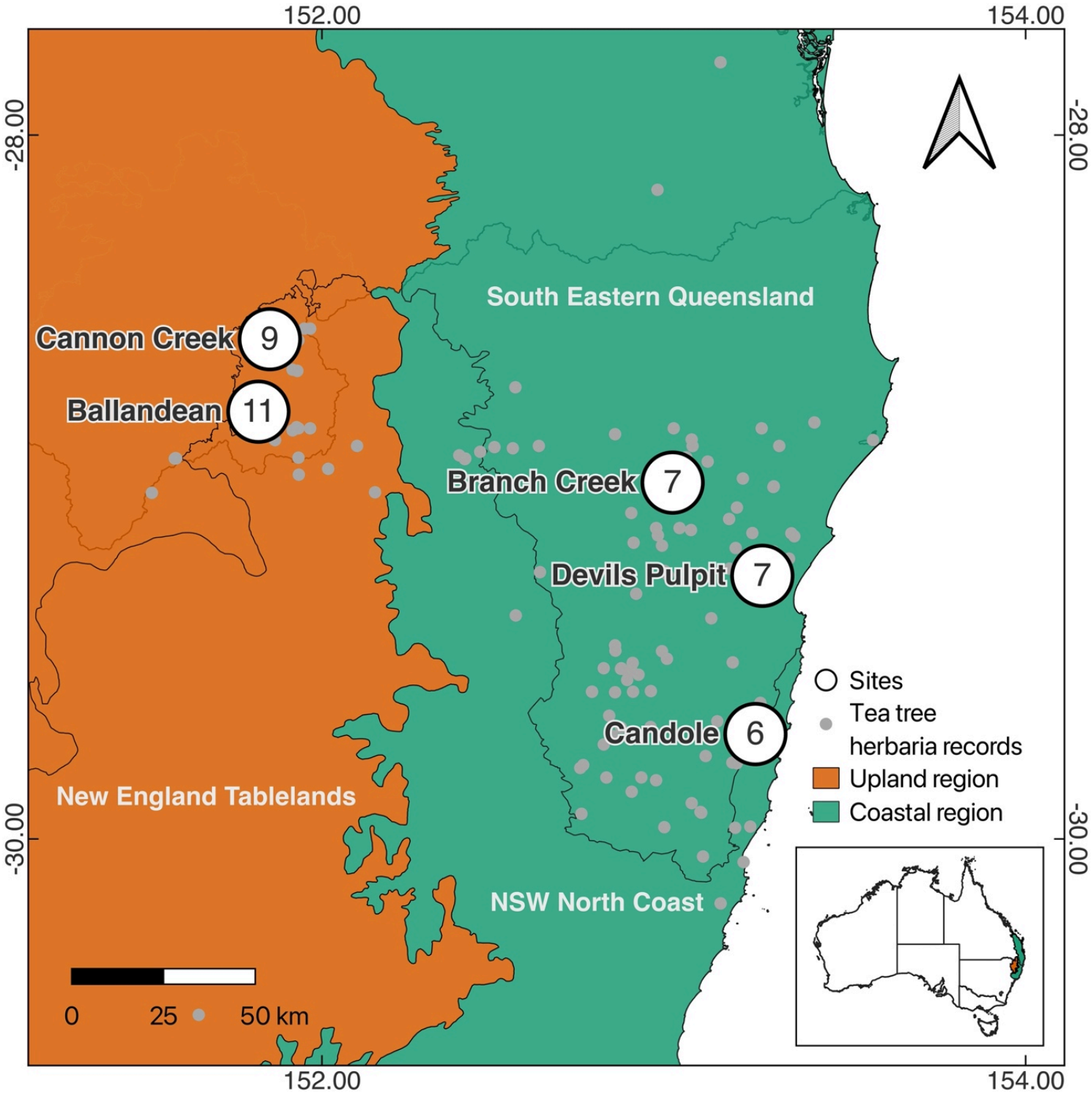

Figure 1

Distribution of tea tree (*Melaleuca alternifolia*) from native upland and coastal populations. Herbaria records include all tea tree voucher specimens (*n* = 177) on Atlas of Living Australia (2024a). We excluded seven outlier points that occurred up to 400 km south of the map extent. Numbers within site icons indicate tea tree sample sizes in our study.

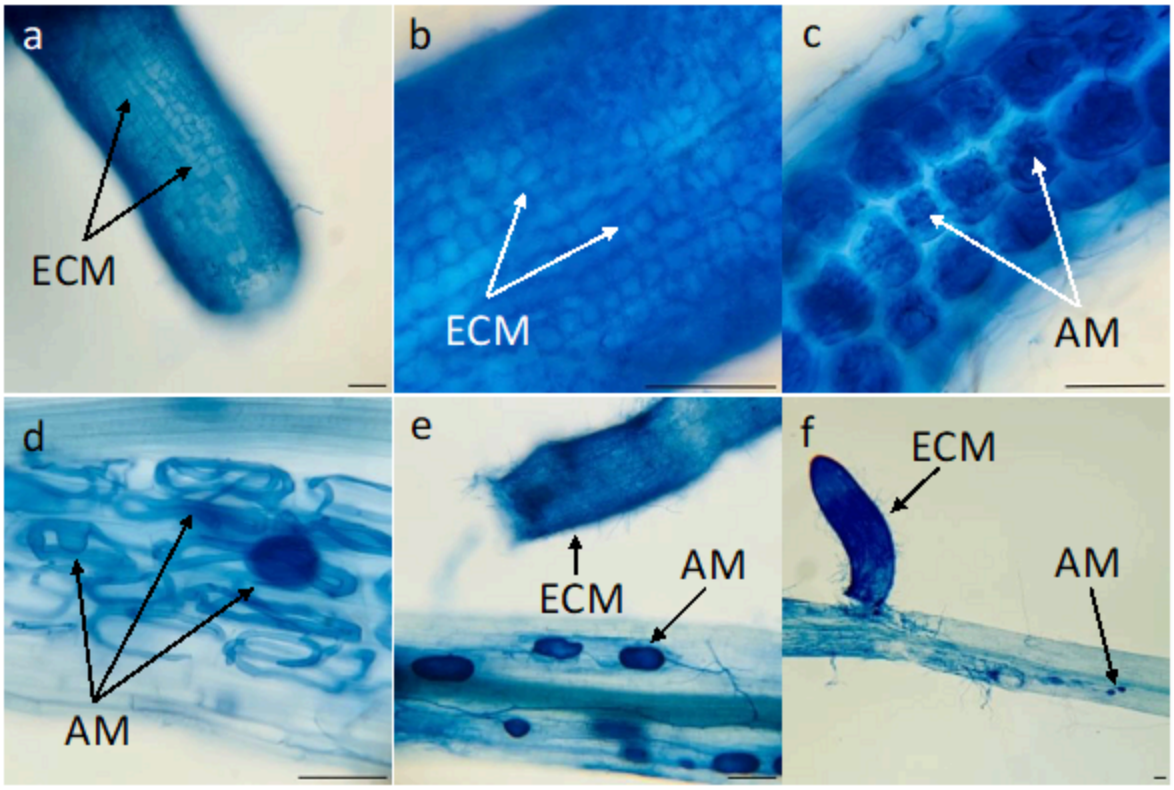

Figure 2

Dual mycorrhizal colonisation on tea tree roots from upland and coastal populations. Arrows showcase characteristic ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) features from tea tree in our study: **a**–**b** poorly developed thin mantle; **c** Paris-type arbusculate coils; **d** hyphal coils and spore; **e**–**f** mantles (ECM) and vesicles (AM) illustrating simultaneous AM/ECM infection within individual root segments. Scale = 100 mm.

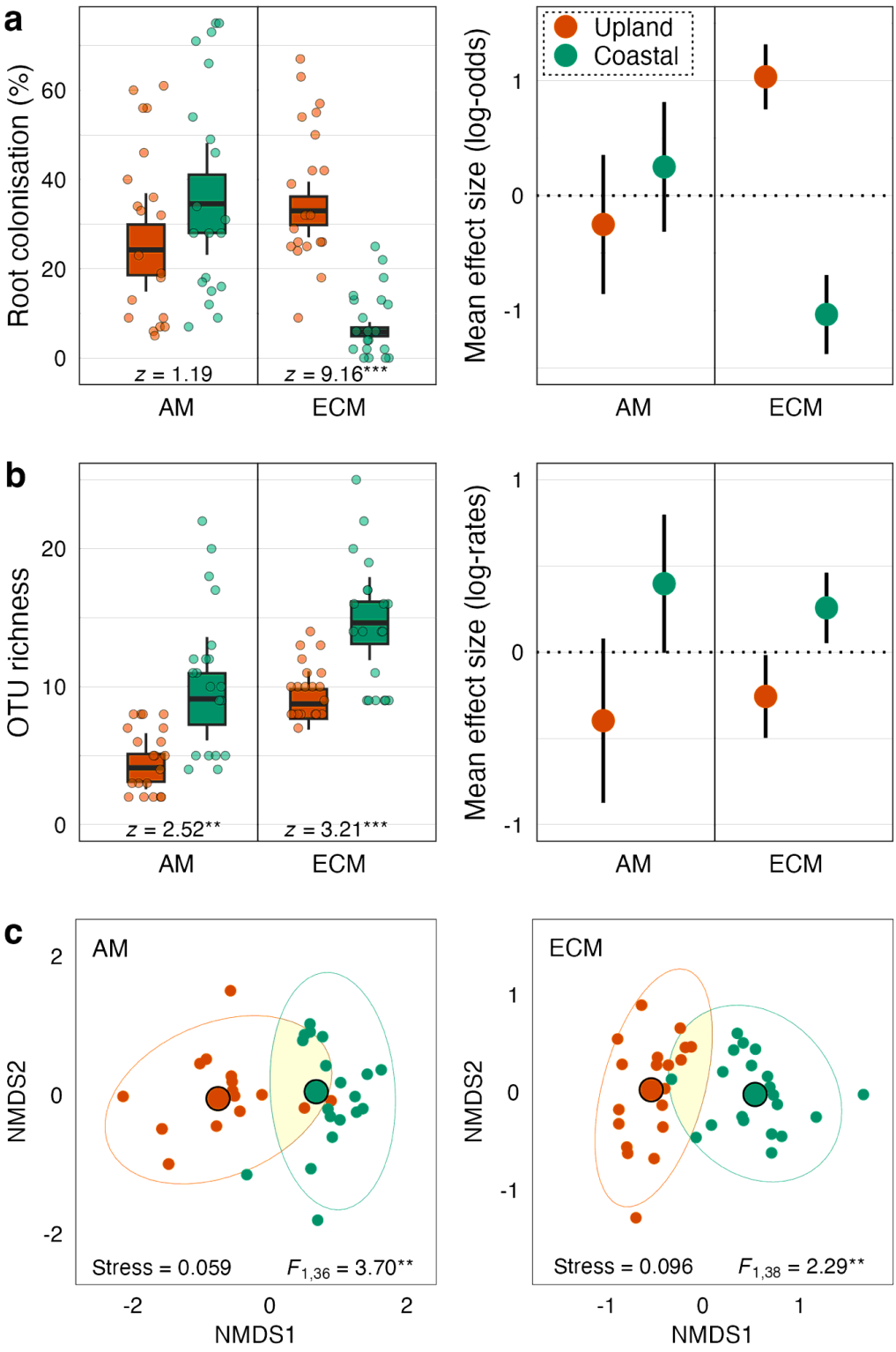

Figure 3

Differences in arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungal root colonisation and diversity on tea tree roots from coastal and upland populations: **a** root colonisation (%), **b** operational taxonomic unit (OTU) observed richness, **c** fungal community composition based on Bray–Curtis dissimilarity. **a**–**b**Left panels show scatterplots of raw data, and boxes representing marginal means with standard errors and 95% confidence intervals (CIs); right panels show mean differences in effect size

around the grand mean (dotted line) with 95% CIs; statistical tests were performed independently for AM and ECM association types. **c** Enlarged points are centroids and ellipses represent 95% CIs. Significance values: ** ≤0.01, *** ≤0.001.

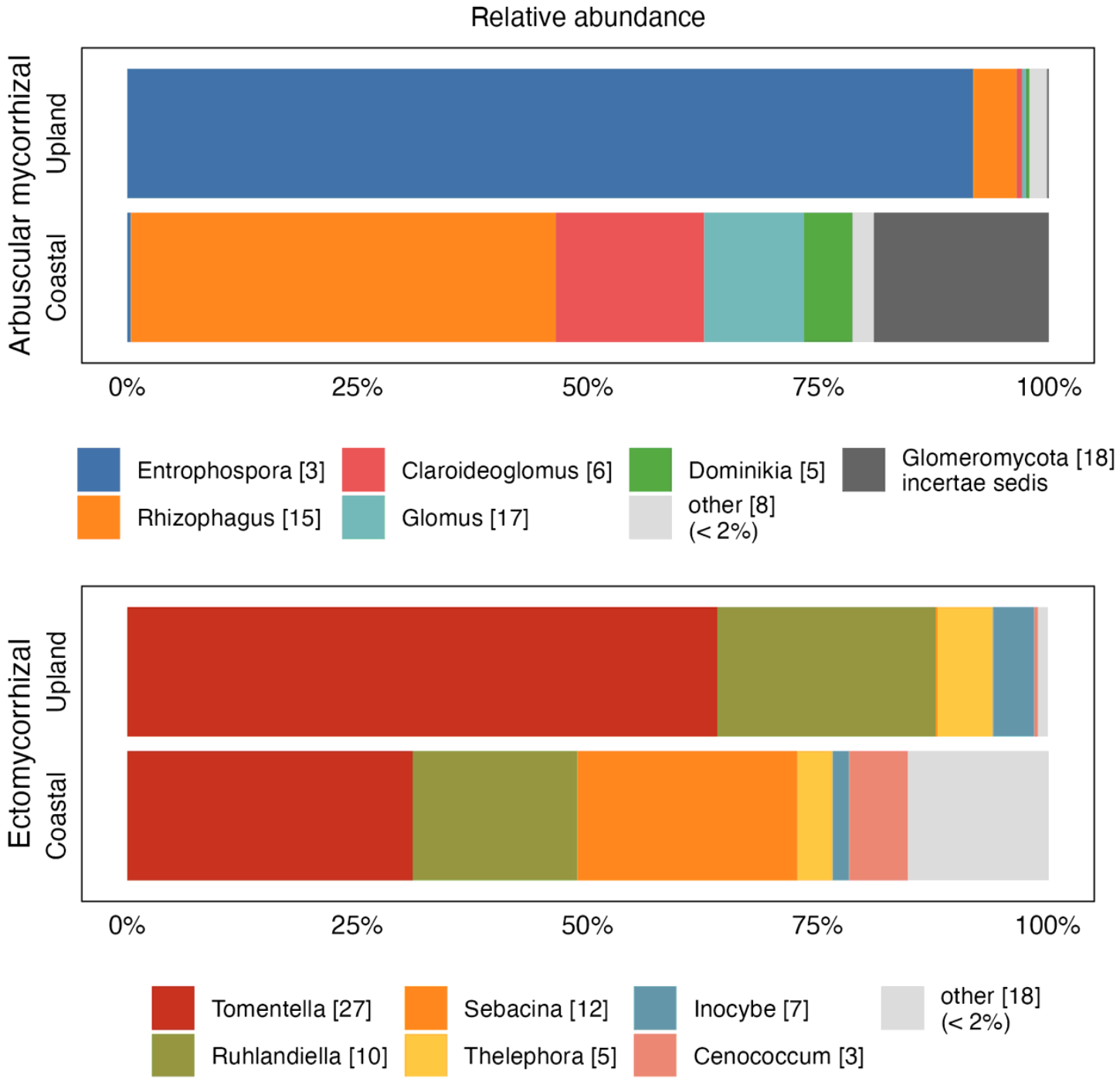

Figure 4

Relative abundance of read counts and operational taxonomic unit (OTU) richness of mycorrhizal fungal genera from coastal and upland tea tree populations. Numbers in brackets are cumulative OTU richness

across coastal and upland populations. Detailed relative abundance and richness values can be found in Supporting Information: Tables S3–S4.

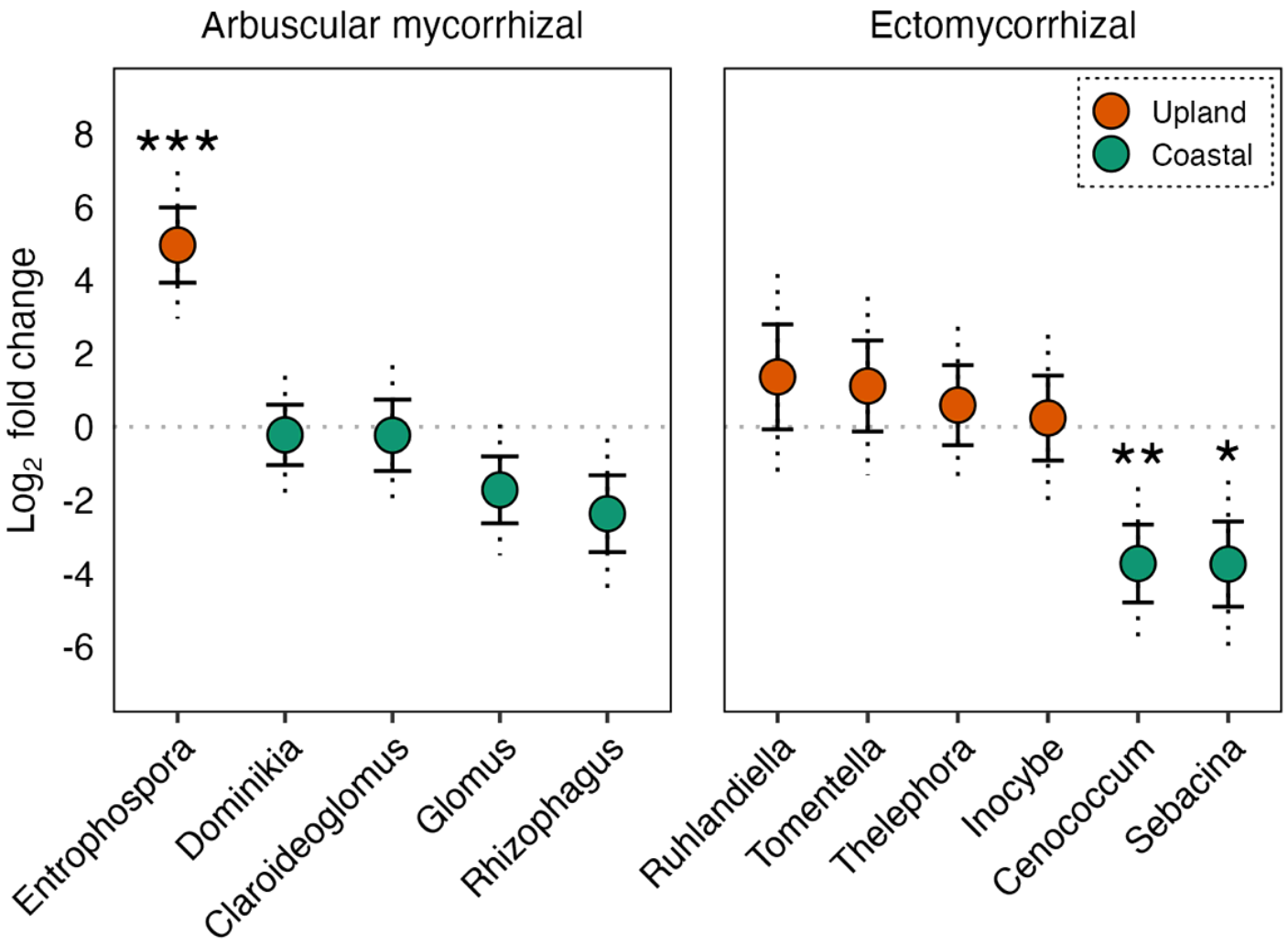

Figure 5

Differential abundance of mycorrhizal fungal genera in upland and coastal tea tree populations. Log_2 fold change above the dotted line indicates genera with higher abundance in upland communities, and below the line represents higher abundance in coastal communities. Bars are standard errors, and dotted lines are 95% CIs. Detailed test statistics can be found in Supporting Information: Tables S5–S6. Significance values are Benjamini–Hochberg adjusted: * ≤0.05, ** ≤0.01, *** ≤0.001.

Supplementary Files

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