

# Comparative analysis of genetic diversity in wild and restored subtropical rainforest trees: *Argyrodendron trifoliolatum*, *Ficus watkinsiana* and *Diploglottis australis*

Sally L Cooper (✉ [scooper@ecosure.com.au](mailto:scooper@ecosure.com.au))

Southern Cross University

Peter C Bundock

Southern Cross University

Catherine Nock

Southern Cross University

---

## Research Article

### Keywords:

**Posted Date:** February 1st, 2023

**DOI:** <https://doi.org/10.21203/rs.3.rs-2531630/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

---

## Abstract

Since European settlement of Australia, substantial areas of subtropical rainforest have been cleared. More recently, effort is being made to restore small areas of this biodiverse ecological community. The financial investment and increasing reliance on ecological restoration in biodiversity conservation justifies a need for a better understanding of how to improve success at the genetic level. This research measures the levels of genetic diversity (allelic richness ( $R_s$ ), expected heterozygosity ( $H_e$ ), and inbreeding using Wright's coefficient ( $F_{IS}$ )) for expected neutral DNA markers in three characteristic Australian subtropical rainforest trees – white booyong (*Argyrodendron trifoliolatum*), Watkin's fig (*Ficus watkinsiana*) and native tamarind (*Diploglottis australis*). Wild and restored populations of these species were sampled and compared in two neighbouring regions in Northeast New South Wales (the Tweed Caldera and the Big Scrub). There were no statistically significant differences in allelic richness or expected heterozygosity between wild and restored populations for any of the target species. Inbreeding was significantly higher in one of the restored versus wild population comparisons: *F. watkinsiana* in the Tweed (restored  $F = 0.20$ ; wild  $F = -0.02$ ;  $p = 0.01$ ). Small but statistically significant genetic differentiation between restored and wild populations ( $F_{ST} < 0.17$  Big Scrub,  $F_{ST} < 0.053$  Tweed) was detected for some of the comparisons between these cohorts. The comparable levels of genetic diversity observed in restored and wild populations targeted in this study are likely due to informed practitioner perspectives and practices, multiple introductions and high genetic diversity and low genetic differentiation within source populations.

## Introduction

Compensatory restoration is increasingly relied upon for the persistence of ecological communities especially where remnant communities are small, fragmented and under threat of further degradation (Bull & Strange 2018). Restoration often requires considerable financial outlay (Collard et al. 2020). Consequently, it can be prudent to study genetic diversity in restored and remnant communities to inform future restoration efforts and better equip restoration plantings for survival into the future (Thomas et al. 2014).

Assessing measurable indicators against pre-determined goals and objectives is a key principle underpinning ecological restoration that allows for adaptive management and information sharing (Gann et al. 2019). When employed, monitoring programs for restoration projects typically focus on indicators at a macro scale such as: plant survival rate, structural and species diversity of surviving plants, plant height/growth rates, native/weed vegetation cover, natural recruitment/stem counts and habitat features (e.g. fallen log length and leaf litter cover) (Biodiversity Conservation Trust (BCT) 2020). Assessment of genetic indicators of restoration success are rarely employed due to the financial and time constraints involved and specialist skills required for genetic research. However, biodiversity encompasses all species and populations; including the genetic variation among them; and their complex assemblages of communities and ecosystems (CBD 2021). Maintenance of genetic diversity in restored communities is a measure of fitness for a variety of ecological functions (Bischoff et al. 2010; Hughes et al. 2008; Kettenring et al. 2014; Reynolds et al. 2012). It is a significant determining factor to the long-term sustainability of a restored community (Kardos et al. 2021) and is of importance and relevance to rainforest restoration practitioners, land managers and the broader community (Cooper et al. 2018). A wholistic assessment of ecological restoration success should include analysis of genetic diversity, where possible.

Ecological restoration typically takes place in landscapes that have been subject to large-scale habitat clearing and fragmentation. Remnant populations in these types of landscapes face genetic pressures such as lower mate availability and restrictions to gene flow, which can lead to decreased genetic diversity, increased population differentiation and elevated inbreeding (Lowe 2004). Fragmentation and a reduction in flowering-tree density can affect plant mating systems through increased self-pollination and breeding between related individuals, leading to increasingly inbred populations (Breed et al. 2013; Konuma et al. 2000). In outbreeding species, inbreeding will typically reduce fitness. This is due to an increase in the proportion of homozygous loci, which in turn increases the chance of expression of rare deleterious recessive alleles, which are usually masked by heterozygosity. This is especially the case with progeny resulting from self-fertilisation, where there is a 25% chance for each heterozygous deleterious recessive allele in the parent to become homozygous (and therefore expressed) in each generation (Allendorf 2013). Creating restored populations with germplasm collected from inbred individuals has the potential to affect the genetic fitness and the long-term viability of restored populations (Broadhurst 2011; Broadhurst 2013).

There are numerous seed sourcing strategies, also referred to as provenancing strategies, utilised in ecological restoration (Standards Reference Group 2017). Provenancing strategies include 'local,' 'composite' (Broadhurst, Lowe et al. 2008), 'admixture' (Breed, Stead et al. 2013), 'predictive' (Crowe and Parker 2008, Sgrò, Lowe et al. 2011), 'climate-adjusted' (Prober, Byrne et al. 2015) and 'site-adjusted' (Carvalho et al. 2021). Restoration practitioner's adherence to a chosen seed sourcing strategy requires an understanding, or more commonly an assumption, of population genetic structure. Practitioners opting for a local provenance strategy, for example, need to understand the geographical boundaries of local populations. Similarly, those wishing to utilise a composite seed sourcing strategy need to understand how different populations in different locations are genetically related to each other to fulfil the provenancing requirements. However, defining seed provenancing boundaries is not a mere matter of applying a geographical distance.

Genetic differentiation amongst populations is highly variable and often not predictable based on simple Isolation by Distance (IBD) analysis (Nazareno et al. 2021). Landscape features such as altitude, topography, and ground cover as well as phenological factors such as flowering synchrony and environmental factors such as habitat heterogeneity all influence gene flow, genetic connectedness, and ultimately genetic population structure (Gao et al. 2012; Rivest et al. 2021; Storfer et al. 2007). Guidelines recommending propagule collections from within specific distances can be overly restrictive for species with little genetic structure and can be ecologically detrimental if this leads to seed being collected from nearby isolated fragments with high levels of inbreeding at the expense of more genetically diverse seed from intact habitat which is further afield (Broadhurst et al. 2008; SERA 2021). However, a lack of representation of local allelic diversity in restored communities has the potential to lead to a loss and/or change of alleles represented locally over time. This is especially so in highly degraded landscapes where undisturbed habitat is limited in extent, may be highly fragmented and where restored habitat contributes a high proportion of the extent of the community represented in an area.

This research provides a comparison of genetic diversity and inbreeding between restored and wild populations for three characteristic tree species in subtropical rainforest in Northeast New South Wales (NE NSW), Australia. Genetic indicators including allelic richness ( $R_s$ ), expected heterozygosity ( $H_e$ ), and inbreeding using Wright's coefficient ( $F_{IS}$ ) were estimated using expected neutral DNA markers. The hypothesis tested was that there is a reduction in genetic diversity for restored rainforest tree species relative to those from wild communities. The null hypothesis was that there is no difference in the genetic diversity of restored populations relative to wild populations. A secondary question was, "Are trees in restored communities more inbred than those in the wild reference communities?". We also assessed genetic differentiation ( $F_{ST}$ ) of restored and wild populations and populations from two geographic regions within NE NSW.

## Materials And Methods

### Site selection and leaf sample collection

The two study regions comprise the Big Scrub area of the Richmond River catchment (-28.602/153.251; -28.911/153.551) and the Tweed area of the Tweed River catchment (-28.158/153.590; -28.544/153.120), which are situated on the far north coast of New South Wales. Under the Interim Biogeographic Regionalisation for Australia (IBRA) classification system (version 7), (Thackway & Cresswell 1995), both study regions are within the South East Queensland Bioregion, and the Burringbar-Conondale Ranges (SEQ03), Scenic Rim (SEQ10) and Clarence Lowlands (SEQ13) subregions. Refer to Fig. 1.

These regions are characterised by high rainfall (> 1200 mm/year) and fertile volcanic soils, the combination of which has enabled the establishment of unique and diverse subtropical rainforest communities. Large tracts were cleared with the introduction of colonial settlers, circa early to mid 1800's, with < 1% of the pre-Colonial settlement extent of the Big Scrub remaining (Parkes et al., 2012). Subtropical rainforest is now listed as a Critically Endangered ecosystem under the Commonwealth *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act) and an Endangered ecosystem under the NSW *Biodiversity Conservation Act 2016*. Despite extensive clearing throughout both study regions, generally small remnant patches of subtropical rainforest persist (Parkes et al. 2012). More recently, an effort has been made to restore rainforest in small areas on previously cleared land.

The three species targeted in this study were sampled from restored and wild communities within the Tweed and Big Scrub regions. It became apparent early in this study that there were too few individuals of the target species at any single restoration site or wild site to collect sample sizes that would be meaningful in their own right. Therefore, samples for each cohort were a composite of individuals collected from a variety of sites spread throughout the study regions (refer to Figs. 2–3 and Table 1). This approach ensured the restored population spanned a variety of different restoration sites and likely a mixture of different seed sources. The target sample size was 25 as, according to Hale et al. (2012), 25–30 samples is considered adequate to represent allelic diversity of the population/cohort for population genetic studies using microsatellite loci. This is consistent with the sample size recommended by Brown and Marshall (1995). An average of 23 individuals was sampled, per species, per test community, per region (min 21; max 25). Within the Big Scrub region, samples were collected across nine wild sites and seven restored sites. Within the Tweed, samples were collected across fifteen wild sites and eight restored sites. A considerably higher number of wild sites in the Tweed was required due to the lower prevalence of the study species in this region compared to the Big Scrub. The taller and broader wild trees were selected to represent a mature age class of wild trees, compared to the smaller and younger more recently established restored trees.

Table 1  
Sample locations and numbers

| Region | Site ID | Lat / long         | No. of samples from restored population |            |            | No. of samples from wild population |            |            | Region | Site ID | Lat / long         | No. samples from restored population |            |            | No. of samples from wild population |            |            |
|--------|---------|--------------------|-----------------------------------------|------------|------------|-------------------------------------|------------|------------|--------|---------|--------------------|--------------------------------------|------------|------------|-------------------------------------|------------|------------|
|        |         |                    | <i>A.t</i>                              | <i>F.w</i> | <i>D.a</i> | <i>A.t</i>                          | <i>F.w</i> | <i>D.a</i> |        |         |                    | <i>A.t</i>                           | <i>F.w</i> | <i>D.a</i> | <i>A.t</i>                          | <i>F.w</i> | <i>D.a</i> |
| Tw     | 1       | -28.1874, 153.4626 | 7                                       | 0          | 0          | 0                                   | 0          | 0          | Tw     | 19      | -28.4155, 153.3355 | 0                                    | 0          | 0          | 2                                   | 1          | 0          |
| Tw     | 2       | -28.2034, 153.4254 | 0                                       | 1          | 1          | 0                                   | 0          | 0          | Tw     | 20      | -28.4558, 153.3494 | 0                                    | 0          | 0          | 0                                   | 1          | 0          |
| Tw     | 3       | -28.2272, 153.4691 | 9                                       | 7          | 6          | 0                                   | 0          | 0          | Tw     | 21      | -28.5252, 153.2977 | 0                                    | 0          | 0          | 3                                   | 5          | 0          |
| Tw     | 4       | -28.2538, 153.4386 | 0                                       | 6          | 3          | 0                                   | 8          | 1          | BS     | 22      | -28.6091, 153.4588 | 0                                    | 0          | 0          | 0                                   | 1          | 0          |
| Tw     | 5       | -28.271, 153.3788  | 0                                       | 0          | 0          | 0                                   | 0          | 1          | BS     | 23      | -28.6511, 153.4264 | 0                                    | 4          | 0          | 0                                   | 2          | 0          |
| Tw     | 6       | -28.2509, 153.3131 | 0                                       | 0          | 0          | 0                                   | 1          | 0          | BS     | 24      | -28.6378, 153.3446 | 1                                    | 1          | 2          | 0                                   | 0          | 0          |
| Tw     | 7       | -28.2676, 153.2789 | 0                                       | 0          | 0          | 0                                   | 0          | 3          | BS     | 25      | -28.6713, 153.5859 | 0                                    | 0          | 0          | 2                                   | 3          | 4          |
| Tw     | 8       | -28.2565, 153.2631 | 1                                       | 2          | 0          | 5                                   | 2          | 0          | BS     | 26      | -28.6871, 153.4397 | 0                                    | 0          | 0          | 0                                   | 1          | 0          |
| Tw     | 9       | -28.1846, 153.2583 | 0                                       | 0          | 0          | 0                                   | 1          | 0          | BS     | 27      | -28.6962, 153.4242 | 0                                    | 0          | 0          | 3                                   | 4          | 2          |
| Tw     | 10      | -28.3322, 153.1883 | 0                                       | 0          | 0          | 4                                   | 1          | 0          | BS     | 28      | -28.7214, 153.503  | 7                                    | 1          | 7          | 1                                   | 0          | 1          |
| Tw     | 11      | -28.3238, 153.1592 | 0                                       | 0          | 0          | 0                                   | 2          | 10         | BS     | 29      | -28.7438, 153.4468 | 0                                    | 0          | 0          | 6                                   | 1          | 7          |
| Tw     | 12      | -28.2744, 153.4977 | 1                                       | 2          | 5          | 0                                   | 0          | 1          | BS     | 30      | -28.7481, 153.5006 | 7                                    | 7          | 6          | 0                                   | 0          | 0          |
| Tw     | 13      | -28.2948, 153.456  | 0                                       | 4          | 0          | 0                                   | 0          | 0          | BS     | 31      | -28.7696, 153.5227 | 0                                    | 5          | 0          | 0                                   | 0          | 0          |
| Tw     | 14      | -28.3611, 153.3541 | 8                                       | 4          | 10         | 0                                   | 0          | 0          | BS     | 32      | -28.8155, 153.3999 | 5                                    | 5          | 3          | 0                                   | 0          | 0          |
| Tw     | 15      | -28.3783, 153.2973 | 0                                       | 0          | 0          | 0                                   | 0          | 7          | BS     | 33      | -28.7834, 153.3083 | 5                                    | 0          | 7          | 0                                   | 0          | 0          |
| Tw     | 16      | -28.4449, 153.1968 | 0                                       | 0          | 0          | 7                                   | 0          | 2          | BS     | 34      | -28.8658, 153.4043 | 0                                    | 0          | 0          | 8                                   | 4          | 2          |
| Tw     | 17      | -28.3688, 153.4669 | 0                                       | 0          | 0          | 0                                   | 1          | 0          | BS     | 35      | -28.9029, 153.4113 | 0                                    | 0          | 0          | 0                                   | 1          | 0          |
| Tw     | 18      | -28.3877, 153.4003 | 0                                       | 0          | 0          | 1                                   | 0          | 0          |        |         |                    |                                      |            |            |                                     |            |            |

Note: Tw = Tweed, BS = Big Scrub, *A.t* = *Argyrodendron trifoliolatum*, *F.w* = *Ficus watkinsiana*, *D.a* = *Diploglottis australis*

## Target species

The three subtropical rainforest species targeted in this study have varied mating and pollen/seed dispersal systems, which capture some of the variability employed by subtropical rainforest species. *Ficus watkinsiana* occurs in two disjunct populations along the east coast of Australia (Atlas of Living Australia 2022c; Harden 2014). This species can be dioecious or monoecious, is wasp-pollinated with seed predominantly dispersed by medium to large frugivorous birds and flying foxes (Dixon 1999; Peel 2010; Williams & Adam 2010). *Diploglottis australis* is distributed throughout the subtropical east coast of Australia and extending south into temperate climates in NSW with a disjunct population recorded in North Queensland (Atlas of Living Australia 2022b). Seed is primarily dispersed by birds and flying foxes (Peel 2010). *Argyrodendron trifoliolatum* is distributed throughout the subtropical and tropical east coast of Australia (Harden 2014). A disjunct population is recorded in Papua New Guinea (Atlas of Living Australia 2022a). Seed of this species is a samara adapted for wind dispersal. Species-specific information on pollinators could not be found for *D. australis* or *A. trifoliolatum*, however, these species are likely pollinated by a diverse guild of insect taxa, as is typical for most canopy species in subtropical rainforests of Australia (Williams & Adam, 2010). Pollen of these species is also likely dispersed by nectarivorous birds and flying foxes. Information regarding the mating systems of these species was unavailable prior to this study.

## DNA extraction, amplification and genotyping

Once collected, leaf samples were refrigerated and then frozen at -20°C for storage. DNA was extracted using a variety of methods, including the CTAB-based method described by Xin and Chen (2012), QIAGEN DNeasy Plant Mini Kits, and Lucigen Epicentre QuickExtract™ DNA Extraction Solution. Extracted DNA was

quantified using a Qubit fluorometer.

The genetic analysis was undertaken using Simple Sequence Repeats (SSR) markers, which were species specific and developed for each of the three target species by Bottaro (2017). Fluorescent labels were covalently attached to the forward primers for each SSR marker for analysis purposes. Refer to supplementary information for details on SSR markers and primers. Samples were amplified using Polymerase Chain Reaction (PCR) and genotyped using capillary electrophoresis. Of the microsatellites tested, those best performing were included in this study, amounting to seven, six and four useable loci for *A. trifoliolatum*, *F. watkinsiana* and *D. australis*, respectively. Genetic diversity measures were made on a total of 279 samples (21–25 leaf samples/cohort x 3 species x 2 treatment populations x 2 regions).

PCR testing and optimization were undertaken for each locus to determine the optimal conditions. The PCR solution used comprised the following: 1.5 µl 10 x Platinum Buffer, 0.2 mM of combined dNTPs, 0.25 µM fluorescently-labelled forward (tagged with FAM, NED, PET or VIC) and reverse primers, 10% bovine serum albumin (BSA), 2 mM MgCl<sub>2</sub>, 5 U/µl platinum Taq and 2.5 µl of DNA template. PCRs were amplified on Applied Biosystems Veriti 96-Well Thermal Cyclers and Corbett Gradient Palm–Cyclers using the following generalized program: 95°C for 2 minutes; 94°C for 20 seconds; 70°C for 30 seconds (with a touchdown of -1°C per cycle for six cycles); 72°C for 1 minute; 94°C for 20 seconds; 56–62°C (depending on the locus) for 30 seconds (for 35 cycles); 72°C for 1 minute; 72°C for 5 minutes and 4°C hold at the completion of cycling. Genotyping of amplified SSRs for each sample was undertaken by capillary electrophoresis using an ABI PRISM 3730 Genetic Analyser (Applied Biosystems Inc.). Allele size was scored in reference to ABI PRISM GS (Liz) internal size standards using Geneious Prime version 2020.1.1 software.

## Data analysis

Allelic richness ( $R_s$ ), expected heterozygosity ( $H_e$ ), and a measure of inbreeding based on Wright's coefficient ( $F_{IS}$ ) were calculated for each SSR locus for the three tree species. The number of alleles ( $N_a$ ), number of effective alleles ( $N_e$ ), observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_e$ ), average unbiased expected heterozygosity ( $uH_e$ ), and inbreeding coefficient ( $F$ ) were calculated using GeneAIEx v6.503 (Peakall & Smouse 2012; Peakall & Smouse 2006). Allelic richness ( $R_s$ ) and private allelic richness ( $Apr$ ) were calculated using HP-Rare version June-6-2006, which applied a rarefaction formula as described in Kalinowski (2004) to account for differing sample sizes.

The presence of null alleles was tested using Micro-Checker v2.2.3 software (Van Oosterhout et al. 2004). Where null alleles were identified and accepted, corrections were applied using the 'Brookfield 2' estimator (Brookfield 1996), as recommended by Girard and Angers (2008). Departures from Hardy-Weinberg Equilibrium (HWE) were assessed for each locus in each population with an exact test using the Markov Chain method (dememorization = 1000, batches = 300; iterations per batch = 1000) using Genepop (web version 4.7.5) (Raymond M. & Rousset F 1995; Rousset 2008). Self-fertilization rates were estimated using RMES (robust multilocus estimate of selfing) software as described in David et al. (2007) and confirmed by independently examining the number of homozygous loci per individual within each population. A paired two-tailed t-test was applied to each of the genetic diversity measures to compare between the control (wild) cohort and test (restored) cohort. Genetic differentiation between wild and restored populations and the Big Scrub and Tweed regions was investigated using an AMOVA assessment of the fixation indices (a measure of the proportion of shared alleles) implemented in GenAIEx v6.503 (Peakall & Smouse 2012; Peakall & Smouse 2006) (permutations = 999, pair-wise population permutations = 999). A more fine-scale 'individual-level' investigation of genetic heterogeneity was employed using spatial auto-correlation in GenAIEx v6.503 (Peakall & Smouse 2012; Peakall & Smouse 2006). This analysis was based on the heterogeneity test (Omega) described by Smouse et al. (2008) (permutations  $n = 999$  and bootstrapping  $n = 999$  were used to estimate variance). The p value was set at 0.01 as recommended by Banks and Peakall (2012) to minimise the chance of Type I errors (false positives). This analysis assessed the correlation between genetic distance and geographic distance within and between each region's population.

## Results

### Data checking

Analysis using Micro-Checker v2.2.3 software identified the potential presence of null alleles for both *A. trifoliolatum* and *D. australis* due to heterozygosity deficits from HWE at certain loci. There was no evidence of null alleles in *F. watkinsiana* samples. The rationale and assumptions applied in determining the occurrence of null alleles and the subsequent adjustments to allele frequencies is provided in supplementary information. No evidence of large allele dropout or scoring error due to stuttering was detected at any loci for any species, according to the Micro-Checker v2.2.3 analysis.

### Genetic variability

For all populations, observed heterozygosity ( $H_o$ ) values were equal to or within one SE range of the expected heterozygosity ( $H_e$ ), based on data adjusted for null allele frequency estimates. These values are indicative of populations that are achieving panmixia (random mating). The exception was the restored Tweed *F. watkinsiana* population with observed heterozygosity ( $H_o$ ) substantially lower than expected heterozygosity ( $H_e$ ) and a high inbreeding coefficient ( $F = 0.20$ ) (Table 2). A significant departure from HWE was detected in six of the seven loci for the restored *A. trifoliolatum* population in the Tweed and in three of the four loci in the restored *D. australis* population in the Big Scrub. These departures from HWE were observed even when null allele adjusted genotype frequencies were used (as estimated by Micro-Checker v2.2.3 software).

Table 2

Comparison of genetic diversity indices<sup>a</sup> for wild and restored populations by species and region

| Species                 | Region    | N           |             | Na          |             | Ne         |            | Rs    |       | Ho                |                   | He         |            | uHe        |          |
|-------------------------|-----------|-------------|-------------|-------------|-------------|------------|------------|-------|-------|-------------------|-------------------|------------|------------|------------|----------|
|                         |           | Wild        | Rest.       | Wild        | Rest.       | Wild       | Rest.      | Wild  | Rest. | Wild              | Rest.             | Wild       | Rest.      | Wild       | Rest.    |
| <i>A. trifoliolatum</i> | Big Scrub | 23.43(0.30) | 22.86(0.14) | 12.57(1.31) | 11.14(1.58) | 6.02(0.90) | 5.76(1.08) | 11.65 | 10.35 | 0.73(0.04)        | 0.76(0.04)        | 0.81(0.03) | 0.78(0.04) | 0.83(0.03) | 0.8(0.0) |
|                         | Tweed     | 21.00(0.44) | 24.86(0.14) | 11.86(1.61) | 11.00(1.50) | 5.11(0.50) | 5.91(1.21) | 11.52 | 10.26 | 0.80(0.04)        | 0.74(0.04)        | 0.79(0.02) | 0.79(0.04) | 0.81(0.02) | 0.8(0.0) |
| <i>F. watkinsiana</i>   | Big Scrub | 24.00(0.00) | 21.83(0.17) | 13.00(2.03) | 12.00(1.37) | 8.55(1.60) | 7.76(1.18) | 12.45 | 11.78 | 0.88(0.03)        | 0.86(0.04)        | 0.84(0.05) | 0.85(0.03) | 0.86(0.05) | 0.8(0.0) |
|                         | Tweed     | 21.00(0.00) | 23.50(0.50) | 12.33(1.71) | 13.83(1.58) | 7.83(1.27) | 8.60(1.06) | 12.23 | 13.34 | <b>0.86(0.04)</b> | <b>0.70(0.05)</b> | 0.84(0.04) | 0.87(0.02) | 0.86(0.04) | 0.8(0.0) |
| <i>D. australis</i>     | Big Scrub | 22.75(0.95) | 23.75(0.25) | 17.25(1.65) | 17.00(2.04) | 9.27(2.25) | 8.56(1.56) | 15.62 | 15.13 | 0.84(0.05)        | 0.83(0.04)        | 0.87(0.03) | 0.87(0.03) | 0.89(0.03) | 0.8(0.0) |
|                         | Tweed     | 22.75(0.95) | 20.50(0.87) | 17.00(4.34) | 14.75(2.93) | 9.48(3.10) | 5.01(1.02) | 14.67 | 13.51 | 0.93(0.03)        | 0.74(0.11)        | 0.85(0.05) | 0.77(0.05) | 0.87(0.05) | 0.7(0.0) |

<sup>a</sup> N = average sample size; Na = Average No. of Alleles per locus; Ne = Average No. of Effective Alleles per locus =  $1 / (\sum p_i^2)$ ; Rs = Average Allelic Richness Heterozygosity across all loci = No. of Hets / N; He = Average Expected Heterozygosity across all loci =  $1 - \sum p_i^2$ ; uHe = Average Unbiased Expected Heterozygosity; F = Fixation Index =  $(He - Ho) / He = 1 - (Ho / He)$ ; Apr = Private allelic richness; Statistically significant ( $p < 0.05$ ) differences between wild and restored (F highlighted in bold. Value in parentheses is Standard Error.

## Self-fertilisation

Following the identification of a heterozygote deficit in the restored Tweed *F. watkinsiana* population, selfing rates were investigated further. Potential selfing was detected in the restored Tweed *F. watkinsiana* population ( $s = 0.26$ ), likely a consequence of high homozygosity in at least three of the 24 individuals sampled. This suggests that the stock used at some of the restoration sites may be a product of self-fertilisation, contributing to the relatively high inbreeding detected in this population ( $F = 0.20$ ). Selfing was not detected in the Tweed or Big Scrub wild *F. watkinsiana* populations ( $s = 0$ ). These results indicate *F. watkinsiana* may be self-compatible (in monoecious trees). However, the lack of selfing detected in the wild and Big Scrub populations suggests this species is preferentially out-crossing.

Selfing analysis of *A. trifoliolatum* samples indicated low levels of self-fertilisation in the Tweed populations ( $s = 0.04$  for the wild and  $s = 0.07$  for the restored populations). Inspection of the number of homozygous loci per individual suggests one of the individuals sampled in each of the wild and restored Tweed populations was likely a product of self-fertilisation ( $N = 22$  and  $N = 25$ ). No selfing was detected in the Big Scrub populations for this species ( $s = 0$ ).

Selfing analysis of *D. australis* samples identified low levels of self-fertilisation in the restored Tweed population ( $s = 0.06$ ). Inspection of the number of homozygous loci per individual suggests one of the 22 individuals sampled from this population may be the product of self-fertilisation. No selfing was detected in any other populations for this species ( $s = 0$ ).

The low levels of selfing detected in *A. trifoliolatum* and *D. australis* suggest these species are also preferentially outcrossing. The results indicate self-fertilised individuals are occasionally occurring in restored populations of all three study species. *Argyrodendron trifoliolatum* was the only species for which selfing was detected in the wild populations, albeit at low levels.

## Comparison of genetic diversity in wild versus restored populations

Average allelic richness across all loci was higher in the wild populations compared with restored populations in five of the six comparisons within each species and region (Table 2). However, no statistically significant differences in genetic diversity were detected (Table 2 and Figs. 4–6).

In the case of *A. trifoliolatum*, the allelic richness and expected heterozygosity of the restored population was lower than that of the wild population in the Big Scrub. Whilst this difference was not significant at  $p > 0.05$ , it was at the 0.1 level (Rs,  $p = 0.079$ , He  $p = 0.068$ ). No significant difference in allelic richness or expected heterozygosity was detected for this species in the Tweed (Rs,  $p = 0.289$ ; He,  $p = 0.882$ ). For *F. watkinsiana*, the difference in allelic richness and expected heterozygosity between the wild and restored populations was not significant in the Big Scrub (Rs,  $p = 0.505$  and He,  $p = 0.707$ ), or the Tweed (Rs,  $p = 0.377$  and He,  $p = 0.282$ ). For *D. australis*, the difference in allelic richness and expected heterozygosity between the wild and restored populations was also not significant in the Big Scrub (Rs,  $p = 0.737$  and He,  $p = 0.826$ ) or the Tweed (Rs,  $p = 0.378$  and He,  $p = 0.533$ ).

Allelic richness and expected heterozygosity results for all restored populations were at least 80% and 90% respectively of that recorded in the corresponding wild reference populations. Differences in genetic diversity between wild and restored populations were not strongly correlated with a particular species as no difference was found to be significant. The Big Scrub *A. trifoliolatum* population was the only restored population where reductions in both allelic richness and heterozygosity relative to the wild population were close to significant (Rs,  $p = 0.079$ ; He  $p = 0.068$ ). Whilst the Tweed *A. trifoliolatum* restored population had lower allelic richness than the wild population this was not statistically significant (Rs,  $p = 0.289$ ) and there was no detectable difference in expected heterozygosity (He,  $p = 0.882$ ).

With regards to the secondary hypothesis that inbred individuals would be more common in restored compared to wild communities, the inbreeding coefficient was found to be significantly higher ( $p = 0.01$ ) in the restored Tweed *F. watkinsiana* population ( $F = 0.20$ ) compared to the wild population ( $F = -0.02$ ) in that region. This was associated with a significant difference in observed heterozygosity (Ho,  $p = 0.037$ ) due to three highly homozygous individuals which were

probably the result of self-pollinations ( $s = 0.26$  for this population). When an estimate of the rate of selfing for this population was carried out after excluding these three individuals, the rate was estimated to be zero ( $s = 0$ ). No detectable differences in inbreeding levels were identified in the Big Scrub *F. watkinsiana* populations, or in the other species - *A. trifoliolatum* and *D. australis* - in either region.

## Genetic differentiation between restored and wild populations and regions

Results from the AMOVA assessment of fixation indices, found most molecular variance occurred within individuals (91%, 87% and 86% for *F. watkinsiana*, *A. trifoliolatum* and *D. australis*, respectively). Molecular variance between individuals was 6%, 12% and 11% for *F. watkinsiana*, *A. trifoliolatum* and *D. australis*, respectively. A relatively small proportion of variance was found between populations (3%, 1% and 3% for *F. watkinsiana*, *A. trifoliolatum* and *D. australis*, respectively). No molecular variance was detected between regions (0% for all species).

Small but statistically significant genetic differentiation was detected in four of the six (66.7%) intra-region assessments between wild and restored populations. In the case of *F. watkinsiana*, the Tweed restored population was found to be genetically differentiated from all other populations, including the wild Tweed population ( $\theta = 0.053$ ;  $p = 0.001$ ) and both wild and restored communities in the Big Scrub ( $\theta = 0.061$ ;  $p = 0.001$  and  $\theta = 0.048$ ;  $p = 0.001$ , respectively) (Table 4). In the case of *A. trifoliolatum*, the individuals collected from the Tweed restored communities were found to be significantly genetically differentiated from the wild Tweed population ( $\theta = 0.021$ ;  $p = 0.002$ ) and the restored Big Scrub population ( $\theta = 0.027$ ;  $p = 0.001$ ), but not from the wild Big Scrub population ( $\theta = 0.008$ ;  $p = 0.062$ ) (Table 3). In the case of *D. australis*, the Tweed restored population was found to be genetically differentiated from the wild population in the Tweed ( $\theta = 0.041$ ;  $p = 0.001$ ) and both wild ( $\theta = 0.053$ ;  $p = 0.001$ ) and restored ( $\theta = 0.049$ ;  $p = 0.001$ ) Big Scrub populations. The restored *D. australis* population in the Big Scrub was also found to be differentiated from the wild populations in this region ( $\theta = 0.017$ ;  $p = 0.001$ ) as well as the Tweed wild population ( $\theta = 0.024$ ;  $p = 0.001$ ) (Table 5).

Table 3 Pairwise  $F_{ST}$  between *A. trifoliolatum* populations.  $F_{ST}$  values below diagonal. Probability,  $p$  (rand  $\geq$  data) based on 999 permutations is shown above diagonal. Statistical significance indicated by bold text.

| Big Scrub wild | Big Scrub restored | Tweed wild | Tweed restored |                    |
|----------------|--------------------|------------|----------------|--------------------|
| 0.000          | 0.265              | 0.259      | 0.062          | Big Scrub wild     |
| 0.002          | 0.000              | 0.001      | 0.001          | Big Scrub restored |
| 0.003          | 0.019              | 0.000      | 0.002          | Tweed wild         |
| 0.008          | 0.027              | 0.021      | 0.000          | Tweed restored     |

Table 4. Pairwise  $F_{ST}$  between *F. watkinsiana* populations.  $F_{ST}$  values below diagonal. Probability,  $p$  (rand  $\geq$  data) based on 999 permutations is shown above diagonal. Statistical significance indicated by bold text.

| Big Scrub wild | Big Scrub restored | Tweed wild | Tweed restored |                    |
|----------------|--------------------|------------|----------------|--------------------|
| 0.000          | 0.291              | 0.389      | <b>0.001</b>   | Big Scrub wild     |
| 0.002          | 0.000              | 0.339      | <b>0.001</b>   | Big Scrub restored |
| 0.001          | 0.002              | 0.000      | <b>0.001</b>   | Tweed wild         |
| 0.061          | 0.048              | 0.053      | 0.000          | Tweed restored     |

Table 5. Pairwise  $F_{ST}$  between *D. australis* populations.  $F_{ST}$  values below diagonal. Probability,  $p$  (rand  $\geq$  data) based on 999 permutations is shown above diagonal. Statistical significance indicated by bold text.

| Big Scrub wild | Big Scrub restored | Tweed wild   | Tweed restored |                    |
|----------------|--------------------|--------------|----------------|--------------------|
| 0.000          | <b>0.001</b>       | 0.088        | <b>0.001</b>   | Big Scrub wild     |
| 0.017          | 0.000              | <b>0.001</b> | <b>0.001</b>   | Big Scrub restored |
| 0.006          | 0.024              | 0.000        | <b>0.001</b>   | Tweed wild         |
| 0.053          | 0.049              | 0.041        | 0.000          | Tweed restored     |

The spatial auto-correlation test found no correlation between genetic distance and geographic distance within wild populations of *A. trifoliolatum* (Tweed:  $r = -0.013$ – $0.018$ ,  $p > 0.01$ ; Big Scrub:  $r = -0.019$ – $0.022$ ,  $p > 0.01$ ). Further, no significant correlation was detected between Tweed and Big Scrub wild populations of *A. trifoliolatum* (Total  $\omega = 2.085$ ;  $p = 0.862$ ). No correlation between genetic and geographic distance was detected within wild populations of *F. watkinsiana* either (Tweed:  $r = -0.019$ – $0.017$ ,  $p > 0.01$ ; Big Scrub:  $r = -0.015$ – $0.022$ ;  $p > 0.01$ ). Nor was there a significant correlation between Tweed and Big Scrub wild

populations of *F. watkinsiana* (Total  $\omega = 2.614$ ;  $p = 0.731$ ). Results of the spatial auto-correlation test did detect a statistically significant though weak correlation between genetic and geographic distance within wild populations of *D. australis* (Tweed:  $r = -0.065$ – $0.077$ ,  $p < 0.01$ ); Big Scrub:  $r = -0.028$ – $0.054$ ;  $p < 0.01$ ). The correlation between Tweed and Big Scrub wild populations of *D. australis* was also weak though statistically significant (Total  $\omega = 9.281$ ;  $p = 0.016$ ). Refer to supplementary information for correlograms).

## Discussion

Genetic analysis comparing wild and restored cohorts has been undertaken for several plant species, both Australian and abroad, however, no such comparison has previously been undertaken for rainforest species. This research provides the first assessment of the health of subtropical rainforest restoration at the genetic level by comparing the genetic diversity and inbreeding of three subtropical rainforest tree species from restored and wild populations.

## Are restored populations capturing the genetic diversity of their wild reference populations?

This study suggests that the restored subtropical tree populations substantially match the genetic diversity of their wild source populations. Whilst most of the restored populations had reduced allelic richness in comparison to wild populations, none of these comparisons was statistically significant and a high proportion of the allelic richness present in the wild populations was present in restored populations. Large and significant reductions in genetic diversity (akin to founder events) in the restored populations were not observed in this study, as they have been in others, for example Salas-Leiva et al. (2009).

Previous comparative analyses of this kind have produced variable results with the number of studies providing evidence for reductions in genetic diversity in restored populations only marginally higher than those providing evidence for increases in genetic diversity in restored populations (Jordan et al. 2019). According to Jordan et al. (2019), studies reporting higher levels of genetic diversity in revegetation compared to natural stands were more likely to have been established using multiple seed sources, whereas those that found reduced levels of genetic diversity in revegetation than in natural stands were more likely to have been sourced from a single site. Bottaro (2017) found that allelic richness could be improved to above wild population levels when seedlings were sampled from several different nurseries. The seed sources used to establish the restored populations studied here are unknown. However, it is known that seed collectors and native nursery workers operating in these regions value the importance of genetic diversity in ecological restoration (Cooper et al. 2018). In a survey that asked these seed collectors and native nursery workers for the most effective approach to increasing genetic diversity in restored rainforest communities, the following response was most favoured: 'obtaining seed from more than one remnant patch' (Cooper et al. 2018). This awareness of the importance of maximizing genetic diversity in ecological restoration and an understanding of the importance of collecting from multiple sites may have contributed to the results of this study that suggests genetic diversity has been maintained in restored populations.

Results of this study suggest that restoration efforts at the studied sites have, for the most part, been collectively successful in capturing the available high levels of genetic diversity found in the wild populations for each of the three species across each region. The data gathered from this study does not refute the null hypothesis that genetic diversity is equivalent in restored versus wild populations of the target subtropical tree species. Genetic diversity for each cohort across both regions of the Tweed and Big Scrub were not significantly different. Given that ecological restoration involves translocation of a sample of genotypes from a source population (or populations), it is considered a founder event. So why was a substantial reduction in genetic diversity (strong founder effect) in restored subtropical rainforest populations not observed in this study? An explanation may be found in plant invasion genetic theory.

Invasive plant populations have been found to counter expectations of reduced genetic diversity despite establishing from smaller founder populations (Helsen et al. 2013; Shirk et al. 2014). This seemingly unexpected result seen in numerous invasive populations has been attributed to multiple introductions as well as low genetic differentiation over the source population range (Dlugosch & Parker 2008; Shirk et al. 2014). Low genetic differentiation within source populations reduces founder effects by enabling more effective capture of allelic diversity in any sample of individuals going on to establish a new population (Helsen et al. 2013; Shirk et al. 2014). Multiple introductions bring genetic variation and novel genetic combinations to a population (Dlugosch & Parker 2008), and act to augment genetic diversity by increasing both the founder population size ( $N_e$ ) and growth rate of the population (Dlugosch & Parker 2008; Nei et al. 1975). It follows that founder effects are expected to be stronger for introduced populations with few introductions of few individuals that have low within-population diversity in their native range (Shirk et al. 2014). Whilst it is typical in subtropical restoration for few individuals of each species to be introduced in a single restoration project, the restored population is made up of many restoration projects and therefore many introductions. The combination of the apparent intrinsically high within-population genetic diversity of the studied species' source populations, the lack of strong genetic differentiation throughout the study regions, as well as the logistical and financial necessity of creating the restored population from numerous small restoration projects spread over many years, may inadvertently protect the restored populations from strong founder effects and therefore limit losses in genetic diversity. However, it is acknowledged that the conclusion that these restored populations are comparable to the wild populations, in terms of genetic diversity, assumes that the various restoration sites collectively making up the restored population are capable of interbreeding. That is, it is assumed they are not genetically isolated from one another and are in fact operating as a true population. Considering the pollination and seed dispersal mechanisms of these species, gene flow between individuals throughout each of the study regions is considered plausible. However, *A. trifoliolatum* would be expected to have the greatest challenge to region-wide genetic exchange on account of its wind seed dispersal (Lowe et al. 2018) and would likely depend on pollen mediated gene flow (Heslewood et al. 2014). Regardless, the spatial auto-correlation results support this assumption with no strong correlation between genetic and geographic distances detected. Further research into gene flow between these restored patches and between restored and wild patches would add clarity to the conclusions drawn here. Furthermore, research on the genetic diversity of natural recruits within these populations would yield valuable information on the levels of genetic diversity and inbreeding over successive generations.

## Inbreeding

The hypothesis that inbred individuals would be more common in restored compared to wild communities was supported by one comparison in the Tweed (*F. watkinsiana*). However, no other comparison was statistically significant (Table 2). Kinship of planted propagules has been detected in other restored populations (e.g. Salas-Leiva et al. (2009)). However, it is possible that inbred individuals planted at the restoration sample sites had already been selected against, even in relatively young, restored forest. Overall, the subtropical rainforest populations in this study were found to have relatively high levels of genetic diversity and limited inbreeding, except for one restored *F. watkinsiana* population that included a few inbred individuals that were probably derived from self-fertilised seed.

High levels of genetic diversity coupled with high inbreeding coefficients, as recorded in the restored Tweed *F. watkinsiana* population, has been reported in other restored populations including those established from multiple genetic founders, with population sizes equivalent to native populations and a positive growth rate (Fant et al. 2013; Salas-Leiva et al. 2009). The high inbreeding coefficients in these genetically diverse restored populations were attributed to either true inbreeding or a restoration-induced Wahlund Effect (Wahlund 1928). The Wahlund Effect is a deficiency in the number of heterozygotes compared with Hardy-Weinberg expectations caused by combining samples from different subpopulations in a structured population (Garnier-Géré & Chikhi 2013; Wahlund 1928). This arises due to variance in allele frequencies amongst different sub-populations (see Garnier-Géré and Chikhi (2013) and references therein). The Wahlund Effect tends to decline over time as the combined subpopulations interbreed (Fant et al. 2013; Lloyd et al. 2012). In this study, a Wahlund Effect could have potentially been created if genetically distinct seed sources (seed from different subpopulations) had been sampled across the different collection sites. However, this sampling strategy was employed across all populations and only one was found to exhibit inbreeding – the restored Tweed *F. watkinsiana* population. In addition, low levels of population subdivision were identified for all three species, both between regions and between wild and restored cohorts ( $F_{ST} < 0.053$  Tweed,  $F_{ST} < 0.017$  Big Scrub, Tables 3 to 5). The AMOVA assessment found that most molecular variance occurred within individuals, with  $\leq 12\%$  between individuals and  $\leq 3\%$  between cohorts (sample populations). The spatial auto-correlation test did not find any strong correlation between genetic and geographic distance within and between wild populations of these species. These results are not suggestive of a structured population. The evidence of selfing in a single Tweed *F. watkinsiana* population and lack of evidence of population structuring suggests that this may be the result of true inbreeding.

Of the three highly likely selfed individuals from the restored *F. watkinsiana* population in the Tweed, two were from the same restoration site. An additional individual from this restoration site was 50% homozygous and is also likely to be the result of selfing, so overall three of the four *F. watkinsiana* individuals sampled from this one restoration site are likely selfs. Enquiries into the source of the *F. watkinsiana* individuals planted at the restoration site found that these *F. watkinsiana* seedlings were propagated from seed collected from a single mature fig which was cut down during a road upgrade in the Tweed Shire (G. Newland, personal communication, January 27, 2021; M. Bloor, personal communication, January 27, 2021). The data suggests this source fig was self-pollinating, presumably due to its isolation and lack of available pollen donors. The deployment of progeny from this source to restoration sites throughout the Tweed may be the cause of the elevated level of inbreeding recorded for the entire restored population in this region. This case demonstrates how impactful seed collection decisions can be on the genetic health of restored populations. The more inbred individuals are deployed to a restoration site, the greater the chance of deleterious alleles being expressed with potentially unfavourable ecological outcomes (e.g. lower fecundity and disease resistance). Individuals may self-fertilise for reproductive assurance in response to a lack of pollinators and/or mates (Barrett 2013). The lack of evidence for trees resulting from self-pollination in the wild *F. watkinsiana* populations suggests that there may be selective pressures towards a preferentially outcrossing mixed mating system in this species or that progeny created from self-fertilization have been selected against prior to reaching maturity. Evidence of selection against selfed progeny has been previously reported in tree species at various stages of their lifecycle, including embryo, seedling and sapling stages (Hufford & Hamrick 2003; Konuma et al. 2000; Naito et al. 2005; Williams 2008). Possible reasons for observed losses of selfed individuals as they progress through their life cycle includes smaller seed mass (Hufford & Hamrick 2003; Naito et al. 2005) and expression of deleterious recessive alleles (Kuang et al. 1998). Given the demonstrated selfing potential of *F. watkinsiana*, it is important that seedlings of this species are obtained from large populations or multiple sources to avoid potentially swamping a restoration site with inbred and, therefore, likely poorly performing stock with low genetic diversity. Seed collectors could assist in minimizing the chance of collecting selfed seed by limiting collection from highly isolated trees, such as paddock trees, despite their appeal due to ease of collection. Given the unknown factors regarding the mating system of many species employed in restoration works, obtaining stock from large populations and multiple sources is a sensible safeguard to apply to all species to minimise the chance of introducing large numbers of highly inbred stock.

## Genetic differentiation between restored and wild populations and regions

Whilst most of the restored communities maintained comparable genetic diversity to wild populations, they were not always genetically representative of their wild reference communities. This is likely influenced by the variability in restoration history (e.g., where, and how seed/stock is sourced, whether the site had supplementary planting over time, etc.). Strong genetic differentiation between wild and restored communities would be expected if only a small portion of the wild gene pool was deployed to the restoration sites. This could be a consequence of several factors individually or combined: seed collection from few and/or inbred individuals; strong genetic selection in the nursery production stage, or any process that results in over/under representation of certain alleles in stock transferred to restoration sites.

Strong differentiation would also be expected if seed/seedlings were predominantly sourced from non-local stock. The restored *F. watkinsiana* population in the Tweed is characterized by high genetic diversity, high inbreeding, a high representation of selfed individuals, low but statistically significant genetic differentiation and a high number of private alleles. In summary, this population differs genetically from the other populations of *F. watkinsiana* in this study and shows signs of inbreeding. This could be explained, for example, if the selfed individuals from the restored population were propagules from a mature fig not sampled in the wild population, which anecdotal evidence suggests is true in this case. This may also occur if nursery stock for this species is being produced on a large scale from non-local and/or non-representative parent stock (e.g. a few selected seed trees). The genetic differentiation between the restored Tweed *F. watkinsiana* population and the other *F. watkinsiana* populations is partially attributable to the presence of selfed individuals. When these selfed individuals were removed from the analysis the degree of genetic differentiation between the restored Tweed population and other *F. watkinsiana* populations was reduced ( $F_{ST} = 0.048\text{--}0.061$  with selfs included and  $F_{ST} = 0.033\text{--}0.046$  with selfs excluded), although still statistically significant ( $p = 0.001$ ),

suggesting other factors are at play. The use of non-local stock is potentially contributing to this small but statistically significant differentiation. Considering the anecdotal account of the history of the inbred stock deployed into the restored Tweed *F. watkinsiana* population, for which the adult no longer exists, the results are considered a snap-shot of a random collection event. Deployment of inbred stock may not necessarily be a persistent problem for this species in the Tweed. However, this case serves as a warning. If seed collectors favour collection from particular trees over long periods of time, especially isolated source trees, the deployment of inbred stock to restoration sites remains a risk. The genetic differentiation detected among some restored populations is small, however, it does reinforce the importance of genetic considerations in community restoration planning and suggests that restored communities may not necessarily be representative of wild communities at the genetic level. Relying on restored communities to replace loss of wild communities could potentially result in a change in genetic composition of local populations. This could be desirable in some circumstances where restoration seedlings are strategically selected for their adaptation to future predicted environmental conditions or to maximise adaptive potential, for example in a climate-adjusted or admixture provenancing scenario. However, there is no evidence to suggest provenancing strategies other than local provenancing have been employed in these study regions (Cooper et al. 2018). This suggests the small divergence from the genetic composition of local wild populations detected in restored populations is unplanned and circumstantial rather than strategic.

## Local provenance

No molecular variance was detected between the wild populations of different study regions ( $F_{ST} = 0\%$  for all species). The spatial auto-correlation heterogeneity test did not detect any strong relationship between genetic and geographic distance which would be suggestive of genetic structuring between wild populations from the Big Scrub and Tweed region. These results suggest that there has been genetic connectedness, at least in the relatively recent past, due to gene flow between these two neighbouring regions. These results suggest that local provenance for these three species of subtropical rainforest trees may encompass both neighbouring regions of the Tweed and Big Scrub, spanning at least 80 kms within NE NSW. This distance of local provenance is consistent in scale with the Zucchi et al. (2018) study of four tropical semi-deciduous species which found distinct gene pools in wild individuals occurring > 250 kms away. Similarly, no population differentiation was found in Australian rainforest trees from the *Elaeocarpus* genus in Northern NSW across the study area which extended ~ 250 kms (Rossetto et al. 2004). The Restore and Renew webtool (The Royal Botanic Garden 2021) estimates a local seed collection zone for various species based on genetic information, species distribution ranges and climate models. One of the target species from this study – *D. australis* is addressed by this resource. For this species, the local seed collection zone, based on a site located in NE NSW (both Murwillumbah and Lismore were investigated), spans ~ 315–350 km north suggests that the statistical significance detected for the spatial auto-correlation result for *D. australis* is unlikely representative of true population structuring. Rainforest species with highly-mobile seed dispersal vectors and a history of broadscale connected habitat are expected to have more extensive local provenance boundaries than species from different habitats which have been historically isolated.

Propagules of *A. trifoliolatum*, *F. watkinsiana* and *D. australis* sourced from anywhere within the neighbouring regions of the Tweed and Big Scrub would be considered to be within the bounds of local provenance for revegetation projects situated within these regions. However, ensuring a mixture of nearby local, more distant local, and possibly even further afield propagules, may assist in maximising genetic diversity and minimising inbreeding while maintaining local alleles in the gene pool. This approach is expected to provide the broadest genetic base to allow natural selection, mutation and recombination to act upon.

## Conclusion

This study provides results from a comparative genetic analysis of restored versus wild populations of three subtropical rainforest species. It provides a health check and measure of the effectiveness of restoration efforts, at the genetic scale, something rarely practicable for most restoration projects. This study demonstrates how restoration activities can influence the genetic composition of plant populations, sometimes creating populations which are genetically different to the local wild populations. The results are a reminder of the importance of genetic considerations to ecological restoration as the collective ancestry of the plants translocated to revegetation sites influence the levels of genetic diversity and adaptive potential of the restored population and the ecological community throughout the landscape more broadly.

## Declarations

### Acknowledgements

Tweed Shire Council, Big Scrub Landcare and Skyrail Rainforest Foundation provided financial assistance for this research without which the research would not have been possible. The many hours of field work could not have been completed without the site knowledge and access provided by the various landowners. Greg Lollback (Tweed Shire Council) provided advice on research design and data analysis. Much gratitude to Frances Elliott and Michael Cross from Southern Cross University's School of Plant Science for their invaluable assistance in the laboratory.

## Funding

Financial support was received for this research from the following funding bodies: Tweed Shire Council (Biodiversity Grant), Big Scrub Landcare and Skyrail Rainforest Foundation Limited (Skyrail Rainforest Research Fund).

## Competing Interests

The corresponding author Sally L Cooper declares she was employed at Tweed Shire Council at the time this research was conducted. Tweed Shire Council was also a funding body for this research.

## Author Contributions

The research was designed by Sally L Cooper and Dr Peter Bundock. The field work was undertaken by Sally L Cooper with assistance from family, friends, colleagues and a volunteer student. The laboratory work was carried out by Sally L Cooper with assistance from Southern Cross Plant Science staff members - Frances Elliott and Michael Cross. The data analysis was carried out by Sally L Cooper, Dr Peter Bundock and Dr Catherine Nock. The manuscript was written by Sally L Cooper with peer review and advice provided by Dr Peter Bundock and Dr Catherine Nock.

## Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

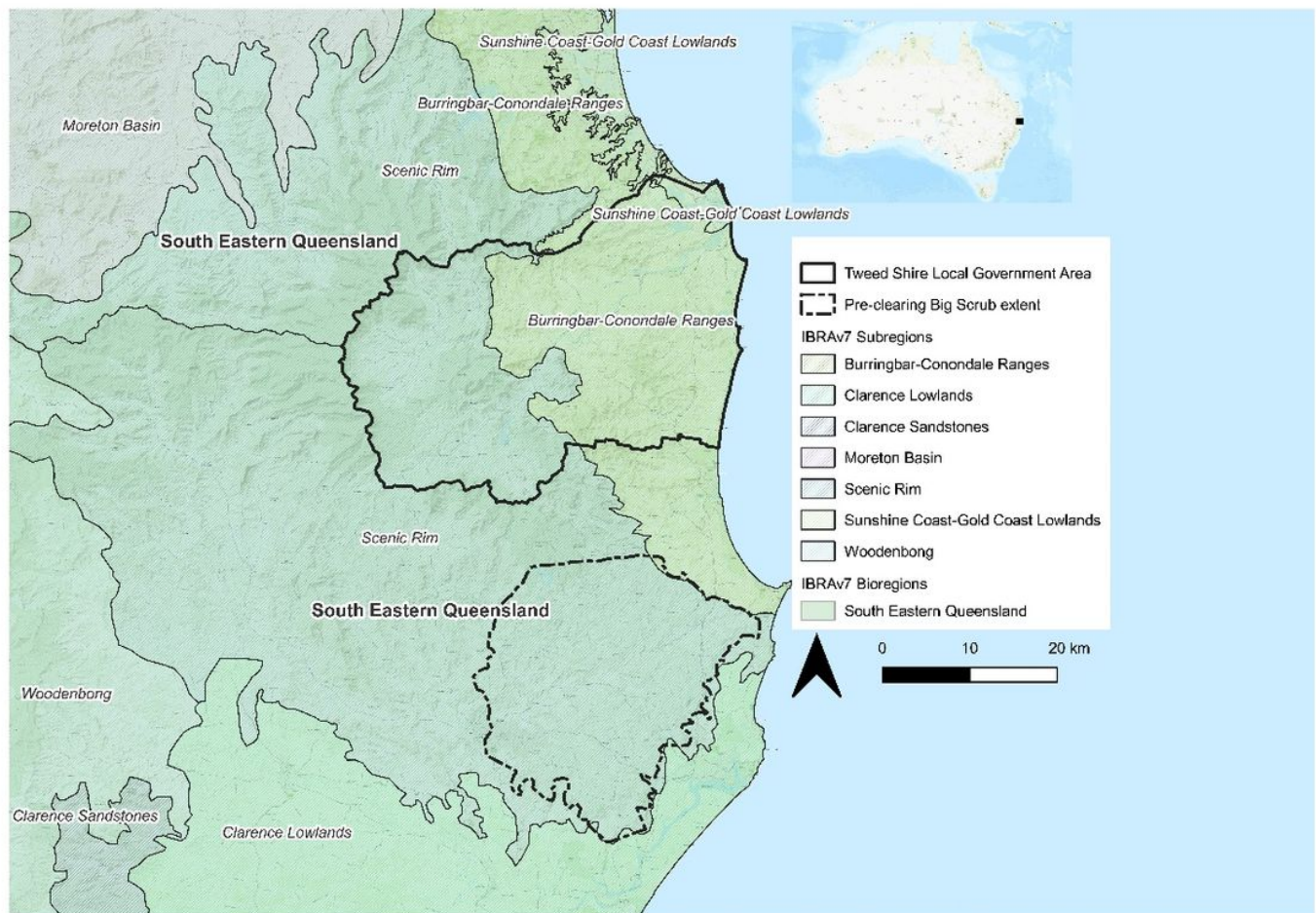
## References

1. Allendorf FW, Luikart GH, Aitken SN (2013) Conservation and the genetics of populations, 2nd edn. ed.): Wiley
2. Atlas of Living Australia (2022a) *Atlas of Living Australia occurrence records for *Argyrodendron trifoliolatum**
3. Atlas of Living Australia (2022b) Atlas of Living Australia occurrence records for *Diploglottis australis*. doi:<https://doi.org/10.26197/ala.33a68184-35b0-4b65-9068-185eb8913b28>
4. Atlas of Living Australia (2022c) Atlas of Living Australia occurrence records for *Ficus watkinsiana*. doi:<https://doi.org/10.26197/ala.ce8c3aa4-6146-4b63-83cb-3159cfab74c6>
5. Banks SC, Peakall ROD (2012) Genetic spatial autocorrelation can readily detect sex-biased dispersal. *Mol Ecol* 21(9):2092–2105. <https://doi.org/10.1111/j.1365-294X.2012.05485.x>
6. Barrett S (2013) *IV.8. Evolution of Mating Systems: Outcrossing versus Selfing*
7. Biodiversity Conservation Trust (BCT) (2020) *Ecological Monitoring Module*
8. Bischoff A, Steinger T, Müller-Schärer H (2010) The Importance of Plant Provenance and Genotypic Diversity of Seed Material Used for Ecological Restoration. *Restor Ecol* 18(3):338–348. 10.1111/j.1526-100X.2008.00454.x
9. Bottaro M (2017) *Genetic diversity of remnant rainforest trees compared with nursery seedlings for revegetation*. Honours Minor Thesis Institute of Innovative Agriculture. Southern Cross University. Lismore, Australia
10. Breed MF, Stead MG, Ottewell KM, Gardner MG, Lowe AJ (2013) Which provenance and where? Seed sourcing strategies for revegetation in a changing environment. *Conserv Genet* 14(1):1–10. 10.1007/s10592-012-0425-z
11. Broadhurst LM (2011) Genetic diversity and population genetic structure in fragmented *Allocasuarina verticillata* (Allocasuarinaceae) – implications for restoration. *Aust J Bot* 59:770–780
12. Broadhurst LM (2013) A genetic analysis of scattered Yellow Box trees (*Eucalyptus melliodora* A.Cunn. ex Schauer, Myrtaceae) and their restored cohorts. *Biol Conserv* 161:48–57. <http://dx.doi.org/10.1016/j.biocon.2013.02.016>
13. Broadhurst LM, Lowe A, Coates DJ, Cunningham SA, McDonald M, Vesk PA, Yates C (2008) Seed supply for broadscale restoration: maximizing evolutionary potential. *Evol Appl* 1(4):587–597. 10.1111/j.1752-4571.2008.00045.x
14. Brookfield JF (1996) A simple new method for estimating null allele frequency from heterozygote deficiency. *Mol Ecol* 5(3):453–455. 10.1111/j.1365-294x.1996.tb00336.x
15. Brown AHD, Marshall DR (1995) A basic sampling strategy: theory and practice. *Collecting plant genetic diversity: technical guidelines*. CAB International, Wallingford, 75–91
16. Bull JW, Strange N (2018) The global extent of biodiversity offset implementation under no net loss policies. *Nat Sustain* 1(12):790–798. 10.1038/s41893-018-0176-z
17. Carvalho CS, Forester BR, Mitre SK, Alves R, Imperatriz-Fonseca VL, Ramos SJ, Jaffé R (2021) Combining genotype, phenotype, and environmental data to delineate site-adjusted provenance strategies for ecological restoration. *Mol Ecol Resour* 21(1):44–58. <https://doi.org/10.1111/1755-0998.13191>
18. CBD (2021) Why is Biodiversity Important? *United Nation Decade on Biodiversity 2011–2020*. Retrieved from <https://www.cbd.int/2011-2020/about/biodiversity>
19. Collard SJ, O'Connor PJ, Prowse TAA, Gregg D, Bond AJ (2020) Objectives versus realities: Spatial, temporal, financial and social deficiencies in Australia's public revegetation investment model. *Ecol Manage Restor* 21(1):35–41. <https://doi.org/10.1111/emr.12398>
20. Cooper SL, Catterall C, Bundock PC (2018) Local provenancing in subtropical rainforest restoration: For better or worse? A review of practitioners' perspectives. *Ecol Manage Restor* 19(2):156–165. <https://doi.org/10.1111/emr.12305>
21. David P, Pujol B, Viard F, Castella V, Goudet J (2007) Reliable selfing rate estimates from imperfect population genetic data. *Mol Ecol* 16(12):2474–2487. 10.1111/j.1365-294X.2007.03330.x
22. Dixon DJ (1999) Figuring out the figs: systematic studies in *Ficus*, *Moraceae*, (*Urostigma* sect. *Malvanthera*). In
23. Dlugosch KM, Parker IM (2008) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Mol Ecol* 17(1):431–449. <https://doi.org/10.1111/j.1365-294X.2007.03538.x>
24. Fant JB, Kramer A, Sirkin E, Havens K (2013) Genetics of reintroduced populations of the narrowly endemic thistle, *Cirsium pitcheri* (Asteraceae)1. *Botany* 91(5):301–308. 10.1139/cjb-2012-0232

25. Gann GD, McDonald T, Walder B, Aronson J, Nelson CR, Jonson J, Dixon KW (2019) International principles and standards for the practice of ecological restoration. Second edition. *Restoration Ecology*, 27(S1), S1-S46. doi:<https://doi.org/10.1111/rec.13035>
26. Gao L, Tang S, Zhuge L, Nie M, Zhu Z, Li B, Yang J (2012) Spatial Genetic Structure in Natural Populations of *Phragmites australis* in a Mosaic of Saline Habitats in the Yellow River Delta, China. *PLoS ONE* 7(8):e43334. 10.1371/journal.pone.0043334
27. Garnier-Géré P, Chikhi L (2013) Population Subdivision, Hardy–Weinberg Equilibrium and the Wahlund Effect. *eLS*. doi:<https://doi.org/10.1002/9780470015902.a0005446.pub3>
28. Girard P, Angers B (2008) Assessment of power and accuracy of methods for detection and frequency-estimation of null alleles. *Genetica* 134(2):187–197. 10.1007/s10709-007-9224-8
29. Hale ML, Burg TM, Steeves TE (2012) Sampling for Microsatellite-Based Population Genetic Studies: 25 to 30 Individuals per Population Is Enough to Accurately Estimate Allele Frequencies. *PLoS ONE* 7(9). 10.1371/journal.pone.0045170
30. <http://dx.doi.org/10.1371/journal.pone.0045170>
31. Rainforest Plants of Australia - Rockhampton to Victoria, Harden GN, McDonald H (2014) Bill; Nicholson, Nan; Tame, Terry and Williams, John [Mobile application software]
32. Helsen K, Jacquemyn H, Hermy M, Vandepitte K, Honnay O (2013) Rapid Buildup of Genetic Diversity in Founder Populations of the Gynodioecious Plant Species *Origanum vulgare* after Semi-Natural Grassland Restoration. *PLoS ONE* 8(6):e67255. 10.1371/journal.pone.0067255
33. Heslewood MM, Lowe AJ, Crayn DM, Rossetto M (2014) Contrasting levels of connectivity and localised persistence characterise the latitudinal distribution of a wind-dispersed rainforest canopy tree. *Genetica* 142(3):251–264. 10.1007/s10709-014-9771-8
34. Hufford KM, Hamrick JL (2003) Viability selection at three early life stages of the tropical tree, *Platypodium elegans* (Fabaceae, Papilionoideae). *Evolution* 57(3):518–526. <https://doi.org/10.1111/j.0014-3820.2003.tb01543.x>
35. Hughes AR, Inouye BD, Johnson MTJ, Underwood N, Vellend M (2008) Ecological consequences of genetic diversity. *Ecol Lett* 11(6):609–623. 10.1111/j.1461-0248.2008.01179.x
36. Jordan R, Breed MF, Prober SM, Miller AD, Hoffmann AA (2019) How well do revegetation plantings capture genetic diversity? *Biol Lett* 15(10):20190460. 10.1098/rsbl.2019.0460
37. Kardos M, Armstrong Ellie E, Sarah F, Hauser W, Philip SHedrick, Miller Joshua W, Funk M (2021) W. C. The crucial role of genome-wide genetic variation in conservation. *Proceedings of the National Academy of Sciences*, 118(48), e2104642118. doi:10.1073/pnas.2104642118
38. Kettenring KM, Mercer KL, Adams R, C., Hines J (eds) (2014) EDITOR'S CHOICE: Application of genetic diversity–ecosystem function research to ecological restoration. *Journal of Applied Ecology*, 51(2), 339–348. doi:10.1111/1365-2664.12202
39. Konuma A, Tsumura Y, Lee CT, Lee SL, Okuda T (2000) Estimation of gene flow in the tropical-rainforest tree *Neobalanocarpus heimii* (Dipterocarpaceae), inferred from paternity analysis. *Mol Ecol* 9(11):1843–1852. <https://doi.org/10.1046/j.1365-294x.2000.01081.x>
40. Kuang H, Richardson TE, Carson SD, Bongarten BC (1998) An allele responsible for seedling death in *Pinus radiata* D. Don. *Theor Appl Genet* 96(5):640–644. <http://dx.doi.org/10.1007/s001220050783>
41. Lloyd MW, Burnett RK, Engelhardt KAM, Neel MC (2012) Does genetic diversity of restored sites differ from natural sites? A comparison of *Vallisneria americana* (Hydrocharitaceae) populations within the Chesapeake Bay. *Conserv Genet* 13(3):753–765. 10.1007/s10592-012-0324-3
42. Lowe A, Harris S, Ashton P (2004) Ecological genetics: design, analysis and application, vol 95. Blackwell Publishing, Oxford, 4
43. Lowe AJ, Breed MF, Caron H, Colpaert N, Dick C, Finegan B, Cavers S (2018) Standardized genetic diversity-life history correlates for improved genetic resource management of Neotropical trees. *Divers Distrib* 24(6):730–741. <https://doi.org/10.1111/ddi.12716>
44. Naito Y, Konuma A, Iwata H, Suyama Y, Seiwa K, Okuda T, Tsumura Y (2005) Selfing and inbreeding depression in seeds and seedlings of *Neobalanocarpus heimii* (Dipterocarpaceae). *J Plant Res* 118(6):423–430. 10.1007/s10265-005-0245-z
45. Nazareno AG, Knowles LL, Dick CW, Lohmann LG (2021) By Animal, Water, or Wind: Can Dispersal Mode Predict Genetic Connectivity in Riverine Plant Species? *Front Plant Sci* 12(31). 10.3389/fpls.2021.626405
46. Nei M, Maruyama T, Chakraborty R (1975) THE BOTTLENECK EFFECT AND GENETIC VARIABILITY IN POPULATIONS. *Evolution* 29(1):1–10. <https://doi.org/10.1111/j.1558-5646.1975.tb00807.x>
47. Parkes T, Delaney M, Dunphy M, Woodford R, Bower H, Bower S, McDonald T (2012) Big Scrub: A cleared landscape in transition back to forest? *Ecol Manage Restor* 13(3):212–223. 10.1111/emr.12008
48. Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinf (Oxford England)* 28(19):2537–2539. 10.1093/bioinformatics/bts460
49. Peakall ROD, Smouse PE (2006) genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* 6(1):288–295. <https://doi.org/10.1111/j.1471-8286.2005.01155.x>
50. Peel B (2010) Rainforest restoration manual for south-eastern Australia. CSIRO Publishing, Collingwood, Victoria
51. Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Heredity* 86:248–249
52. Reynolds LK, McGlathery KJ, Waycott M (2012) Genetic Diversity Enhances Restoration Success by Augmenting Ecosystem Services. *PLoS ONE* 7(6):e38397. 10.1371/journal.pone.0038397
53. Rivest S, Lajoie G, Watts DA, Vellend M (2021) Earlier spring reduces potential for gene flow via reduced flowering synchrony across an elevational gradient. *Am J Bot* 108(3):538–545. 10.1002/ajb2.1627

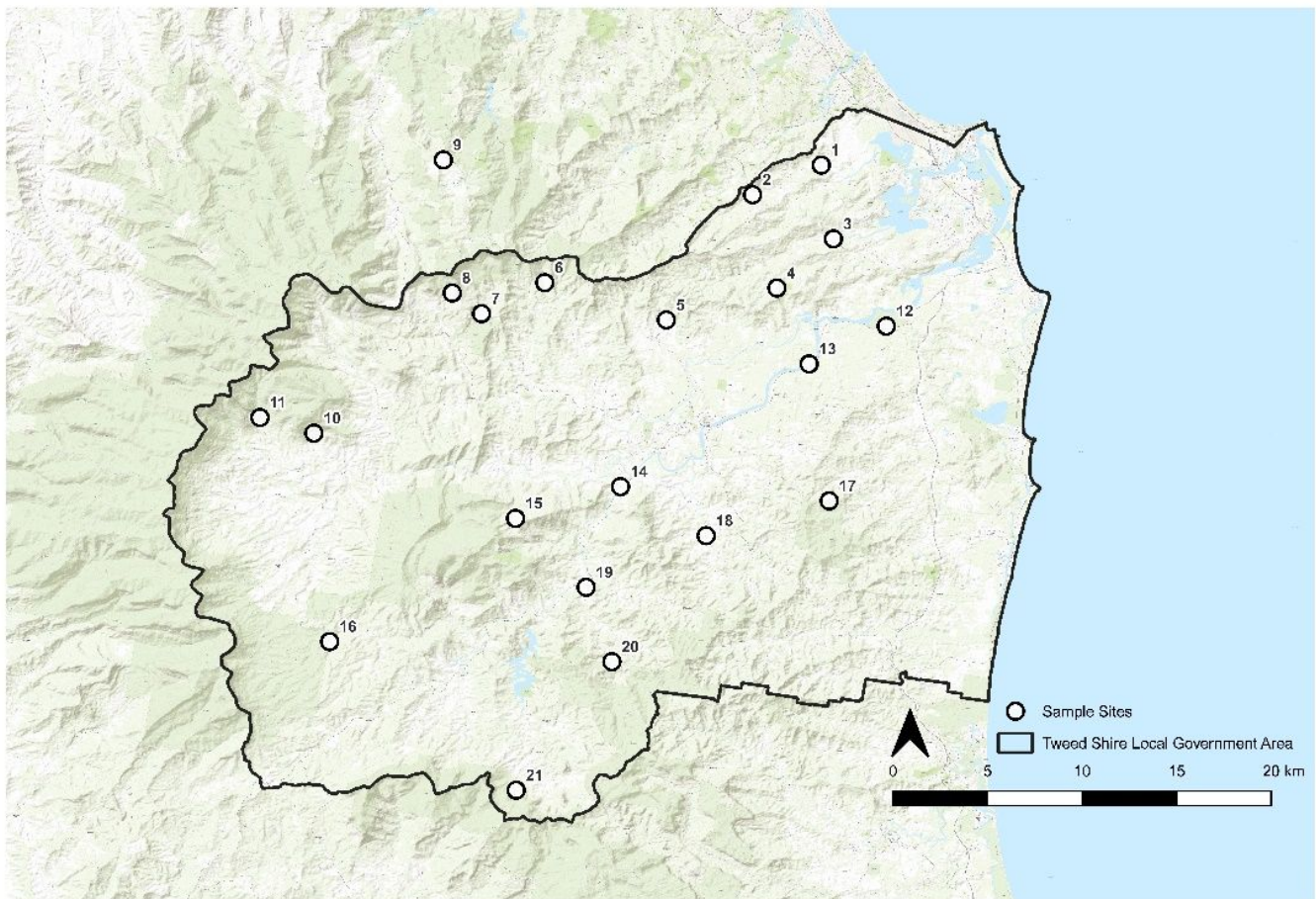
54. Rossetto M, Jones R, Hunter J (2004) Genetic effects of rainforest fragmentation in an early successional tree (*Elaeocarpus grandis*). *Heredity*, 93(6), 610–618. Retrieved from <http://ezproxy.scu.edu.au/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=mnh&AN=15367910&site=ehost-live>
55. Rousset F (2008) Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. *Mol Ecol Resources* 8:103–106
56. Salas-Leiva DE, Mayor-Durán VM, Toro-Perea N (2009) Genetic diversity of black mangrove (*Avicennia germinans*) in natural and reforested areas of Salamanca Island Parkway, Colombian Caribbean. *Hydrobiologia* 620(1):17–24. 10.1007/s10750-008-9611-x
57. SERA SRG (2021) Edition 2.2.. National Standards for the Practice of Ecological Restoration in Australia. In: Society for Ecological Restoration Australasia
58. Shirk RY, Hamrick JL, Zhang C, Qiang S (2014) Patterns of genetic diversity reveal multiple introductions and recurrent founder effects during range expansion in invasive populations of *Geranium carolinianum* (Geraniaceae). *Heredity* 112(5):497–507. 10.1038/hdy.2013.132
59. Smouse PE, Peakall ROD, Gonzales EVA (2008) A heterogeneity test for fine-scale genetic structure. *Mol Ecol* 17(14):3389–3400. <https://doi.org/10.1111/j.1365-294X.2008.03839.x>
60. Standards Reference Group, S. f. E. R. A (2017) National Standards for the Practice of Ecological Restoration in Australia. Second Edition. In
61. Storfer A, Murphy MA, Evans JS, Goldberg CS, Robinson S, Spear SF, Waits LP (2007) Putting the "landscape" in landscape genetics. *Heredity* 98(3):128–142. 10.1038/sj.hdy.6800917
62. Thackway R, Cresswell I (1995) *An interim biogeographic regionalisation for Australia: a framework for setting priorities in the National Reserves System Cooperative Program / edited by R Thackway and I D Cresswell*. (0642213712). Canberra: Australian Nature Conservation Agency, Reserve Systems Unit
63. The Royal Botanic Garden, S (2021) Restore and Renew webtool. Retrieved from <https://www.restore-and-renew.org.au/webtool/>
64. Thomas E, Jalonen R, Loo J, Boshier D, Gallo L, Cavers S, Bozzano M (2014) Genetic considerations in ecosystem restoration using native tree species. *For Ecol Manag* 333:66–75. <https://doi.org/10.1016/j.foreco.2014.07.015>
65. Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) micro-checker: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes* 4(3):535–538. <https://doi.org/10.1111/j.1471-8286.2004.00684.x>
66. Wahlund S, ZUSAMMENSETZUNG VON POPULATIONEN UND KORRELATIONSERSCHEINUNGEN VOM STANDPUNKT DER VERERBUNGSLEHRE AUS BETRACHTET (1928) *Hereditas* 11(1):65–106. <https://doi.org/10.1111/j.1601-5223.1928.tb02483.x>
67. Williams CG (2008) Selfed embryo death in *Pinus taeda*: a phenotypic profile. *New Phytol* 178(1):210–222. <https://doi.org/10.1111/j.1469-8137.2007.02359.x>
68. Williams G, Adam P (2010) *The Flowering of Australia's Rainforests: A Plant and Pollination Miscellany*. CSIRO Publishing, Collingwood, VIC
69. Xin Z, Chen J (2012) A high throughput DNA extraction method with high yield and quality. *Plant Methods* 8(1):26. 10.1186/1746-4811-8-26
70. Zucchi MI, Sujii PS, Mori GM, Viana JPG, Grando C, Silvestre E d., Brancalion A (2018) P. H. S. Genetic diversity of reintroduced tree populations in restoration plantations of the Brazilian Atlantic Forest. *Restoration Ecology*, 26(4), 694–701. doi:<https://doi.org/10.1111/rec.12620>

## Figures



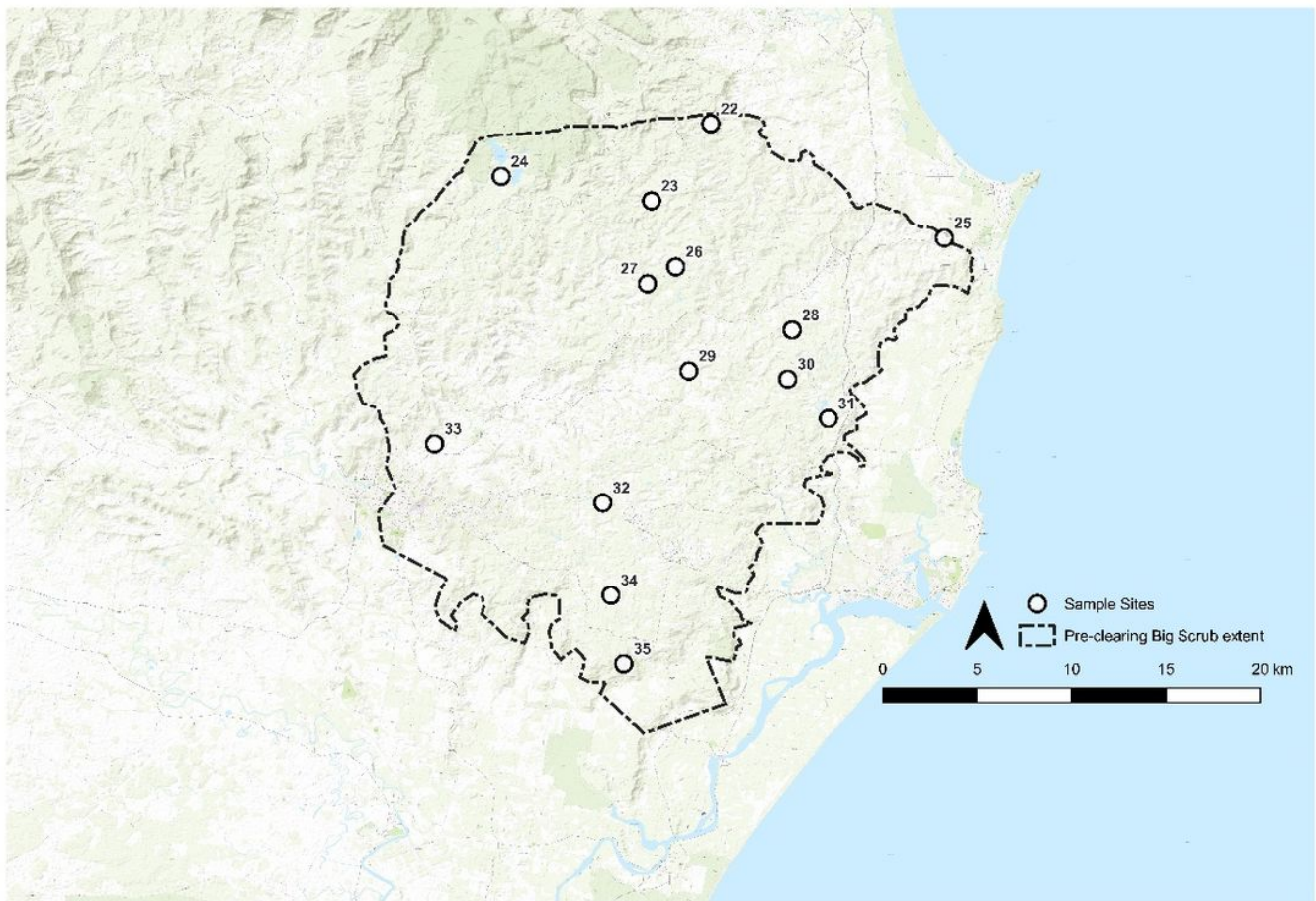
**Figure 1**

Study area indicating the two regions of the Big Scrub and Tweed Caldera situated within the IBRA v7 South-East Queensland Bioregion and Sunshine Coast-Gold Coast Lowlands, Burringbar-Conondale Ranges and Scenic Rim Sub-regions



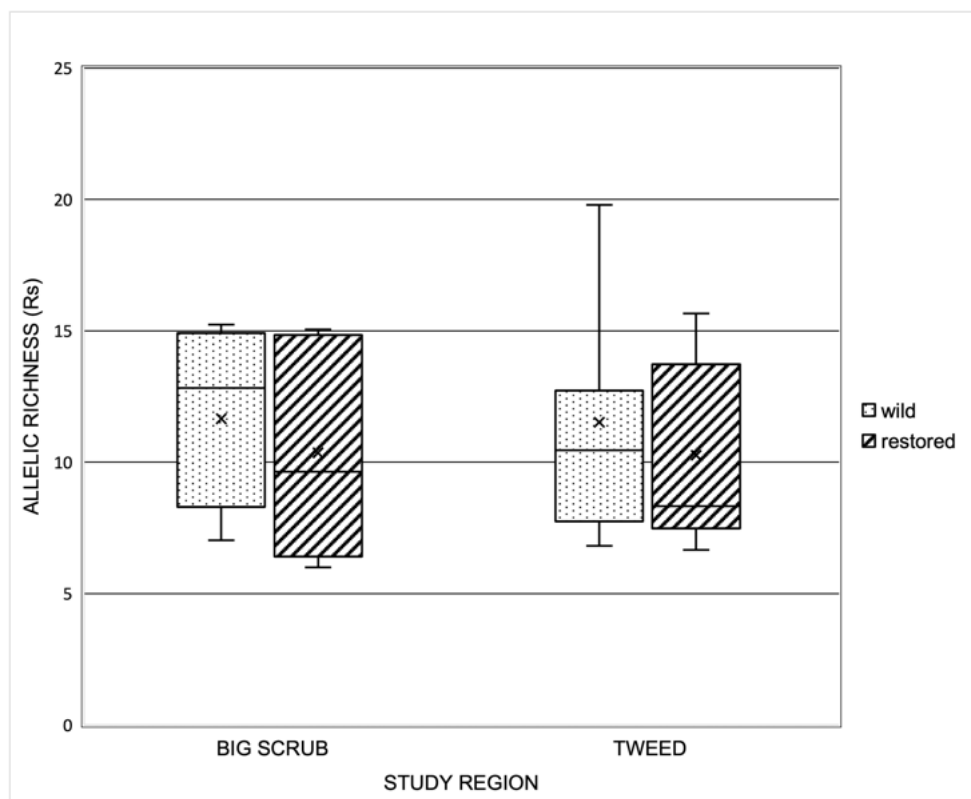
**Figure 2**

Restored and wild population sample sites from the Tweed



**Figure 3**

Restored and wild population sample sites from the Big Scrub



**Figure 4**

Comparison of Allelic Richness ( $R_s$ ) across seven SSR loci between wild and restored populations of *A. trifoliatum* in the Big Scrub and Tweed regions

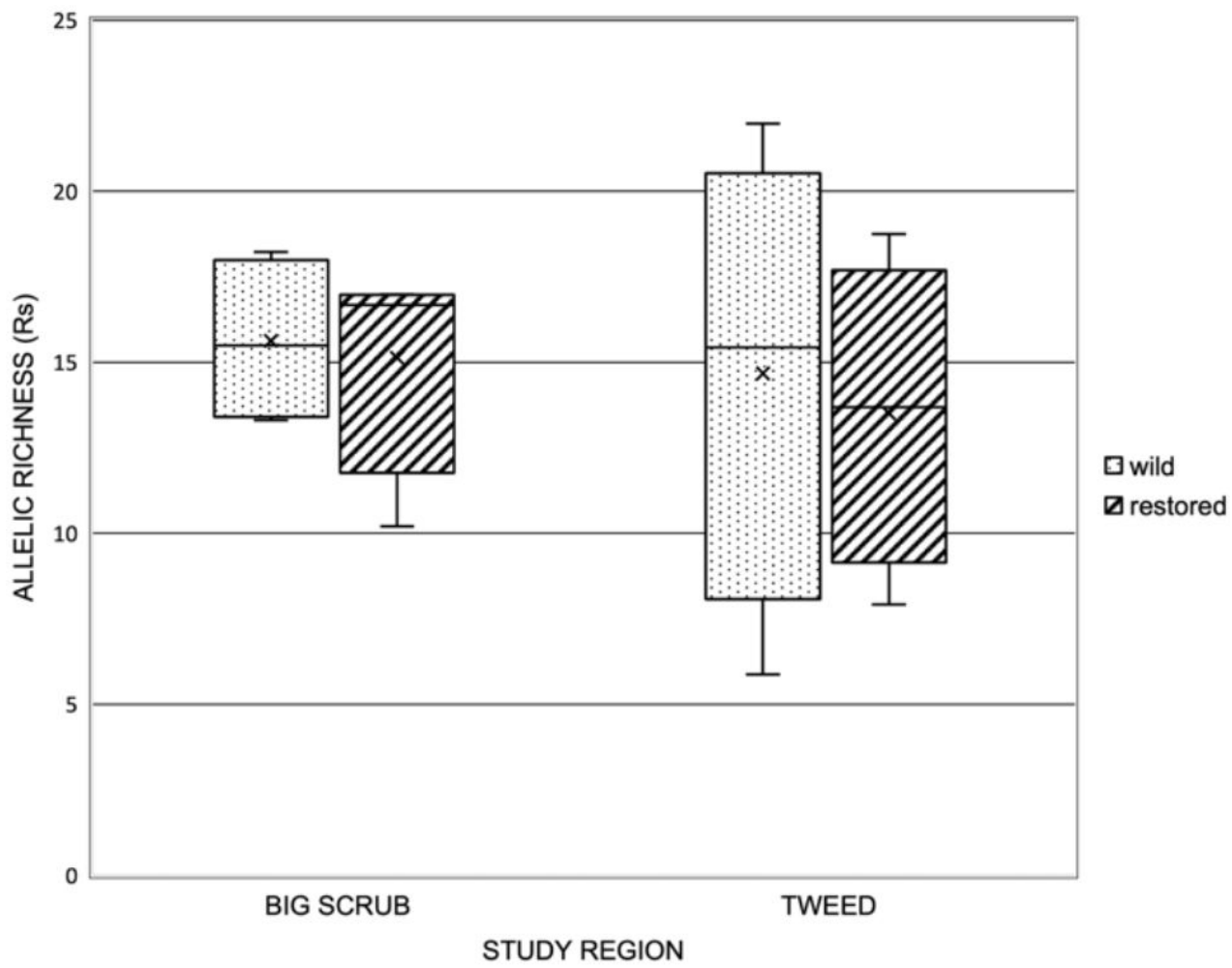
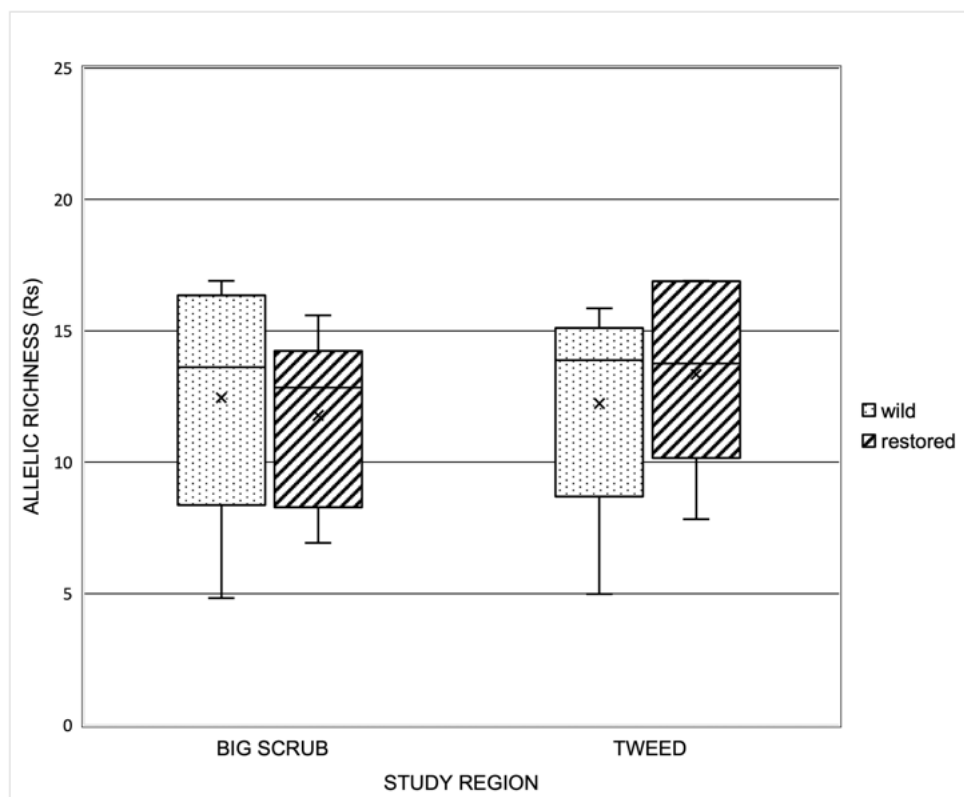


Figure 5

Comparison of Allelic Richness (Rs) across four SSR loci between wild and restored populations of *D. australis* in the Big Scrub and Tweed regions



**Figure 6**

Comparison of Allelic Richness (Rs) across four SSR loci between wild and restored populations of *D. australis* in the Big Scrub and Tweed regions

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ConGenSupplInfoSLCooper19Jan23.docx](#)