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Evaluating the rarity and genetic structure of populations of the endangered Australian endemic plant *Trioncinia retroflexa* (Asteraceae): potential consequences for management and conservation.

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Abstract

Trioncinia retroflexa is an endangered endemic plant species found in the Bluegrass grasslands of central Queensland Australia. The recent degradation and fragmentation of this ecosystem is commonly assumed to have driven this species to become rare and persist only in isolated populations. Despite a lack of empirical evidence supporting this assumption (and equivocal anecdotal evidence), conservation and restoration plans for this species are generally based on it. Using microsatellites, the genetic diversity, structure and differentiation of all known populations of these species were examined to determine if there is evidence for recent isolation of remaining populations. Populations had high genetic differentiation, with little heterozygosity, minimal gene flow and few migrants, reflecting long-term population isolation well beyond the scope of modern fragmentation. High genetic differentiation also suggests that all known populations of this species maintain a similar proportion of the species' total genetic diversity, despite varying extensively in the number of individuals they support. As such, the loss of any population may drastically reduce the species' adaptive potential in the future. In the context of long-term population isolation, seed and germination traits related to species dispersal and establishment were examined among these long isolated populations. Though some significant differences in traits among populations were found, there was not strong evidence of trait divergence. Given the findings of this study, this species has likely persisted in isolated populations for at least the last 158 years (time since identification) and may have been rare prior to this time. A large restoration experiment completed in association with this project failed to establish a new population over a two-year period. The difficulty of establishing new populations of *T. retroflexa* highlights the lack of information we have about this species' environmental requirements and the importance of maintaining the remaining natural populations of this species.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

No publications

Publications included in this thesis

No publications included

Contributions by others to the thesis

Dr. Mayfield and I worked together in the development of the ideas, concepts and specific projects completed for this thesis. Dr. Daniel Ortiz-Barrientos worked closely with me on the genetics component of this thesis, teaching me the skills required for the genetic data set and was instrumental in the analysis and interpretation of data trends. Dr. Roderick Fensham contributed to guiding field surveys and sample collection. I am solely responsible for all data collection and data analysis and I wrote this thesis with guidance and editorial assistance from Dr. Mayfield.

Statement of parts of the thesis submitted to qualify for the award of another degree

The populations utilized for this thesis were identified and surveyed as part of a Master of Environmental Management. All research conducted from these population is unique to the current thesis.

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Keywords

Conservation genetics, germination, Australian bluegrass grasslands, rare species conservation, *Trioncinia retroflexa*

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FoR code: 0604, Genetics, 60%
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List of Abbreviations

***T. retroflexa* –** *Trioncinia retroflexa*

SC – Southern Clermont population

RC – Retro Creek population

C – Cardbeign population

MD – Meteor Downs population

MDR – Meteor Downs Road population

SMD – South Meteor Downs population

DNA - Deoxyribonucleic acid

ICBR – Interdisciplinary Centre for Biotechnology Research

PCR – Polymerase Chain Reaction

IDT – Integrated DNA Technologies

Exo-AP – Exonuclease I/Antarctic Phosphatase

dNTPs - Deoxynucleotide triphosphates

AMOVA – Analysis of molecular variance

F_{st} – F-statistic

PCoA – Principal coordinates analysis

IBD – Isolation-by-distance

PhiPT - Partitioning of among-group genetic variation

H_o – Observed heterozygosity

GA - Gibberillic acid

ANOVA – Analysis of variance

Tukey's HSD – Tukey's honestly significant differences

IAM – Infinite alleles model

TPM – Two phase model

SMM – stepwise mutation model

INTRODUCTION:

The protection and restoration of endangered and rare species is one of the biggest conservation challenges conservationists face (Nicoletti et al. 2012; Sork and Smouse 2006; Walters 2007; Young et al 1996). This problem is exacerbated by a lack of information about the ecology of most rare and endangered species under previous or current conditions. This issue is even more severe for plants than vertebrates as they receive less research attention and conservation money (Escaravage, et al. 2011; Schemke, et al. 1994; Shapcott et al. 2009; Zhao et al. 2006).

Most conservation and restoration plans for rare and endangered plant species are based on assumptions about how human activities have impacted these species over time. A common assumption underlying this type of conservation plan is that threats such as habitat loss, habitat fragmentation and habitat degradation have caused the rare status of the species (Aguilar et al. 2008; Nicoletti, et al. 2012). Further, it is often assumed that anthropogenic environmental changes have led to retractions of rare species' distributions, from assumed large- to highly restricted and often fragmented ranges since human activities started in such species' ranges. Though this is undoubtedly sometimes the case (Bartgis, 1997; Cozzolino, et al. 2003; Jacquemyn, et al. 2007; Shapcott, et al. 2009; Young et al. 1999), there are known examples of plant species with naturally high fragmented, restricted and isolated distributions (Gilani, et al. 2009; Shepherd and Perrie 2011). Because so few plant species have been well studied, it is not surprising that it is often unclear whether plant species have become rare due to human activities, or are simply persisting in their natural condition under global change. To ensure that conservation efforts are well designed for the specific species in question, it is important to assess whether a species' rarity is caused by human activities in the recent past or not.

Trioncinia retroflexa (Asteraceae), or the Belyando Cobblers Peg*,* is a rare endemic herb historically restricted to the bluegrass grasslands of central Queensland (Fensham 1999). First documented in 1856, by Ferdinand von Mueller, the species common name is derived from the species' rigid elongated seed shape and barbed structures which are common to Cobblers Peg species. Since identification, *T. retroflexa* is generally assumed to have undergone substantial population declines in the past 150 years (Fensham et al. 2002; Fensham and Fairfax 2005) and was listed as extinct in the 1930s (QPWS 2000). Rediscovered in 1996, *T. retroflexa* is currently list as rare; with as few as 10,000 individuals known to persist today (Fensham and Fairfax 2005).

This species has many characteristics in common with rare plants of conservation interest around the world, making it ideal for studying from a rarity perspective. *Trioncinia retroflexa* was initially discovered during a two day exploration into central Queensland over 150 years ago. Though no complete survey of *T. retroflexa* exists as part of this first description, the fact that it was collected and commented on at all during this short survey, especially given its unremarkable form and habitat, has been considered, by some, to be evidence that it may have been common in 1856. What makes this leap of logic seem doubtful is that *T. retroflexa* has been difficult to find ever since (though again no proper surveys were available until 1996). The difficulty in finding this species after its initial description has been cited as further evidence that the land use changes associated with European settlers may have led to its decline, rather than as a reason to question whether it was ever widespread at all. The decline of many other plant species from this region of Queensland have been much more carefully assessed following the introduction of cattle, sheep, and agriculture in the region, which started around the time of the species' original description (Fensham et al. 2002). Further evidence that *T. retroflexa* is negatively impacted by anthropogenic land use changes over the past 150 years is that the known extant populations of this species are predominantly found in road edges, which are largely protected from grazing and other agricultural production activities common in the Bluegrass grasslands of central Queensland.

While previous management has been structured around the assumption of human-driven decline and population fragmentation, ambiguities surrounding *T. retroflexa*'s ecology and historical distribution may contribute to the difficulties faced in the conservation of this species (Fensham and Fairfax 2005). In order to develop the best conservation plans possible for this species, it is important to determine whether its current fragmented distribution is a recent change to this species population dynamics or not. Assessment of population genetic structure and genetic differentiation among extant populations has been used to identify losses of genetic diversity and increased genetic differentiation among populations resulting from previous anthropogenic changes to population connectivity (Young et al. 1996, Young et al. 1999). Multiple studies have also used genetic tools to examine the current status of rare (Cozzolino, et al. 2003; Escaravage et al. 2011; Shepherd and Perrie 2011; Young et al. 1996), endangered (Rosas et al. 2011; Young et al. 1999) and common (Jacquemyn et al. 2007; Schmidt et al. 2009) plant species around the world. Based on such studies, a range of management strategies have be suggested

with some stressing the importance of genetically unique smaller populations (Jacquemyn et al. 2007), some highlighting the importance of the largest populations, (Cozzolino, et al. 2003; Young et al. 1999) and others disregarding population size in exchange for an increase in connectivity (Escaravage et al. 2011; Rosas et al. 2011; Shepard and Perrie 2011) depending on study findings.

If populations of this species have been isolated for a substantial period of time, the phenotypic response to the observed genotypic differentiation would be of additional interest, as it could reflect important ecological differences among these populations. As phenotypic and genetic diversity can similarly influence population persistence and extirpation (Oka 1983; Luquet et al. 2011), identifying those traits undergoing differentiation is pivotal to the conservation of current populations and future species' management. Multiple studies have considered the relevance of traits to populations in isolation (Johansson et al 2011; Kolb and Diekmann 2005; Saar et al. 2012; Sutton and Morgan 2009), but with varying results. Due to the presence of clear advantageous trade-offs across the continuum of any one trait (Jakobsson and Eriksson 2000), the relationship between individual life-history traits and isolated population persistence cannot be generalized (Kolb and Diekmann 2005). As such, focal traits must be identified based on a case by case basis.

For the case of *T. retroflexa,* while many functional traits may have differentiated in its isolated populations*,* changes in traits relating to dispersal and establishment (germination) may be particularly important. From a conservation perspective, such traits can provide valuable insights into the species' regenerative potential (Kolb and Diekmann 2005; Lindborg 2007; Lindlborg et al. 2012; Sutton and Morgan 2009; Tremlová and Münzbergova 2007) and thus explain why, post-fragmentation, *T. retroflexa* has maintained its current landscape population structure (Lindborg 2007). From a managerial perspective, such traits may direct how to best to develop new populations so as to encourage self-perpetuating population growth and connectivity within the modern landscape.

In order to improve our understanding of the population structure of *T. retroflexa* and to aid in the development of more appropriate conservation strategies for this species, I asked the following questions with this study:

1. Is there evidence to support the hypothesis that *Trioncinia retroflexa* was a once widespread species with a largely continuous distribution across the region where it now persists in small isolated populations?
2. Is there evidence of genetic differentiation of known populations of *T. retroflexa*?
3. Is there evidence of significant differences in key life history traits among the remaining known populations of *T. retroflexa*?

METHODS:

Trioncinia retroflexa

This study focused on *Trioncinia retroflexa* (Asteraceae), a rare and endangered short-lived perennial forb found in the Bluegrass grasslands of central Queensland (Figure 1). This species is characterized by a ground-lying basal rosette and a fleshy rootstock, which may allow *T. retroflexa* to remain dormant below ground during the periods of dry weather. During *T. retroflexa*'s growing season (October- March) plants develop a cauline-leaved reproductive stem that grows approximately 80cm tall. Reproductive stems bear terminal yellow inflorescences, which bloom December – February, and produce 17 – 28 barbed achenes (Fensham et al. 2002; Fensham and Fairfax 2005) (Figure 2). Based on these barbed appendages seeds are most likely dispersed through animal fur attachment; with kangaroo and cattle respectively being the most likely dispersal vectors pre and post European settlement. Individuals are thought to live for approximately five years and can produce up to 1,200 seeds a year (Fensham and Fairfax 2005). Seed are estimated to survive in the soil for up to eighteen months, and have improved germination rates if exposed to a 2-3 month after-ripening period (Haller unpublished data). All known populations of *T. retroflexa* are on tertiary basalt and Permian shale vertosol soils (Fensham 1999). Regional annual rainfall averages for the central highlands, where all known *T. retroflexa* populations persist, is 534.1 mm; approximately 75% of which falls from October to March. Mean annual maximum and minimum temperatures are 29.8°C and 16.4°C, respectively, with respective peaks and valleys in January and July (BOM 2009).

Historically, the bluegrass grasslands of the Queensland central highlands occupied approximately 1.1 million hectares and dominated by Queensland bluegrass (*Dichanthium sericeum*). Since European settlement of the region in the 1850's Bluegrass grasslands have been extensively impacted by habitat conversion to pastures, grazing, mineral mining

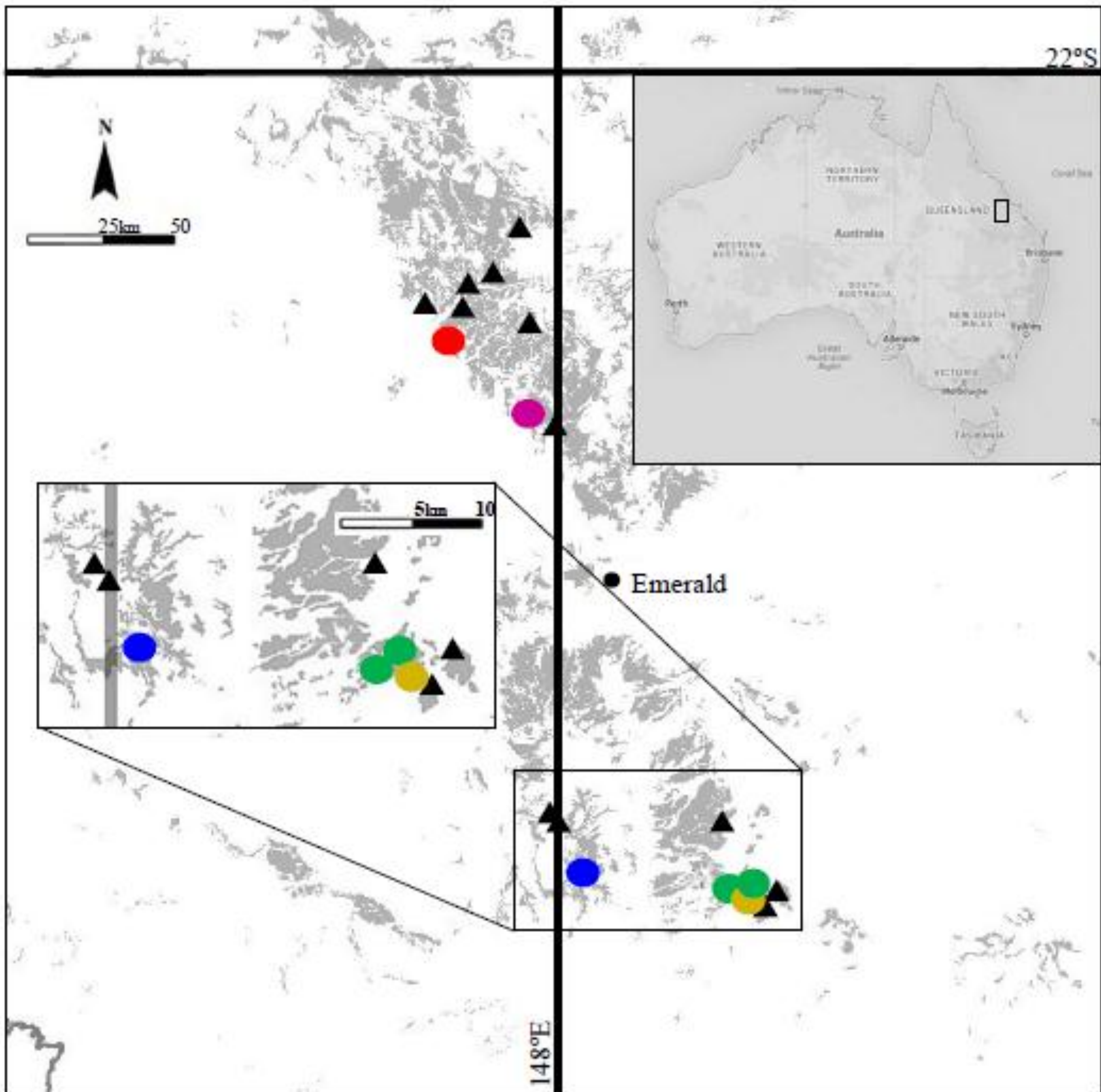

Figure 1 Map of study area. Map of sampled *Trioncinia retroflexa* populations in the central highlands of Queensland, Australia. Colors refer sampled populations with unique population structures, as determined in this study. Red = Southern Clermont, purple = Retro Creek, green = Meteor Downs and Meteor Downs Rd, yellow = South Meteor Downs and blue = Cardbeign. Grey patches on the map indicate areas of the Bluegrass (*Dichanthium*) grassland habitats. Populations of *Trioncinia retroflexa* with different colored circles were identified as distinct genetic populations in this study. Black triangles indicate populations previously identified by Dr. Rod Fensham, that could not be located for this study.

and crop cultivation (Fensham 1997; QPWS 2000; Fensham et al. 2002). Today only 36% (TSSC 2007) of these grasslands persist in the shires of: Emerald, Belyando, Jericho, Peak Downs, Bauhinia, Broadsound and Duaringa (QPWS 2000); with additional dominant grasses including: white speargrass (*Aristida leptopoda*), native millet (*Panicum decompositum*), yabila grass (*Panicum queenslandicum*) and King bluegrass (*Dichanthium queenslandicum*) (Fensham 1999a; QPWS 2000; Fensham et al.2002).

Figure 2 Images of *Trioncinia retroflexa* seed. Images depict barbed appendages used to disperse via attachment to animal fur.

Study sites

From November-February 2008-2009, the 16 *T. retroflexa* populations identified by Dr. R. Fensham (personal communications 2008) were visited but living individuals were only found in five of these sites. Thus, this study was restricted to these five populations, plus an additional population identified in 2011 (Figure 1, Table 1). Across these six populations, leaf material was collected from between 10 and 36 individuals, depending on population size. 165 individuals were sampled in total. Leaves from the five original populations were collected from September 2008 to February 2009 and from the new population in November 2011. Leaf samples were frozen immediately after collection and maintained at -18°C for use in genetic analyses (described below). In addition to leaf samples, approximately, 1080 mature seeds from 360 individuals in the three largest populations, SC, MD and C were collected from November 2012 to February 2013. Seeds were collected from 13 - 49 individuals for the remaining populations, due to a limited number of reproductive individuals in them, from October 2010 to February 2012. Seeds were stored under dry conditions at room temperature until germination experiments commenced (details below).

Table 1 Sampled *T.retroflexa* populations. A) Details of the six populations used in this study. Abbr. refers to the abbreviation used throughout the thesis to indicate each population. GPS units are in an Universal Transverse Mercator (UTM) coordinate system. Population coordinates indicate the epicenter of the total extent of individuals in each population. Area refers to the total area within which individuals were located as defined by a convex envelope. N is the number of mature individuals in each population. Land use refers to the most consistent land use of the site over at least the past decade. B) Relative distances in kilometers (Km) between population pairs.

A

Name	Abbr.	GPS	Area (ha)	N	Land Use
South Clermont	SC	S22° 44’ 55.16”, E147° 37’ 58.59”	8.05	~892	Cattle stock route
Retro Creek	RC	S22° 59’ 13.02”, E147° 50’ 40.19”	0.0003	13	Cattle stock route
Cardbeign	C	S24° 16’ 25.96”, E148° 7’ 3.13”	7.05	>1000	Cattle grazing/Hay production
Meteor Downs	MD	S24° 16’ 52.61”, E148° 19’ 18.95”	1	~438	Cattle stock route
Meteor Downs Road	MDR	S24° 17’ 14.71”, E148° 19’ 16.77”	0.001	49	Cattle stock route
South Meteor Downs	SMD	S24° 17’ 40.82”, E148° 20’ 27.16”	0.001	45	Cattle stock route

B

	SC	RC	MD	MDR	C	SMD
South Clermont	0.000					
Retro Creek	34.234	0.000				
Meteor Downs	184.330	151.918	0.000			
Meteor Downs Road	184.937	152.544	0.685	0.000		
Cardbeign	176.648	145.778	20.734	20.710	0.000	
South Meteor Downs	186.446	153.952	2.430	2.140	22.755	0.000

Population genetics

In the lab, approximately 100µl of genomic leaf tissue DNA was extracted from each frozen leaf sample using the QIAGEN DNeasy Plant mini kit (QIAGEN 2006). The NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc. 2008) was used to estimate A_{260}/A_{280} ratios and determine DNA concentrations. For DNA samples outside acceptable A_{260}/A_{280} ratios (1.7< 2.0 <), the Promega Wizard SV Gel and PCR Clean-Up System (Promega Corporation 2009b) was used for purification.

DNA products from forty-five individuals were recombined into two sets of twelve samples and shipped to the Interdisciplinary Centre for Biotechnology Research (ICBR), at the University of Florida, for microsatellite development. Pyrosequencing was conducted using the Roche Titanium 454 instrument. Approximately 30,000 reads were produced and mined for di-, tri- and tetranucleotide repeat DNA with appropriate flanking sequences for primer development. Identified sequences were manually scanned, selected and reviewed to prevent multiple hits on single loci. Tri- and tetranucleotide repeat DNA were preferentially selected and submitted to Batch $Primer_3$ for development; 46 of which were selected for primer synthesis (completed commercially by Eurofin Oligo). Diluted primers were Polymerase Chain Reaction (PCR) optimized and the most promising primers were then tested for polymorphism.

Fourteen primers were selected and synthesized by Integrated DNA Technologies (IDT). Primers were diluted in TE (10mM Tris Cl, 1 mM EDTA, pH 8.0) to obtain 50 µL 10x primer mix stock solutions with 2µM of each primer. PCR amplifications were performed for each of the 165 sampled plants using a thermal cycler in a reaction mixture (50 µL) containing 1 µg of template DNA, 25µL of 2x QIAGEN Multiplex PCR master mix with 3 mM $MgCl_2$ and 5 µL 10x primer mix (QIAGEN 2008). PCR conditions include an initial 15 min activation stage at 95°C, 24-40 cycles of 30 s at 94°C, 90 s at a yet defined (57-63°C) annealing temperature and 60 s at 72°C and followed by 30 min at 60°C.

PCR products were visualized in a 2% agrose gel to ensure amplification success. Successfully amplified PCR products were purified prior to sequencing using Exonuclease I/Antarctic Phosphatase (Exo-AP). Exonuclease I was used to digest single stranded DNA without contamination from any leftover primer. Anatactic Phosphatase was used to

render deoxynucleotide triphosphates (dNTPs) from the PCR and Exonuclease I primer digestion inert through the removal of 3'phosphate groups from single nucleotides.

PCR precipitation was carried out using a thermal cycler with a reaction mixture (22.5 µL) containing 0.5 µg of Exonuclease I, 2.0µL of Antarctic Phosphatase and 20 µL of PCR product. PCR precipitation conditions included a 1 hour digestion stage at 37°C, followed by a 20 minute deactivation stage at 80°C to prevent digestion of sequencing reactions. Microsatellite analysis was conducted with an Applied Biosystems 3730xl DNA analyzer (ABI3730XL).

Analysis:
A Bayesian cluster analysis was performed, using the program STRUCTURE v 2.3.4 (Pritchard et al. 2000), to estimate the proportion of each sampled genome is derived from an ancestral genetic group and to define an optimal number of genetic clusters. An admixture model, with correlated allele frequencies among populations, was chosen with 10,000 burn-in periods, 500,000 Markov chain Monte Carlo repetitions, 10 iterations and K (the assumed number of populations) ranging from 1-10. The optimal number of K clusters was determined following Prohl et al. (2010), using the mean values for the log likelihood of K (*L(K))*(Rosenberg et al. 2001) and the ad hoc quantity Δ*K* (Evanno et al. 2005), which is calculated utilizing the rate of change in the log probability of the data between successive K values. Optimal number of K clusters were further verified using STRUCTURE HARVESTER (Earl and vonHoldt 2012).

GenAlEx v 6.4 was used to assess the partitioning of the genetic variance within and across populations and among regions. Analysis of molecular variance (AMOVA, Excoffier et al., 1992) was calculated to hierarchically partition genetic variation among regions and populations. AMOVA was further applied to estimate F-statistic (F_{st}). Based on pairwise population F_{st} values, significant genetic differentiation between populations was identified for populations where F_{st} was significantly greater than zero. Individuals were assigned to their most likely source population to estimate gene flow and migration between populations. Assignment tests mirrored the allele frequency method of Paetkau et al. (1995). Population assignment involved the calculation of expected allele frequencies for each individual's genotype, across the six populations, and the assignment of individuals to the population with the highest frequency. To visualize and interpret the pairwise F_{st} matrix, a Principal coordinate analysis (PCoA) was used to identify major patterns within

the multivariate data set and plot them along axes of variation in descending order of relevance to the total genetic variation. As the absence of genetic differentiation among populations over large geographical scales is unlikely, isolation-by-distance (IBD) was tested by submitting a matrix of PhiPT statistics (a Euclidean metric of among-group genetic variation analogous to F_{st}) and a second matrix of geographical distances to a Mantel Test for Matrix Correspondence. Observed heterozygosity (H_o) (the proportion of the population samples that were heterozygous at the given loci) was also calculated for each population.

To detect a genetic bottleneck signature, a sign test and an allele frequency distribution test was run in BOTTLENECK 1.2.02 (Piry et al. 1999) for all microsatellite loci. The sign test compares the number of loci with excess heterozygosity to the number of expected number of such loci under random chance. Three mutational models were considered for this test: the infinite alleles model (IAM), the stepwise mutation model (SMM) and the two phase model (TPM)(Di Rienzo et al. 1994). The allele frequency distribution test examines and compares all allele frequencies within a population to the expected distribution at mutation-drift equilibrium.

Germination traits

To assess whether there were differences in germination traits among populations of *T. retroflexa*, the effects of water availability, temperature, light, smoke, heat shock and scarification on germination rates of seed from the three largest populations (SC, MD, C) were examined. Germination experiments ran from April, 2013 to June, 2014.

To test the effects of water availability on germination, 144 individuals were selected to source seed from; 48 individuals from each of the largest populations (SC, MD and C). All seed was collected under permit. All regulations and restrictions associated with the harvest of an endangered plant were adhered to. From each population's pool of selected individuals (48), eight individuals were randomly assigned to one of the six watering treatments. Four germination trays were assigned to each watering treatment; 24 trays in total. Within each germination tray, two of the six wells were randomly allocated to each population. Within each well, an individual from the corresponding population was selected and three arbitrarily selected seeds from that individual were placed in the well. This provided 24 seeds, from eight individuals from each population for each of the six treatments (Figure 3).

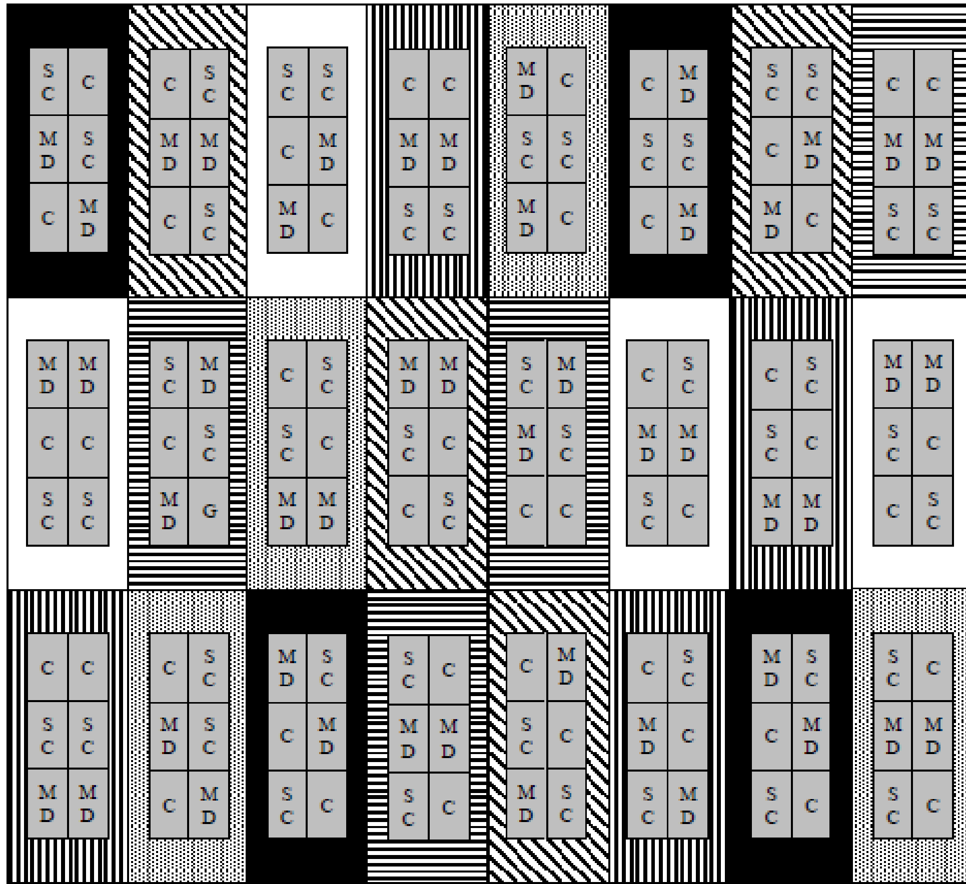

Figure 3 Water availability germination experimental design Larger, patterned rectangles indicate germination tray water treatments: white = 0.8ml, dots = 1.6ml, vertical lines = 2.4ml, horizontal lines = 3.2ml, diagonal lines = 4.0ml and black = 4.8ml. Smaller, lettered rectangles indicate the population seeds were sourced from for individual tray wells: SC = South Clermont, MD = Meteor Downs and C = Cardbiegn.

A circular insert (~962mm^2) of Kimpac germination paper was used as the germination substrate in clean six-well cell culture trays. Each treatment consisted of moistening the germination paper weekly with the following water volumes: 0.8, 1.6, 2.4, 3.2, 4.0, or 4.8ml. The number of seeds that germinated was assessed daily, with germination defined as the presence of the radical outside of the seed coat. Once a seed was germinated it was removed from the tray. To avoid any position bias within growth chambers, plates were randomly repositioned within growth chambers daily.

The importance of temperature, light and seed coat scarification on germination rates was assessed in a similar fashion to water but in a separate trial run from May to June of 2013. Based on the results of the watering experiment, a watering treatment of 3.2ml (the water amount that yielded the highest germination rate) was used in in the larger trait trial. In this experiment, 72 individuals were randomly selected from each population, 36 of which were allocated to one of two temperature treatments (low and high, details below). Individuals selected for each temperature treatment, were further divided among three light treatments

(dark, shade and full light), with twelve individuals from each population allocated to each light treatment. Finally, within each light treatment, two of the twelve individuals, per population, were allocated to one of six seed modification treatments: control, GA, excised embryo, seed coat scarification, heat shock and smoke. Three seeds were taken arbitrarily from each individual for their allocated treatment. As this experiment employed a nested design (Figure 4), a total of 108, 36 and 6 seeds, from each population, were respectively allocated to each treatment level (temperature, light, seed modification) in descending order.

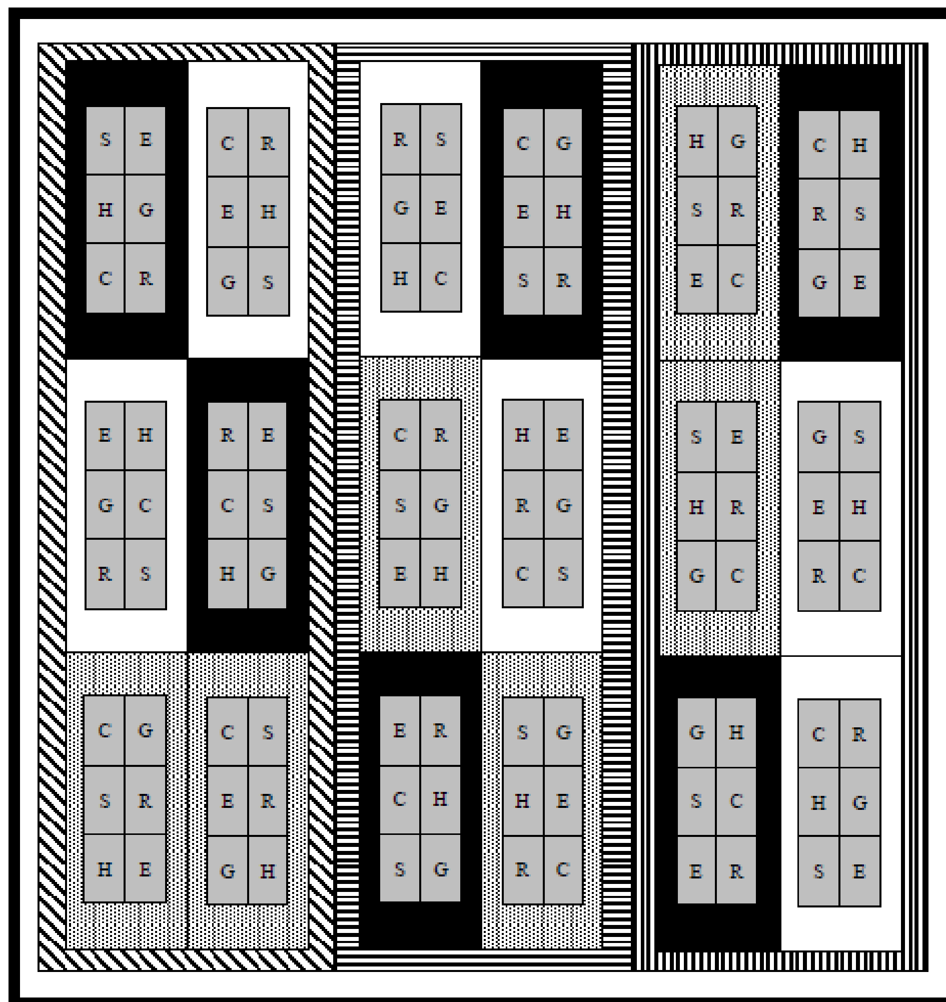

Figure 4 Germination experimental design. Nested block design. Thick black outer line represents temperature treatment. The three large, vertical rectangles indicate germination trays allocated to each population (SC = diagonal lines, C = horizontal lines and MD = vertical lines), light treatments are indicated by the smaller black (dark), patterned (shade) and white (full light) rectangles. Smallest, lettered rectangles indicate seed modification treatments: S = Smoke, E = excised, H = heat shock, G = gibberillic acid, R = seed coat scarification and C = control.

To assess differences in germination rates under different light intensities, the maximum light setting in the growth chamber was used. Light levels were then reduced with shade cloth and aluminum foil. Light treatments were: 100% light (225 μmoles/m^2/s), 70% (157 μmoles/m^2/s) and dark, 0% light intensity. There were two temperature treatments were:

15ºC and 25ºC. The six pre-sowing seed treatments: smoke (Regen 2000 Smokemaster), heat shock, gibberillic acid (GA), scarification and embryo excision. Embryos were excised from their seed coats using a dissecting microscope and scalpel. Longitudinal cuts were performed to expose the embryo which was then gently clasped and removed with forceps. GA treated seeds were soaked for 24 hours in a 200mg/L solution of GA and permitted to air dry overnight prior to sowing. GA is a naturally produced plant hormone that stimulates germination and has been shown, upon application, to overcome dormancy mechanisms (Bell et al. 1995). Smoke was simulated using the liquid smoke product Regen 2000 Smokemaster. Seeds were soaked in a 5% smoke water solution for 24hrs, drained and air dried, as per manufacturer instructions, prior to placement in tray wells. The heat shock treatment was designed to simulate fire and involved placing seeds in a pre-heated oven at 80°C, for 0.5hrs, on open glass Petri dishes (Cromer 2007; Rawson et al. 2012). Post treatment, petri dishes were immediately sealed and stored under dry conditions for 24hrs until sowing. GA treated seeds were soaked for 24 hours in a 200mg/L solution of GA and permitted to air dry overnight prior to sowing. Mechanical scarification was achieved by placing seeds in a cylinder lined with coarse sandpaper, sealing both ends and rolling the cylinder for 10min. Post-scarification, seeds were soaked in a 1% NaCLO solution for 10min to sterilize them before rinsing them with deionized water. Embryo excision was done by removing seed coats from seeds, using a dissecting microscope and scalpel. Longitudinal cuts were performed to expose the embryo, which was then gently clasped and removed with forceps.

Once in the germination trait trial, seeds were checked daily and seeds that germinated were counted and removed. Plates were randomly repositioned within growth chambers daily to avoid any position bias within growth chambers. Germination trials were conducted in Conviron Adaptis- A1000 controlled environmental chambers.

Analysis

Across all germination trails, seed germinability (%) and mean germination time (t) were calculated. Mean germination time ($t = \sum n_i . t_i / \sum n_i$) was calculated as per Meiado et al. (2010), where t_i indicates the duration from experiment commencement to the i_{th} day and ni is the number of observed germinations in the time i (germinations associated with the i_{th} day). Statistically significant differences in germination parameters, across all treatments, were tested using one-way analysis of variance (ANOVA) and supported by Tukey's honestly significant difference (HSD) test. Assumptions of normality and

homogeneity of the variances were tested utilizing Shapiro-Wilk (Shapiro and Wilk 1965) and Brown–Forsythe tests (Brown and Forsythe 1974), respectively. All statistical test were run using R v 2.14.2.

Seed dispersal traits

As a plant's capacity to disperse is vital to species persistence, seed morphological traits were examined across *T. retroflexa* populations to see if there were significant phenotypic differences among populations. Specifically, I measured: seed mass, seed dimensions (length, width, and height), seed terminal velocity, initial drop velocity, a seed shape index and diaspore surface structure. For dispersal trait measurements, between 13 and 49 individuals, from the three smallest populations and from 50-75 individuals, for the three largest population, were sourced for seed. Seeds were collected from October 2010 to February 2012. Seeds from multiple years were used for these measurements to capture the natural variation in seed traits that likely reflect the different environments of these years.

To quantify seed mass, seed structure, terminal velocity and initial drop speed, 30 seeds from 10 arbitrarily select individuals from SC, C and MD were selected and 20 seeds from five individuals were selected from RC, MDR and SMD (due to low population sizes). Diaspores were oven-dried at 40°C for one week before being weighing and measuring.

As the diversification of agricultural processes in the region may have restricted native fauna population movements, it is possible that contemporary seed dispersal has been heavily dependent on introduced livestock. As remnant populations of *T.* retroflexa persist in road verges and stock routes that may experience infrequent livestock movements, terminal velocity was ascertained to estimate the capacity for seed dispersal in the absence of such faunal movements. Terminal velocity was calculated using the equation v = the square root of $((2*m*g)/(\rho*A*C))$, where; m = mass of the falling seed, g = the acceleration due to gravity (9.8m/sec), ρ = the density of the medium the seed is falling through, *A* = the projected area of the seed and c = the drag coefficient. ρ was calculated for each population based on its government-surveyed elevation and historical averages of local temperature and humidity. *A* was calculated using prior measures of seed height and width. The drag coefficient (c) was uniformly set at 0.045 which is indicative of the seeds elongated shape.

Since terminal velocity is only reached after a particular time of acceleration, the inherent error that seeds would unlikely reach terminal velocity (based on seed release height) needed to be considered. To consider this error, initial drop velocity was estimated. From each population, 20 random individuals were selected. From each individual, three arbitrary seeds were selected for trials. Initial drop velocity was estimated as unassisted seed acceleration for seeds dropped from a height of 3.15m. Every seed was tested three times; and averaged. Trials were conducted under still laboratory conditions to prevent the influence of air currents. Fall time was measured to the millisecond. In preliminary trials, ten arbitrarily selected seeds was measured 25 times each to quantify measuring error. Measuring error was determined to be negligible. .

Of the initial 30 seeds selected for seed measurements, ten seeds (one from each selected individual) were chosen to measure seed appendages; with the exception of smaller populations from which two seeds were selected per individual. Seed appendages were characterized by the coarseness of the diaspore surface structure (Römermann et al. 2005) and barb circumference.

Analysis

Size was quantified as the mean seed dry weight (g). Seed measurements were utilized to calculate a seed shape index, which indicates the degree of seed shape variation from a sphere. The seed shape index was calculated according to Bakker et al. (1996) using the variance of seed length (*l*), width (*w*) and height (*h*) after all measurements were transformed. The summed squares of the deviation was divided from the mean (x') by n = 3: $Vs = \sum(x - x')^2/n$, where Vs is variance of seed dimension, to calculated the variance of measurement values (Römermann et al. 2005). Variance value ranged from zero to 0.2, where 0 is spherical and 0.2 is flat.

Diaspore surface structure was calculated by dividing the diaspore contour from the convex envelope perimeter. Resulting ratio values ranged from 0 to 1, reflecting simplified (smooth) or highly complex (rough) seed surface structures, respectively; which suggest as to the potential for seeds to attach to animal fur upon contact. As diaspore surface structure is absent from many life history trait databases, classifications of seed dispersal

morphology and diaspore exposure within the infructescence (Will et al. 2007) were also used for comparative analyses.

RESULTS:

Population genetics

Population structure

Most population structure was embodied by dividing all populations into two unique genetic lineages (Figure 5a). However, further subdivisions, into three (Figure 5b) four (Figure 5c) and five (Figure 5d) sub-clusters, revealed the additional isolation of population SMD from the southern cluster and the separation of the northern populations (SC and RC), respectively. Populations MD and MDR remained undivided regardless of K value with high proportions of admixed individuals. The likelihood of increased population clustering occurred when K was increased from K=3 to K=4 and from K= 4 to K=5 (Figure 6a); each of these increases Pr(X|k) was non-significant (Figure 6b). Furthermore, when K was increased to 6 the assignment of individuals became visibly less clear (Figure 5e); a trend that is increasingly evident for higher values of K (data not shown). As such, an optimal K of 5 was selected as the most probable population structure. It should be noted, however, that there is evidence to suggest that K=4 also provides a possible population structure.

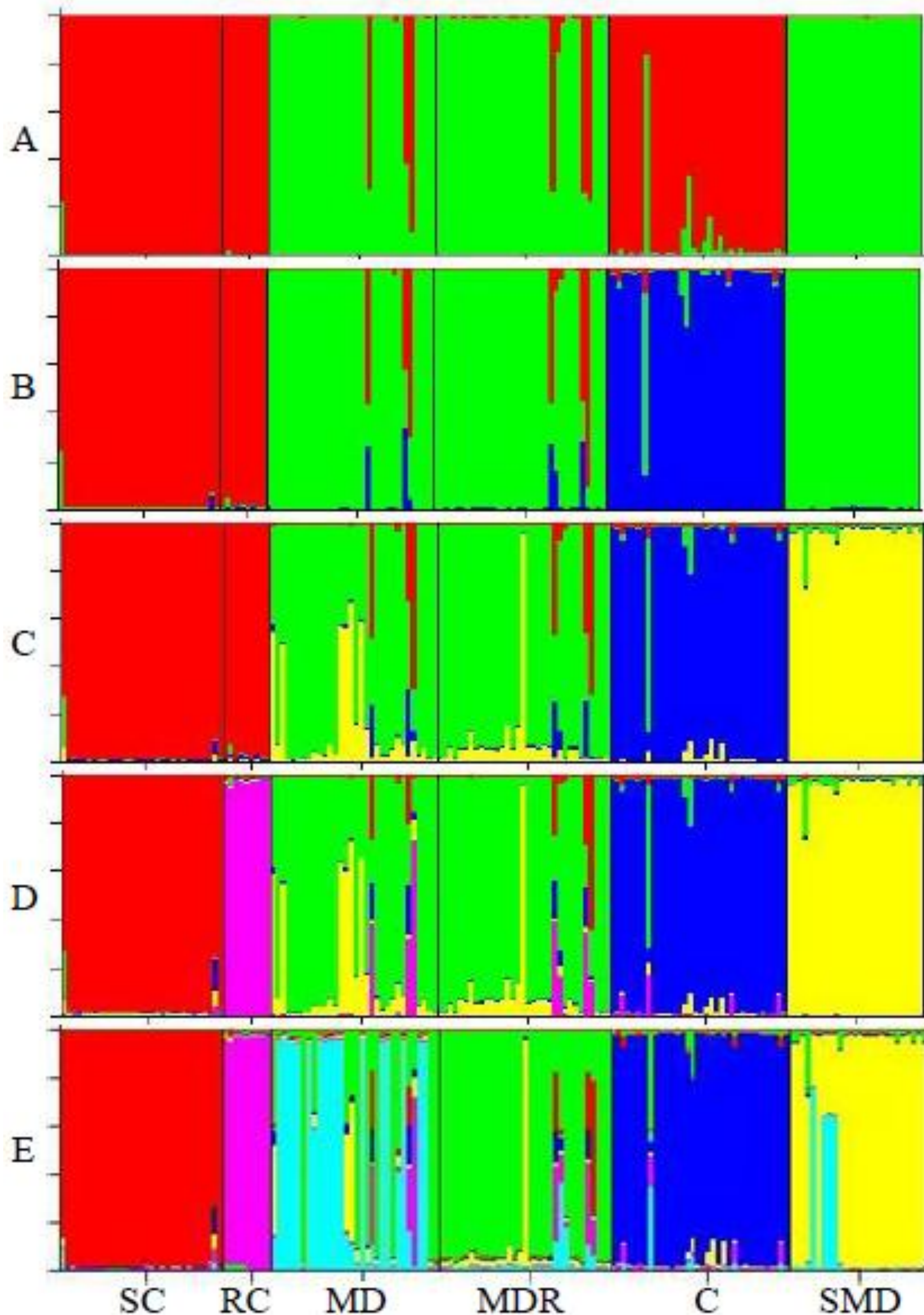

Figure 5 Population Structure of *Trioncinia retroflexa*. Results of the STRUCTURE Bayesian cluster analysis for identifying population genetic structure for: A) K=2, B) K=3, C) K=4, D) K=5 and E) K=6. K indicates the hypothesized number of genetic populations. Each vertical band indicates a sampled individual's genotype. Colors indicate genotypes from the same source genetic population. Sampled individuals are organized by their geographical population along the x-axis.

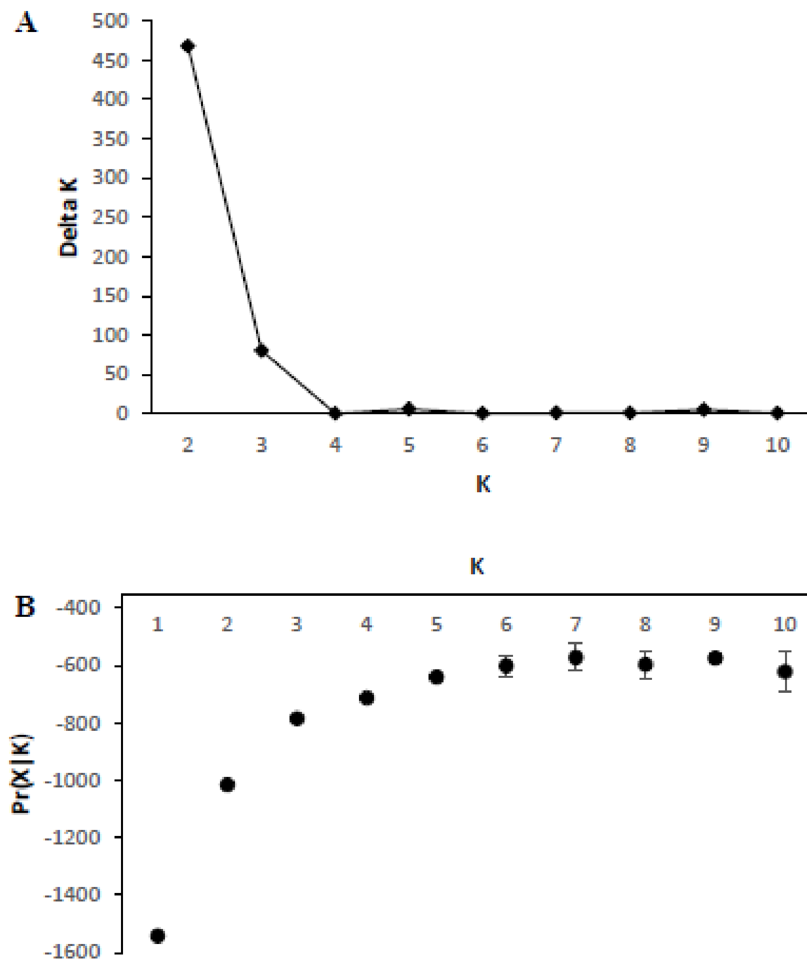

Figure 6 Posterior probability for population clusters. A) ΔK in response to the number of clusters (K). The graph indicates that ΔK is highest when K=2. However, relevant change, in the [Pr(X|k)], is still evident by increasing to K=3, K=4 and K=5. B) The log likelihood [Pr(X|k)] for assumed number of population clusters (K) for *T. retroflexa*. The graph depicts the majority of the population structure being encompassed by setting K=4 or K=5. For higher K values, the likelihood only slightly increases, if at all

AMOVA revealed all differences to be statistically significant (Table 2). Variation among regions (29.6%) and among populations (29.3%) were relatively similar and explained the majority of the variance. Differences among (21.2%) and within (19.9%) individuals were also relatively similar, respectively encompassing 21.2% and 19.9% of the variance. F_{st} values (Table 3) were significant for all population pairs. The Northern populations (SC and RC) and population C were more divergent than all other populations. F_{st} was highest between SMD and the northern populations (the geographically furthest clusters). F_{st} was lowest for MD/MDR and MD/SMD. Notably this trend was not observed for SMD and MDR.

Table 2 Analysis of Molecular Variance. Analysis of molecular variance (AMOVA) of six microsatellite loci in the six populations of *Trioncinia retroflexa*.

Source of variation	Degrees of freedom	Sum of squares	Estimated variance	Percentage variation (%)	P-value
Among Regions	1	118.41	0.67	29.6	0.001
Among Populations	4	147.42	0.66	29.3	0.001
Among Individuals	159	223.02	0.48	21.2	0.001
Within Individuals	165	74.00	0.45	19.9	0.001
Total		562.85	2.25		

Table 3 Population pairwise comparisons of F_{ST}. Pairwise F_{ST} estimates among the six populations of *Trioncinia retroflexa*.

	SC	RC	MD	MDR	C	SMD
SC	-					
RC	0.581	-				
MD	0.578	0.543	-			
MDR	0.621	0.606	0.110	-		
C	0.542	0.501	0.451	0.433	-	
SMD	0.723	0.726	0.304	0.311	0.527	-

Gene flow and migration

Across all populations 90% of individuals reached the highest assignment score for their source population. The MD population was the exception with 52.4% of individuals being assigned to another population. Of the total individuals assigned to another source population 64.7% were from MD, 29.4% were from MDR and 5.9% were from C. Of these individuals 35.3% were assigned to northern populations. No individuals from the northern populations were assigned to populations other than their source population.

Population genetic differentiation

Of the five coordinates obtained in the PCoA, the first four coordinates explained 95.38% of the genetic variation. The first and second axes constituted the majority (61.91%) of the total variation; individually explaining 37.52% and 24.39%, respectively. The third and fourth axes respectively displayed 18.02% and 15.45% of the variation. The first axis corresponded to differentiation across the southern populations and between southern and northern populations. The second axis reflects separation of the northern populations (Figure 7).

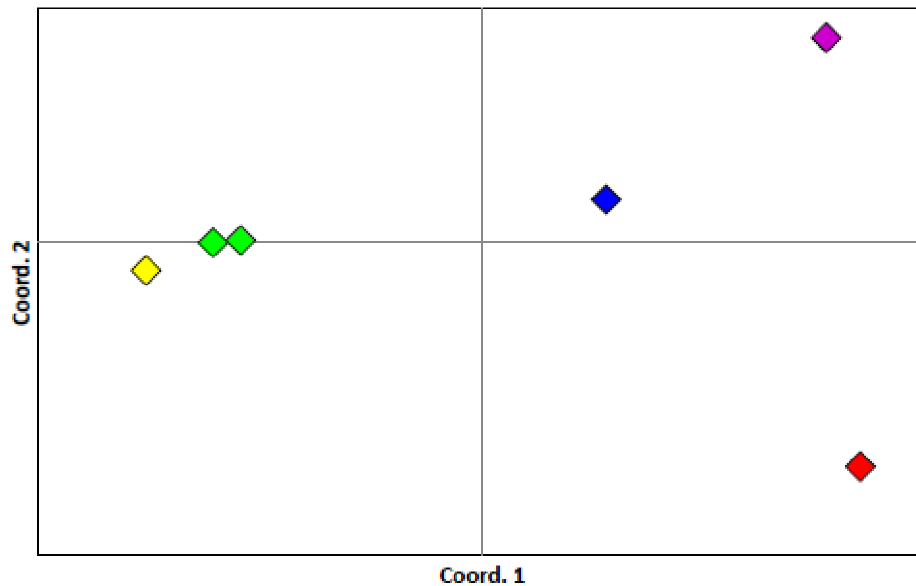

Figure 7 Principal coordinates analysis. Scatter plot of the first and second principal coordinates obtained from six microsatellite primer combinations from six *Trioncinia retroflexa* populations.

Isolation by distance

Across the sampled population range, genetic distance (PhiPT) and geographic distance were highly correlated (r = 0.809, p = 0.008). Geography explained 65.5% of the genetic differences between populations (R^2 = 0.655). When isolating the southern populations, the correlation coefficients were lower and non-significant (r = 0.685, p = 0.161, R^2 = 0.4688, Figure 6).

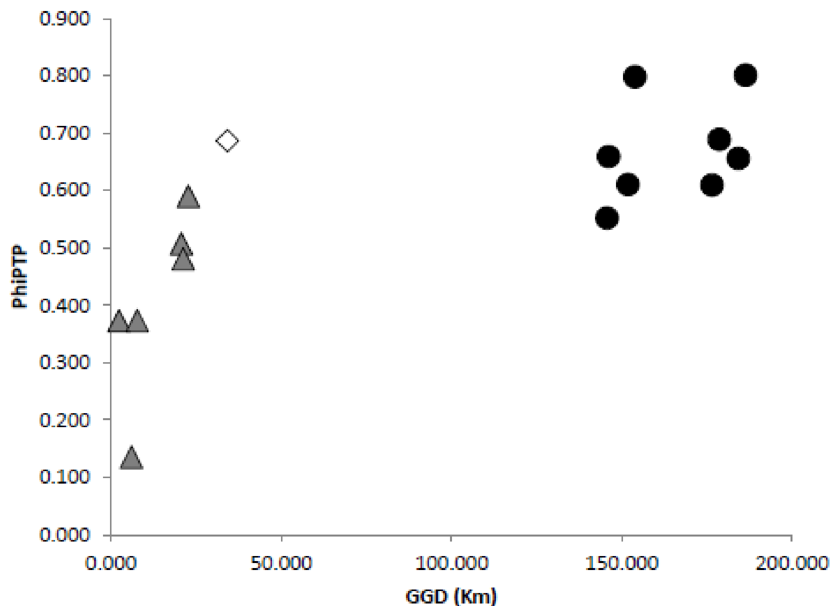

Figure 8 Isolation by Distance. Correlation of genetic distance (PhiPTP values) against geographic distance (GGD), in Km, as population pairwise comparisons for *Trioncinia retroflexa*. White diamonds represent distances between two northern populations; Grey triangles represent distances between two southern populations, and black circles represent distances between a northern and a southern population.

Population genetic bottleneck

The mean number of alleles was: 1.83 for SC, 1.50 for RC, 1.83 for C, 2.17 for MD, 1.50 for MDR and 1.67 for SMD. In the sign test, all populations were found to be at mutation-drift equilibrium across all three mutational models ($p > 0.05$). The allele frequency distribution test detected no shift (normal L-shape) in the distribution for populations SC, RC, C and MD. A mode shift was detected for populations MDR and SMD (Table 4).

Table 4 Genetic diversity of *Trioncinia retroflexa* populations. Genetic diversity averaged over six microsatellite loci calculated from mature individuals of *Trioncinia retroflexa* from each study population. H_e = Expected heterozygosity, H_o = Observed heterozygosity, IAM = Infinite alleles model, TPM = Two phase model, SMM = Stepwise mutation model.

			Allelic	Gene	Sign test			
Populations	H_e	H_o	**Diversity**	**Diversity**	**IAM**	**TPM**	**SMM**	**Allele frequency distribution**
SC	0.194	0.169	1.833	0.197	0.609	0.660	0.678	L-shaped
RC	0.113	0.139	1.500	0.122	0.533	0.546	0.483	L-shaped
MD	0.314	0.196	2.167	0.321	0.255	0.306	0.355	L-shaped
MDR	0.131	0.128	1.500	0.135	0.377	0.427	0.493	Mode-shift
C	0.215	0.183	1.833	0.222	0.262	0.289	0.688	L-shaped
SMD	0.207	0.188	1.667	0.214	0.220	0.299	0.667	Mode-shift

Germination traits

Water scarcity

Mean percent seed germination was significantly lower across all water treatments ($p < 0.001$) for population C (34.02%, SE±4.31%), than SC (66.66%, SE±6.26%) and MD (65.97%, SE±6.06%), which were not significantly different from each other. Within populations there were no significant differences among percent seed germinability across water treatments, except for when germinated in 3.2 mg water. At this treatment, populations SC and MD had significantly higher germination rates (79.15%, SE±4.15%, $p = 0.009$ and 66.67%, SE±6.8%, $p = 0.044$, respectively) than C (33.5%, SE±11.78%), but did not differ from each other.

Mean time to germination was significantly higher, across all water treatments, (8.56, SE±0.62days $p < 0.01$) for population C, than for populations SC (6.08, SE±0.6days, $p = 0.001$) and MD (7.18, SE±0.63days, $p < 0.001$), which did not differ significantly. Within populations, significant differences in germination time were detected for population SC ($P<0.021$) but not for populations MD or C. Within population SC, time to germination was negatively correlated with available water (r = -0.6361, $p = 0.021$, $R^2 = 0.3571$) with significantly lower mean germination times for 4.0 (3.24, SE±1.05days p < 0.01) and 4.8 (4.38, SE±0. 22days, $p < 0.01$) ml than the 0.8 ml water treatment (9.42, SE±1.9 days).

Seed Treatments

Within populations, significant differences ($P<0.05$) in percent seed germinability were found among dark (SC= 69.44 SE±6.93%, C= 75.0 SE±7.02%, MD= 69.44 SE±5.65%) and shade (SC= 66.67 SE±5.68%, C= 69.44 SE±6.93%, MD= 62.5 SE±7.32%) treatments but not among temperature or pre-sowing treatments. Across populations, treatments did not differ significantly with dark and shade treatments uniformly having the highest percent seed germination. No significant differences among populations were detected for mean germination time.

Seed dispersal traits

Significant differences among populations were found for: seed index ($F(5,144) = 3.72$ $p = 0.0034$, Figure 9 a), seed mass ($F(5,144) = 5.13$, $p < 0.001$, Figure 9b), terminal velocity ($F(5,144) = 3.34$, $p < 0.0069$, Figure 9c) and initial drop velocity ($F(5,144) = 3.72$ $p < 0.001$, Figure 9d). A marginally significance in barb circumference was also found ($F(5,54)$

= 2.20, p = 0.0681). No significant differences were found for seed structure (F(5,54) = 0.71, p = 0.6209).

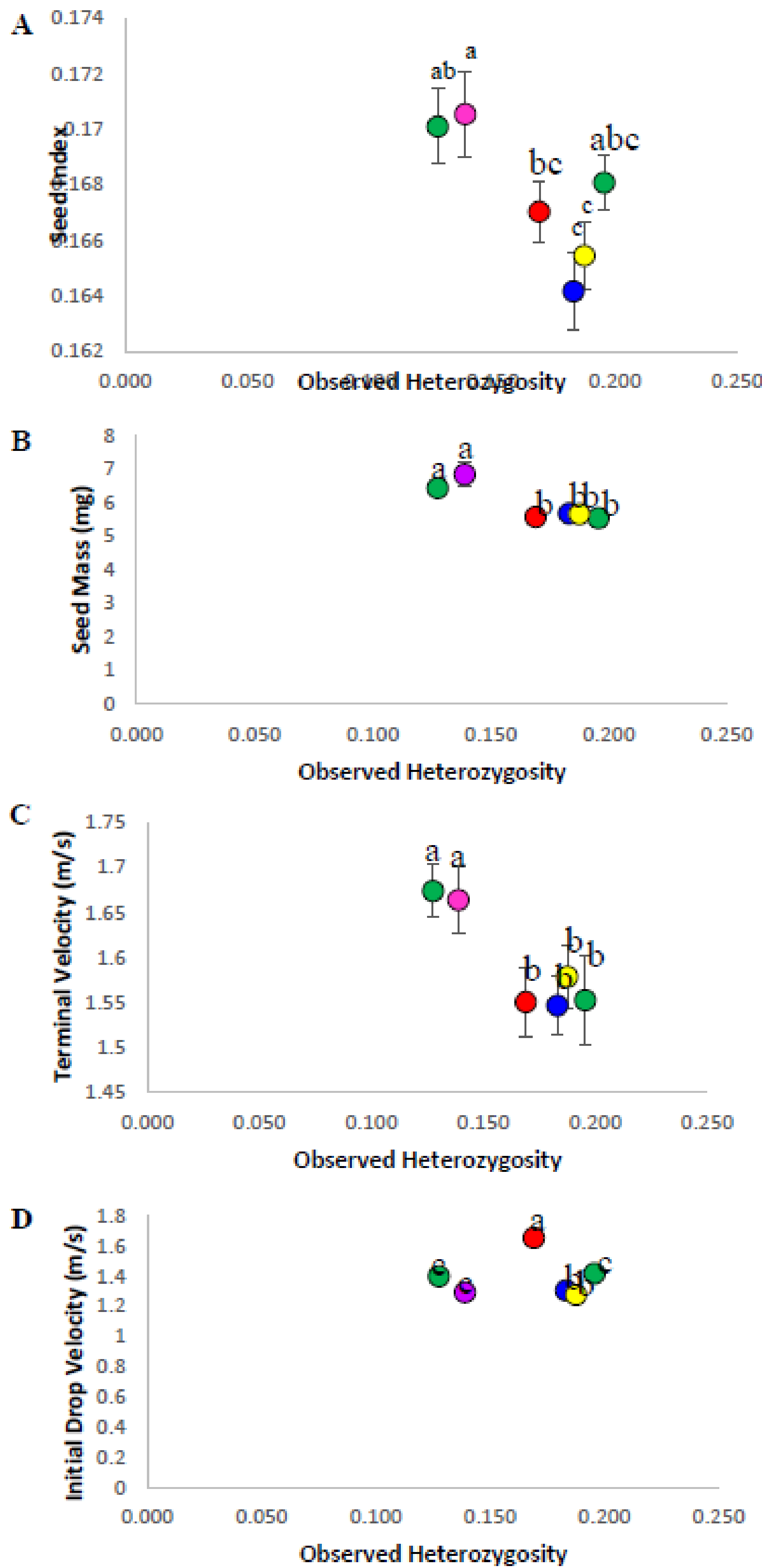

Figure 9 Seed Dispersal Traits by Observed heterozygosity. Comparison of seed dispersal traits across sampled *Trioncinia retroflexa* populations by observed heterozygosity (H_o). Red = SC, Purple = RC, Green = MD/MDR, Yellow = SMD, Blue = C. Error bars indicate ± SE. A) Seed index, B) Seed mass, C) Seed terminal velocity and D) Seed initial drop velocity. Letters above populations indicate significant differences as determined by Tukey's HSD test.

For seed index, population MDR and RC were the only populations to consistently differ significantly from other populations. MDR (0.17, SE±0.002), was significantly larger (elongated) than populations C (0.16, SE±0.002, $t(46.69) = 3.3$, $p = 0.0018$), SMD (0.17, SE±0.002, $t(37.98) = 3.03$, $p = 0.0045$) and SC (0.17, SE±0.001, $t(42.16) = 2.28$, $p = 0.0277$). Population RC (0.17, SE±0.002) also had significantly larger seeds than populations C ($t(41.08) = 2.70$, $p = 0.01$) and SMD ($t(36.39) = 2.45$, $p = 0.0193$), but not from SC.

Populations RC (6.83, SE±0.35mg)and MDR(6.43, SE±0.21mg) had significantly greater mean seed mass (SC: $t(28.75) = 3.2$, $p = 0.0033$ and $t(41.93) = 3.12$, $p = 0.0033$, C: $t(24.05) = 3.1$, $p = 0.0049$ and $t(32.68) = 3.07$, $p = 0.0043$, SMD: $t(35.91) = 2.63$, $p = 0.0125$ and $t(35.54) = 2.22$, $p = 0.0326$, MD: $t(35.81) = 3.05$, $p = 0.0043$ and $t(47.74) = 2.8$, $p = 0.0073$, respectively) than all other populations (SC: 5.57, SE±0.18mg, C: 5.67, SE±0.13mg, MD: 5.53, SE±0.24mg, SMD: 5.66, SE±0.28mg), but did not differ significantly from each other. Seed mass for all other population pairs, excluding RC and MDR, did not differ significantly.

Terminal velocity results matched those for seed mass with RC and MDR having significantly higher terminal velocities (SC: $t(46.82) = 3.05$, $p = 0.0038$ and $t(41.93) = 2.41$, $p = 0.0212$, C: $t(48.0) = 3.47$, $p = 0.0011$ and $t(32.68) = 3.07$, $p = 0.0043$, SMD: $t(37.72) = 2.63$, $p = 0.0125$ and $t(30.91) = 2.20$, $p = 0.0351$, MD: $t(47.99) = 2.63$, $p = 0.0116$ and $t(41.97) = 2.44$, $p = 0.0189$, respectively) (1.72, SE±0.04m/s and 1.69, SE±0.03m/s, respectively), but not significantly differ from each other. Populations SC (1.55, SE±0.04m/s), C (1.55, SE±0.03m/s), MD (1.55, SE±0.05m/s) and SMD (1.58, SE±0.04m/s) did not differ significantly amongst each other.

Drop velocity differed significantly among most populations. SC (1.65, SE±0.04m/s) was significantly faster than all other populations (RC: $t(44.92) = 7.7$, $p < 0.001$, C: $t(51.31) = 4.87$, $p < 0.001$, MD: $t(51.61) = 5.22$, $p < 0.001$, MDR: $t(34.61) = 8.31$, $p < 0.001$ and SMD: $t(47.42 = 7.65$ $p < 0.001$). Population C (1.42, SE±0.03m/s) and MD (1.4, SE±0.03m/s) did not differ significantly from each other, but had faster drop velocity than the other population (RC: $t(47.65) = 3.38$, $p = 0.0014$ and $t(47.73) = 2.87$, $p = 0.006$, MDR: $t(39.98) = 3.73$, $p < 0.001$ and $t(39.79) = 3.11$, $p = 0.0035$ and SMD: $t(44.85) = 3.55$, $p < 0.001$ and $t(45.09) = 3.08$, $p = 0.0035$, respectively), excluding SC. Populations RC (1.29,

SE±0.02m/s), MDR (1.3, SE±0.01m/s) and SMD (1.28 SE±0.03m/s) did not differ significantly from each other and had the slowest drop velocity.

DISCUSSION:

Anthropogenic habitat fragmentation is a recent but pervasive characteristic of the central Queensland landscape. In response to agricultural expansion throughout this area, reduced habitat, increased isolation and modified ecological boundaries are but a few of the challenges faced by plant species native to this ecosystem. The first objective of this study was to evaluate the extent to which this contemporary landscape has impacted the historical distribution of *T. retroflexa* based on its modern population genetic structure. While four of the six populations clearly exhibit the higher genetic variation, all six populations are genetically different. Considering how recently anthropogenic fragmentation has occurred in the region (~160 years), species life expectancy (~5 years), and thus the number of elapsed generations since fragmentation (~32), it is unlikely that such a high level of genetic differentiation would be observed if *T. retroflexa* possessed a continuous distribution at the time of European settlement. Furthermore, since all populations maintain relatively unique genetic structures, it is possible that they have persisted in small isolated states, with minimal gene flow, long before anthropogenic fragmentation. Such conclusions are further supported by the presence of IBD and the observed deficit of heterozygotes; as genetic diversity decreases as a consequence of small population size, isolation and random genetic drift.

While it is concluded that populations were historically isolated, alternative interpretation of results should be addressed. First, strong genetic differentiation can be observed in remnant populations of species proposed to historically have maintained continuous distributions. Furthermore, associations between genetic and geographic distance could be indicative of clinal variation in what was once a continuous distribution prior to habitat destruction. However, such the interpretations propose the impact of vicariance (Shepherd and Perrie 2011) and or the presence of an inhospitable landscape matrix (Shapcott et al. 2009). As *T. retroflexa* lacks an isolating geological event, within the species' range, and continues to persist in marginal habitats (road verges and stock routes), the relevance of such explicative scenarios is up to interpretation. *Trioncinia retroflexa* has been found to be absent from a plethora of surveyed areas that have been identified potential habitats. This is would not be expected under a clinal variation

scenario, as there would be an expected increase in population isolation as intermediate populations were replaced with an otherwise inhospitable matrix. While it cannot be conclusively determined as to whether any such sites once supported *T. retroflexa*, the lack of remnant populations across the contemporary landscape of suitable natural (bluegrass grasslands) and disturbed (road verges and stock routes) habitats does call to question the likelihood of a continuous distribution. Secondly, it must be considered that the genetic values obtained here may reflect common aspects of the biology of Asteraceae. To determine this, values obtained here were compared to document genetic values for the wide spread Asteraceae *Senecio lautus* (Roda, et al. 2013). F_{ST} value for *T. retroflexa* were significantly higher than those documented for *S. lautus.* Based on this comparison of genetic differentiation, it is unlikely that values obtained in this study are representative of Asteraceae commonalities.

The lower differentiation between the sampled southern populations (MD and MDR), is consistent with Isolation by Distance (IBD) results that demonstrated a significant relationship between genetic differentiation and geographical distance across the species' total range but not within the regions. Considering how close these populations are to each other (0.67km), it is likely that they were functionally a single population in more recent historical time than the other populations. While geographical distance did explain the majority of genetic variation across the species' entire distribution, the lack of correlation within the southern populations (C, MD, MDR and SMD) would suggest limitations to the resolution of IBD. The loss of significant correlation between genetic and geographic distance at small scales may suggest spatial extents within which gene flow is more likely. As the species is likely dispersed via animal fur, this loss of correlation could be associated with livestock movements, as movements between paddocks and/or adjacent properties would be more frequent than long distance movements. Such conclusions, are further supported by F_{st} values which demonstrated less differentiation between the geographically closest populations (MD, MDR and SMD).

While populations demonstrated significant genetic differentiation, the identification of relatively recent migrant may provide insights into *T. retroflexa* seed dispersal in the contemporary landscape. As 47.1% of the total migrations were between populations MD and MDR, this could further indicate a scale within which the potential for seed dispersal is more likely. As F_{ST} values have suggested that MD and MDR were recently, relative to other population pairs, the same population, this migration could further support

conclusions that the modern landscape does not completely negate localized dispersal. Beyond this local migration, 54.5% of the remaining migrants originated in northerly populations before dispersing to southern populations. This migration suggests that modern agricultural practices may promote migration among populations in a directional flow (Auffret and Cousins 2013). As sampled migrants were fully mature individuals, all would have established within the last five years (lifespan), suggesting fairly routine migration events; potential via cattle movements along the road verges and stock routes where these populations are located. A confounding element to these observations is the apparent lack of northern genes in the genetic structure of southern populations in light of the potential frequency of these dispersal events. This suggests that dispersal is not the most limiting factor for *T. retroflexa* and that some other post dispersal and establishment factor is limiting the transference of northern genes into the southern population.

While the variation observed in this study is selectively neutral, the potential loss of these neutral loci, should populations be lost in the future, may have substantial conservation implications. Studies have suggested that the loss of neutral loci may indicate a parallel loss in species' potentially adaptive genetic variability (Lee and Olds 2011). Such loss is of major interest to conservation efforts, as it may lead to increased extirpation potential should population sizes remain small and/or environmental conditions shift in the future. As almost all *T. retroflexa* populations embody a unique genetic structure, conservation efforts should consider the protection and maintenance of all populations of *T. retroflexa.* The loss of any populations would lead to the complete loss of those unique genotypes, from the species' gene pool, and potentially compromise the species' adaptive capacity in the future. As population long-term isolation is a driver of this species' current population structure, multiple reintroduction efforts should be locally applied, across the species' distribution, utilizing seed from local populations to bolster the size of these genetically unique populations, while not encouraging loss of local alleles to outbreeding.

While varying across populations, the overall low germination rate of *T. retroflexa* may be indicative of the deleterious effects of a population bottle neck in this species (Piquery et al. 2011; Soons and Heil 2002). In particular, the significantly lower germination rate of seeds from population C, may indicate long-term deleterious effects of a past bottleneck in this particular population. As this is currently the largest population the question remains as to how such a small population with limited germination capacity can have reestablish so effectively. One possible explanation is the distinct land use experience at this site.

The frequency of hay production and cattle grazing may increase suitable habitat (intertussock space) and reduce competition with dominant grasses. *Trioncinia retroflexa's,* low lying growth form is likely to spare it from extensive browsing and/or damage from hay harvest practices. Furthermore, as the species is dispersed via attachment to animal fur, the increased frequency in cattle may permit additional opportunities for dispersal/establishment (Hobbs & Yates 2003) away from parent plants.

The lack of germination response to water treatment across all populations is consistent with the water requirements of other species persisting in semi-arid (Kos and Poschlod 2007) and grassland systems (Maze et al. 1993). Water constraints on germination have been theorized to be advantageous in these types of systems as they permit earlier establishment and onset of flowering, increasing reproductive success when conditions are optimal (Maurer et al. 2003; Stanton et al. 2000).

As the significantly longer time until germination observed in population C does not readily present an adaptive advantage one possible explanation is the fixation of deleterious mutation due to increased genetic isolation. Genetic isolation has been attributed to declines in the fitness of rare and previously common species (Aguilar et al. 2008) through a diversity of biological and physiological responses. While MD did have significantly shorter mean germination times, the lack of variation across water treatments for both populations, C and MD, further accentuates the species' water stress adaptations as mentioned above and illustrates the persistence of this trait in population C. The negative correlation between germination time and water treatment, for population SC, suggests a modified seed response to water availability. As the oscillation of extended wet and dry periods are natural to the study region, this decrease in time to germination, under higher water availability, prolongs the exposer of seedlings to hospitable, wet conditions (Kos and Poschlod 2007). The nature of black cracking clay soils across *T. retroflexa*'s range, further encourages such early germination response. The high presence of water in the soil at the time of germination further prolongs the time seedlings have to establish root systems that are deep enough to bypass the eventual hardening of the top soil's crust.

Counter to observed increases in germination, multiple studies of grassland species have documented substantial declines in germination rates under light restrictions from dense living biomass and thick litter layer accumulation (Morgan 1998a, Partzsch et al. 2011; Zia and Khan 2004). *Trioncinia retroflexa* germination increased under dark and shade

conditions, suggesting that this species may germinate best under tussock grasses native to its grassland habitats, though this micro-environmental preference has yet to be tested. Another explanation could be the deep cracking nature of the soils associated with *T. retroflexa* (Fensham 2003). If seeds are better adapted to germinate in shady or dark conditions, these cracks would shelter seeds from dry, full light conditions; enhancing germination rates. From a conservation perspective, the inherent lack of germination under full light treatments may stress the necessity to promote tussock grass species and the routine maintenance of the intertussock space through occasional disturbance, such as light grazing. To date, two studies have attempted the reintroduction of *T. retroflexa* within its native range (Fensham and Fairfax 2005, Haller, unpublished data). In both cases, efforts had resoundingly poor success rates, with relatively little new recruitment after initial reintroduction efforts, and few individuals persisting beyond two years. Such results, indicate additional abiotic and biotic factors that will need to be considered to improve conservation returns.

The significant variation in seed index, mass, terminal velocity and initial drop velocity supports the third hypothesis. The observed difference in seed dispersal traits suggests that populations do maintain unique phenotypes in addition to their unique genetic structure. From a conservation perspective multiple studies have considered the correlation between life history traits and species persistence in fragmented systems (Johansson et al 2011; Kolb and Diekmann 2005; Sutton and Morgan 2009) with varying conclusions. With regards to the traits measured here, studies have suggested that species with seeds that have lower terminal velocity, seed mass (Saar et al. 2012) and exozoochoric dispersal modes (William et al. 2005) are particularly susceptible to local extinction. That said trait-based life history trade-offs (Jakobsson and Eriksson 2000; Lindborg and Eriksson 2004) are common and as such there is an appreciated need to consider traits on a case-by-case basis.

The significant differences in seed index and mass may have several significant implications for the dispersal and survival capacity of this species. Smaller, rounder seeds have demonstrated increased longevity in soil seedbanks (temporal dispersal) (Maurer et al 2003), spatial dispersal (Thompson et al. 1993) and frequency of dispersal through increased seed production (Jakobsson and Eriksson 2000) in other species. Considering the frequency of disturbance in the region, traits that permit species to wait out inhospitable periods (like longer longevity in the seedbank) may be important to

maintaining *T. retroflexa* populations over the long-term (Thompson et al. 1993; William et al. 2005). Smaller seeds, however, also produce smaller, less competitive seedlings and thus decreasing the probability of recruitment success (Jakobsson and Eriksson 2000), with evidence suggesting that larger seeds are beneficial for recruitment, particularly under disturbed, competitive, light-eficient (Jakobsson and Eriksson 2000) and drought environments (Kos and Poschlod 2007; Westoby et al. 1996), all of which *T. retroflexa* experiences. Overall, many different pressures may be involved in the selection of seed and germination traits in this species, and there are no findings from our study to suggest that the different *T. retroflexa* populations in this study are experiencing strong and distinct selection pressures for the seed traits I measured.

Populations of *T. retroflexa* are often found in restricted portions of seemingly homogenous grasslands, suggesting local dispersal limitation or the importance of yet unidentified microclimate factors in defining this species' realized niche. The identification of what these microclimate factors are and the use of seed with traits that will maximize dispersal among suitable habitat patches in restoration efforts are likely critical for the successful establishment of new populations of this species.

CONCLUSIONS:

The results of this study suggest a historical distribution of *T. retroflexa* counter to previous assumptions. While results from this study do not eliminate the possibility that the species' may have been widespread at some point in the past, the large genetic differentiation coupled with the lack of heterozygotes within populations suggests that populations have persisted in isolation at least beyond the time frame for anthropogenic fragmentation in the region. As such, current management strategies need to adapt to this new insight. As demographically unique populations closely parallel unique genetic populations, conservation agendas need to consider the preservation of multiple populations, regardless of size, in order to preserve overall genetic diversity. Furthermore, efforts should concentrate equally on both large and small populations as they embody a similar proportion of the overall genetic diversity. Smaller population should be more strictly monitored as they may be at greater risk of extirpation due to pollen limitation, dispersal limitation and stochastic extinction. Finally, gene flow between populations should not be artificial enhanced beyond current levels. As populations are genetically differentiated, the artificial enhancement of gene flow between these populations could have deleterious effects due to out breeding and the loss of local adaptations.

In addition to the monitoring and maintenance of current remnant populations, the population genotypic and phenotypic variation identified in this study needs to be considered in the application of future reintroduction efforts. In both prior reintroduction efforts, seeds were sourced from multiple remnant populations with varying distances to the reintroduction sites. As the variation captured in this study could indicate local adaptations, future reintroduction efforts should source seed from geographically close remnant populations with similar abiotic and biotic environmental conditions. This may increase the effectiveness of establishment efforts as seed may be embody genetic variation for ecologically important traits in the novel environment.

This study highlights the importance of population genetics in the development of effective management schemes. In the absence of such initial studies of the genetic structure of rare or endangered plant populations, the potential for ineffective conservation efforts increases. Trait analyses can be helpful but may not be sufficient alone for determining why species’ may be rare within their natural habitats. There are still many puzzling questions about this species’ rarity status, including how microclimate conditions impact this species demographic success, but this genetic and trait assessment has provided valuable insights that will aid in the protection of this species into the future.

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APPENDIX