

The geno-geo-climate nexus: contributions of geographic and ecological factors in shaping the genomic divergence of two closely related threatened rainforest species of *Fontainea* Heckel (Euphorbiaceae)

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Abstract

Context

Processes that shape genomic and ecological divergence can reveal important evolutionary dynamics to inform the conservation of threatened species. *Fontainea* is a genus of rainforest shrubs and small trees including critically endangered and threatened species restricted to narrow, but complex geographic and ecological regions.

Objectives

Our aim was to compare spatial patterns of genetic variation between two, closely related species, the Vulnerable *Fontainea australis* (Southern Fontainea) and Critically Endangered *F. oraria* (Coastal Fontainea), endemic to the heterogeneous subtropical region of central, eastern Australia, where large-scale clearing in the study region has severely reduced rainforest habitat to a fraction (<1 %) of its pre-European settlement extent. These two species are subject to spatially explicit ecological conditions and experience limited intra-specific gene flow, likely generating genetic differentiation and local adaptation. Here, we explored the genetic and ecological mechanisms underlying patterns of diversification in the two species.

Methods

We used a set of 10,000 reduced-representation markers to infer genetic relationships and the drivers of spatial genetic variation across the two species. In addition, we employed a multivariate genome-environment association analysis using a set of topo-climatic variables to explore potential patterns of local adaptation as a factor impacting on genomic divergence.

Results

Our study revealed that Coastal Fontainea have a close genetic relationship with Southern Fontainea. We showed a combination of isolation by distance and by environment have played a key role in their genetic variation, indicating that vicariance and adaptation to local environments can explain the spatial genetic distribution of the two species. Genotype-environment analyses showed a strong association with average summer temperatures and topographic features, suggesting adaptation to localised thermal environments. We used a multivariate redundancy analysis that controls for spatial effects to identify a range of putatively adapted loci associated with local environmental conditions. Searches of functional responses for putatively adaptive loci identified a range of genes linked to abiotic stressors shared with other Australian rainforest species and Euphorbiaceae allies.

Conclusions

Divergent selection at the local-habitat scale as a result of dispersal limitations and environmental heterogeneity (including physical barriers), are likely contributors to adaptive divergence between the two

Fontainea species. Our findings have presented evidence to indicate that Southern and Coastal *Fontainea* were comprised of distinct genetic groups and ecotypes, that together may form a single species continuum, with further phenotype research suggested to confirm the current species boundaries. Proactive conservation actions, including assisted migration to enhance the resilience of populations lacking stress-tolerant single nucleotide polymorphisms (SNPs) may be required to secure the long-term future of both taxa. This is especially vital for the critically endangered Coastal *Fontainea* given projections of habitat decline for the species under future climate scenarios.

Introduction

Incorporating genetic, environmental, and ecological data can enhance our understanding of how ecology, evolution, and geography contribute to the processes that drive spatial distribution (Balkenhol et al., 2017). Exploring how environmental factors influence population genetic differentiation can also improve our knowledge of the elements responsible for speciation, which are central to the diversification of species (Barracough and Nee, 2001, Manel and Holderegger, 2013). Species with narrow geographic distributions within heterogeneous environments represent ideal examples with which to examine the function of environmental factors in shaping genotypic variation. In this context, the rainforests of central, eastern Australia provide the opportunity to explore these elements in one of the most complex and diverse subtropical biomes in the world (Chester and Bushnell, 2005).

Fontainea Heckel (Euphorbiaceae) is a genus of dioecious rainforest shrubs and small trees with links to the Gondwana landmass (Brunton et al., 2023) that is patchily distributed among rainforest fragments of Australia, Papua New Guinea, New Caledonia, and Vanuatu. Several *Fontainea* species have low to moderate genetic diversity and occupy restricted environments as a result of historic rainforest contraction and expansion cycles, coupled with more contemporary effects of habitat loss, modification and fragmentation from human disturbance (Lamont et al., 2016, Conroy et al., 2019, Brunton et al., 2022). The critically endangered *F. oraria* (Coastal *Fontainea*) and Vulnerable *F. australis* (Southern *Fontainea*) belong to a closely related, subclade that occurs across the heterogeneous landscape of the Wollumbin Caldera in central, eastern Australia (Act, 1999, Brunton et al., 2022). Rossetto et al. (2000) and more recently, Brunton et al. (2022) showed that Southern *Fontainea* and Coastal *Fontainea* have shared plastid genotypes and that morphological differences between species may represent the influence of distribution across the highly variable environment of the Wollumbin Caldera. Another hypothesis is that the two species are at various points along a speciation continuum as a result of geographic isolation and differing environments (Brunton et al. 2022).

Local adaptation to specialised habitats can be a key driver of genetic differentiation between species (Holderegger et al., 2006, Li et al., 2017) and can develop in populations in response to environmental factors such as climate and topography (Lajoie et al., 2018, Kooyman et al., 2012). Such responses can result in changes to the genetic structure of populations over multiple generations, leading to Isolation by Environment (IBE) (Wang and Bradburd, 2014). IBE is the relationship between genetic divergence among populations and environmental differentiation, outside of geographic distance. Genetic differentiation

can also be related to geographic distance due to limited gene flow between distant populations in the phenomenon known as Isolation by Distance (IBD) (Wright, 1943). For example, *Elaeocarpus sedentarius*, a species endemic to the Wollumbin caldera, has a restricted range as a result of dispersal limitations rather than highly specialised habitat requirements, although fine-scale environmental factors do affect different populations of the closely related *E. grandis* (Rossetto et al., 2008). By contrast, using reduced-representation genome sequencing, Rutherford et al. (2018) showed geneflow was not a strict constraint to population differentiation for six closely related *Eucalypt* species. Overall, the study showed the various *Eucalypt* lineages were in different stages along a continuum of speciation, indicating a number of mechanisms such as vicariance, divergent selection between environments (ecological speciation) and origins from partially merged lineages (reticulate evolution) acting in combination were likely responsible.

Recent evidence suggests that Southern Fontainea and Coastal Fontainea are poorly conserved, with most populations located on private land, and are at risk of decline from limited habitat suitability under future climate projections (Brunton et al., 2023). Therefore, investigating the influence of spatial drivers of population genetic differentiation is crucial to understanding how the effects of geographical and environmental isolation have shaped the current distribution and genetic composition of these threatened *Fontainea* species. This will aid in identifying geographical barriers to geneflow and the role that local environment has played in the genetic divergence of their populations. Furthermore, exploring genome-wide data for evidence of loci correlated with local adaptation, and identifying the processes of selection, can ultimately be used to inform conservation management decisions to preserve populations of Southern Fontainea and Coastal Fontainea and to guide potential genetic rescue efforts.

Using reduced-representation single-nucleotide polymorphism (SNP) markers, we utilised a multiple matrix regression approach to examine the contributions of geographic and environmental distance to spatial genetic variation of the sister species, Southern Fontainea and Coastal Fontainea. As such, we aimed to disentangle the individual and combined effects of IBD, IBE on the patterns of genetic differentiation both within and among the two species. In addition, we explored genotype-environment interactions by applying a multivariate partial redundancy analysis (pRDA) to identify putative loci under selection and identify key ecological drivers of population differentiation between these two closely related, threatened rainforest species.

Methods

Study system and field sampling

The Wollumbin caldera is the largest erosion caldera in the southern hemisphere, covering an area of approximately 2,400 km², with its northern extent spanning the border of Queensland (QLD) and New South Wales (NSW), central eastern Australia (Solomon, 1964). Intermittent lava flows from the eruption cycles of Mount Warning (Wollumbin) ~ 23 million years ago, have generated a banded geology that has undergone variable weathering to form a complex, geodiverse landscape with a unique range of landforms in a circular arrangement around the central mass (Willmott and Blight, 2004). This system is

thus characterised by distinct habitat shifts marked by steep slopes, cliffs, gullies, and ridges, which influence rainfall, temperature, and topographical gradients at a local and landscape scale. Essentially, the caldera represents an area with distinct physical boundaries and microclimates formed by natural processes and the more contemporary effects of land-clearing, which has created the isolated, patchy distribution of subtropical rainforest in the region (Laidlaw et al., 2016).

Here, we focussed our analyses on the two closely related sister species (Brunton et al., 2022) Southern *Fontainea* (Fig. 1a) and Coastal *Fontainea* (Fig. 1b), endemic to the rainforest fragments across the Wollumbin caldera (Fig. 1c). Similar to most *Fontainea* species, Southern *Fontainea* occur in small, clumped and isolated populations (Forster, 1997) with recent surveys estimating there are < 200 mature, wild individuals of this taxon from 13 known populations (Brunton et al., 2023).

Coastal *Fontainea* is presently only found on the outer, southeastern margin of the Wollumbin caldera within several, small scattered littoral (coastal) rainforest fragments on basaltic soils (Brown et al., 2016) derived from the Wollumbin volcano lava flows in a 600 m radius at Lennox Head, northeast NSW. With just 10 mature individuals, it is considered at-risk of extinction which has prompted in-situ and ex-situ conservation management to enhance the long-term survival of the species (Rossetto et al., 2000). Little is known about the dispersal syndromes for *Fontainea*, however gravity and hydrochory are likely key vectors for the transport of diaspores (Conroy et al., 2019, Lamont et al., 2016). Reproductive biology of *F. picrosperma*, from far-north QLD showed pollen-mediated gene flow of is generally limited to short distances of ~ 30 m. Since all *Fontainea* species share similar reproductive traits, we may expect that pollen movement events for Southern and Coastal *Fontainea* are also spatially restricted.

Genetic evidence indicates limited genetic distance between some populations of these two geographically proximate and phenotypically similar *Fontainea* species. In addition, phenotypic distinctions between the species are expected to be driven by local adaptation to variable habitats (Rossetto et al., 2000, Brunton et al., 2022). In order to further understand the genetic distinctions between Southern *Fontainea* and Coastal *Fontainea*, samples from across the broader species' range are necessary for a more complete representation of the genetic profile for each respective taxon. Therefore, leaf samples were collected from 139 Southern *Fontainea* individuals from 12 sites and seven mature individuals of Coastal *Fontainea* throughout the natural distribution of the two species across the Wollumbin Caldera between the years 2020 and 2021 (Supplementary Table 1 and Fig. 1c). Leaf samples were dried with silica in zip-lock plastic bags in preparation for genotyping.

Reduced-representation Genotyping And Snp Data Filtering

Silica-dried leaf samples were genotyped by Diversity Arrays Technology Pty Ltd (DART, Canberra, Australia), who performed DNA extraction and generated reduced-representation SNP markers using a proprietary Next Generation Sequencing method, known as DARTseq (Kilian et al., 2012). DARTseq has been used for a variety of plant phylogenetic and population studies including commercial and natural populations (Brunton et al., 2022, Steane et al., 2011, Iqbal et al., 2021). We applied the same series of

genotype quality filtering steps on the raw DArTseq SNP markers as outlined in Brunton et al (2022). Due to the large number of loci recovered post-filtering (> 17 000), we retained a sub-sample of 10,000 loci using a down-sample approach for all downstream analyses to maintain computational efficiency. This method randomly samples the filtered SNP dataset so that all classes have the same frequency as the minority class.

Phylogenetic Relationships Of Southern Fontainea And Coastal Fontainea

We used decipher (Wright, 2016) to generate FASTA alignments of the SNP dataset, followed by phangorn (Schliep, 2011) and phytools (Revell, 2012) to construct a maximum-likelihood tree with the best-fit GTR + I model and nearest-neighbour branch arrangements with 100 permutations to generate bootstrap support, representing the phylogenetic relationships for all 146 samples across both *Fontainea* species. For the final phylogeny, we pruned tips to the node which represented the position of divergence for each population.

Genetic Structure And Differentiation

To explore patterns of genetic structure we conducted a discriminant analysis of principal components (DAPC) using the R package adegenet (Jombart et al., 2020) to identify posterior probabilities of individual assignment to K -means groups. DAPC analyses were conducted twice to examine the influence of *a priori* groupings on the results. In the first analysis, sampling locations were used as *a priori* groups. In the second analysis, the *find.clusters* function was used to determine the number of groups (K) *de novo*, with optimal K selected as that with the lowest BIC value. For both analyses, the optimal number of principal components (PCs) to use in the DAPC was determined using the *optim.a.score* function.

For comparison to the DAPC, we also investigated admixture analysis using the R package LEA (Frichot and Francois, 2015), which applies a sparse non-negative matrix-factorization (snmf) algorithm to generate ancestry coefficients. In this case, to estimate the best representation of K -means groups, we ran a cross-entropy analysis between $K = 1-20$ values, with 10 runs for each value of K . The values of K were then visualized in a cross-entropy plot with the lowest value of K prior to an increase used to indicate the closest estimate for the number of K -means groups.

We evaluated patterns of genetic differentiation using F_{ST} , which we calculated in the R package StAMPP version 1.6.3 (Pembleton et al., 2013) with 1,000 bootstraps to generate 95% confidence intervals and to evaluate whether the observed differentiation was statistically significant ($p\text{-value} \leq 0.05$).

Environmental Data

For the assessment of spatial genetic variation and to identify candidate SNPs for environmental adaptation we utilised eight explanatory variables: slope, aspect, hillshade (Hs), isothermality, mean temperature of the wettest quarter (TWQ), mean temperature of the driest quarter (TDQ), precipitation seasonality and precipitation of the warmest quarter (PWQ) downloaded from WorldClim database (Hijmans et al., 2005) at 30-arc second resolution ($\sim 1 \text{ km}^2$ grids). Brunton et al (2023) identified this set of environmental factors were relevant to the distribution of Southern *Fontainea* and Coastal *Fontainea* from a broader set of 23 variables using a stepwise procedure to exclude correlated factors and reduce overfitting for species distribution model predictions. In this way, the combination of landscape and climate factors may accurately represent the complex environmental characteristics across the study region.

Quantifying Spatial Genetic Variation Of *Fontainea*

To explore the influence of landscape heterogeneity on spatial genetic variation of *Fontainea*, we used a multiple matrix regression with randomization (MMRR) analysis on genetic, geographic, and environmental distance matrices. This method is similar to Mantel and partial Mantel tests, but is extended to incorporate multiple regression, which allows us to quantify how the dependent variable (i.e., genetic distance) changes in response to the independent variables (i.e., environmental distance, geographic distance; Wang, 2013). Therefore, we can estimate and disentangle the relative influences of IBD and IBE on genetic variation (Wang, 2013). Moreover, we can estimate the significance of individual variables as well as their combined effects on genetic variation (see Wang, 2013 for details). We tested for IBD, IBE, and the combined effects of geographic and environmental distance by implementing MMRR on different matrices of pairwise genetic distance and Euclidean distances for geographic and environmental factors between all sampling locations. Genetic and geographic matrices were generated using the R package *adeigenet* (Jombart et al., 2020) and the environmental matrix was scaled and generated using *vegan* (Oksanen et al., 2013).

MMRR was implemented in R using the function *MMRR* (Wang, 2013) with 10,000 permutations to assess the additive effect of both independent factors (geographical distance and environmental). All matrices were scaled to a mean of zero and unit standard deviation using the *scale* function implemented in R before running the MMRR. With simulated data, this approach has produced robust results across a range of dispersal rates and proved accurate in situations of low-to-moderate gene flow (Wang 2013). This is especially relevant with the potential dispersal limitations suggested of extant *Fontainea* species (Lamont et al., 2016, Conroy et al., 2019).

Genome-environment associations and functional relationships of *Fontainea*

To detect genetic-environment relationships of the two *Fontainea* species, we tested the association between the set of climate and topographic variables described above, and the genetic variability across the sampled populations of each species. Although differentiation-based detections of statistical outliers have been frequently applied to identify signals of adaptive differentiation, multivariate GEA methods are

increasingly favoured over population differentiation-based methods for their improved potential to detect weak selection signals (Forester et al., 2018, De Mita et al., 2013). Multivariate GEA methods are especially valuable for the ability to simultaneously model the contribution of a range of environmental factors on large genetic datasets such as reduced representation SNPs (Xuereb et al., 2018). As such, to identify putative signals of local adaptation, we identified loci associated with environmental factors using a multivariate genome-environment association approach (GEA). We performed the GEA using a partial redundancy analysis (pRDA), a multivariate analogue of linear regression that examines the proportion of genetic variation that can be explained by environmental variables while controlling for spatial structure. Recent studies have identified multiple advantages of redundancy analysis: (i) it exhibits low false-positive and high true-positive rates in simulation studies (Forester et al., 2018, Farleigh et al., 2021), and (ii) constrained redundancy analysis allows for the identification of selective gradients in environmental variables (Capblancq et al., 2018, Byer et al., 2021) making it an ideal GEA in this system. We considered six environmental variables found to maximize the explanatory power of pRDA while retaining uncorrelated predictors. Latitude and longitude were included as conditioning variables to represent the spatial structure in the genetic variation. We evaluated the overall pRDA significance and significance of each pRDA axis using permutation tests with 999 permutations and a *p-value* threshold of 0.05. We then performed pRDA in the vegan package (Oksanen et al., 2016) following the methods of Capblancq et al. (2018) to identify candidate SNPs under selection, and to identify selective gradients associated with pRDA axes. Briefly, SNP loadings are recovered on each significant axis and a Mahalanobis distance is calculated for each SNP using the R package robust (Wang et al., 2009). *P-values* were calculated from the Mahalanobis distances, and candidate SNPs under selection were identified after correcting for multiple tests using a False-Discovery Rate of 10% (Benjamini and Hochberg, 1995). Selective gradients associated with each pRDA axis were calculated by correlating the environmental variables with each axis.

Functional annotations of the candidate loci of the pRDA SNPs were investigated to examine if putative SNPs with a strong relationship to the environmental variables could be associated with relevant biological processes. This method can identify gene ontology (GO) terms linked to candidates that are associated with molecular, biological and cellular functions. This is especially suitable for non-model species without a reference genome (Conesa et al., 2005) and was performed using the default settings of BLAST2GO (Conesa et al., 2005) to provide functional annotations of the novel SNP sequences and assign GO terms.

Additive Polygenic Score (Dup: Abstract ?)

We used additive polygenic scores (APS) to evaluate the cumulative effect of candidate SNPs identified from GEA and to assess if there were differences between Southern Fontainea and Coastal Fontainea. Additive polygenic scores were calculated as the total number of adaptive alleles within a particular environment. We identified the adaptive allele for each candidate SNP as the allele that exhibited a positive relationship with increasing values of a given environmental variable (Xuereb et al., 2018). Then

we compared the average APS between *F. australis* and *F. oraria* to determine if there was a significant difference in APS between the two species using Wilcoxon Mann Whitney tests.

Results

Genetic relationships of Southern Fontainea and Coastal Fontainea

The maximum-likelihood tree we constructed was generally congruent with previously published assessments of these taxa (Rossetto et al., 2000, Brunton et al., 2022), with (LW) weakly clustering separately from other Southern Fontainea populations and Coastal Fontainea. However, with our broader set of populations, LW was nested within a subclade of the Southern Fontainea populations; NB, CC, CRY, MTC and EHR (Fig. 2a) at the northern area of the Wollumbin caldera (Fig. 2b). Three Southern Fontainea populations; MNP, KRR and MRR formed a well-supported (100) subclade. Additionally, the population of NC, which is a notable geographic outlier for its position at a much higher elevation (> 750 m) compared to the remaining populations (~ 50–380 m), and WG had weak support as genetically distinct populations. Interestingly, HB, which is found in a gorge north of WG (Fig. 2b), was nested with Coastal Fontainea (FO).

Results from the DAPC and snmf algorithms to indicate genetic assignments for Southern Fontainea and Coastal Fontainea had incongruent conclusions. The DAPC assignment without predefined groups (Supplementary Fig. 1a) supported four genetic groups. However, in contrast the snmf cross-entropy was less definitive, with separate plateaus at $K = 6$ and $K = 8$ clusters (Supplementary Fig. 1b). Due to the potential of the snmf algorithm to over-split, and therefore over-estimate the number of genetic clusters under a strong influence of IBD (Bradburd et al., 2018), we used a combined approach to explore outputs from the DAPC results, phylogenetic tree relationships and F_{ST} values to estimate a biologically appropriate K value for Southern Fontainea and Coastal Fontainea. Based on this collection of population differentiation analyses, we determined $K = 4$ as the suitable representation of genetic clusters for the two *Fontainea* species.

When sampling locations were used as a priori groups, DAPC results (Fig. 2c) supported a number of clear genetic groups that mostly reflected the geographic-genetic relationships indicated by the maximum-likelihood tree and F_{ST} values (Fig. 2d). Without predefined groups, DAPC analysis showed genetic assignments that also represented the F_{ST} results, which identified four distinct genetic clusters (based on optimal K value, see Supplementary Fig. 1a), to indicate that generally, genetic differentiation and structure of the two *Fontainea* species could be strongly associated with the geographic location of the sampled populations (Fig. 2b).

Comparisons of pairwise genetic differentiation (F_{ST}) between all population pairs indicated significant differentiation among Southern Fontainea and Coastal Fontainea, highlighting a number of discrete

populations (Fig. 2d). Although the difference in F_{ST} values were significant between all pairs of Southern *Fontainea* populations (p -values = < 0.001), the effect size of differentiation between some populations was relatively small and typically reflected their geographical associations. For example, CRY had low genetic differentiation values compared to LW, NB, MTC, EHR and CC, which are all located around the northern area of the Wollumbin caldera. This pattern of geographic association and low relative magnitude of genetic differentiation was observed amongst several other Southern *Fontainea* populations, including between those in the Mooball area (MNP, MRR, and KR). Interestingly, perhaps the most geographically isolated Southern *Fontainea* population of Nightcap National Park showed strong levels of genetic differentiation only in comparison with the Mooball populations. Importantly, F_{ST} values of FO (Coastal *Fontainea*) showed relatively high genetic differentiation from all the Southern *Fontainea* populations (Fig. 2d).

Contributions of geography and ecology to the genomic divergence of *Fontainea*

Overall, the explanatory power of the MMRR was significant ($R^2 = 0.424$, $p < 0.001$), in explaining the geographic and ecological variables that influence genetic divergence among Southern *Fontainea* and Coastal *Fontainea*. In particular, the MMRR showed that IBD ($\beta = 0.424$, $p < 0.001$) played a significant role in genetic differentiation between Southern *Fontainea* populations and among the Southern *Fontainea* and Coastal *Fontainea* (Fig. 3a). The regression coefficient for IBE was marginally associated ($\beta = 0.330$, $p = 0.069$) with genetic differentiation. However environmental distance was not strongly associated with geographic distance (Fig. 3b). The joint effects of geographic and environmental distance (Fig. 3c) were low but significantly correlated with genetic distance ($\beta = 0.100$, $p < 0.001$) to indicate both IBD and IBE could plausibly represent the influence of geoclimate variation within the study region on the genetic structure of the two *Fontainea* species.

Evidence of divergent selection of *Fontainea* and functional associations

GEA using pRDA identified 136 candidate loci (Fig. 4a; Supplementary Table 2) that were correlated with six environmental variables (Fig. 4b). Nearly 60% of loci inferred to be under selection were associated with mean temperature in the wettest quarter (Supplementary Table 2). Additionally, the topographic variables Hillshade (17% of total candidates) and Aspect (15% of total candidates) also showed significant association with candidate SNPs to indicate putative signals of local adaptation across Southern *Fontainea* and Coastal *Fontainea* populations. Within the pRDA, six of the constrained axes were significant (Supplementary Table 3), with the first two axes found to explain 48% of the genetic variation within the SNP dataset (adjusted $R^2 = 0.12$).

In this study, the ecological variables chosen reflected the distinct environmental gradients related to the physical complexity of the landscape across the study region. Thus, it is especially interesting to consider the molecular functions of both *Fontainea* species as they relate to a range of stress responses and other growth traits in common with Euphorbiaceae allies (Fig. 4c) as well as some examples of Australian rainforest species. A range of candidate loci from our GEA matched gene annotations for drought, low-

light, salt tolerance, heat radiation, growth regulation and pathogen defence (Fig. 4d) that indicated the two *Fontainea* species have developed adaptations to cope with the distinct, discrete environments found across the Wollumbin caldera. Although summer temperatures in the region are ameliorated by the unique relief and microhabitats that support rainforest habitat, a large part of the area, especially in the southeast section experiences temperatures up to 6 °C warmer than where most of the Southern *Fontainea* populations are found. Therefore, increased temperature regimes, in addition to the warm trade-winds could explain the large number of temperature related candidate loci especially associated with Coastal *Fontainea*.

Functional annotation with BLAST2Go identified 11 candidates with gene ontology (GO) terms relevant to the adaptation potential of Southern *Fontainea* and Coastal *Fontainea*, with no blast hits or GO terms found for the remaining 125 putative candidates (Supplementary Table 4). Generally, there were a broad series of annotations that indicated both *Fontainea* species have putative candidate SNPs aligned with associated abiotic stressors that could be associated with historical climate fluctuations and local climate conditions. For example, there were gene functions associated with candidate loci of Southern *Fontainea* and Coastal *Fontainea* related to drought stress, fruit and flower production and heat radiation (Supplementary Table 4). Notably, several salt-tolerant GO were identified, which were likely to be associated with Coastal *Fontainea*.

Additive Polygenic Score

Estimates of APS did not identify a significant difference between the average number of adaptive alleles between Southern *Fontainea* and Coastal *Fontainea* ($p\text{-value} = 0.46$).

Discussion

The capacity for local adaptation among small, threatened plant populations and the migration of advantageous alleles to track suitable habitats is critical to their long-term survival, especially in the context of rapidly changing and extreme climate projections (Kooyman et al., 2012, Leimu and Fischer, 2008, Weeks et al., 2011). Our study indicated that the combined influence of geographic isolation (IBD) and ecological differentiation (IBE) are the key drivers of genetic divergence of natural populations of Southern *Fontainea* and Coastal *Fontainea* across the environmentally heterogeneous region of the Wollumbin caldera. Additionally, we found putative signals of local adaptation for the two *Fontainea* species to a range of topo-climatic gradients that could be linked to important biological functions. These findings also suggest that the number of putatively adaptive alleles varies between populations, degree of adaptive potential of individual populations to certain factors. We also showed that 12 populations of Southern *Fontainea* and the single, natural population of Coastal *Fontainea* together, likely form part of a broader species continuum, that have become genetically differentiated as a result of long-term geographic isolation and recent adaptation to local habitats. Based on genetic associations we found four distinct genetic groups that require separate conservation management to preserve the unique

genetic resources and adaptive potential within each group. Importantly, dispersal dynamics are critical to the conservation of both Southern *Fontainea* and Coastal *Fontainea*, particularly in cognisance of human-induced habitat loss and fragmentation. Consequently, to preserve these two threatened species into the future, a strategy that considers the adaptation potential of the distinct genetic groups, their local habitats and assisted migration is suggested.

Our study showed that Southern *Fontainea* and Coastal *Fontainea* exhibit habitat-linked geographic divergence, with geography the best predictor of genomic variation when compared to the single model of environmental variation. To better reflect the diverse environments of the Wollumbin caldera, we extended our analysis to incorporate the interaction between geographical features and environmental gradients, which we expected to impose selective filtering across both *Fontainea* species. Genetic differentiation and IBD among most populations indicated vicariance has been the key driver of genomic divergence of Southern *Fontainea* and Coastal *Fontainea*. In addition, the combined effect of IBD and IBE suggests some ecological divergence between species. These results generally reflected the geographic relationships of both *Fontainea* species, to indicate the likely existence of multiple isolated refugia or 'microrefugia' for the populations during paleoclimatic fluctuations. Microrefugia are a characteristic of complex, mountainous environments (Holderegger and Thiel-Egenter, 2009, González-Martínez et al., 2007), which are a prominent feature of the Wollumbin caldera that contains numerous isolated valleys, gorges, steep slopes, and mountain ranges. This region is a noted climate refugia for a variety of species endemic to the area (Weber et al., 2014) including Southern *Fontainea* (Brunton et al., 2023), and the orographic features have had a major influence on the phylogeographic processes within the system (Kooyman et al., 2013). For example, some Southern *Fontainea* populations occur in largely continuous areas of complex, subtropical rainforest (LW, NB, EHR, CRY, CC), while others (MTC, KR) are found in regrowth which can be dominated by sclerophyll species such as Brushbox (*Lophostemon confertus*). Certain populations (MNP and MRR) survive in forest margins exposed to edge effects with the latter found in a small, narrow strip of road reserve vegetation. HB is comprised of isolated individuals of regenerating rainforest located in a valley, while WG is found mid-slope in a deep gorge with a shallow, but wide, permanent creek that can swell above its banks during significant rain events. NC could be considered the most isolated population, located atop a steep, narrow escarpment at an elevation > 750 m in a once heavily logged forest that is now a protected area. Finally, Coastal *Fontainea* occur in littoral rainforest fragments exposed to salt-bearing winds. With these observations, we can begin to understand how local adaptation and dispersal limitations could influence genetic heterogeneity along the range of the distribution for Southern *Fontainea* and Coastal *Fontainea*.

We propose that the current distributions of Southern *Fontainea* and Coastal *Fontainea* are a consequence of local expansion from glacial refugia of the mountain ranges spanning the Queensland and NSW border and subsequent contraction to their present ranges as conditions became less favourable for rainforest. Further, over evolutionary time, dispersal potential has declined, leading to increased genetic drift, progressively smaller, more fragmented, and genetically distinct populations that have also adapted to the variable environments found across a fine, geographical range. In the Eocene to early-Miocene, large, fleshy-fruited species were widely distributed across the Australian continent,

alongside a range of large vertebrate dispersers (Greenwood and Christophel, 2005). Extreme climate fluctuations following glacial cycles in the Quaternary led to severe contraction of rainforest habitats and extirpation of subtropical species (Kershaw et al., 2007). The subtropical biome within which Southern *Fontainea* and Coastal *Fontainea* occur today, has relied on the survival or re-colonisation of fauna to assist in the re-establishment and expansion of these large, fleshy-fruited species. However, whilst some habitat was able to withstand the Quaternary climate cycles, some refugial areas were too small and fragmented to support frugivorous, vertebrate dispersers (Rossetto et al., 2008, Rossetto et al., 2015) necessary for the long-distance movement of Southern *Fontainea* and Coastal *Fontainea*. Even though current dispersal vectors of *Fontainea* remain a critical knowledge gap, we may assume our evidence of clear population differentiation, strong signal of geographic divergence and highly clumped distribution indicates both species are dispersal limited. Generally, the population dynamics of other *Fontainea* taxa were similar to our findings, with geographically isolated populations displaying strong genetic differentiation and minimal evidence of geneflow among some adjacent populations (Grant et al., 2019, Conroy et al., 2019). Two previous studies on two QLD congeners used microsatellite markers which makes direct comparison of genetic differentiation to Southern *Fontainea* and Coastal *Fontainea* data difficult. However, their population genetic analysis of *F. picrosperma* and *F. rostrata* identified low genetic diversity for both taxa, alongside some regional difference in genetic profiles and also identified population clusters related to gene flow (Lamont et al., 2016, Conroy et al., 2019).

Phylogenetic topology and structure analyses suggested a strong genetic structure related to geographical and ecological barriers in Southern *Fontainea* and Coastal *Fontainea*. There were limited signs to indicate geneflow between most of the Southern *Fontainea* populations and Coastal *Fontainea* to suggest the two species have been reproductively isolated for some time. Admixture was present in some geographically proximate Southern *Fontainea* such as between NB, CC, CRY, LW, MTC, and EHR to support the notion that genetic exchange has occurred among these neighbouring populations. This finding was somewhat unexpected given the NB, EHR and MTC populations are found at relatively higher elevations at the northern edge of the caldera, whilst CC and CRY are located south of the other three populations, in different sub-catchments at the foot of steep cliffs, which was an anticipated physical barrier to geneflow. These five populations formed a genetic cluster that occur across a largely intact area of vegetation, which may signal the importance of habitat corridors in maintaining the potential for genetic exchange among this group. Although directionality of geneflow has not been tested here, this implies there are two possible genetic interchange scenarios: 1) propagules have been transported by gravity or hydrochorous dispersal from the populations at higher elevations (NB, EHR, MTC), in a southerly trajectory down the steep escarpment to where the present LW, CC and CRY populations are found or, 2) a series of dispersal events in either direction (north or south) over the cliffs via animal dispersal. It is widely acknowledged the movements of a single disperser seldom effect the final outcome of a seed's transport (Hämäläinen et al., 2017). As such, it is possible a combination of both scenarios or an alternative version is involved in this case, which likely applies to the broader species' and genus' dispersal dynamics. If we also consider the limitations of pollen-transport as a major impediment to geneflow among spatially isolated *Fontainea* populations (Grant et al., 2019), we could therefore

hypothesise Southern *Fontainea* and Coastal *Fontainea* populations have likely experienced genetic bottlenecks and drift, a pattern frequently observed in small, isolated populations (Hewitt, 2004). This leads us to infer both *Fontainea* species have survived the extreme paleoclimate fluctuations, probably including glaciation events, in discrete pockets of climate refugia within the Wollumbin caldera, with a decreased post-Quaternary genetic exchange driving contemporary population differentiation.

Three additional Southern *Fontainea* population, MRR, MNP, and KR, which are within a ~ 3 km radius of each other, had a level of population admixture and formed another genetically distinct group. Notably, these populations occur on the southern side of the Tweed valley and substantial Tweed River, in a habitat corridor that spans both protected areas and private land, with limited physical barriers to geneflow. The remaining Southern *Fontainea* populations (NC, HB, WG) and Coastal *Fontainea*, are all generally geographically discrete and separated by prominent physical barriers, which could impede geneflow between them. Even though Coastal *Fontainea* is genetically differentiated from some Southern *Fontainea* populations, phylogeny and structure analyses showed that Coastal *Fontainea* shared genotypes with certain Southern *Fontainea* populations. This included HB and WG, to indicate that these populations were once possibly part of a much larger area of rainforest habitat that has since been cleared following European settlement. Genetic associations between the two species have been highlighted in previous research (Rossetto et al., 2000, Brunton et al., 2022). Our results have provided further evidence to support a consideration of classifying Southern *Fontainea* and Coastal *Fontainea* as ecologically distinct, but genetically aligned species. These findings are incongruent with the current species concepts, therefore until a formal taxonomical revision confirms this theory, we advise caution against a formal conclusion.

Despite environmental selection not being identified as the leading driver of genetic differentiation, environmental gradients could be a good predictor of genomic variance of Southern *Fontainea* and Coastal *Fontainea*. A combination of environmental factors, especially temperature in the austral summer, aspect and hillshade, were identified as key drivers of spatial patterns of genetic divergence and putative adaptive genetic variation for the two *Fontainea* species. These factors could also play an important function that influences local adaptation across the heterogeneous landscape of the Wollumbin caldera and surrounds.

We found numerous candidate loci that showed clear signatures of local adaptation after accounting for spatial structure. This implies that ecological divergence resulting from geoclimate gradients across the species' range may play a significant role in driving continued divergence in Southern *Fontainea* and Coastal *Fontainea*. Among ecological gradients, thermal tolerance is considered a major factor in shaping species diversity and determining plants species' range (Reyer et al., 2013). Putative loci under selection were strongest for temperature gradients for Coastal *Fontainea*. This strengthens initial suggestions by Rossetto et al. (2000) that morphological distinctions between the two *Fontainea* species is likely a product of adaptation to variable habitats, which may have generated morphological plasticity in key features applied to taxonomical treatments. Clearly, the environment, where Coastal *Fontainea* is situated in a unique littoral rainforest fragment, which is within 1 km of the ocean, would typically be

considered unsuitable for Southern *Fontainea*. Indeed, the species composition and structural diversity at this site is less complex than most Southern *Fontainea* habitats. Specifically, Coastal *Fontainea* is found in a vegetation community that is dominated by *Cupaniopsis anacardiodes* (Tuckeroo) and is exposed to harsh coastal conditions, with a low, wind-sheared canopy shaped by the prevailing, salt-laden south-easterly trade winds and annually warmer conditions than inland areas of the region. Unlike many examples of Australian littoral rainforest, which have a sandy substrate (Howard et al., 2016), Coastal *Fontainea* is found on basalt-derived soil (Brown et al., 2016) formed from the Wollumbin lava flows ~ 20 mya (Willmott and Blight, 2004). Even with a suitable substrate, salt-tolerance is necessary for the coastal environment where salt-spray can cause leaf necrosis for maladapted populations and be a factor in higher salt concentrations in soil (Lowry et al., 2009).

However, while Coastal *Fontainea* shows greater adaptation to the local conditions (e.g., salt spray), it does not imply that it is a distinct lineage altogether. More plausible, is that Coastal *Fontainea* is an example of one *Fontainea* population that has undergone differential environmental filtering through time, that is currently geographically separated and has undergone pronounced genetic drift, while potentially still being part of a broader species continuum that includes Southern *Fontainea*. The extremely small, patchy habitat area that contains Coastal *Fontainea* likely represents the eastern margin of the once-widespread rainforest vegetation that covered a larger extent known as the 'Big Scrub'. However, the large-scale clearing in the region known as the Big Scrub has severely reduced rainforest habitat to a fraction (< 1%) of its pre-European settlement area. As such, without the capacity to sample geographically intermediate populations to provide evidence to support reticulate evolution, we suggest that a combination of allopatric (geographic) separation and ecological divergence has shaped the genetic variation of Southern *Fontainea* and Coastal *Fontainea*.

Although our overall findings seem to challenge the current species boundaries between Southern *Fontainea* and Coastal *Fontainea*, a conclusive judgement would benefit from an analysis of the phenotypic variation associations, ideally in a common-garden experiment where the local conditions are unlikely to induce plastic responses. Consequently, we recommend genotype-phenotype sampling is necessary and should include representatives of the two species populations as well as an ecological outlier such as the *Fontainea* congener *F. rostrata*, which is found in drier rainforest habitats (Forster, 1997). This would bridge taxonomic and geographical gaps in our dataset and could also provide greater information on the relative strength of selection and drift in the two southern *Fontainea* species, which is of importance given that the current inter-population and inter-species isolation and fragmentation is likely to remain at current levels or most likely be further exacerbated.

We also highlight our use of a robust analysis to indicate SNPs under putative selection and minimise false-positive rates. However, it is possible the individual SNP loci we identified may be unique to this method, therefore significance must be interpreted carefully. Also, the evidence of local adaptation we uncovered does not necessarily support a role of ecological opportunity in initiating divergence between the two *Fontainea* species as adaptation may have occurred sometime after taxa became isolated from one another. Nevertheless, the overall pattern presented clear evidence of spatial climatic and

topographic variation that appear to be important factors shaping local adaptation and possibly endemism between the two *Fontainea* species.

Identifying populations with adaptive loci may reveal sites of adaptive diversity, which may aid in highlighting peripheral populations with a history of selection in extreme environments (Keller et al., 2018). Therefore, it may suggest populations with low rates of adaptive loci reflect areas of long-term stable habitat and indicate sites at risk of extirpation from habitat degradation. As such, identifying genomic regions of local adaptation in peripheral populations is essential to conserving genetic resources of *Fontainea* in the context of habitat loss and climate change. Several geographically and genetically distinct population clusters were found to support the genetic divergence between the northern locations (EHR, MTC, NB, CC, CRY, LW) of Southern *Fontainea* and the other Southern *Fontainea* and Coastal *Fontainea* populations. This enhances the importance of this region providing critical climate refugia for a host of ancient plant groups, which were spared the extreme conditions associated with paleoclimate fluctuations (Weber et al., 2014). Further, the strong association of Coastal *Fontainea* to increased temperatures suggests although this species has some level of thermal tolerance, it may still be vulnerable to projections of increased thermal conditions.

Adaptive potential is an important factor to predict how threatened species will respond to a changing climate. If such species are found to have a narrow range of tolerance to projected shifting biomes, the ability to evolve in a rapidly changing environment may place species from southern rainforest lineages at significant risk of extinction (Crisp et al., 2009) and active conservation strategies could be necessary. Our investigation of the two sister *Fontainea* species revealed evidence for four highly differentiated genetic clusters, including between Southern *Fontainea* populations and Coastal *Fontainea*, that represent the currently defined taxonomic units. In addition, we found further support that Coastal *Fontainea* shares some genetic structure with some Southern *Fontainea* populations and the physical distinctions between species are likely a combined result of historical biogeographical processes and more recently, localised adaptation to a coastal environment. More detailed ecological and phenotypic studies (including closely related congeners) are needed to determine whether these taxa should each be considered their own species, or as varieties or ecotypes of a single species; other genetic studies of *Fontainea* suggest that either scenario may be appropriate based on observed values of genetic variation (Rossetto et al., 2000) and phylogenetic topology (Brunton et al., 2022). The recent discovery of a proposed new *Fontainea* species, *F. sp. Coffs Harbour* A.S. Benwell (PlantNET), also suggests a taxonomic revision of the genus could be timely to resolve uncertainties presented from our genetic data.

Irrespective of taxonomic classification, conservation and management efforts could be enhanced by conserving the genetic and adaptive traits of all genetic groups highlighted in this study. Both Southern *Fontainea* and Coastal *Fontainea* face an elevated risk of population decline due to genetic factors (low genetic diversity, strong genetic differentiation) as well as ongoing threats from continued habitat decline and fragmentation, under-representation of sites within protected areas, land use modification and climate change (Brunton et al., 2023, Rossetto et al., 2000). Although both *ex-situ* and *in-situ* Coastal *Fontainea* populations have been established for conservation purposes, there is an opportunity to refine

management objectives that include adaptive potential outlined in this study. Equipped with the information from this research and previous studies (Rossetto et al., 2000, Brunton et al., 2022), we advocate for immediate action to preserve Southern *Fontainea* populations, using a similar strategy proposed for *F. rostrata* or Deep Creek *Fontainea* (Conroy et al., 2019) to ensure the long-term survival of both species. Specifically, for longstanding conservation of both *Fontainea* species, assisted migration of populations to suitable habitats may be necessary but should consider the genetic composition, adaptation potential and local environment conditions of each population. Considering an adaptation potential perspective will provide environmental managers an improved understanding of the broader ecology of the two species, which can be used to review and identify significant conservation units. This approach also aids in developing conservation plans that consider the capacity to survive with the rapid onset of changing environments, and consequently enhance the conservation of Southern *Fontainea* and Coastal *Fontainea*. Collectively, our study has provided critical insights into the combined contributions of geo-climate and local adaptation on the diversification of these two narrow-range, endemic species, which could also have consequences for the conservation of other rare, threatened rainforest species.

Conclusion

While it is well-recognised that local adaptation is crucial for species' survival in a complex, changing environment and plays a central role in diversification (Kawecki and Ebert, 2004, Hereford, 2009), there are few studies investigating the evolutionary dynamics of narrow-range, rainforest endemics. Closely related species such as Southern *Fontainea* and Coastal *Fontainea* provide ideal models to explore this subject, owing to their geographically discrete distribution across a fine, but complex landscape, while their threatened status adds to the significance of this study. We presented molecular evidence that the spatial and temporal isolation of Southern *Fontainea* and Coastal *Fontainea* has led to local adaptation by some populations to their distinct environments. Survival of rainforest species through paleoclimate oscillations relied upon tracking suitable conditions, however this necessitates adequate dispersal capacity and accessibility to appropriate habitats. All *Fontainea* species produce a fleshy-fruit suitable for animal-mediated dispersal, however our data support the proposed dispersal limitations of *Fontainea* to indicate gravity and hydrochory remain the key mode of seed transport and genetic movement. Crucially, we showed that Southern *Fontainea* and Coastal *Fontainea* may be part of larger, albeit spatially segregated species continuum, but suggest that distinct genetic clusters should be managed as separate units due to putative signals of local adaptation within some populations. Small, disjunct populations are expected to continue to decline from increased genetic differentiation as a result of genetic drift, bottlenecks and limited adaptive potential to climate projections (Lesica and Allendorf, 1992). Threatened species such as the two focal study taxa face an elevated risk of extirpation from a variety of stochastic events including wild-fires, droughts or demographic fluctuations that can lead to a build-up of deleterious alleles. Since Southern *Fontainea* and Coastal *Fontainea* are confined to narrow, isolated areas of suitable habitat, mostly lacking formal protection, they are at a high-risk of extirpation caused by these stochastic processes, as such immediate conservation action is required to ensure their long-term survival.

Declarations

Statements & Declarations

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Permits

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Conflict of Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AB reports financial support was provided by QBiotics Group Limited Australia. SO reports a relationship with QBiotics Group Limited Australia that includes: board membership, employment, and equity or stocks.

Data Availability

The datasets generated during and/or analysed during the current study are available in the GitHub repository, [PERSISTENT LINK TO DATASETS will be attached upon successful notification]

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Figures

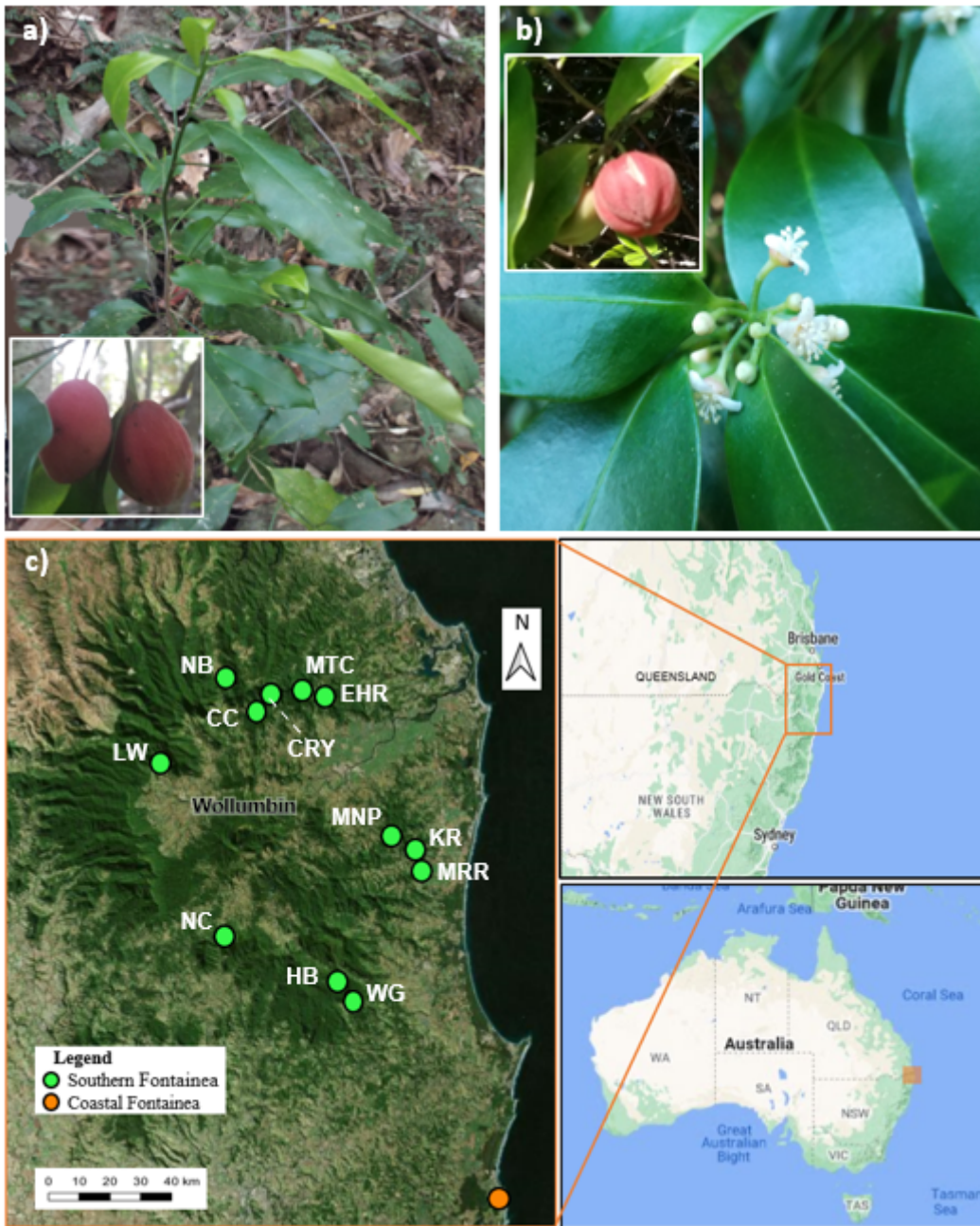


Figure 1

Images of two sister species of threatened *Fontainea*; a) *Fontainea australis*(Southern Fontainea), with inset image of fruit and; b) *F. oraria*(Coastal Fontainea), including male flowers and inset image of fruit and; c) satellite image of the Wollumbin caldera region and sampling locations throughout the distribution of the two study species in central, eastern Australia. Inset maps indicate relative area (in orange) to larger map extents.

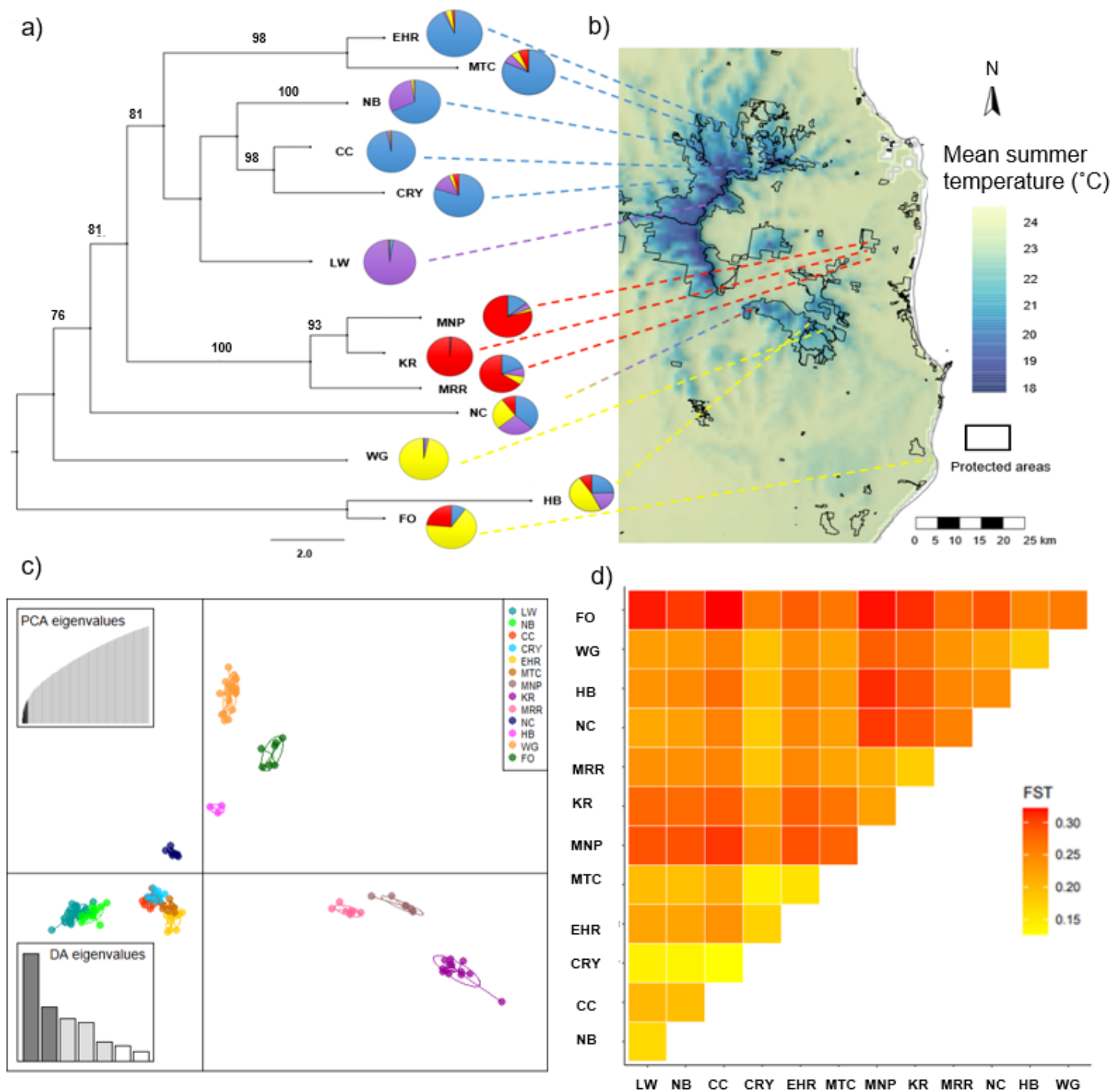


Figure 2

Population differentiation in the two sister-species, Southern Fontainea and Coastal Fontainea from central, eastern Australia, including; a) population-species relationships based on a GTR + I, maximum-likelihood tree generated with 10,000 reduced-representation markers with bootstrap values >50 represented at branch nodes, with pie charts indicating $K=4$ genetic clusters; b) position of sampling locations in the Wollumbin caldera overlaid with current mean summer temperatures; c) *A priori* discriminant analysis of principal components (DAPC) results from seven principal components identified using optimal a score and; d) F_{ST} heatmap for comparing the defined Southern Fontainea

populations and Coastal Fontainea. For figures a), c), and d) **FO** represents Coastal Fontainea, with all other abbreviations representing Southern Fontainea populations.

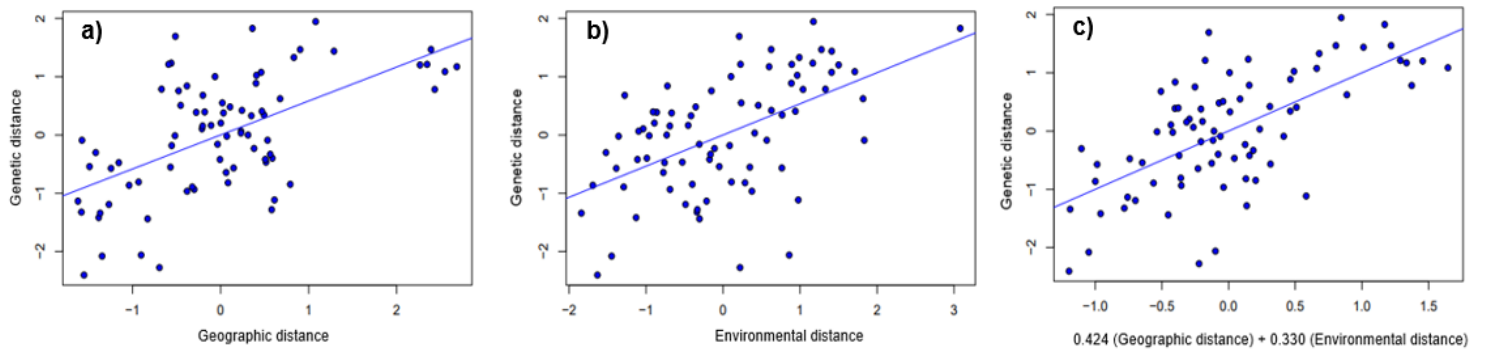


Figure 3

Scatterplots generated by multiple matrix regression with randomisation (MMRR) analysis showing relationships of isolation by distance a); isolation by environment b); and the joint effects of geographical and environmental distance c); for Southern Fontainea and Coastal Fontainea.

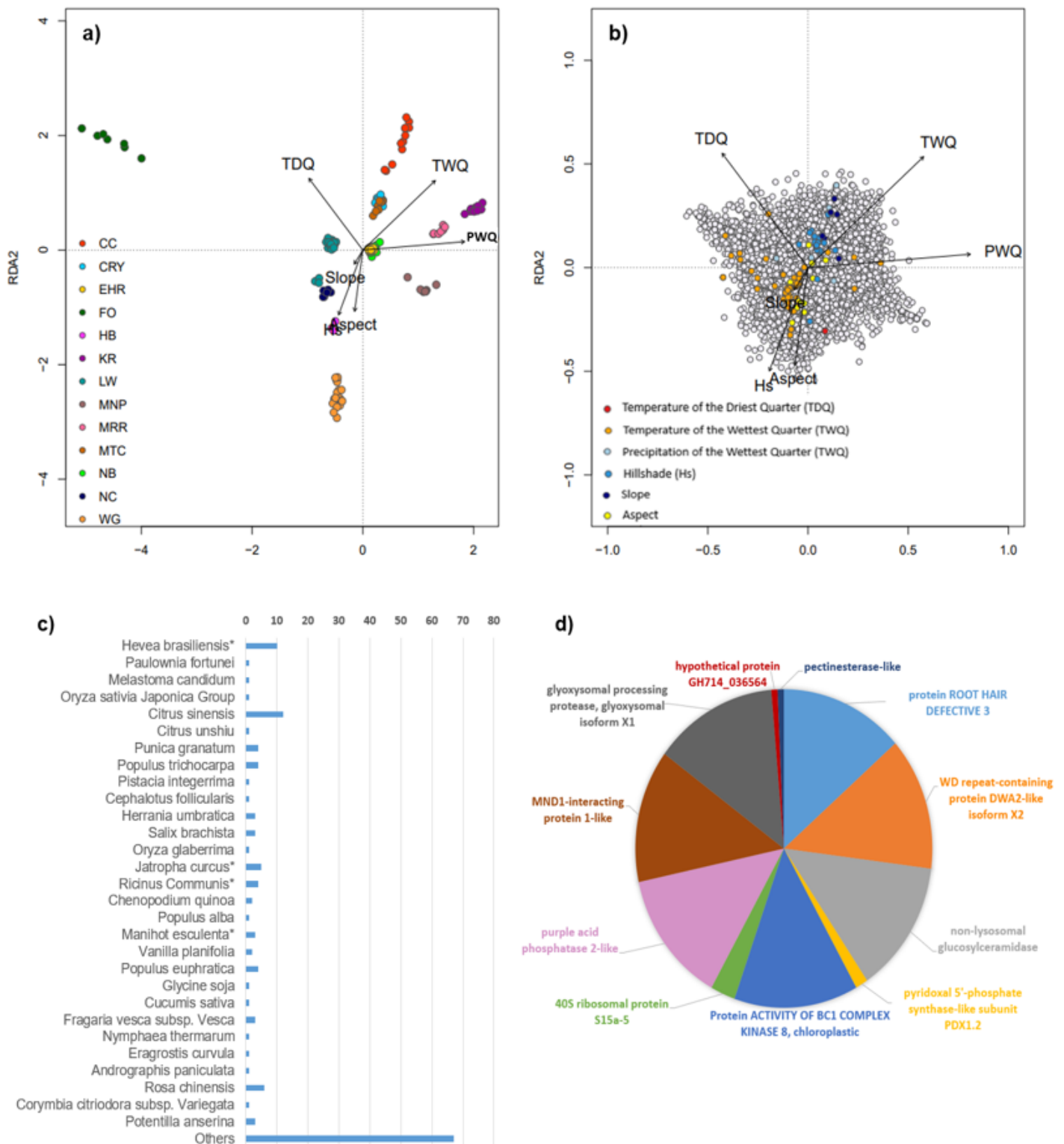


Figure 4

Partial redundancy analysis (pRDA) for the first two constrained ordination axes showing: a) relationship between individuals from Southern Fontainea and Coastal Fontainea populations (colour coded circles) and directionality of association with six correlated environmental variables (arrows) and; b) candidate loci (colour-coded to environmental predictor) and neutral loci (grey circles) with six environmental variables (arrows) detected from 10,000 reduced representation SNPs. c) Number of successful BLAST

hits with gene ontology terms from 136 putative candidate loci against plant species detected from BLAST2GO analysis (* denotes species from Euphorbiaceae family), d) pie chart with summary of 148 hits from the BLAST2GO functional classifications from the 11 candidates with gene ontology terms.

Supplementary Files

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