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Genetic Diversity and Population Structure of *Olearia flocktoniae* Maiden & Betche (Asteraceae) – A Comparison of Aboveground and Seed Bank Populations

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Declaration

I certify that the contents and substance of this thesis have not already been submitted and is not currently being submitted for any other degree. All assistance received in the preparation of this thesis and all sources used have been acknowledged.

Lorelie Flood

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Abstract

Olearia flocktoniae Maiden & Betche (Asteraceae) is an endangered shrub found within a 45 km x 45 km area in the Dorrigo District of northern NSW, Australia. A genetic diversity study using allozyme loci was conducted comparing 14 aboveground and 6 seed bank (2 extinct and 4 extant) populations. The degree of polymorphism (P) and allelic frequencies (A) for aboveground populations (0.32 and 1.32, respectively) was comparable to that found for endemic species; expected heterozygosity (H_e) (0.15) was closer to values expected for widespread species. The fixation index (F_{ST}) (0.15) indicated moderately substructured populations while observed heterozygosity to expected heterozygosity H_o/H_e (1.35) and the inbreeding coefficient (F_{IT}) (-0.13), suggested an excess of heterozygotes. The age of aboveground populations was significantly related to the number of effective alleles (A_e) and H_e. No significant differences between extant and extinct populations were found in terms of P, A and A_e. Significant differences were found between seed bank populations and their respective aboveground populations in three out of four allozyme loci. Seed bank populations were more homozygous (F_{IT} = -0.09) than aboveground ones (F_{IT} = -0.59) indicating selection against homozygote individuals from surviving to the next life stages. Both aboveground and seed bank populations were marked with high genetic identities (0.9677 and 0.9596, respectively). Allelic richness (A_e) in populations was significantly related to population age. Though there was a general clustering of the populations' genetic distances in relation to geographical distance, some populations from the opposite sides of the metapopulation had high identities whereas some neighbouring populations had low identities. This supports previous suggestions that seed are moved by vehicles associated with logging activities. The rapid decline of *Olearia flocktoniae* populations in the last five years as reported in annual field surveys may be a natural pattern for this rare species. However, maintaining populations for extended periods (5 years +) through active management is likely to increase the number of effective alleles. Routine genetic assays (c. every 5 years) of the populations should be a part of its management plan.

Table of Contents

Chapter 1 – Introduction

Biodiversity is biological wealth. – A. J. Beattie (1995).

Australia is a large island continent with 80 biogeographic regions (Thackway and Cresswell, 1995) and thus a diversity of habitats. It is the home to more than a million species including at least 18,000 species of vascular plants and over 12,000 species of non-vascular plants (Australian Bureau of Statistics, 1996). The Australian flora has a biological affluence marked by endemism and species diversity, with 33 per cent of genera and 85 per cent of species of all its vascular plants being endemic (Williams, 2001). One such species is *Olearia flocktoniae*, an endangered shrub endemic to northern New South Wales. It is a species at risk of extinction in the next 10-20 years if the current level and types of threatening processes that besiege it continue (Briggs and Leigh, 1996; IUCN, 2002). This thesis is concerned with the conservation genetics of *O. flocktoniae* and it will be demonstrated in this introduction that determining key aspects of the species' conservation genetics will assist with its management.

1.1. Biodiversity Conservation.

With the extent of Australia's biodiversity in mind, a "high burden of responsibility" is placed on Australians to conserve the existing biological diversity by not rendering species to become deliberately extinct or endangered through neglect (Leigh *et al.*, 1984). Habitat fragmentation, environment modification and introduction of exotic species were identified as main factors that severely affect the biodiversity of Australia's native species (IUCN, 2002). Leigh *et al.* (1984) identified woodland vegetation as the habitat with the most numbers of endangered and extinct species; agriculture, followed by domestic and feral grazing, are the three highest causes of extinction and endangerment. Eastern, south eastern and southern Australia were identified as having the highest level of disturbance due to European settlement (Leigh *et al.*, 1984) coinciding with their present status as areas containing the most number of threatened ecosystems and species (see Figs. 1.1 and 1.2; Sattler and Creighton, 2002). *Olearia flocktoniae* is found within a 45 km x 45 km area in eastern Australia (Fig. 1.3).

A conservation classification scheme for species was introduced in order to understand individual species' needs for conservation. Plant and animal species in Australia needing conservation were classified as either presumed extinct, endangered, vulnerable, rare

or threatened (Briggs and Leigh, 1996). Of about 18,000 formally classified vascular plants in Australia (Leigh and Briggs, 1992), over 5,000 are in the conservation list with 47.2% of them tagged as 'poorly known' (which could be rare or threatened), 31.2% as 'rare', 14.1% as 'vulnerable', 6.0% as 'endangered' and 1.5% as 'presumed extinct' (Briggs and Leigh, 1996). As a guide, a rare tree species has fewer than 5,000 individuals occurring in the wild while a rare herb species has fewer than 10,000 (Leigh and Briggs 1992).

More updated inventories were produced through The Environment Protection and Biodiversity Conservation (EPBC) Act 1999. As of July 2001, 1245 vascular plant species were listed as extinct (61 species), critically endangered (37 species), endangered (492 species) and vulnerable (655 species). Section 179 of the act lists 5 criteria for a native species to meet in order to be classified as critically endangered, endangered or vulnerable. They include: 1) impending reduction in number (very severe, severe, substantial); 2) geographic distribution (very restricted, restricted, limited); 3) the estimated number of mature individuals (very low, low, limited) and showing evidence that the numbers will continue to decline (very high rate, high rate, substantial rate); 4) the estimated total number of mature individuals (extremely low, very low, low); and 5) the probability of its extinction in the wild (50% in the immediate future, 20% in the near future, 10% in the medium-term future). The EPBC Act, providing for the conservation of biodiversity has, from its conception to present, allowed for the declaration of 14 key threatening processes, the production of recovery plans where the federal government has made them available to the public for comments and for governments to adopt and implement, and the regular update of inventories of listed threatened species (see www.deh.gov.au/biodiversity/threatened).

Recovery plans outline the research and management actions needed to stop the decline and support the recovery of listed threatened species or threatened ecological communities with the aim of maximising their long term survival in the wild (EPBC Act 1999). Compliance to these plans is taken very seriously such that no Commonwealth agency can take any action that contravenes these plans (Section 268 of the EPBC Act 1999).

Recovery plans are manuals for effective biodiversity conservation. A recovery tool often promoted by managers is the incorporation of conservation genetics. Table 1.1 outlines several recovery plans where conservation genetic studies are to be employed.

Table 1.1. Recovery Plans of Threatened Species That Employ Conservation Genetics.

Name of Species	Status	Recommendations derived from genetic studies OR reasons for recommending genetic studies
Grevillea beadleana	Endangered (EPBC Act 1999)	Continue research into the genetic variation of *G. beadleana* (NSW NPWS 2002b).
Epacris hamiltonii	Endangered (ESP Act 1992)	Determine genetic variation in known populations in different catchments (NSW NPWS 2001).
Acacia pubescens	Vulnerable (EPBC Act 1999)	Genetic variability study proved essential for management of the species (NSW NPWS 2002a).
Grevillea kennedyana	Vulnerable (EPBC Act 1999)	Within and among population genetic variability estimates will provide information about the number of clones in the populations and will infer the nature of the species' reproduction (NSW NPWS 2000a).
Hakea pulvinifera	Endangered (EPBC Act 1999)	The one population of this species consists of one clone and the establishment of *ex-situ* populations are recommended (NSW NPWS 2000b).

Conservation genetics employs the disciplines of ecology, molecular biology and population genetics in investigating organisms in their populations. Such populations are usually threatened or endangered and knowledge of their genetic makeup and interactions are usually useful in drafting management decisions. For example, the Recovery Plan for *Acacia pubescens* (NSW NPWS, 2002a) lacks substantial information on the biology, ecology and distribution of the species. A genetic assay of individuals in its known 124 sites would reveal the extent of identity or similarity of the populations as well as identify which ones are genetically divers. With this information in hand priorities in management could be made (which sites need the most urgent protection). *Grevillea kennedyana* individuals are found in clumps which could be due to horizontal roots or seedling

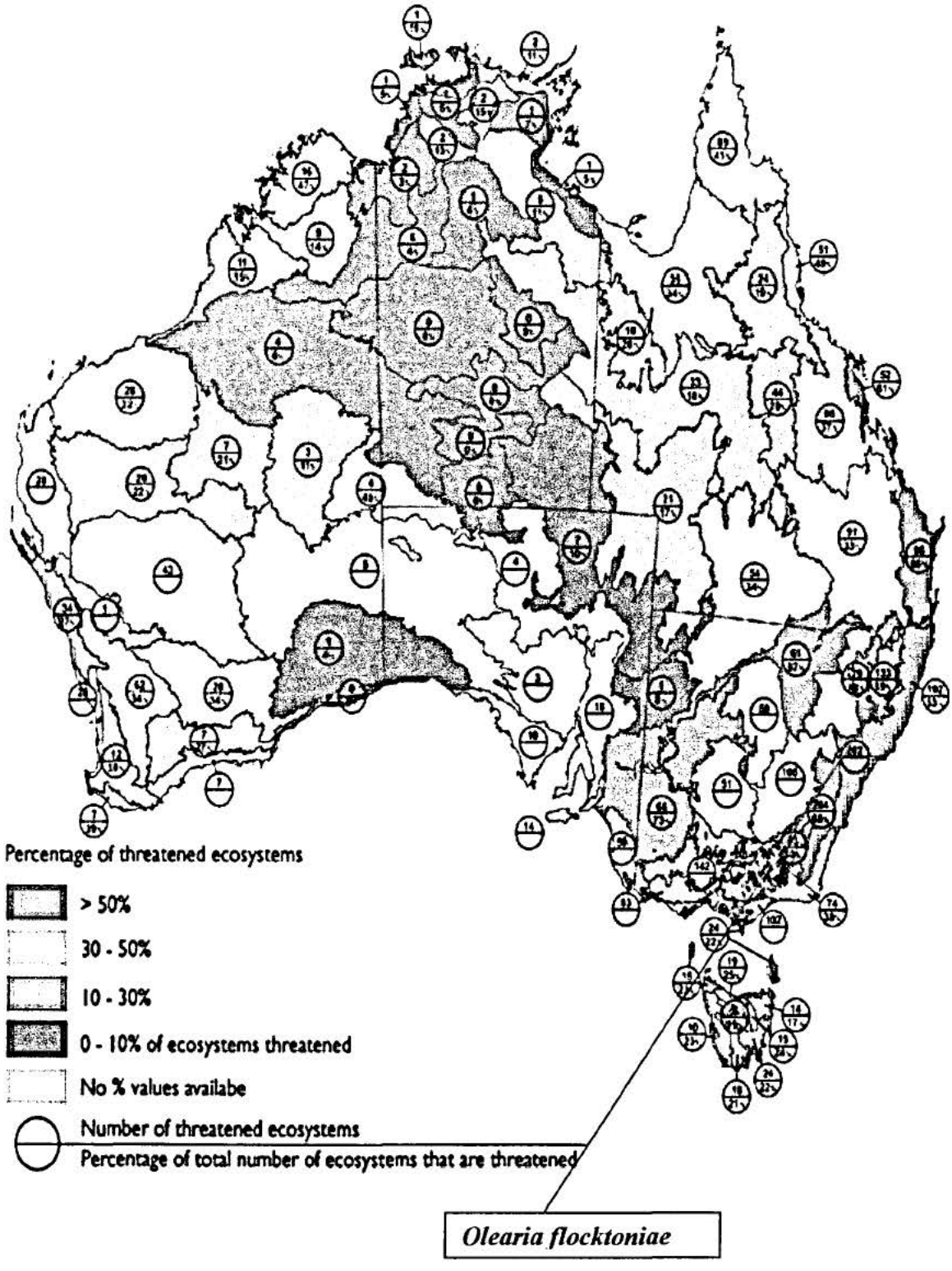

Fig. 1.1. The number and percentage of threatened ecosystems and other ecological communities identified across bioregions. Adapted from Australian Natural Resources Atlas V2.0.

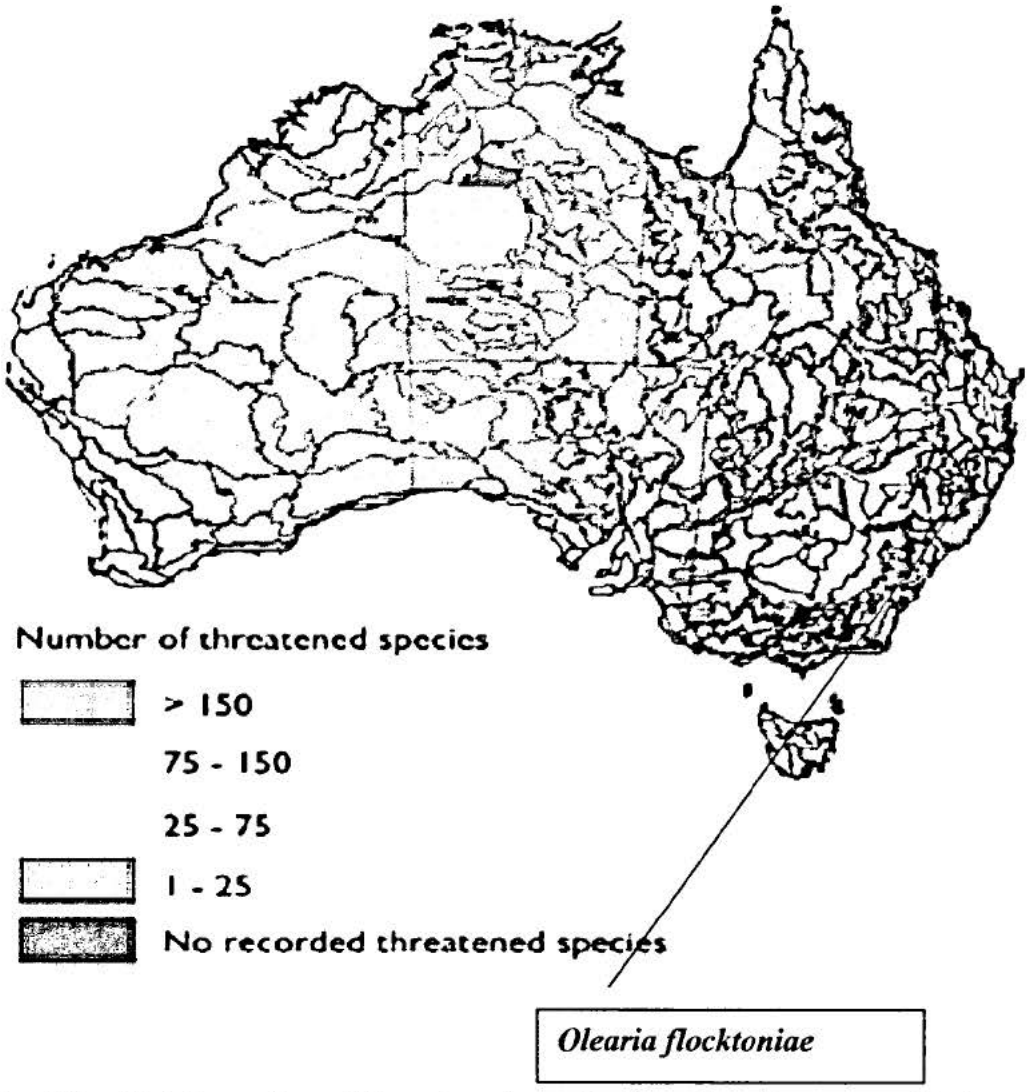

Fig. 1.2. Total number of threatened species by subregion as per State and Territory listings. Adapted from Australian Natural Resources Atlas V2.0.

establishment and a genetic assay would reveal the number of clones there are in each population or whether the populations are a single clone (NSW NPWS 2000a). Internationally, many published studies have made management recommendations from genetic data. Cultivated stocks of *Tulipa sprengeri,* a bulb species extinct in the wild, were sampled and individuals from a botanical garden were found to be the most genetically diverse. It was then recommended that seedling populations be established from that population under near natural conditions (Maunder, 2001). Populations of the rare mallee eucalypt, *Eucalyptus curtisii* covering a 500-km range were found to have very low levels of genetic diversity but high levels of interpopulation variation. It was recommended that, after preserving each of the population's natural habitats, they should be managed and treated

separately (Smith *et al.*, 2003). The endangered *Eriogonum ovalifolim* var. *vineum* was found to possess substantial genetic variation. Because its populations were not highly substructured, choosing sites to protect could be based on other factors (such as ecological) provided that sufficient numbers of sites are protected (Neel and Ellstrand, 2003).

A recovery plan has been drafted for the study species, *Olearia flocktoniae*, for 2003. The results and recommendations from this study may prove useful in the management actions that will be undertaken for the species' recovery in the wild.

1.2. The Study Species - *Olearia flocktoniae* Maiden and Betche (Asteraceae).

Olearia is from the Latin *olea* meaning olive. Some species of this genus resemble the foliage of the olive tree. Most members of this genus are called Daisy Bushes (Leigh *et al.*, 1984).

1.2.1. Taxonomic Description.

Olearia flocktoniae Maiden and Betche (Asteraceae) or the Dorrigo daisy is an endangered species found only on the northern fall of the Dorrigo district of northern NSW. It is a short-lived semi-herbaceous shrub (Figs. 1.4 and 1.5) with a physical description from Lander (1992) and Gross (unpub data) as follows:

> 1-2.5 m high, single to multi-stemmed near base. Leaves: sessile or sub-sessile, alternate, crowded; lamina linear, 20-90 mm long, 1-5 mm wide; apex acute, blunt; margins entire or occasionally with a few small scattered teeth, revolute; both surface glabrescent; lateral venation indistinct. Heads terminal, in simple corymbs, 19-25 mm in diameter. Peduncle to 55 mm long. Ray florets 30-48, white and often tinged violet. Disc florets 39-50, yellow. Achenes silky; pappus with 36-50 bristles in one series.

1.2.2. Ecology.

O. flocktoniae is a pioneer of disturbed margins of the Dorrigo rainforests and wet schlerophyll forests. In 1909, individuals were observed in clearings that had been disturbed especially by logging activities. It was presumed extinct for more than half a century before rediscovery in 1984 (Gross and Mackay, 1998). Most populations are found along verges of roads that are used in logging operations (other sites include power line areas and gravel pits) (Gross and Mackay, 1998). *O. flocktoniae* performs poorly with other plant species in comparison as observed in the wild and revealed by glasshouse experiments (Gross and Mackay, 1998). Dorrigo daisy individuals are reproductively mature on their

Fig. 1.3. Map of the Dorrigo District and vicinity showing the 14 *O. flocktoniae* populations. Source: www.wilmap.com.au/ausmaps.

Fig. 1.4. ***Olearia flocktoniae*** **capitulae (and single leaf at left foreground).** Source: C. L. Gross.

second year and can survive for up to three years in the wild. Older individuals (4-5 years) have been observed in areas with open vegetation (Gross and Mackay, 1998). These tend to disappear in sites where dense vegetation encroaches on them (Gross & Mackay, 1998).

Gross *et al.* (1998) found *O. flocktoniae* as predominantly selfing and capable of effecting seed set without the aid of biotic vectors. Ten insect species were observed floral visitors; they may be acting as pollinators. Recent work suggests that self-incompatibility may be more widespread (Gross, unpub data). *O. flocktoniae* seeds germinate readily (ten days under glasshouse conditions). Seed viability studies among populations revealed a wide range of 12-96% germinability (Gross, unpub. data). Seed longevity studies are under way however 7 year-old seeds exhibit 50% germinability (Gross, unpub data).

Populations of this species have been observed to fluctuate and more than half of them surveyed in 1997 had ten or less individuals. In the most recent census 37% of known individuals from the previous year have disappeared. Of the 83 sites surveyed, only 46 of them were populated. At this rate of decline, the species is predicted to reach extinction in the wild within 5–8 years (Mackay & Gross, 2001).

1.2.3. Other *Olearia* Species.

Understanding the distribution patterns (whether common worldwide or endemic) of congeneric species is useful for the specific species under study.

Of the 130 *Olearia* species worldwide (Bremer, 1994) around 75 are found in Australia (Leigh *et al.*, 1984) and one third of which (23 species) are listed in the 1997 IUCN Red List of Threatened Plants (Walter and Gillett, 1998). Fifteen of them are rare, 4 are vulnerable, 3 are endangered and 1 is extinct (Table 1.2). This pattern of rarity and endemism is substantially high for this genus.

Table 1.2. Threatened *Olearia* species found in Australia. Source: 1997 IUCN Red List of Threatened Plants.

Species	Status	Location
O. adenophora	Rare	Victoria
O. allenderae	Rare	Victoria
O. archeri	Rare	Tasmania
O. atroloba	Vulnerable	Victoria
O. ballii	Rare	Lord Howe Island
O. cordata	Vulnerable	NSW
O. ericoides	Rare	Tasmania
O. flocktoniae	Endangered	NSW
O. frostii	Rare	Victoria
O. hetrocarpa	Rare	NSW, Queensland
O. hookeri	Rare	Tasmania
O. hygrophila	Endangered	Queensland
O. lasiophylla	Rare	NSW
O. macdonnellensis	Vulnerable	NT
O. microdisca	Endangered	SA
O. montana	Rare	NSW
O. mooneyi	Rare	Lord Howe Island
O. oliganthema	Extinct	NSW
O. pannosa ssp *cardiophylla*	Rare	SA
O. pannosa ssp *pannosa*	Vulnerable	SA, Victoria
O. quercifolia	Rare	NSW

O. rhizomatica	Rare	ACT, NSW
O. stilwelliae	Rare	NSW

1.3. Allozyme Genetics.

To preserve biodiversity, conservationists are not only concerned with species richness or the number of species in a given environment, but also with genetic richness of individual species. An evolutionary unit (be it species or populations) can be equipped with a lot more chances to cope with evolutionary changes if its heritable units (genes) are variable (Lewontin, 1974). A fixed gene is almost at a dead end although mutations can still change the fate of this gene (Kimura, 1983). However, a gene at a certain locus with varying alleles (gene forms) has a greater chance to adapt to different evolutionary pressures. Alleles become the unit of biological diversity (Butlin and Tregenza, 1998). Even if an allele is recessive and its frequency is very low, Mendel's law of segregation will still allow it to be useful in due "evolutionary" time.

How do we measure genetic diversity? Diversity is measured by counting the number of forms (alleles) and subsequent frequencies of genes, gene segments (DNA), gene products (proteins) and other segments of the genome of a species. Accumulated mutations enable this polymorphism to exist though not all mutations dramatically affect gene function or product (Hartl and Clark, 1989). The choice of what segments of the genome to use can be affected by several factors. Genes or loci that are dispersed throughout the genome optimise sampling and give a fair representation of the entire genome.

Allozymes are variants of an enzyme, having the same function and location in the cell (Markert and Moller, 1959). These variants are the alleles of an enzyme-coding gene or isozyme (e. g. ADH-1 and ADH-2) normally inherited in a Mendelian fashion and can be compared against Hardy-Weinberg ratios of neutral alleles (Gottleib, 1977). This inheritance mechanism in the (sexual) reproduction process is a crucial element in performing allozyme studies. Allozymes are a result of the different mutation events that have taken place at particular isozyme loci throughout the evolutionary history of a species (Gottlieb, 1977). These events modify DNA sequences for a particular locus and give rise to polymorphic forms of the DNA product. If one form is fixed the locus becomes monomorphic. A polymorphic locus in a diploid organism will express more than one allele (reviewed by Brown and Weir, 1983).

Isozyme alleles or allozymes are expressed by the differences in the final phenotype, e.g. varying protein sub-unit sizes and charges (Gottlieb, 1977). Because diploid organisms have two copies of any gene at each locus, the copies can either be the same allele (homozygous) or one of each (heterozygous). The expression of these alleles (genotypes) varies. Dominance results from the expression of a dominant homozygous allele and the heterozygote that contains an undetected recessive allele. Co-dominance however has a detectable phenotype for the heterozygotes that is intermediate between or a mixture of the two homozygotes. The latter can be detected by electrophoresis where different sub-units of the different inherited forms (allozymes) are laid out on an electrophoretic gel as banding patterns called zymograms. Furthermore these enzymes, usually glycolytic or tri-carboxylic, are believed to be dispersed throughout the genome of any species and assume linkage equilibrium among loci (Gottlieb, 1977; Brown and Weir, 1983). Hence, allozyme data become a collection of genetic information from multiple loci and can be treated as a gauge in estimating genetic diversity or genetic distance of individual plants in a population or among populations.

Enzymes, as gene products or proteins, have a structural heirarchy. The fundamental structure consists of a polypeptide chain of amino acids held together by carboxyl bonds. The sixteen different amino acids have positive, negative or neutral side chains. The charge, size and hydrophilicity of the side chains determine the secondary structure of the enzyme—the folded structure. The tertiary structure involves further folding due to thiol bonds, etc. The quaternary structure is characterised by the existence of more than one peptide chain (monomer). A dimer is made up of two peptide chains, a trimer, three peptide chains and so on (reviewed by Acquaah, 1992). The knowledge of the quaternary structure of an enzyme is crucial in reading the banding patterns that different allozymes leave on the electrophoretic gel. For example, an individual homozygous for a monomeric isozyme will display one allozyme band; a heterozygous individual will display two bands (Gottlieb, 1977).

There are two possible causes of allozyme variation: neutral mutations (e.g. Eanes *et al.*, 1993) and maintenance of balancing selection (e.g. Wayne *et al.*, 1996). The neutral theory as proposed by Kimura (1983) says that evolution at the molecular level is driven by random genetic drift of neutral mutations (mutations that has the same fitness as the other allele(s) at the same locus). The equilibrial level of polymorphism or heterozygosity is a balance between its elimination by genetic drift and creation by mutation. Neutral variation therefore arose through molecular evolution caused by events that are of little or no selective significance (Kimura, 1983). One evidence that Kimura (1983) cited was the constant rate

of evolution seen across taxa. The selectionist theory on the other hand claims that some evolutionary events are mainly caused by natural selection, a process where individuals in an environment best adapted to it increases in frequency after a number of generations relative to the ones not as well adapted.

Based on the assumption that allozymes are selectively neutral (Kimura, 1983), genetic drift should influence all isozyme loci equally (Gottlieb, 1977; reviewed by Linhart and Grant, 1996). Studies that show significant variation in leaf shape and size, and lack of allozyme variation in populations with fragmented distributions suggest that the morphological variation is influenced by selection and protein heterozygosity more by genetic drift or neutral evolution (Matolweni *et al.* 2000). However, using a large data base on allozyme variation in over 1500 species, Skibinski *et al.* (1993) found a positive correlation between the amount of polymorphism within species and evolutionary rate thereby concluding that a major percentage of protein variation or heterozygosity can be explained by variation in the rate of neutral mutation and some of it by strong selection. As more and more studies on enzyme evolution claim either theory or both theories the cause of enzyme variation (Dykhuizen and Hartl, 1983; Skibinski *et al.* 1993; Eanes *et al.* 1996; Terauchi *et al.* 1997; Eanes, 1999) the use of allozymes in population genetic studies remains a useful tool. [There was an initial concern about the extent of representation of the entire genome by allozyme data considering that they are usually enzymes in the glycolytic or tri-carboxylic acid pathways. When two-dimensional analysis of proteins associated with the cell membrane, ribosomes and other structural proteins gave lower heterozygosities than allozyme data and more recent DNA sequence analysis data provided compatible results to allozyme studies, the apprehension regarding misrepresentation abated (reviewed by Avise, 1994)].

1.4. Starch Gel Electrophoresis in Allozyme Studies.

Allozyme studies involve the genetic analysis of genetic groups (populations and species) using the estimation of population parameters such as degree of polymorphism and heterozygosity and allele count. Smithies (1955a and 1955b) first developed starch gel electrophoresis. Hunter and Markert (1957) further advanced its utility by using histochemical staining. Hubby and Lewontin (1966) showed that the differences in electrophoretic mobility seen in *Drosophila* were due to allelic variation. They were the first to use genetic parameters such as percentage polymorphism and heterozygosity (Hubby and Lewontin, 1966; Lewontin and Hubby, 1966).

Table 1.3. Comparison of the attributes of the four primary methods of protein electrophoresis on gel support media.[a] Adapted from Murphy *et al.* (1996).

Attribute	SGE	PAGE	CAGE	AGE
Separates by charge	Yes	Yes	Yes	Yes
Separates by size	Yes[b]	Yes[b]	No	No
Number of slices per gel	<6[b]	1	1	1
Toxic	No[b]	Yes	No[b]	No[b]
Running time	4-24 hrs	4-6 hrs	0.3-3 hrs[b]	3-4 hrs
Minimum amount of sample required per gel	2 µl	2µl	0.5µl[b]	1µl
Maximum amount of sample possible per gel	>50 µl[b]	>50µl[b]	5µl	>50µl[b]
Amount of stain required	5-50 ml	10-50 ml	1-3 ml[b]	10-50 ml
Electroendosmosis[c]	Yes	No[b]	Yes	Yes
Voltage required (V/cm)	1-10	5-10	<3[b]	20
Cooling required	Yes	At times	No[b]	Yes
Gel easily handled	Usually[b]	No	Yes[b]	Yes[b]
Simultaneously resolves cationic and anionic proteins	Yes[b]	No	Yes[b]	Yes[b]
Allows counterstaining of adjacent slices	Yes[b]	No	No	No

[a] SGE= starch gel electrophoresis; PAGE= polyacrilamide gel electrophoresis; CAGE= cellulose acetate gel electrophoresis; AGE= agarose gel electrophoresis.
[b] Perceived advantage
[c] Characterised "by a 'backwash' of buffer solution caused by gel charge groups that accelerates the mobility of cationic isozymes but retards or reverses the anionic isozymes" (Murphy *et al.*, 1996)

Starch gel electrophoresis has been used in plant population and breeding studies for more than four decades and its popularity is not only due to its value for money but also to its various advantages over other types of electrophoresis. Murphy *et al.* (1996) compares the different attributes of four types of protein electrophoretic methods that use a gel support medium in Table 1.3.

Morphological or phenotypic comparisons of individuals in and between populations were used to measure genetic diversity prior to the advent of gel electrophoresis. The former however is significantly affected by environmental factors (reviewed by Linhart and

Grant, 1996). More recent studies use morphological and quantitative trait analyses in conjunction with even more sophisticated tools like multilocus genotyping (e.g. random amplified polymorphic DNA and restriction fragment length polymorphism analyses) and single locus fragment analysis (e.g. allozymes and microsatellites of simple repeats and single locus minisatellites (Burdon *et al.*, 1980; Savolainen and Hedrick, 1995; Comes and Abbott, 1999; Hardig *et al.*, 2000). Both positive (Ouborg *et al.*, 1991; Linhart and Grant, 1996) and negative (Leeton and Fripp, 1991; Podolsky, 2001) relationships had been found between allozyme heterozygosity and morphological variance.

Apart from measuring diversity, quantitative traits and allozyme electrophoretic studies have been collectively used to: identify pure from hybrid lines (e.g. Rieseberg *et al.*, 1989), accurately discriminate taxa (e.g. Case *et al.*, 1998), determine phylogenetic relationships between wild and cultivated populations (e.g. Jenczewski *et al.*, 1999; Colunga-GarciaMarin *et al.*, 1999) and establish an easily reproducible method of identifying morphological forms of a species (Burdon *et al.*, 1980).

More novel molecular techniques using nuclear sequence data from RAPD or randomly amplified polymorphic DNA (e.g. Esselman *et al.*, 2000), internal transcribed- and external transcribed spacers of nuclear ribosomal DNA (Clevinger and Panero, 2000), non-coding chloroplast DNA (Bayer *et al.*, 2000) and other microsatellite (RFLPs and other molecular markers) analyses may report more accurate degrees of genetic diversity (Esselman *et al.*, 2000). Compatibility, however, is usually observed between molecular and allozyme data (Isabel *et al.*, 1995; O'Brien, 1995; Ross *et al.*, 1999) even though the former usually report higher diversity in terms of the degree of polymorphism and heterogeneity (e. g. Baruffi *et al.*, 1995; Isabel *et al.*, 1995; Esselman *et al.*, 2000). Nevertheless allozyme data, as in any study that provides a genetic diversity yardstick, are still considered a valuable conservation tool (Hannan and Orick, 2000; Lutz *et al.*, 2000).

1.5. Allozymes and Genetic Diversity Measures.

Allozyme diversity, as an estimate of genetic diversity, is measured by a) counting the proportion of isozymes that are polymorphic over all the isozymes assayed for, b) measuring the degree of heterozygosity of each of the polymorphic isozyme and c) measuring the frequency of all the alleles (allozymes) in a polymorphic enzyme system. The following analyses were used in this study.

1.5.1. Percentage of Polymorphic Loci (Nei, 1987).

Percentage of polymorphic loci in a population is measured by counting the number of loci that have multiple types of alleles (polymorphic) over the entire set of loci (monomorphic and polymorphic) multiplied by 100. A locus is considered "polymorphic" if the frequency of the most common allele is less than 95 % and the least common one is not less than 5% (Brown and Weir, 1983; Hartl and Clark, 1989; Ridley, 1996).

1.5.2. Expected and Observed Heterozygosity Within Populations (Nei, 1978).

Heterozygosity is the general measure of genetic variation at a locus in a population. In the Hardy-Weinberg equation, $p^2 + 2\,pq + q^2 = 1$, heterozygosity is represented by 2pq (the rest of the expression, $p^2 + q^2$ is the homozygosity). Expected heterozygosity is the fraction of the population that would be heterozygous if it is mating randomly while observed heterozygosity reflects the actual number of heterozygotes. Differences in expected and observed heterozygosity are due to deviations from Hardy-Weinberg equilibrium (including non-random mating, selection, mutation, drift and migration). Heterozygosity is calculated by averaging over all loci including loci that are not polymorphic. Allele frequencies are sufficient to calculate expected heterozygosity.

1.5.3. Allele Frequency (Hartl and Clark, 1989).

Alleles are the copies of genes inherited from two parents. In a population, allele frequency is the fraction of all the alleles in a locus that are of the same type. When a gene or locus is monomorphic there is only one type of allele (that allele is "fixed" at that locus) in the population or species. In a population of diploid organisms, there are at least two alleles in a polymorphic locus.

1.5.4. Genetic Divergence and Cluster Analysis (UPGMA) [Nei, 1972].

The calculation of a genetic distance between two populations within a species estimates the time that has passed since the populations have existed as a single unit. Genetic distance/identity measures such as that of Nei's (1972), counts the number of accumulated allele differences per locus and the results become the basis of the hierarchical (grouped similar items) arrangement called a dendogram.

A genetic distance value close to 0 indicates population substructuring due to isolation and a value close to 1 indicates gene flow among populations.

1.5.5. Population Differentiation or Subdivision (Wright, 1978).

1.5.5.1. Wright's F-statistics.

Wright's fixation indices (1951) employs three levels of genetic variation: (1) observed heterozygosity per locus for a population, denoted by H_o, (2) expected

heterozygosity of a randomly mating population, H_e (here 2pq becomes 2p*i*q*i*, where *i* refers to the *i*th population) and (3) expected heterozygosity per locus of all the randomly mating populations combined together, denoted by H_t.

The f-statistics derived from the above are:

$F_{IS} = (H_e - H_o)/ H_e$

$F_{IT} = (H_t - H_o)/ H_t$

$F_{ST} = (H_t - H_e)/ H_t$.

F_{IS} or inbreeding coefficient, measures the reduction in heterozygosity in an individual due to non-random mating within its population. A positive value suggests inbreeding or heterozygote deficiency while a negative value, outbreeding or heterozygote excess. Inbred populations usually have reduced heterozygosity because of the increased probability that individuals carry alleles that are identical by descent. Random mating gives values close to 0 and overdominance (see 1.3.5.2) gives negative values.

F_{IT} is the overall inbreeding coefficient of all individuals relative to the total population. It reflects both non-random mating within populations (F_{IS}) and subdivision among populations (F_{ST}).

F_{ST} or fixation index, measures the reduction in heterozygosity of a population or the effect of subdivision of populations. When averaged over all populations, it becomes the proportion of genetic diversity that resides among populations. F_{ST} is always a positive value. When it is closer to 0 it denotes random mating and the absence of subdivision among populations, when closer to 1 it suggests extreme subdivision among populations or complete isolation. [All three parameters are related by this equation: $F_{ST} = (F_{IT} - F_{IS}) / (1-F_{IS})$].

1.5.5.2. Heterozygote Deficiency or Excess.

Similar to Wright's (1951) F-statistics, the inbreeding coefficient or fixation index $f = [H_e-H_o]/H_e$ (Hedrick, 1983) also estimates the degree of inbreeding from the level of heterozygote deficiency/excess as compared to Hardy-Weinberg expectations. Heterozygote deficiency is related to inbreeding caused by selfing and mating between related individuals. There are two theories as to the cause of heterozygote excess: overdominance and dominance. In overdominance (or heterozygote advantage) the heterozygote genotype has a greater fitness. In the absence of selection, genotype frequencies (with two alleles A and a, there are three possible genotypes: AA, aa and Aa) are in Hardy-Weinberg equilibrium. The H-W frequencies in diploid organisms when there are two alleles are: $AA = p^2$, $aa = q^2$ and $Aa = 2pq$; all the frequencies add up to 1. When

selection operates, a genotype's chance of survival is altered depending on which allele or genotype is favoured. When A has a greater chance of survival, AA and Aa will have a fitness designation of 1 and aa will have a fitness designation of 1 – s (s is the selection coefficient). The H-W frequencies will be AA = p^2, Aa = 2pq and aa = $q^2(1-s)$ and only Aa has a fitness = 1. This is called heterozygote advantage. Dominance on the other hand proposes that the loci examined are neutral and the heterozygosity observed is the actual genomic heterozygosity (reviewed by Ridley, 1996).

1.5.5.3. G_{ST} (Nei, 1973).

F_{ST} is also equivalent to Nei's (1973) genetic diversity statistics, G_{ST}, which is calculated as: $G_{ST} = (H_T - H_S) / H_T$ (where H_T = total genetic diversity of pooled populations, and H_S = mean diversity within each population).

1.6. Objectives.

The preceding pages have shown that maintenance of biodiversity involves the conservation of genetic diversity (Avise, 1994). Determining the genetic diversity and structure of the *Olearia flocktoniae* metapopulation would give insight as to how to preserve genetic diversity in this endemic and endangered species. There are only 721 individuals left of the species in its natural environment (Mackay and Gross, 2002). Determining conservation priorities as well as planning for the species' future survival is a necessity. These are the main objectives in this thesis.

The objective of Chapter 2 is to determine the genetic diversity and population substructure of 14 above ground *O. flocktoniae* populations. The genetic diversity of each of the studied populations is investigated in terms of its degree of heterozygosity and polymorphism, allelic richness and allelic frequency. Population substructure across the 14 studied populations is estimated by calculating their genetic distance or identity and by using Wright's fixation indices. Results from this chapter will be compared with published averages of genetic parameters to determine whether they coincide or not. A discussion about these datasets will be presented.

The objective of Chapter 3 is to determine the utility of the seed bank for conservation. The genetic diversity and population substructure of 6 seed bank *O. flocktoniae* populations two of which were derived from extinct above ground populations is investigated. Comparisons are made between extinct and extant populations and the differences will be discussed. Seed banks with their corresponding above ground

populations will also be genetically compared and assumptions regarding their differences and similarities will be discussed.

Finally in Chapter 4 the objective is to bring together the results of the two previous investigations and recommended management strategies are given in light of the results and insights garnered from the two previous chapters.

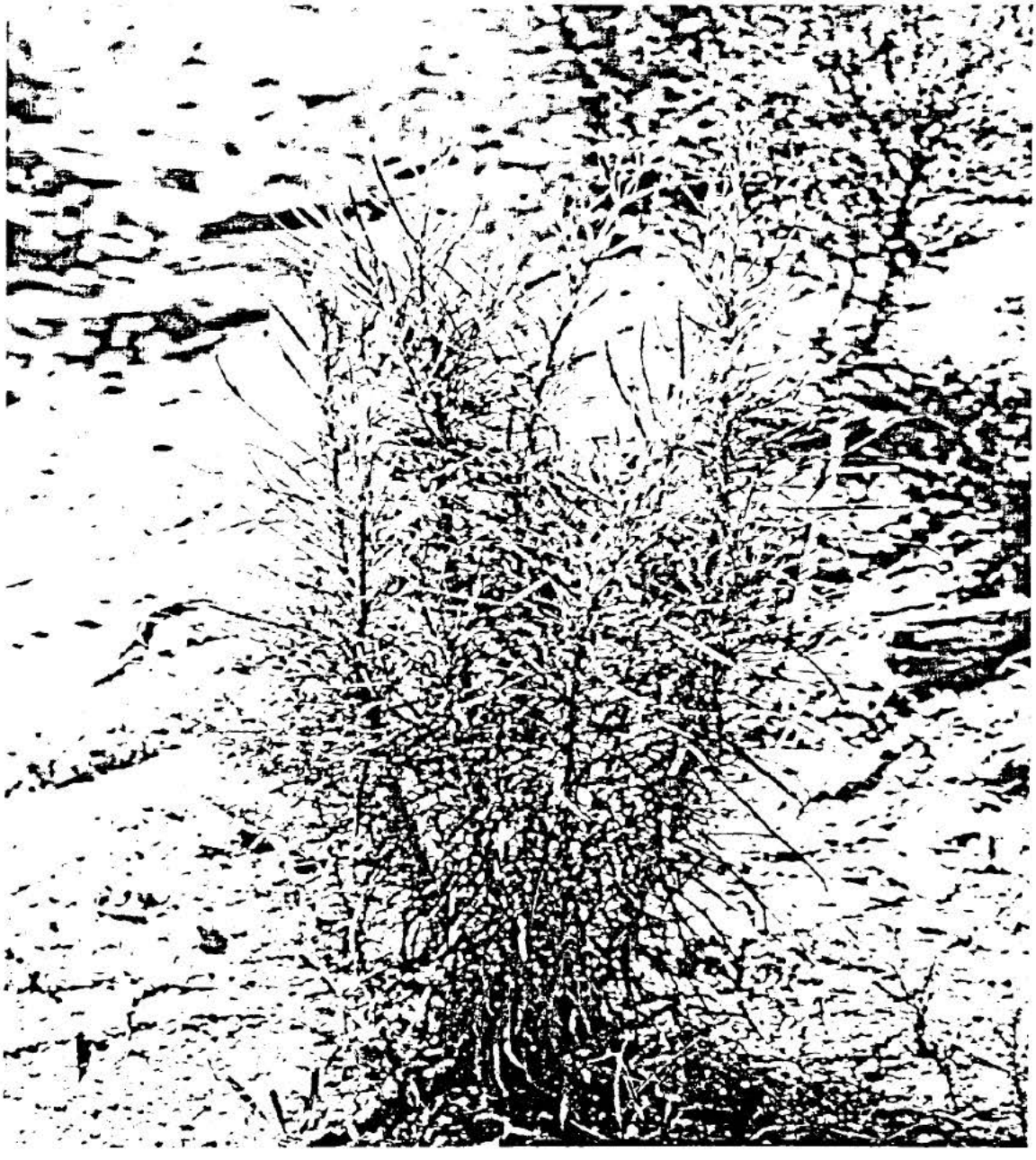

Fig. 1.5. *O. flocktoniae* juvenile plant. Source: C. L. Gross.

Chapter 2 – Patterns of Genetic Diversity Within and Among *Olearia flocktoniae* Aboveground Populations

"...Rarity precedes extinction." -Darwin (1859).

2.1. Introduction

Genetic diversity is the means by which evolutionary potential is optimised in populations (reviewed by Ridley, 1996). High genetic variation allows individuals in populations to possess a potential array of traits that are advantageous over others. These adaptive traits, having evolved at a particular environment, potentially allow populations to survive the dynamic changes that constantly take place (Ellstrand and Elam, 1993). While genetic variation is created by mutations (neutral or deleterious), it is maintained or intensified by migration or gene flow between populations with differing gene frequencies and by selection favouring heterozygotes (Lewontin and Hubby, 1966). [Note: Deleterious mutations are removed from populations by selection because of the 'load' they confer on the genome (Crow, 1993).] Other evolutionary parameters that affect genetic variation include random mating, founder effects and genetic drift. The conservation of diversity thus involves the investigation of genetic parameters in relation to and their connection with historical events, population structure and breeding systems.

This study aims to investigate the genetic structure of 14 *Olearia flocktoniae* populations, a subset of the metapopulation of 39 sites (Mackay and Gross, 1999) [studying the entire metapopulation was not economically and physically feasible]. A metapopulation is a group of interacting populations or subpopulations. These populations, varying in size, interact with each other through gene flow. A more continuous gene flow might have existed among these populations in the past. Historical events (catastrophes, diseases, anthropogenic disturbance), environmental changes (habitat quality deterioration), genetic changes (inbreeding and inbreeding depression) and life history (short annual or long-lived tree) determine the dynamics of a metapopulation (Ballou, 1994). In the case of *Olearia flocktoniae* which was first observed in a clearing in a virgin forest, the extensive subsequent clearings for logging and agriculture (Leigh *et al.*, 1984) may have changed the species' dynamics and interaction. This spatial and temporal dynamics affect extinction and recolonisation events. Through time some populations might go extinct and one or more of the surviving ones may seed or found new populations. Craig (1994) cited some advantages of metapopulations or of subdividing a large group into semi-independent populations. High genetic diversity is maintained when different populations maintain different

alleles. Random changes in the environment may allow the extinction of some populations but other populations will expand or colonise other areas. In times of catastrophe and disease, some populations may survive them and others may not. The existence of a metapopulation reduces the risk of total and complete extinction such as in *O. flocktoniae* where after 70 years of being presumed extinct, thriving healthy populations were rediscovered in 1986 (Gross *et al.*, 1998). However the same cycle of extinction-recolonisation is observed after nearly 20 years where populations are declining (Mackay and Gross, 1999, 2001, 2002) and threatened with extinction (Gross and Mackay, 1998).

In species with declining numbers and distribution such as *O. flocktoniae* a low genetic diversity is expected due to inbreeding. When populations and individuals in populations are low the limited array of alleles lead to reduced genetic variability. This condition of rarity could lead to extinction.

2.1.1. Rarity and Genetic Diversity of Plant Species.

Darwin (1859) noted that usually "rarity precedes extinction". Rarity of species however is a very broad term and in order for rare plants to be managed effectively each case must be studied individually and carefully (Kaye *et al.*, 1997). A clear understanding of the different forms of rarity and their differences is a good initial step. Rabinowitz (1981) outlined a matrix that described eight categories from the combination, seven of which classified the different forms of rarity (Table 2.1). The matrix consisted of geographic range (wide v. narrow), habitat specificity (broad v. restricted) and population size (large v. small).

The types of rare species that attract most conservation genetic studies are type III (in Table 2.1) – restricted geographic range, narrow habitat specificity and large population size (e.g. *Geum radiatum*, Godt *et al.*, 1996; *Lychnis viscaria*, Lammi *et al.*, 1999; *Erigeron parishii*, Neel and Ellstrand, 2001) and type VII – restricted geographic range, narrow habitat specificity and small population size (e.g. *Baptisia arachnifera*, Ceska *et al.*, 1997; *Brighamia insignis* and *B. rockii*, Gemmil *et al.*, 1998; *Gleditsia japonica* var. *koreaiensis*, Huh *et al.*, 1999; *Begonia dregei* and *B. homonyma*, Matolweni *et al.*, 2000; *Eriogonum ovalifolium* var. *williamsae*, Archibald *et al.*, 2001).

Table 2.1. The seven forms of rarity. Adapted from Rabinowitz, 1981 and Rabinowitz *et al.*, 1986.

POPULATION SIZE		HABITAT SPECIFICITY			
		Wide		**Narrow**	
	Large	Common	I. Rare Locally abundant over a wide range in a specific habitat type	II. Rare Locally abundant in several habitats but restricted geographically	III. Rare Locally abundant in a specific habitat but restricted geographically
	Small	IV. Rare Constantly sparse over a wide range and in several habitats	V. Rare Constantly sparse in a specific habitat but over a wide range	VI. Rare Constantly sparse and geographically restricted in several habitats	VII. Rare Constantly sparse and geographically restricted in a specific habitat
		Broad	**Restricted**	**Broad**	**Restricted**
		GEOGRAPHIC RANGE			

To illustrate how restricted the range, narrow the habitat and small the populations a rare species could have, consider the following taxa. *Baptisia arachnifera* is an endangered species restricted to a 16-km strip in southern part of Georgia, USA (Ceska *et al.*, 1997). The natural populations of *Dendroseris ssp.* (Asteraceae) in the Juan Fernandez Islands are almost always fewer than ten plants (Esselman, *et al.*, 2000) while *Centaurea corymbosa* (Asteraceae) from the south of France is endemic to an area of less than three square kilometres and has less than 500 individuals (Colas *et al.*, 1997). *Eucalyptus caesia* populations (2098 individuals in a total of 13 known populations in 1983) are confined to very specialized granite outcrops in south West Australia (Moran and Hopper, 1983). *Fontainea oraria* (Euphorbiaceae) is known to exist as a single population in Lennox Head, NSW (NPWS, 1999). Endemism is indeed often synonymous with rarity.

The subject of this study, *Olearia flocktoniae* (Asteraceae) has 50% of its populations [n=39 (1999), n=46 (2001)] having ten or less individuals in a survey in 1997 (Mackay and Gross, 1997) and declining steadily (Mackay and Gross, 2001; 2002). Like *F. oraria* it is listed as an endangered species in the NSW Threatened Species Conservation Act Schedule 1, 2 and 3 as well

as EPBC Act 1999 (www.ea.gov.au). It is of the type VII form as Rabinowitz described rarity - restricted in geographic range, narrow in habitat specificity and small in population size.

Table 2.2 shows genetic diversity and subdivision values from some rare and endangered species. Though rarity or endemism (being restricted to a particular area) in plant taxa is usually marked by low genetic variation, some exceptions have been observed especially involving short-lived or long-lived perennials. Taxa of recent origin that have arisen from the hybridisation of two parental species may display high levels of variability as seen in five species of *Hemerocallis* (Liliaceae) in Korea (Kang and Chung, 2000) and in two Louisiana *Iris* hybrid populations (Burke *et al.*, 2000). Speciation from a more widespread species (Edwards and Sharitz, 2000) could also result in high genetic diversity. Refugia populations could also harbour high genetic variation (e.g *Asclepias exaltata*, Broyles, 1998). One feature that rare and/or endemic species with high allozyme diversity often have is low gene flow (high G_{ST} and F_{ST} values) as is manifested by high levels of differentiation among their populations. This is maintained especially in isolated populations by genetic drift and mutations that have accumulated since the populations were separated (Martinez-Palacios *et al.*, 1999). Interestingly, the same phenomenon has been observed in populations of an endangered herbaceous perennial *Abronia macrocarpa* (Nyctaginaceae) that were of close geographic proximity. This phenomenon may be due to low vagility of seeds and pollen (Williamson and Werth, 1999) as opposed to the high vagility observed in a common monsoon rainforest tree *Syzgium nervosum* (Myrtaceae). The latter's populations had a relatively low $F_{ST} = 0.118$ (compared to *A. macrocarpa*: $F_{ST} = 0.272$) or high gene flow which was attributed to mobile frugivores (Shapcott, 1999).

2.1.1.1 Geographic Distribution.

Geographic range is one of the many factors that can influence the genetic diversity in plants (Hamrick and Godt, 1989). Hamrick and Godt (1989) compiled data from more than 450 plant species and summarised them into different categories including, geographic range (endemic, narrow, regional and widespread), breeding system (selfing, mixed- animal, mixed- wind, outcross- animal and outcross- wind), life form (annual, short-lived and long-lived/woody), and seed dispersal (gravity, attached, explosive, ingested and wind). Genetic diversity within plant species is most influenced by geographic range with endemics possessing only half the average within species gene diversity (h) of widespread species (0.10 and 0.20, respectively).

Hamrick and Godt's (1989) extensive review of plant genetic data (Table 2.3) and other similar summarised data enabled plant genetic researchers to compare their respective taxa to a broader database. Such comparisons could prove helpful in interpreting data and producing

meaningful conclusions. Karron (1987) however argued that comparisons between a certain species to an averaged value of all studied species might neglect the evolutionary significance of that species' history thereby weakening the interpretative power of the analysis being made (Gitzendanner and Soltis, 2000). A more helpful comparison can be made between a given species and its related taxa (Felsenstein, 1985; Karron, 1987; also see Table 2.5).

Widespread species tend to have more genetic variability when compared to their congeners with narrow distribution (e.g. Thomas and Bond, 1997; Freville *et al.*, 1998; Hannan and Orick, 2000). A similar observation was summarised from available data by Gitzendanner and Soltis (2000). Unfortunately no genetic data are available from any *Olearia flocktoniae* congener whether threatened or common.

2.1.1.2 Population Size.

Population size influences genetic diversity in a population because it determines the effects of random genetic drift. Small populations are most likely to be affected by genetic drift in the same way that small sample sizes tend to give a biased representation of the overall picture. Hence the smaller the population, the greater the genetic drift.

Though smaller populations tend to have lower genetic variation (Godt *et al.*, 1996; Weller *et al.*, 1996; Lammi *et al.*, 1999), population size and genetic variability are not always positively correlated (Berge, *et al.*, 1998; Lutz *et al.*, 2000; Senneville *et al.*, 2001). Populations founded by a single or a few individuals usually have a low genetic variation unless the founding individuals were heterozygous in a number of loci and that rapid population growth followed the bottleneck event (Knapp and Connors, 1999).

Small isolated populations have lower genetic variation because of obstructed gene flow and as a result they become more affected by the consequences of genetic drift. Heterozygote excess can be a result of a reduction in effective population size (Cornuet and Luikart, 1996), the number of individuals likely to contribute genes to the next generation. [Heterozygote excess is discussed in page 17.] Because *Olearia flocktoniae* populations are small, severe genetic changes could easily affect them and a drift towards any direction (whether homozygosity or heterozygosity) could be pronounced.

Table 2.2. Genetic Diversity Comparison of Rare, Endemic or Endangered Species. H_o = observed heterozygosity; H_e = expected heterozygosity; A = number of alleles at a locus. DNG = Data not given.

Species	Within Population			Among Population			F-statistic	Reference
	A	H_o	H_e	A	H_o	H_e		
Abronia macrocarpa (Nyctaginaceae) **Endangered** perennial herb	1.83	0.155	0.197	2.40	DNG	0.269	0.272	Williamson and Werth, 1999.
Agave victoriae-reginae (Agavaceae) **Endangered endemic** perennial shrub	2.2	DNG	0.335	DNG	DNG	DNG	0.236	M-Palacios *et al.*, 1999.
Antennaria soliceps (Asteraceae) **Narrow endemic** perennial herb	No variation							Bayer and Minish, 1993
Begonia dregei (Begoniaceae)[a] **Rare endemic** perennial herb	1.15	0.038	0.054	3.00	DNG	DNG	0.882	Matolweni *et al.*, 2000.
Begonia homonyma (Begoniaceae)[a] **Rare endemic** perennial herb	1.06	0.006	0.018	2.00	DNG	DNG	0.937	Matolweni *et al.*, 2000.
Brighamia insignis (Campanulaceae) **Endangered** perennial	1.1	0.056	0.056	1.4	0.045	0.063	0.635	Gemmil *et al.*, 1998.
Brighamia rockii (Campanulaceae) **Endangered** perennial herb	1.2	0.082	0.057	1.2	0.102	0.063	0.113	Gemmil *et al.*, 1998.
Calamagrostis cainii (Poaceae) **Rare** perennial herb	1.39	DNG	0.056	1.76	DNG	0.089	0.130	Godt *et al.*, 1996.
Calystegia collina (Convolvulaceae) **Endemic** perennial herb	2.11	DNG	DNG	2.63	DNG	DNG	0.392	Wolf *et al.*, 2000.
Carex misera (Cyperaceae) **Rare** perennial herb	1.44	DNG	0.082	1.96	DNG	0.125	0.161	Godt *et al.*, 1996.
Cistus obsbaeckiaefolius (Cistaceae) **Endemic** to Canary islands, shrub	1.27	0.112	0.099	DNG	DNG	DNG	0.299	Batista *et al.*, 2001
Cistus chinamadensis (Cistaceae) **Narrow endemic,** shrub	1.42	0.095	0.103	DNG	DNG	DNG	0.614	Batista *et al.*, 2001
Cypripedium kentuckiense (Orchidaceae) **Rare** perennial herb	1.15	0.044	0.042	1.33	0.045	0.05	0.182	Case *et al.*, 1998

Eriogonum ovalifolium var. *williamsiae* (Polygonaceae) **Narrow endemic** subshrub (single population)	2.6	0.29	0.29	DNG	DNG	DNG	0.01	Archibald *et al.*, 2001.
Geum radiatum (Rosaceae) **Rare** perennial herb	1.29	DNG	0.074	1.44	DNG	0.098	0.191	Godt *et al.*, 1996.
Lychnis viscaria (Caryophyllaceae) **Rare** perennial herb	1.3	0.056	0.05	DNG	DNG	DNG	0.43	Lammi *et al.*, 1999.
Lysimachia minoricensis **Extinct** herb	No variation							Ibanez *et al.*, 1999.
Pinus rzedowskii (Pinaceae) **Endangered** (narrow distribution), tree	DNG	0.162	0.219	1.8	DNG	0.22	0.175	Delgado *et al.*, 1999.
Sagittaria isoetiformis (Alismataceae) **Rare** perennial herb	1.40	0.209	0.218	1.90	0.425	0.399	0.348	Edwards and Sharitz, 2000.
Sagittaria teres (Alismataceae) **Rare** perennial herb	1.17	0.079	0.101	1.30	0.237	0.177	0.473	Edwards and Sharitz, 2000.
Saxifraga azoides (Saxifragaceae) **Rare** perennial herb	2.7	0.41	0.40	DNG	DNG	DNG	0.35	Lutz *et al.*, 2000.
Scutellaria montana (Lamiaceae) **Endemic** perennial herb	2.21	0.190	0.280	DNG	DNG	DNG	0.121	Cruzan, 2001
Tricyrtis flava (Liliaceae) **Restricted** perennial herb	1.45	DNG	0.099	2.05	DNG	0.168	0.409	Maki *et al.*, 1999.
Warea carteri (Brassicaceae) **Endemic** annual herb	1.87	0.018	0.026	DNG	DNG	DNG	0.304	Evans *et al.*, 2000.

[a] Polyacrylamide gel electrophoresis used for isozyme analysis.

Limited migration or gene flow could also result in postdisturbance niches wherein smaller populations, fragments of a bigger more continuous one, become well adapted to their new environments. Such populations may also have low interpopulation genetic diversity (Ceska *et al.*, 1997). Biota on oceanic islands are good examples of this phenomenon as a natural barrier for extensive gene flow exists. It is not surprising that one-third of all known endangered plants exist on islands (reviewed by Francisco-Ortega *et al.*, 2000).

Table 2.3. Allozyme variation within species and within population levels for higher plants. N = minimum number of species in a category; P = proportion of polymorphic loci; A = number of alleles at a locus; h =gene diversity; G_{ST} = F_{ST}. Derived from Hamrick and Godt (1989).

	Within species (S)				Within populations (P)			
Category	**N**	**P**	**A**	**h**	**P**	**A**	**h**	**G_{ST}**
Breeding system								
Selfing	113	42	1.69	0.12	20	1.31	0.07	0.51
Mixed - animal	64	40	1.68	0.12	29	1.43	0.09	0.22
Mixed - wind	9	74	2.18	0.19	54	1.99	0.20	0.10
Outcross - animal	164	50	1.99	0.17	36	1.54	0.12	0.20
Outcross - wind	102	66	2.40	0.16	50	1.80	0.15	0.10
Geographic range								
Endemic	81	40	1.80	0.10	26	1.39	0.06	0.25
Narrow	101	45	1.83	0.14	31	1.45	0.11	0.24
Regional	180	52	1.94	0.15	36	1.55	0.12	0.22
Widespread	85	59	2.29	0.20	43	1.72	0.16	0.21
Life form								
Annual	140	51	2.07	0.16	30	1.48	0.11	0.36
Short-lived	152	41	1.70	0.12	28	1.40	0.10	0.23
Long-lived, woody	110	65	2.19	0.18	50	1.79	0.15	0.08
Seed dispersal								
Gravity	198	46	1.81	0.14	30	1.45	0.10	0.28
Attached	55	69	2.96	0.20	42	1.68	0.14	0.26
Explosive	27	30	1.48	0.09	21	1.25	0.06	0.24
Ingested	67	46	1.69	0.18	32	1.48	0.13	0.22
Wind	105	55	2.10	0.14	43	1.70	0.12	0.14

2.1.1.3. Habitat Fragmentation.

Fragmentation affects the genetic structure of populations because it disrupts gene flow or migration. It may be caused by limited seed dispersal (Matolweni *et al.*, 2000), low pollination effectiveness (Martinez-Palacios *et al.*, 1999; Batista *et al.*, 2001), limited gene flow from source (continent) populations in the case of insular endemics (Maki, 2001), disruption of a once continuous distribution due to evolutionary selective changes in order

to adapt to an ecological niche (e.g. after glaciation, Godt *et al.*, 1996; Allphin *et al.*, 1998; Broyles, 1998; Sarthou *et al.*, 2001; after a geological event, Delgado *et al.*, 1999; due to competition from introduced species (Knapp and Connors, 1999); other unknown ancient isolating event (Moran and Hopper, 1983) or due to anthropogenic reasons (e.g. Godt *et al.*, 1996; Case *et al.*, 1998; Williamson and Werth, 1999; Archibald *et al.*, 2001; Neel and Ellstrand, 2001).

Anthropogenic threats are more than frequently cited as the cause of habitat fragmentation. For example, the only known population of the endangered steamboat buckwheat *Eriogonum ovalifplium* var. *williamsiae* is threatened by an interstate highway due for expansion, planned geothermal development, and other uncertainties brought about by mixed land ownership (Archibald *et al.*, 2001). *Rutidosis leptorrhynchoides* (Asteraceae) of southeast Australia occupies only 0.5% of its original ecosystem due to one and a half centuries of rural development (Brown and Young, 2000). Exotic species often affect the unfortunate demise of once widespread native species. For example, introduced fauna (herbivores) had been the cause of the inclusion of almost all endemic Juan Fernandez Islands' flora on the endangered species list (Ricci and Eaton, 1994).

Some species do not follow any pattern or broad generalizations like that compiled by Hamrick and Godt (1989). Two congeneric species of *Antirrhinum charidemi* and *A. valentinum* are both narrow endemic, out-crossing species with isolated populations. However the genetic structure of their populations was very different. Over 60% of the total variation in *A. valentinum* was distributed among populations while less than 10% in *A. charidemi*'s variation existed among populations (Mateu-Andres and Segarra-Moragues, 2000).

Founding events, where new populations are established by a few individuals or by a single fertilized female (Mayr, 1963), may also be caused by habitat fragmentation. Though these events do not necessarily result in genetically monomorphic populations (e.g. Knapp and Connors, 1999) they may present alleles that are rare in more continuous populations; these alleles can be utilized to measure the extent of migration among populations (Slatkin, 1985). New *Olearia flocktoniae* populations may have been reestablished by a founding event(s) and the presence of any rare allele(s) would point to a founder population.

2.1.2. Pioneer Species and Genetic Diversity.

Olearia flocktoniae as a pioneer species is a disturbance opportunist. The species can be observed appearing in clearings brought about by the logging of undisturbed forests and the construction of transmission power lines. Its disappearance had been observed to coincide with the closing in of the rainforest/wet sclerophyll forest margins (Gross and Mackay, 1998).

Pioneer species establish themselves by taking advantage of the abundance of light due to canopy clearing and disturbed soil. They are also characterized by their rapid growth enabling them to form a new canopy, hence *O. flocktoniae*'s appearance *en masse* as observed by Gross *et al.* (1998). A number of late-succession species found among *O. flocktoniae* plants include native and exotic shrubs and grasses (Gross *et al.*, 1998).

Table 2.4. Genetic diversity of four pioneer plant species. A_e = number of effective alleles; H = genetic diversity and F_{ST} = fixation index. DNG = Data not given.

Species	A_e	H	F_{ST}	Reference
Gentiana pneumonanthe **Rare** perennial herb	1.21	H_o= 0.12	F_{ST} =0.192	Raijmann *et al.*, 1994.
Helicteres brevispira Small tree or shrub **restricted** to tropical regions of South America	1.40	H_e = 0.24	F_{ST} = 0.12 (at 5 m diameter and 0.36 plants/m^2)	Franceschinelli and Kesseli, 1998.
Hippophae rhamnoides[a] Woody plant **common** in Europe, Central Asia and China	DNG	H_o= 0.16	Var $_{\text{between popns}}$ = 0.209	Bartish *et al.*, 1999.
Cecropia obtusifolia **Common** tree in Mexican coasts, Central and South America	DNG	DNG	F_{ST} = 0.029	Alvarez-Buylla and Garay, 1994.

[a]Using RAPD analysis.

A rare perennial herb *Gentiana pneumonanthe* was once common on disturbed open patches in heathlands of the Netherlands. Its current status (threatened with local

extinction) is attributed to soil and water table changes as well as agricultural land use. Population number as well as the number of individuals per population in this species had been in decline. Seedling recruitment has also decreased leaving small populations only with adult plants. *Helicteres brevispira* is a pioneer shrub that appears in the edge and gaps of tropical riparian forests in Brazil and the first to appear after pastures are abandoned. It only lasts for two generations before succession occurs and thus may rely on seed banks for genetic structure. *Cecropia obtusifolia* is an abundant pioneer species in Mexico. It appears in tree fall gaps in primary forests and artificially perturbed areas. Of the four pioneer species that have been assayed genetically, two of them are rare and the other two common.

Pioneer species are prone to local extinctions (Alvarez-Buylla and Garay, 1994; Raijmann *et al.*, 1994; Franceschinelli and Kesseli, 1998). Occasionally, locally abundant populations decline in number; some will reach extinction and subsequently recolonised after some time (Wright, 1940). When the number of colonisers is few, population substructuring is predicted to occur (Wright, 1978). Slatkin (1985) however showed that extinction and recolonisation could reduce population substructuring through augmented gene flow (the island-type model).

2.1.3. Genetic Diversity in the Asteraceae.

The Asteraceae family is distributed worldwide from the frigid to the temperate zones, in sub-tropical and tropical areas. In Australia there are over 200 genera and nearly 1000 species in this family (Clarke and Lee, 1987). Their populations range from narrow (e.g. Freville *et al.*, 1998; Maki and Morita, 1998; Ayres and Ryan, 1999; Torrell *et al.*, 1999; Neel and Ellstrand, 2001) to widespread distributions (e.g. Baskin *et al.*, 1997; Cherniawsky and Bayer, 1998; Freville, *et al.*, 1998; Godt and Hamrick, 1998a; Ayres and Ryan, 1999; Comes and Abbott, 1999; Muller-Scharer and Fischer, 2001). Life form is varied – from herbaceous annuals (e.g. Cronn *et al.*, 1997; Imbert *et al.*, 1999; Kim and Rieseberg, 1999; Muller-Scharer and Fischer, 2001;) to herbaceous (e.g. Godt and Hamrick, 1996; Cherniawsky and Bayer, 1998; Ayres and Ryan, 1999; Maki, 1999; Neel and Ellstrand, 2001; Jesus *et al.*, 2001) and woody (e.g. Schilling, 2001) perennials or a combination of life forms in one species (e.g. Westley, 1993; Bender, *et al.*, 2000). Breeding systems include self compatibility (e.g. Francisco-Ortega *et al.*, 2000; Muller-Scharer and Fischer, 2001), self-incompatibility or outcrossing (e.g. Fore and Guttman, 1999; Morgan, 1999; Cheptou *et al.*, 2000; Francisco-Ortega *et al.*, 2000; Luijten *et al.*,

2000), mixed selfing and outcrossing (e.g. Leeton and Fripp, 1991), apomixis (e.g. O'Connell and Eckert, 1999; Chapman *et al.,* 2000) and combined sexual and apomictic reproduction (e.g. Noyes and Soltis, 1996; Ayres and Ryan, 1999). Table 2.5 presents a few Asteraceae species of varying status with the corresponding genetic diversity data.

Table 2.5. Allozyme variation at species and population levels of some rare and endangered Asteraceae species. P = proportion of polymorphic loci; A = number of alleles at a locus; H = genetic diversity; H_o = observed heteozygosity; H_e = expected heterozygosity; H_T = total genetic diversity of pooled populations; H_S = mean diversity within each population and F_{ST} = fixation index. DNG = Data not given.

Species	Status	Genetic Diversity at Population Level	Genetic Diversity at Species Level	F_{ST}	Reference
Antennaria soliceps	Narrow endemic	No variation	No variation	DNG	Bayer and Minish, 1993.
Aster asa-grayi	Insular endemic	P = 0.11 A = 1.14 H = 0.029	P = 0.21 A = 1.7 H = 0.134	0.292	Maki, 1999.
Centaurea corymbosa	Endemic	P = 0.13 H = 0.05	P = 0.26 H = 0.07	0.35	Freville *et al.*, 2001.
Erigeron parishii	Narrow endemic	A = 2.2 H_o = 0.16 H_e = 0.17	DNG	0.12	Neel and Ellstrand, 2001.
Helianthus occidentalis	Restricted	A = 3.3 - 4.0 H_o = 0.39 - 0.55 H_e = 0.49 - 0.60	DNG	0.007 (between patches)	Fore and Guttman, 1999.
Rutidosis leptorrhynchoides	Endangered	H_S = 0.439 H_T = 0.454	DNG	0.033	Leeton and Fripp, 1991.
Rutidosis leptorrhynchoides	Tetraploids Diploids	A = 3.3 H_o = 0.34 H_e = 0.36 A = 2.5 H_o = 0.22 H_e = 0.25	DNG	DNG	Brown and Young, 2000.
Wyethia reticulata *Wyethia bolanderi*	Narrow endemic Common congener of above	H_o= 0.24 H_o= 0.35	H_T = 0.28 H_T = 0.38	0.18 0.17	Ayres and Ryan, 1999.

In summary, an evaluation of the literature has shown that the genetic diversity of plant species is influenced by its geographic distribution, population size and the existence of gene flow between populations. Rarity, a phenomenon found in *Olearia flocktoniae*, is also defined by the extent of a species' distribution, the specificity of its habitat and the size of its populations. However, it has also been shown that some species do not follow generalised patterns [e.g. Hamrick and Godt's (1989) table] in terms of genetic diversity.

In this chapter, the genetic diversity and population substructure of 14 above ground *O. flocktoniae* populations is investigated in terms of their degree of heterozygosity and polymorphism, allelic richness and allelic frequency. Population substructure across the 14 studied populations is also estimated by calculating their genetic distance or identity and by using Wright's fixation indices. Results from this chapter will be compared with published averages of genetic parameters to determine whether they coincide or not.

2.2. Materials and Methods

2.2.1. Sampling.

The populations of *O. flocktoniae* included for allozyme diversity analysis aimed to represent the geographic range of the species. Some populations that were close proximity-wise (e.g. ERA-110/140 and ERA-095) were selected to make a comparison in genetic relatedness. Since most populations were on roadsides, this particular type of site was over-represented.

Table 2.6. Populations of *Olearia flocktoniae* sampled for genetic assay (adapted from Gross & Mackay, 2001).

Population	Location	Size	Age	Site Description	Region[b]
ERA-110/140	E 460156 N6665478	139 plants	First surveyed 1996.	roadside and log dump	north west
ORC-030/060	E 449032 N 6663070	39[a]	First surveyed 1996.	roadside private property	far north west
EFD-030	E 455900 N 6662903	24	First surveyed 1999.	roadside and log dump	north west
SRW-000/015	E 456825 N 6665760	133	First sighted 1996.	power-line easement	north west
ERA-095	E 460420 N 6665373	3	First sighted 1998.	roadside	north west
SRW-055	E 456450 N 6665750	13	First sighted 2000.	roadside	north west

CRO-090/100	E 448850 N 6663550	35	First sighted 1998.	roadside	far north west
YRS-000/055	E 457119 N 6665853	34	First sighted 1997.	roadside	north west
BBR-175	E 478200 N 6654240	6	First sighted 1988.	roadside	north east
BBR-250	E 477637 N6654115	13	First sighted 1989.	roadside	north east
FHR-GP	E 483542 N 6656490	4[a]	First sighted 1986.	gravel pit	north east
MRR-340/370	E 475478 N 6660037	4[a]	First sighted 1988.	roadside	north
ESF-340	E 454250 N 6663750	78[a]	First sighted 1997.	clearing near log dump	north west
MRW-080	E 478150 N 6657350	26[a]	First sighted 2001.	roadside (forest trail)	north east

[a]From 2001 field survey.
[b]In relation to the town of Dorrigo. All populations are within 40 kms of Dorrigo.

2.2.1.1 Populations Sampled.

Fourteen populations from a metapopulation of forty-six (Mackay and Gross, 2001) were sampled between November 2000 and April 2001. These fourteen were chosen to represent a good spread of the whole distribution as well as populations proximal to each other to compare relatedness of neighbours. Some populations that were originally chosen for a more representative sampling became inaccessible (i.e. a felled tree obstructing a four-wheel drive path) or had become extinct (mass extinction of small clusters of populations had been observed in the previous years (Mackay and Gross, 2001).

In each population all individuals to a maximum of 25 were sampled. Table 2.6 describes the populations as reported in field surveys conducted by Mackay and Gross (2000 and 2001). The sample size was chosen in order to maximise the detection of alleles. To score an allele that occurs in 5% the population, there has to be at least 20 individuals per population.

Individuals in each population (with size greater than 25) chosen for leaf sampling were selected in such a manner that the whole population distribution is well represented (see Fig. 1.3). From each individual at least 10 immature leaves (first ten apical leaves) were picked from the field, stored in a resealable plastic bag with moist paper towel at approximately 4°C then within 8 hours transferred to a -70°C freezer for prolonged storage. Individuals sampled from above ground populations in Dorrigo were physically (as opposed

to reproductively) mature plants (not seedling nor sampling) of varying ages (estimated 6 months+).

2.2.1.2 Isozymes Used in the Study.

A compilation of other allozyme studies in Asteraceae (Table 2.7) assisted in determining which enzyme systems to use first for the genetic assay. Most popular polymorphic systems were tried first followed by monomorphic ones (e. g. malate dehydrogenase or MDH) and enzymes not in the list were tried last (e.g. sorbitol dehydrogenase or SDH). Samples were not assayed for TPI (triose-phosphate isomerase) because of the very high cost involved.

2.2.2. Electrophoresis.

2.2.2.1. Gel Preparation.

Gels were prepared from either 10.5% Sigma starch or 10% Connaught hydrolised starch. The former was prepared by combining 45 grams Sigma hydrolised starch (S-5651) with 120 ml gel buffer (see 2.2.3.1 for components of the buffer) in a flask and set aside. Three-hundred and ten ml of the same buffer was heated in a 1 L flask with an aspirator outlet to almost boiling and the well-mixed starch-buffer mixture added to it while stirring vigorously until a white cloudy even mixture was achieved. The latter was prepared using 41 g Connaught hydrolised starch (discontinued and substituted with Sigma) in 120 ml buffer at room temperature and 290 ml heated buffer. It was left to boil until tiny bubbles covered the flask wall and the whole mixture looked more transparent. The time to achieve this varied from buffer to buffer. Constant flask swirling also helped to achieve an even consistency. This also allowed for easy pouring.

After boiling, the mixture was immediately aspirated for about 20 seconds or until the smaller bubbles were dispersed and replaced by 1-2.5 cm bubbles that rose up the neck of flask (without touching the rubber stopper). Aspiration voids trapped gases and is deemed successful if bubbles are absent after the aspirator is turned off and the vacuum seal released. Further cooking was undertaken if minute bubbles could still be found after aspirating. The aspiration procedure was then repeated. The aspirated mixture was then poured into a gel mould [24 cm (length), 16.5 cm (width) and 1.0 cm (depth)]. A rectangular flat glass lid slightly bigger than the mould was carefully placed over it to ensure that no gas was trapped between the gel and the lid. Weights were placed on the glass lid to end up with a well-moulded gel (Winchester bottles made good weights). The

gel was left to cool at room temperature for an hour before it was carefully moved to the refrigerator without the weights. Gels refrigerated overnight gave better results than those refrigerated for a couple of hours as the former were firmer and thus easier to handle and slice. The volume of the cooked starch was sufficient to fill the gel moulds used in this study.

Slightly frozen gels were saved by leaving them to thaw for an hour on a bench at room temperature or while the leaf extraction procedure was being performed. For best results, the refrigerator temperature was constantly kept at 4°C. Gels were used for electrophoresis within 24 hrs after pouring. Older gels gave unclear bands.

2.2.2.2. Extraction and Loading of Gels.

Frozen leaf samples of c. 0.20 - 0.25 g were each homogenised in a microfuge tube with two drops of cold sucrose extraction buffer pH 7.38 (4.09 g sucrose, 2.03 g Na-ascorbate in 25 ml water, Acquaah, 1992) containing PVP-10. Four grams of PVP-10 (phenol-binding reagent) is added to 10 ml of sucrose extraction buffer and used within a week (Soltis *et al.*, 1980). A stainless steel rod was used for macerating the tissue in the solution.

The homogenate was centrifuged for 5 minutes at 6400 rpm to precipitate coarse matter then replaced in a container of icy water. A 7 mm x 2.5 mm wick made from Whatman paper no. 3 was submerged into a microfuge tube for at least three minutes, blotted onto blotting paper and loaded into gel. Wells were punched using a paper fastener arm 3.0 mm wide and about 0.5 mm thick into a solid gel one-third through its length (about 6 cm from bottom). Care was taken when inserting the blotted wicks so as not to disrupt the walls (about 3.0 mm thick) between wells. This could otherwise result in bleeding of individual samples into each other making the resulting bands not as well defined. Loading gels one-third through its length allowed for sufficient migration of anodal-migrating sub-units. Only anode-proximal gels were used in this work to stain for cathodal running bands (except for PRX- where samples were loaded in the middle of the gel to allow staining for anodal bands as well).

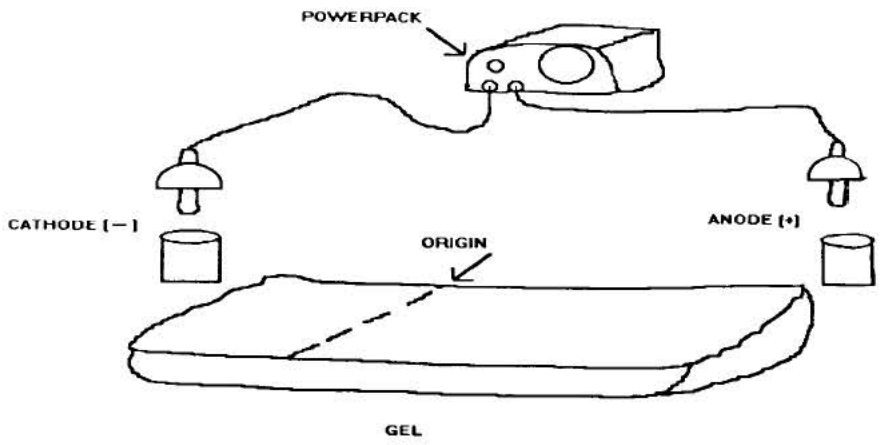

Fig. 2.1. Electrode set-up for starch gel electrophoresis. Source: C.L. Gross.

Bromo-phenol blue was helpful in keeping track of sample migration within the gel but its use was discontinued because of eventual familiarisation of sample migration and its inaccuracy as a tracking tool – with most buffer systems it moved faster than the samples. Some buffer systems allowed for the visualisation of migration in the absence of a tracking dye; this could be observed as a brown line that ran across the gel. When this line was compared to the tracking dye, a space of ~2 cm could be observed.

Table 2.7. Methodology data from selected Asteraceae allozyme studies. Systems in bold were found to be polymorphic. DNG = Data not given.

Reference	Taxa	Enzyme Systems Investigated	Sample	No. of Samples	Starch Concentration	Amperage (constant)
Comes and Abbott (1998)	*Senecio gallicus*	**ACP, AAT, EST, IDH, G3PD, PGI, PGM**	leaves	~10	DNG	DNG
Comes and Abbott (1999)	*S. vernalis* and *S. glaucus*	**AAT, EST, IDH, G3PD, PGI, PGM**	leaves	10	DNG	DNG
Fore and Guttman (1999)	*Helianthus occidentalis*	GPI, PGM	leaves	DNG	15%	DNG
Freville *et al.* (1998)	*Centaurea* spp.	**AAT, ALD, CAT, DIA, EST, LAP (AMP), ME, PGD, PGI**	leaves	16-70	DNG	DNG
Garnatje *et al.* (1998)	*Cheirolophus* spp.	**ACO,** IDH, **LAP,** MDH, **PGI, PGM, 6PGD, SDH**	seedlings	20	11.50%	DNG
Haase *et al.* (1993)	*Helichrysum* spp.	**ACO, ACP, EST, G3PD, IDH, PGI, 6PGD, SKD, SOD**	leaves	30	11.50%	50 mA
Leeton and Fripp (1991)	*Rutidosis* spp.	**ADH, AP,** AMP, **G6PD, GPI,** GDH, **IDH,** MDH, MR, **PGD, PGM, SDH**	leaves	30	12.50%	70 mA (L), 60 mA (H)
Maki (1999)	*Aster asa-grayi*	ACP, ME	leaves	30	DNG	DNG
Reiseberg and Warner (1987)	*Tragopogon* spp.	**ADH, EST, GDH, G6PDH, LAP**	seedlings	10	12.50%	DNG
Sun and Ganders (1988)	*Bidens* spp.	PGI, SKD	seedlings	10	14%	DNG
Warwick and Gottlieb (1985)	*Layia* spp.	ALD, **CAT, G3PDH, HEX, IDH, LAP,** MDH, **ME, PGI, PGM, SKD, TPI**	seedlings, young tissues	~22	DNG	DNG

2.2.2.3 Running Gels.

Electrophoresis was conducted in chilled eskies and in the refrigerator at 4°C. The buffer trays each contained the electrodes (cathode on the left and anode on the right; see Fig. 2.1); they were filled sufficiently for both the platinum wires to be immersed in buffer. The loaded gel was covered with a plastic wrap and placed on and between the buffer trays. The electrode buffer in the tray should be compatible with the gel buffer used, whether it was a continuous or discontinuous system. For example, if the continuous buffer system #1 (histidine-citrate, pH 5.7) was used, the electrode buffer should be histidine-citrate pH 5.7 and the gel buffer made by diluting 1 part of the electrode buffer to 6 parts water. Getting the right constituents in a buffer system is very crucial. Even a change of pH level can affect the running of the gel and thus the resultant zymogram (stained gel).

2.2.2.3.1 Buffer systems used in this study (Wendel and Weeden, 1989):

Buffer system #1. Histidine-citrate, pH 5.7
Electrode buffer: 0.065 M L-Histidine, free base
0.019 M Citric acid, monohydrate
Gel buffer: Prepared by diluting 1 part electrode buffer with 6 parts water.

Buffer system #3. Tris-citrate, pH 7.0
Electrode buffer: 0.135 M Tris
0.043 M Citric acid, monohydrate
Gel buffer: Prepared by diluting 1 part electrode buffer with 14 parts water.

Buffer system #2. Morpholine-citrate, pH 6.1
Electrode buffer: 0.04 M Citric acid, monohydrate
0.068 M N-(3-Aminopropyl)-morpholine
Gel buffer: Prepared by diluting 1 part electrode buffer with 19 parts water.

Buffer system #5. Sodium-borate, pH 8.0 / Tris-citrate, pH 8.6 (discontinuous system)
Electrode buffer: 0.3 M Boric acid (adjusted to pH 8.0 with NaOH)
Gel buffer: 0.076 Tris (titrated to pH 8.6 with citric acid)

Buffer system #6. Lithium-borate, pH 8.3 / Tris-citrate, pH 8.3 (discontinuous system)
Electrode buffer: 0.192 M Boric acid, titrated to pH 8.3 with LiOH
Gel buffer: Prepared by adding 1 volume of electrode buffer to 9 volumes of tris-citrate buffer, pH 8.3.

Table 2.8. Enzyme Systems Studied and Their Recommended Buffer Systems.

Enzyme System	Recommended Buffer System (Wendel and Weeden, 1992)	Buffer System(s) Used in this Study with Corresponding Voltage and Running Times[a]
Aspartate aminotransferase (AAT)	5 or 6	6 (6 hrs @ 400V)
Acid Phosphatase (ACP)	No specific recommendation	3 (5 hrs @ 350V)
Alcohol dehydrogenase (ADH)	5 or 6	6 (6 hrs @ 400V)
Aldolase (ALD)	1 or a modification thereof	3 (5 hrs @ 350V)
Catalase (CAT)[c]	3, 5 or 6	6 (6 hrs @ 400V)
Diaphorase (DIA)	No specific recommendation	6 (6 hrs @ 400V)
Glutamate dehydrogenase (GDH)	None recommended	7 (5 hrs @ 400V)
Glyceraldehyde-3-phosphate dehydrodenase (G3PDH)	1, 2, 3 or 4	3 (5 hrs @ 350V)
Glucose-6-phosphate dehydrogenase (G6PDH)	3, 5, 6 or 7	6 (6 hrs @ 400V)
Isocitrate dehydrogenase (IDH)	1, 2, 3 or 4	2 and 6 (6 hrs @ 500V[b])
Malate dehydrogenase (MDH)	1 or a modification thereof	2 (6 hrs @ 400V)
Malic enzyme (ME)	1	1 (6 hrs @ 500V[b])
Phosphogluconate dehydrogenase (PGD)	1, 3 or 4	1 (6 hrs @ 500V[b])
Phosphoglucomutase (PGM)	1, 3 or 4	3 (5 hrs @ 350V)
Peroxidase (PRX)	1, 2, 3 or 4	3 and 5 (5 hrs @ 350V)
Superoxise dismutase (SOD)	No specific recommendation	6 (6 hrs @ 400V)
Xanthine dehydrogenase	DNG	3 (5 hrs @ 350V)

[a] At constant current of 70 mA.
[b] At a constant 500V, current does not reach 70 mA but ranges from 55-66 mA.
[c] CAT is a tetramer and controlled by a few loci; the various heterozygotes were hard to resolve.

Particular enzyme systems work best with certain buffer systems (Wendel and Weeden, 1989; Acquaah, 1992). For instance samples migrated so fast in buffer system (BS) #3 gels that ADH and DIA could not be resolved properly in them. Both needed a buffer system that allowed samples to migrate slowly so that the bands can be stretched out and distinct zones can be seen. Systems #5 and #6 gave good ADH and DIA zymograms. PRX, a monomer with 1 locus, has bands that appeared just above the wells of origin. Either slow or fast runs did not seem to affect the visualisation of this enzyme.

2.2.2.4 Slicing Gels.

After electrophoresis, gels were immediately stained for visualisation. A 1-cm thick gel was sliced into three or four sheets using a taut string (See Fig. 2.2). [Gels that have been heated up by electrophoresis were not only hard to slice but also very fragile and disintegrated easily. Maintaining electrophoresis at 4°C was crucial.]

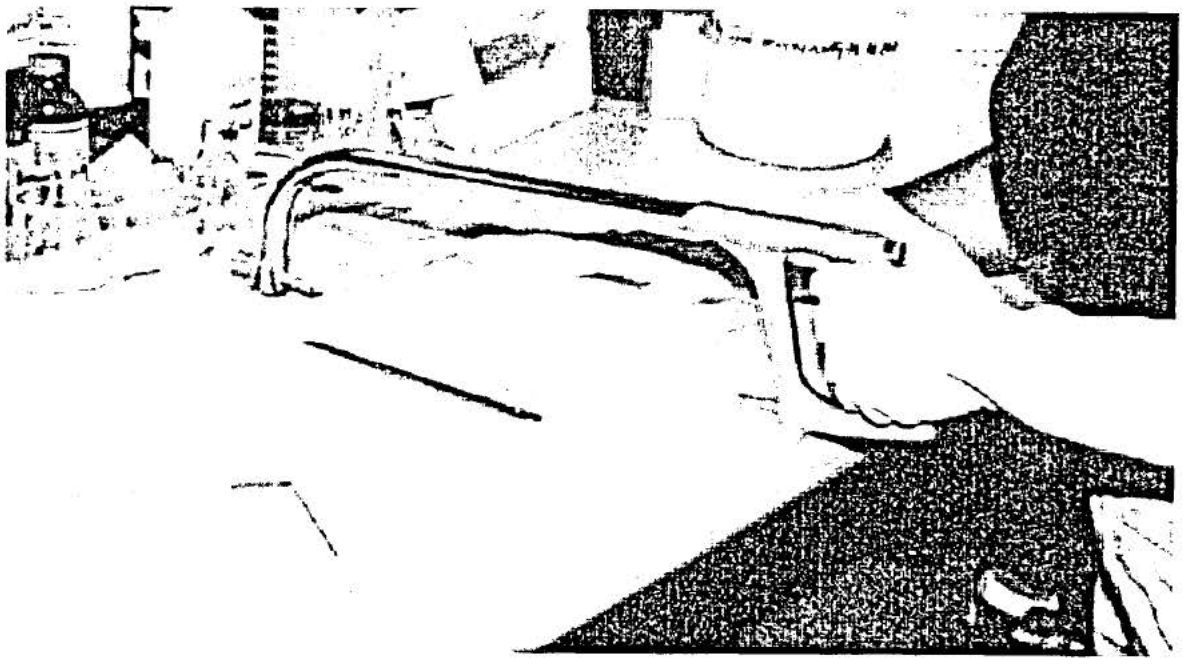

Fig. 2.2. A 1-cm thick gel sliced into three or four sheets using a taut string.
Source: C.L. Gross.

2.2.2.5 Staining Gels.

The optimum methods for staining are outlined in Appendix I.

2.2.2.6 Interpretation of Gels.

The banding patterns discussed below involved cathodal migration unless otherwise stated. No parental assays were conducted to determine inheritance of bands. Since quaternary protein structures are highly conserved in evolution (Weeden and Wendel, 1989) previously published works were relied upon in interpreting banding patterns. However visualizing both homozygote alleles (e.g. fast/fast and slow/slow) as well as the hetrozygote allele (e.g. slow-fast for monomers and slow-medium-fast for dimers) in one gel confirms published results.

Enzyme systems in *underlined italics* were used in the genetic assay (fifteen in all).

AAT.

AAT is a dimer and could be controlled by two or more loci [heteromer] (Weeden and Wendel, 1989). Only heterozygotes were observed in this study (three zones in every banding pattern) and were of different zone intensities. This made scoring difficult. This enzyme system was abandoned.

ACP.

ACP is a monomer or dimer (Acquaah, 1992; Weeden and Wendel, 1989). Fresh young leaves allowed for the visualisation of this enzyme (Fig. 2.3). It proved to be monomeric in seed bank assays where the bands were scorable. Older leaves, whether fresh or frozen at -70°C, gave unresolvable bands (from a pilot study conducted in 1999). Only seed bank samples were assayed for ACP.

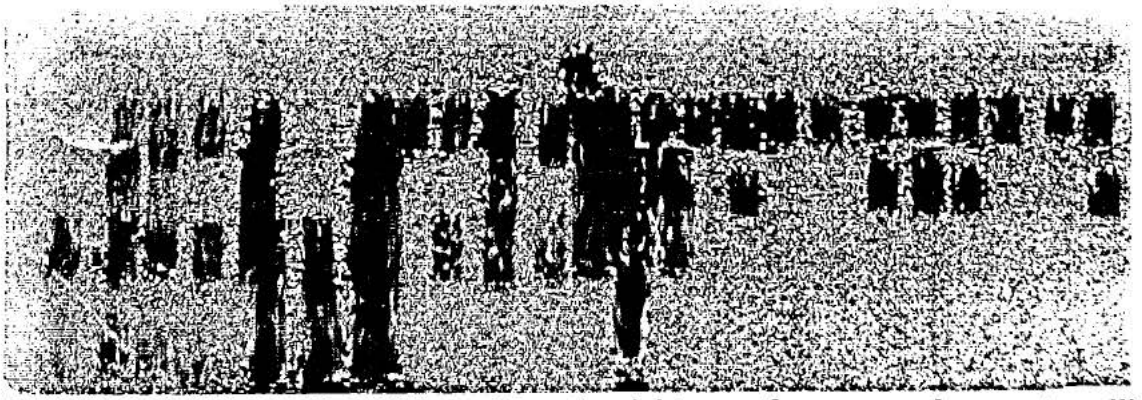

Fig. 2.3. ACP bands: first 10 lanes from the right were from young leaves at seedling or sapling stages. The rest were from adult plant leaves.

ADH.

ADH is a dimer and could be controlled by more than 1 locus (Weeden and Wendel, 1989). Only heterozygous bands were seen. Like AAT, three zones of activities with different intensities were observed. Frozen samples did not give resolvable ADH bands. Because of the aforementioned reasons, ADH was abandoned.

ALD.

Aldolase is a tetramer (Weeden and Wendel, 1989) and was monomeric.

CAT.

Different staining protocols (Weeden and Wendel, 1989; Acquaah, 1992; Murphy *et al.*, 1996) could not give bands clear enough for scoring hence it was abandoned.

DIA.

DIA bands did not separate enough for reliable scoring even though different buffer systems were tried. Fresh samples gave darker bands though. This system was abandoned.

GDH.

GDH is either a dimer or a hexamer (Weeden and Wendel, 1989). Only monomorphic homozygous bands were observed in *O. flocktoniae*. These bands moved about 2 cm from the origin and could also be seen in gels stained for G6PDH. SOD bands could be detected from GDH gels (ascertained by comparing SOD bands found in ADH gels).

GPI 1 and 2.

GPI, a dimer (Weeden and Wendel, 1989), had two isoforms that were difficult to score initially because they were very proximal to each other. Tracing the bands on transparency sheets proved helpful in scoring.

G6PDH 1 and 2.

G6PDH is reported as a dimer (Acquaah, 1992). Monomorphic bands from two isozymes (Weeden and Wendel, 1989) were detected 8 cm from the origin.

G3PDH.

G3PDH is a tetramer (Weeden and Wendel, 1989). All seed bank samples assayed were monomorphic.

IDH.

IDH is a dimer (Weeden and Wendel, 1989; Murphy *et al.*, 1996) and was polymorphic in *O. flocktoniae* populations (Fig. 2.4).

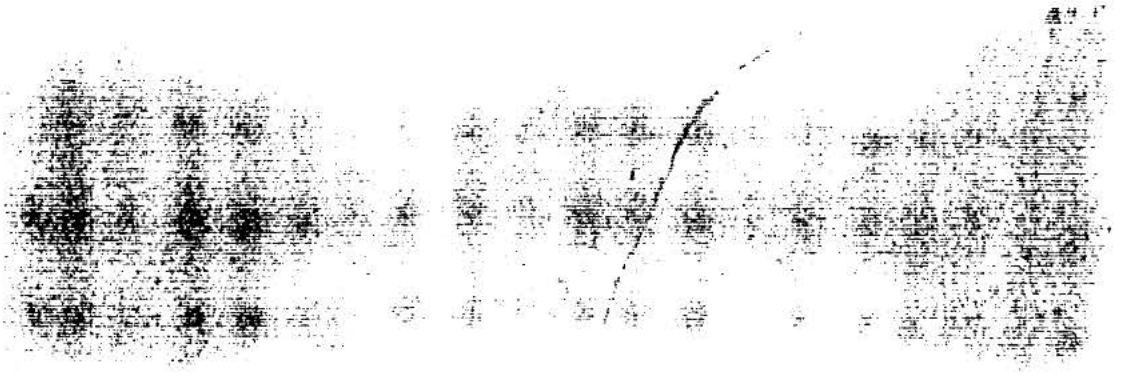

Fig. 2.4. Heterozygous IDH bands from adult and sapling *O. flocktoniae* leaves.

ME.

ME is a tetramer (Weeden and Wendel, 1989). ME bands in *O. flocktoniae* were found cathodally 2 cm from the origin and were monomorphic.

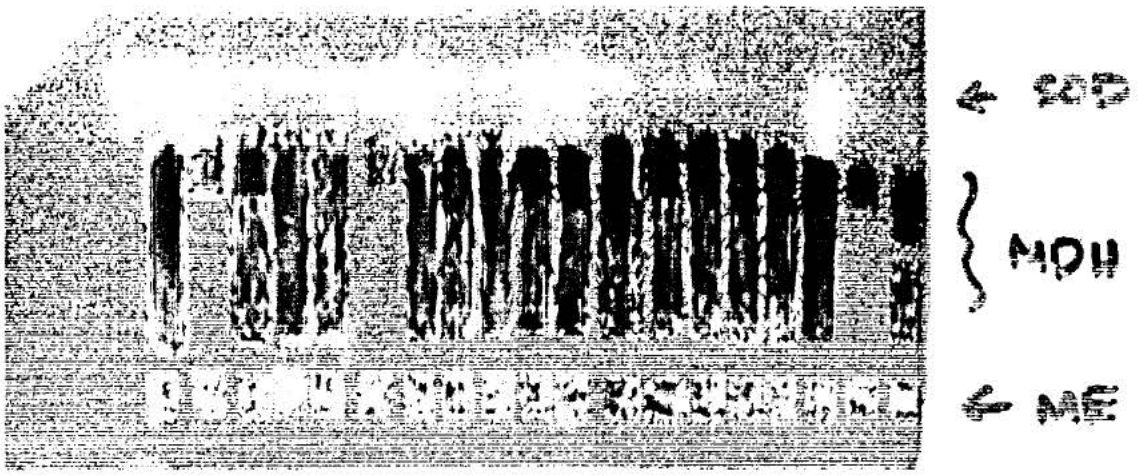

Fig 2.5. ME, MDH and SOD bands from adult and sapling *O. flocktoniae* leaves.

MDH.

MDH is a dimer (Weeden and Wendel, 1989). MDH bands could be observed cathodally more than 6 cm from the origin but no anodal bands were observed despite presence in other species. Overnight runs had been performed to stretch the bands and allow for the visualisation of more than one isozyme. Only one was observed. Four percent sucrose was added to BS2 starch gels when staining for MDH to allow better resolution.

PRX.

There were four PRX bands observed in this study, three of which were cathodal. They were monomeric (Weeden and Wendel, 1989) with zones of heterozygote bands having varying intensities. This made scoring difficult for the PRX+s. One isozyme that migrated anodally gave polymorphic bands with relatively uniformly stained zones (Fig. 2.6).

Fig. 2.6. Anodal PRX bands.

PGD 1 and 2.

PGD, a dimer (Weeden and Wendel, 1989), was monomorphic in all two isoforms (Weeden and Wendel, 1989). Of the three bands that were found in all gels stained for PGD, the lowest was 8 cm from the origin and the next was 1 cm higher.

PGM.

PGM was monomeric (Weeden and Wendel, 1989). Only anodal bands were observed and they were all monomorphic. Only one isozyme of the reported two (Weeden and Wendel, 1989) was observed.

SOD.

Gels stained for MDH and GPI contained SOD bands (see Fig. 2.5). All populations studied (seed bank and wild individuals) were polymorphic for SOD. This enzyme is reported to be either a dimer or a tetramer (Weeden and Wendel, 1989). In *O. flocktoniae*, SOD was dimeric and did not seem to be controlled by more than one locus since zone intensities were fairly even. SOD could be resolved from fresh samples, from samples frozen at –20°C overnight and at -70°C for six months. SOD bands could also be detected in gels stained for GDH and ADH.

XDH.

XDH is a monomer (Murphy *et al.*, 1996). Two bands were seen (possibly fast and slow alleles) but could not be reliably scored.

2.2.3. Statistical Analysis.

Standard measures of genetic diversity were calculated for each population using the computer program GDA [Genetic Data Analysis] (Lewis and Zaykin, 2000). They included percentage of polymorphic loci (P), mean number of alleles per locus (A) and per polymorphic locus (AP), mean observed heterozygosity (H_o) and expected heterozygosity (H_e) within populations and at the species level. Effective number of alleles (A_e) was computed using the formula $A_e = [1/(1-H_e)]$. To calculate deviations from Hardy-Weinberg equilibrium, Wright's fixation index was used for each polymorphic locus within populations. Fischer's exact test analysed each polymorphic locus to determine if differences in allele frequencies existed. Heirarchical population genetic structure was described using f, F and θ_p (Weir and Cockerham, 1984) - also known as F_{IS}, F_{IT}, and F_{ST}, respectively (Wright, 1951) - and accounts for effects of uneven sample sizes and number of populations sampled.

Nei's (1972) genetic distance and identity were computed among populations. TFPGA (Miller, 1997) provided UPGMA analysis- a graphical representation of the genetic distance data. Correlation analyses were performed between genetic diversity parameters and population size and population age using regression analysis.

The spatial study outlined in this section only allows us to endeavour to construct a genetic historical picture of these populations. After being presumed extinct for over 70 years, conducting genetic assays using allozyme data on a subset of the 46 extant populations of this rare species would answer some interesting queries about these populations. How genetically diverse are they? Does differentiation exist within these populations or is it distributed among them (indicating population interaction in terms of gene flow)? How similar are individuals and populations to each other? Is there a founder population?

2.3. Results

2.3.1. Genetic Diversity.

Olearia flocktoniae populations in this study displayed considerably low polymorphism and allele frequency (P = 0.32 and A = 1.32) that are comparable to levels found in endemic species (P = 0.29, A = 1.41; Godt *et al.*, 1996). Of 15 resolvable isozyme loci only 6 were polymorphic (GPI-1 and-2, IDH, MDH, PRX- and SOD). The proportion of polymorphic loci (P) within populations ranged from 0.143 to 0.400 with an average of 0.319 (SE±0.018). The number of alleles per locus ranged from 1.14 to 1.40 with a mean A=1.32 (SE±0.018). Observed heterozygosity (H_o) ranged between 0.066 and 0.310 with an average of 0.203 (SE±0.021). Expected heterozygosity (H_e) ranged from 0.071 to 0.210 and has an average of 0.149 (SE±0.010), slightly higher than Godt *et al.*'s (1996) value of 0.10 for endemic species and more comparable to Hamrick and Godt's (1989) value for widespread species (H_e = 0.16). The number of effective alleles (A_e) per population ranged from 1.08 to 1.26 (mean = 1.17). All populations had a number of alleles per polymorphic locus (A_p) of 2.0. The mean observed heterozygosity was higher than the mean expected heterozygosity giving a mean H_o/H_e of 1.35.

ERA-095, a population with only three individuals has the lowest degree of polymorphism, number of alleles per locus, number of effective alleles and expected heterozygosity (P=0.143, A=1.14, A_e =1.08 and H_e=0.071). SRW-055 has the lowest observed heterozygosity (H_o=0.066). EFD-030 displayed the greatest proportion of polymorphic loci and number of alleles per locus (P=0.40 and A=1.4). BBR-175 has the

highest degree of expected heterozygosity (H_e=0.210) and FHR-GP the highest observed heterozygosity (H_o=0.310).

2.3.2. Wright's F-statistic and Hardy-Weinberg Equilibrium.

An over-all fixation index *f* of –0.340 for all populations suggests heterozygote excess with only two populations (ORC 030/060 and SRW-055) having positive values of 0.180 and 0.316, respectively.

Using Fisher's exact test based on heterozygote excess (Table 2.11), only 45.2% (n = 84) of individual loci were found to be in Hardy-Weinberg equilibrium at $P \leq 0.05$ [or 52.4% (n = 84) at $P \leq 0.01$]. The mean inbreeding coefficient of individuals within a population F_{IS} (-0.344) was significantly different from 0 thus showed departure from Hardy-Weinberg expectations in populations. The mean inbreeding coefficient for all populations F_{IT} (-0.1325) also showed considerable deviation from Hardy-Weinberg expectations over all populations. Genetic differentiation among populations was represented by F_{ST}= 0.15 indicating that 85% of the genetic diversity in this species is distributed among or common to all populations.

Table 2.9. Levels of allozyme variation within 14 populations of *Olearia flocktoniae*: n = number of individuals screened, N = population size, P = proportion of polymorphic loci, A = number of alleles per locus, A_e = number of effective alleles per locus, H_e = expected hereozygosity, H_o = observed heterozygosity and f= fixation index (=[H_e-H_o]/H_e).

Population	n	N	P	A	A_e	H_e	H_o	f
ERA-110/140	26	139	0.357	1.357	1.22	0.179	0.306	-0.734
ORC-030/060	25	39	0.357	1.357	1.19	0.160	0.131	0.180
EFD-030	23	24	0.400	1.400	1.15	0.134	0.157	-0.179
SRW-000	23	133	0.267	1.267	1.13	0.118	0.141	-0.194
ERA-095	3	3	0.143	1.143	1.08	0.071	0.095	-0.529
SRW-055	14	13	0.231	1.231	1.10	0.095	0.066	0.316
CRO-090/100	25	35	0.267	1.267	1.14	0.124	0.187	-0.515
YRS-000/055	26	34	0.357	1.357	1.17	0.148	0.166	-0.122
BBR-175	3	6	0.357	1.357	1.26	0.210	0.286	-0.500
BBR-250	7	13	0.357	1.357	1.21	0.171	0.245	-0.485
FHR-GP	6	6	0.357	1.357	1.24	0.193	0.310	-0.708
MRR-340/370	5	5	0.357	1.357	1.22	0.181	0.258	-0.500
ESF-340	24	78	0.357	1.357	1.18	0.152	0.268	-0.793
MRW-080	21	26	0.308	1.308	1.17	0.146	0.231	-0.599
Mean	**16**		**0.319**	**1.32**	**1.18**	**0.149**	**0.203**	**-0.382**

(SE)			(0.018)	(0.018)	(0.014)	(0.010)	(0.021)	(0.090)
At species level			**0.40**	**2.0**				
Endemic[a]			**0.29**	**1.41**		**0.10**		
Widespread[b]			**0.43**	**1.72**		**0.16**		

[a] Godt *et al.* (1996)

[b] Hamrick and Godt (1989).

Table 2.10. Genetic diversity within populations (H_e), total genetic diversity (H_t) and F-statistics for 6 loci in 14 populations of *O. flocktoniae*.

Locus	H_e	H_t	F_{IS}	F_{IT}	F_{ST}
MDH	0.497976	0.552632	-0.637996	-0.075857	0.343187
SOD	0.491146	0.559471	- 0.221783	-0.131957	0.073520
GPI-1	0.497593	0.634361	-0.326595	-0.270829	0.042037
GPI-2	0.111547	0.117647	-0.228733	0.012425	0.196266
IDH	0.493012	0.64532	-0.400452	-0.300561	0.071328
PRX-	0.380467	0.398148	-0.248985	-0.028542	0.176498
Mean (SE)	0.411957 (0.06293)	0.484597 (0.08177)	-0.34409 (0.06511)	-0.13255 (0.05241)	0.150473 (0.04613)
C.I. (upper)*			-0.244171	-0.052143	0.259643
C.I. (lower)*			-0.505577	-0.254681	0.062240

Table 2.11. Fisher's exact test for Hardy-Weinberg equilibrium.

Population	Locus1	Locus 2	Locus 3	Locus 4	Locus 5	Locus 6
ERA-110/140	0.000	0.021**	0.000	0.000	0.000	0.780*
ORC-030/060	0.377*	0.000	0.008	0.000	0.272*	0.808*
EFD-030	0.616*	0.062*	0.421*	0.814*	0.526*	0.004
SRW-000	0.040**	0.038**	0.757*	0.000	0.004	0.000
ERA-095	0.000	0.000	0.000	0.000	0.656*	0.000
SRW-055	0.000	0.251*	0.001	0.000	0.464*	0.000
CRO-090/100	0.000	0.509*	0.069*	0.000	0.000	0.000
YRS-000/055	0.037**	0.000	0.000	0.000	0.875*	0.377*
BBR-175	0.000	0.844*	0.406*	0.000	0.815*	0.801*
BBR-250	0.316*	0.081*	0.858*	0.000	0.126*	0.573*
FHR-GP	0.500*	0.810*	0.494*	0.000	0.248*	0.126*

MRR-340/370	0.237*	0.754*	0.760*	0.000	0.238*	0.461*
ESF-340	0.129*	0.027**	0.000	0.000	0.000	0.000
MRW-080	0.000	0.000	0.019**	0.000	0.000	0.094*

*P≤5%
** P≤1%

2.3.3. Nei's (1972) Genetic Distance and Identity.

Nei's (1972) genetic identity estimates between populations ranged from 0.8825 (between YRS-000/055 and ERA-095) to 0.9974 (between MRW-080 and ERA-110/140). The most genetically distant population pair, YRS-000/055 and ERA-095, was separated by a genetic distance of 0.1250 and the closest pair, MRW-080 and ERA-110/140, by 0.0026. MRW-080 and ERA-110/140 (the closest pair) were from different regions (north east and north west of Dorrigo, respectively; see Fig. 1.3). The second closest pair (ERA-110/140 and FHR-GP) was also from opposite regions.

The most distant pair (YRS-000/055 and ERA-095) however was from the same area.

Table 2.12. Coefficient of determination (r^2) and standard error (SE) values from regression analyses (at 95% confidence interval).

	r^2	SE
P v logN	0.245 **ns**	0.070
A v logN	0.245 **ns**	0.060
A_e v logN	0.066 **ns**	0.054
H_e v logN	0.025 **ns**	0.040
H_o v logN	0.027 **ns**	0.082
***f* v logN**	0.047 **ns**	0.350
P v Age	0.160*	0.066
A v Age	0.160*	0.066
A_e v Age	0.585**	0.035
H_e v Age	0.544**	0.027
H_o v Age	0.396**	0.064
***f* v Age**	0.118**ns**	0.329

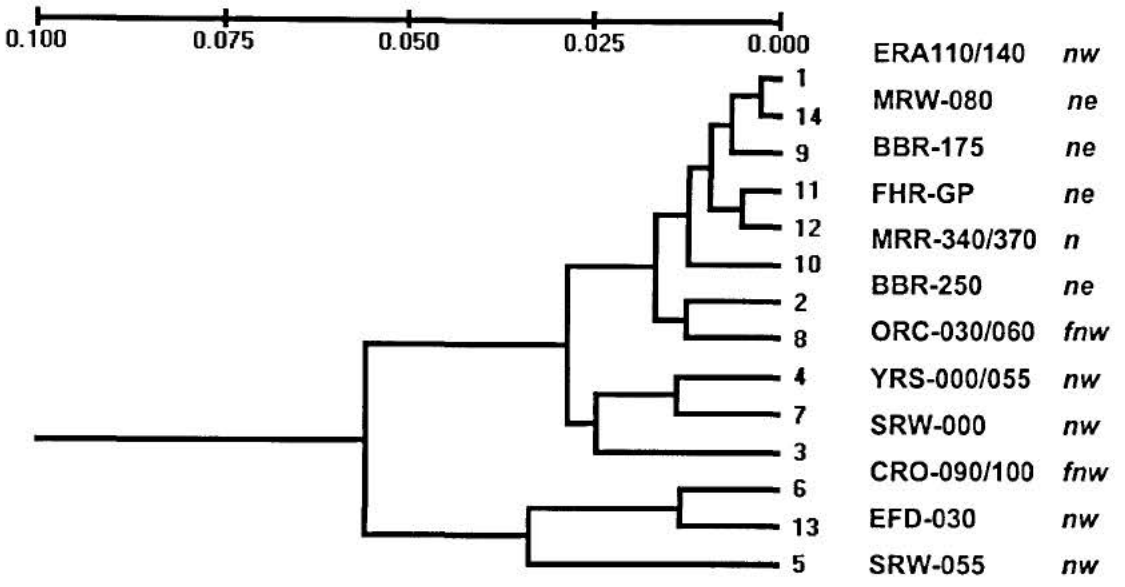

Fig. 2.7. Phenogram for the 14 populations of *O. flocktoniae* based on Nei's (1972) genetic identity. *nw* = northwest; *ne* = northeast; *n* = north and *fnw* = far north east in relation to Dorrigo district.

2.3.4. Regression Analyses.

Regression analyses investigating relationships between number of effective alleles per locus and log population size, expected heterozygosity and log population size, observed heterozygosity and log population size, and fixation index and log population size showed no significant independence between the pairs. Regression analyses between proportion of polymorphic loci and log population size and number of alleles per locus and log population size did not show a significant relationship between variables (Fig. 2.8 and Fig. 2.9).

Regression analyses between population age since reappearance after presumed extinction after 1912 and different genetic diversity measures all showed significant results. There was a 58.5% correlation between age and A_e, 54.4% between age and H_e, and 39.6% between age and H_o. There was a significant relationship between age and P, age and A (Table 2.12) but not between age and inbreeding coefficient, f (Table 2.12).

NEI'S (1972/1978) IDENTITIES/DISTANCES

Nei's original (1972) identity

Popn	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*****													
2	0.9860	*****												
3	0.9728	0.9729	*****											
4	0.9775	0.9696	0.9751	*****										
5	0.9469	0.9048	0.9223	0.9646	*****									
6	0.9657	0.9373	0.9338	0.9503	0.9712	*****								
7	0.9835	0.9718	0.9763	0.9863	0.9639	0.9594	*****							
8	0.9790	0.9877	0.9819	0.9627	0.8825	0.9107	0.9568	*****						
9	0.9959	0.9821	0.9654	0.9745	0.9393	0.9670	0.9675	0.9795	*****					
10	0.9878	0.9805	0.9793	0.9572	0.9216	0.9697	0.9666	0.9790	0.9859	*****				
11	0.9971	0.9893	0.9671	0.9639	0.9202	0.9546	0.9721	0.9847	0.9940	0.9881	*****			
12	0.9906	0.9843	0.9751	0.9602	0.9024	0.9284	0.9690	0.9920	0.9853	0.9826	0.9951	*****		
13	0.9830	0.9522	0.9257	0.9507	0.9627	0.9868	0.9623	0.9277	0.9799	0.9656	0.9748	0.9528	*****	
14	0.9974	0.9902	0.9839	0.9859	0.9499	0.9752	0.9900	0.9774	0.9911	0.9961	0.9924	0.9847	0.9772	*****

Nei's original (1972) distance

Popn	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*****													
2	0.0141	*****												
3	0.0276	0.0274	*****											
4	0.0227	0.0308	0.0252	*****										
5	0.0545	0.1000	0.0809	0.0361	*****									
6	0.0350	0.0648	0.0685	0.0510	0.0292	*****								
7	0.0167	0.0287	0.0240	0.0138	0.0368	0.0415	*****							
8	0.0212	0.0124	0.0182	0.0380	0.1250	0.0935	0.0441	*****						
9	0.0041	0.0180	0.0353	0.0258	0.0626	0.0336	0.0330	0.0207	*****					
10	0.0123	0.0197	0.0209	0.0437	0.0816	0.0307	0.0339	0.0212	0.0142	*****				
11	0.0029	0.0107	0.0334	0.0368	0.0831	0.0464	0.0283	0.0154	0.0060	0.0120	*****			
12	0.0095	0.0158	0.0253	0.0406	0.1027	0.0743	0.0315	0.0080	0.0148	0.0176	0.0049	*****		
13	0.0171	0.0490	0.0772	0.0505	0.0381	0.0132	0.0384	0.0751	0.0203	0.0350	0.0255	0.0484	*****	
14	0.0026	0.0099	0.0162	0.0142	0.0514	0.0251	0.0101	0.0229	0.0089	0.0039	0.0077	0.0154	0.0231	*****

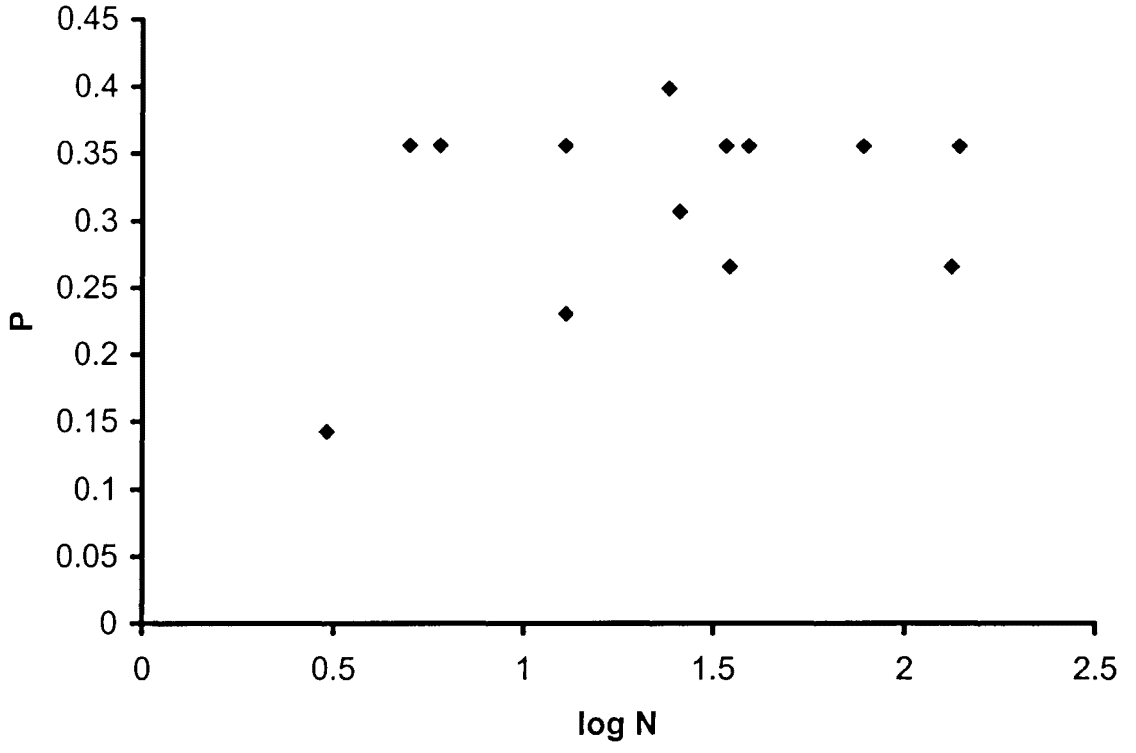

Fig. 2.8. Scatterplot between proportion of polymorphic loci P and log population size logN of 14 *O. flocktoniae* populations. $r^2 = 5.99\%$, p = 0.39.

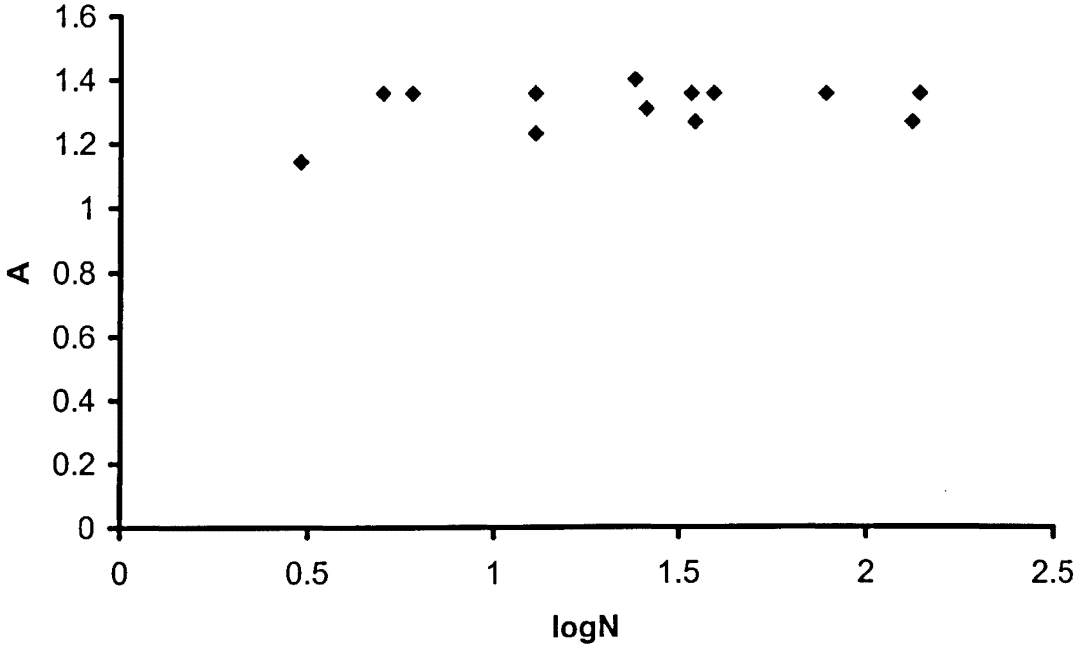

Fig. 2.9. Scatterplot between number of alleles per locus A and log population size logN of 14 *O. flocktoniae* populations. $r^2 = 5.99\%$, p = 0.39.

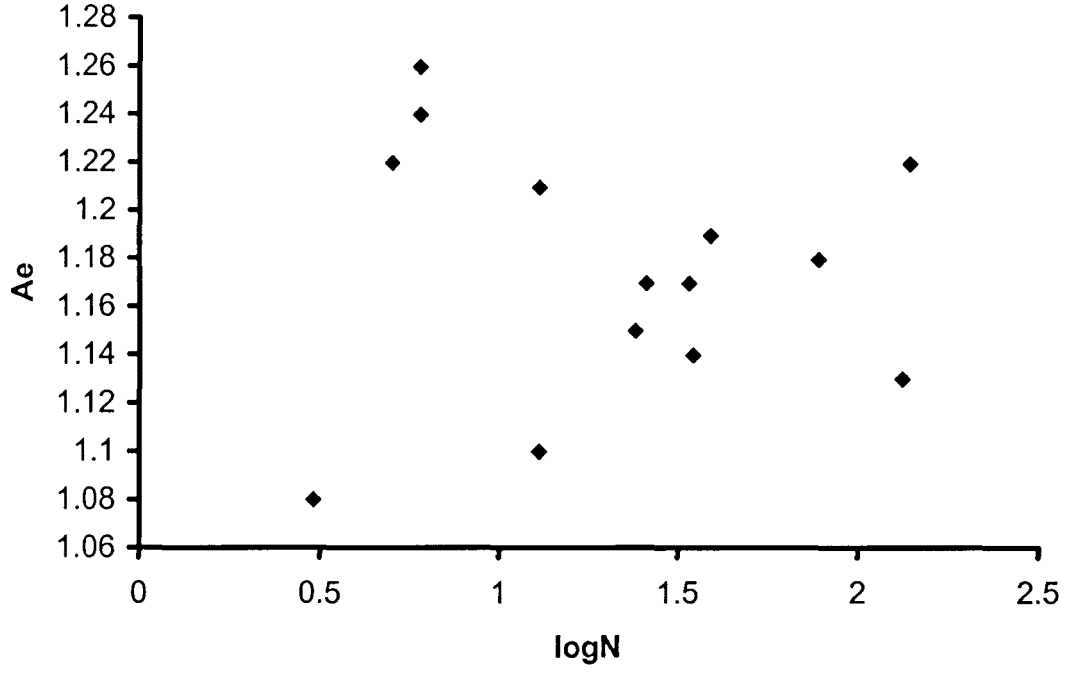

Fig. 2.10. Scatterplot between number of effective alleles A_e and log population size logN of 14 *O. flocktoniae* populations. $r^2 = 0.44\%$, $p = 0.82$.

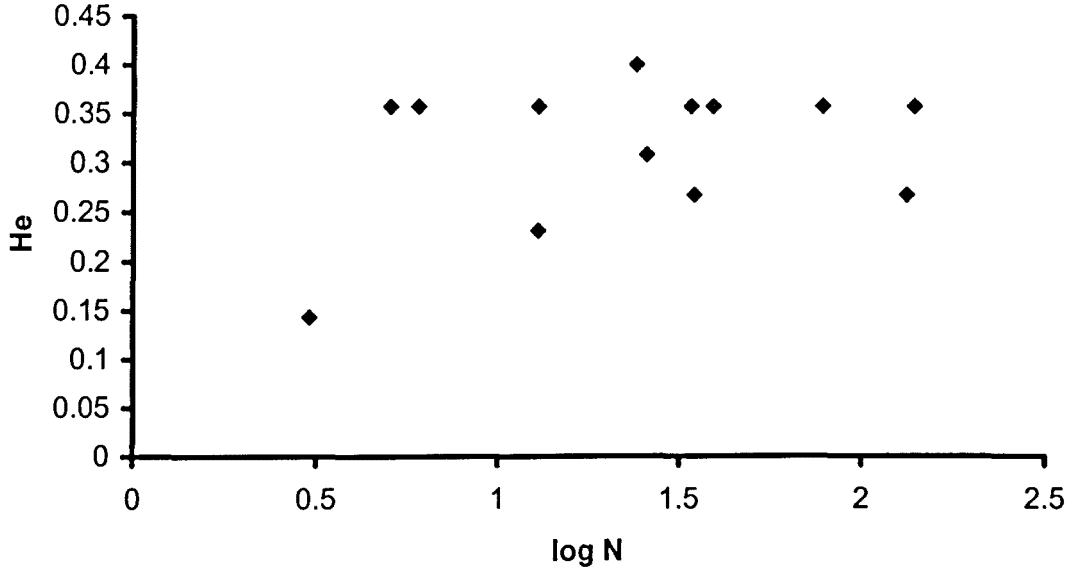

Fig. 2.11. Scatterplot between expected heterozygosity H_e and log population size logN of 14 *O. flocktoniae* populations. $r^2 = 0.06\%$, $p = 0.93$.

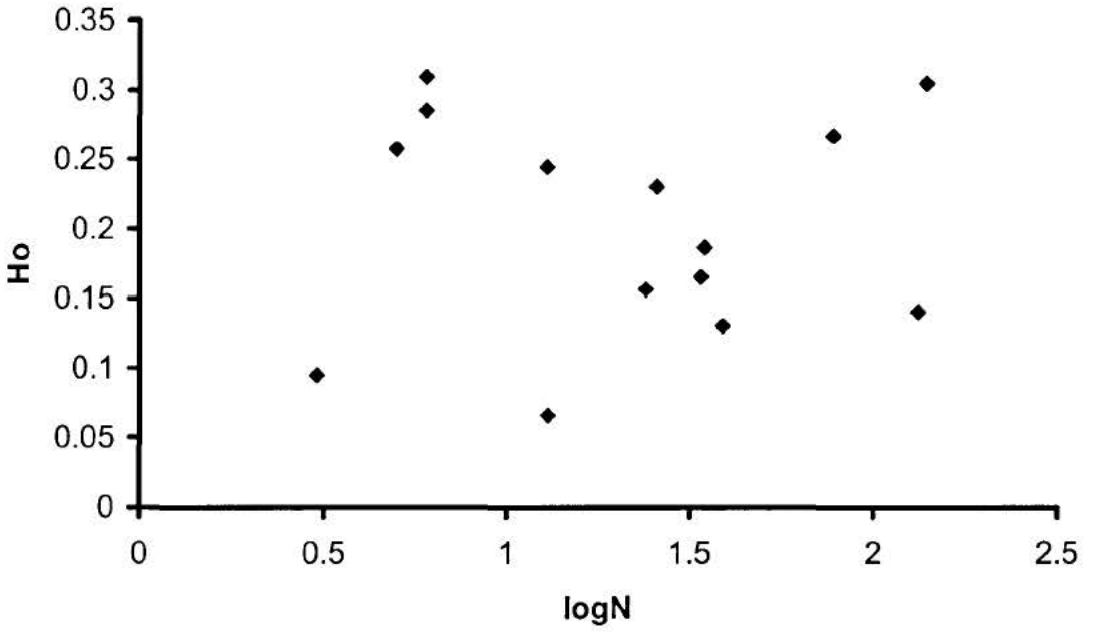

Fig. 2.12. Scatterplot between observed heterozygosity H_o and log population size logN of 14 *O. flocktoniae* populations. $r^2 = 0.07\%$, $p = 0.92$.

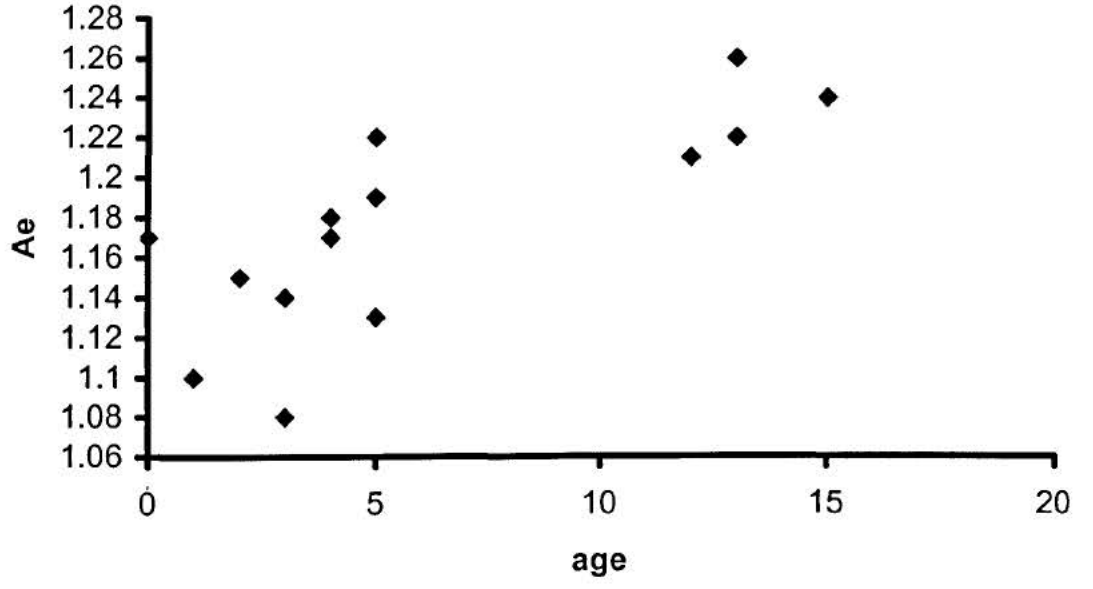

Fig. 2.13. Scatterplot between number of effective alleles A_e and age of the 14 *O. flocktoniae* population. $r^2 = 58.5\%$, $p = 0.0014$.

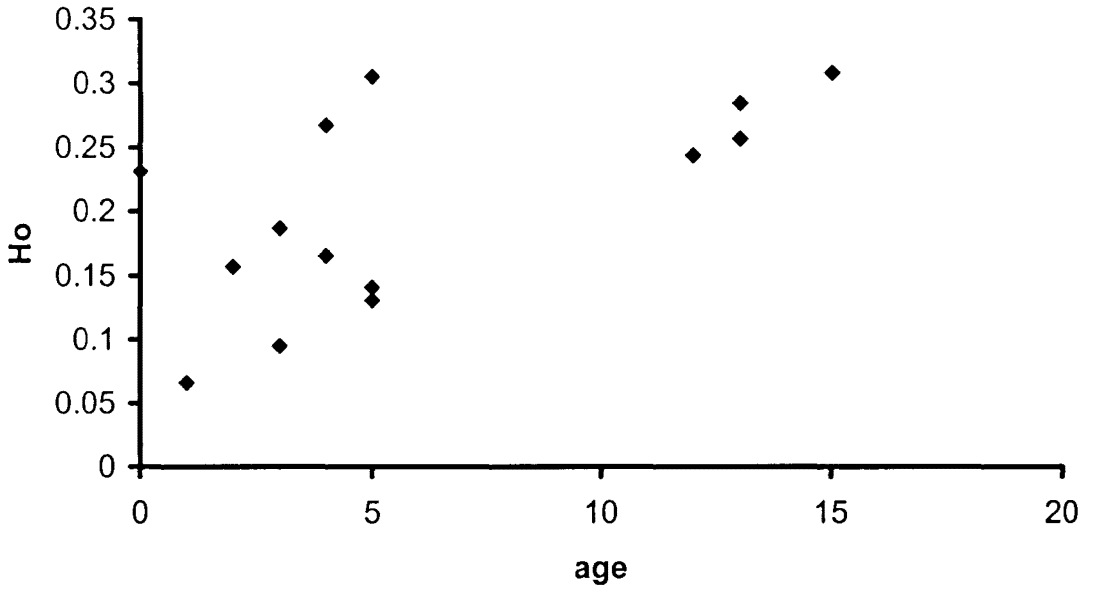

Fig. 2.14. Scatterplot between observed heterozygosity and age of the 14 *O. flocktoniae* populations. $r^2 = 39.6\%$, $p = 0.0158$.

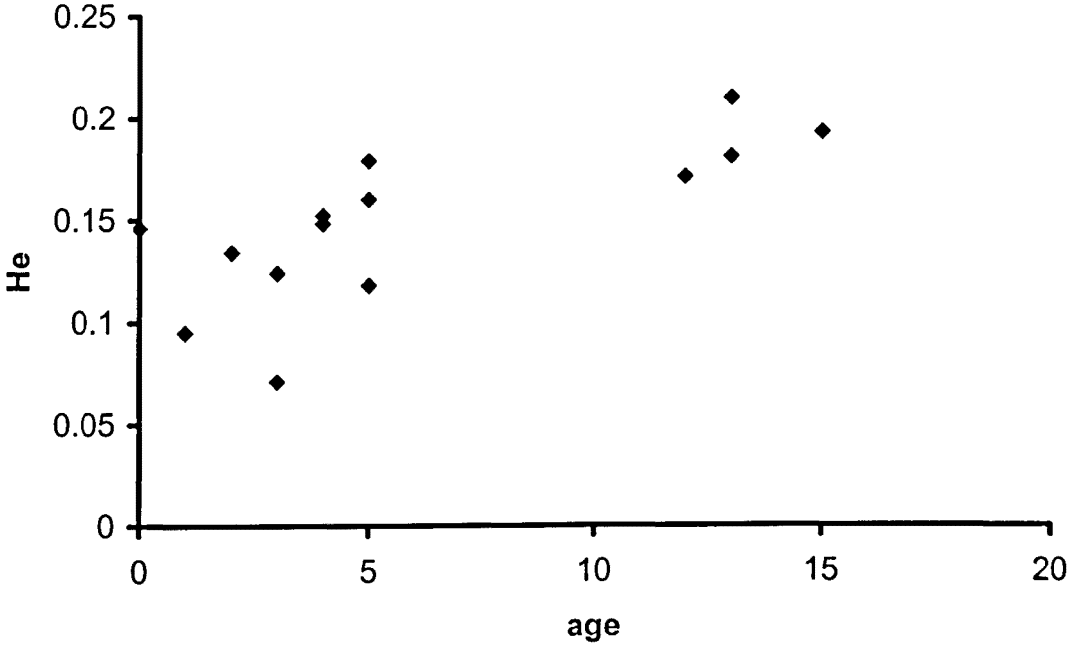

Fig. 2.15. Scatterplot between expected heterozygosity and age of the 14 *O. flocktoniae* populations. $r^2 = 54.4\%$, $p = 0.0026$.

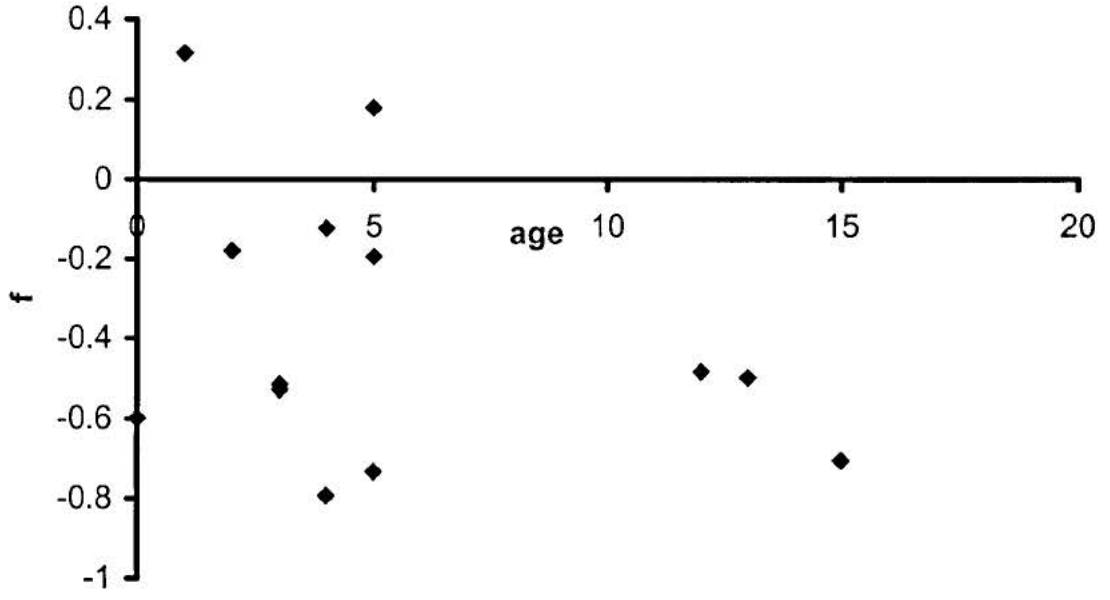

Fig. 2.16. Scatterplot between fixation index f and age of the 14 *O. flocktoniae* populations. $r^2 = 11.8\%$, p = 0.2295.

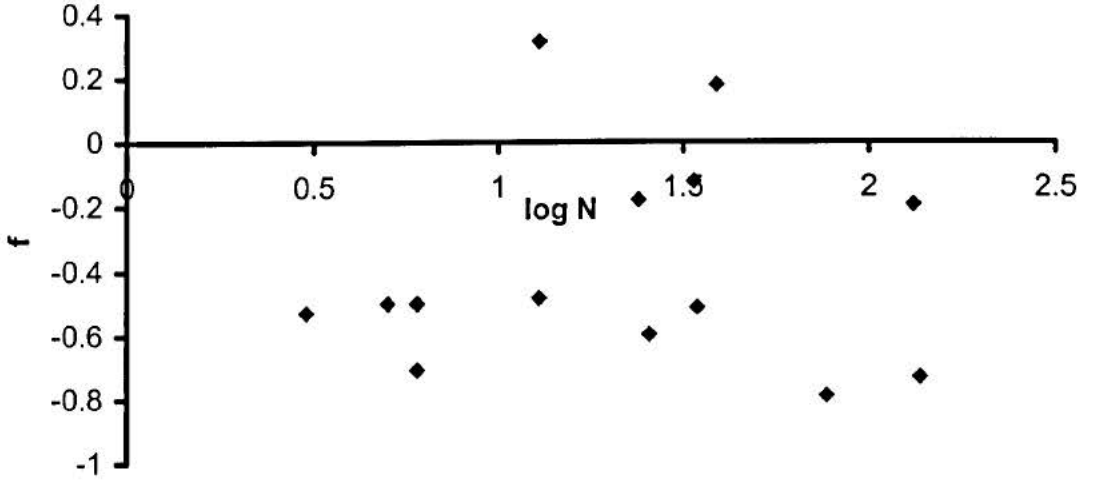

Fig. 2.17. Scatterplot between fixation index f and log population size logN of the 14 *O. flocktoniae* populations. $r^2 = 0.22\%$, p = 0.87.

2.4. Discussion

Contrary to my hypothesis the 14 *O. flocktoniae* populations were moderately differentiated with its F_{ST} value (0.15) similar to the mean G_{ST} (or F_{ST}) value from Hamrick and Godt (1989) for wind-dispersed species (G_{ST} = 0.14; Table 2.3). Though the breeding system of *O. flocktoniae* has not been directly investigated its F_{ST} (0.15) is intermediate between wind pollinated species having mixed and outcrossing breeding systems (both 0.10) and widespread species (0.21). Endemic species have a collective F_{ST} = 0.25 [See Table 2.3]. F_{ST} however is directly affected by heterozygosity [$F_{ST} = (H_t - H_e)/ H_t$] and the discrepancy seen between published averages and the average from this study may be due to the observed heterozygote excess (H_o/H_e =1.35).

Wright's hierarchical F-statistics also showed an excess of heterozygotes (f = -0.34 and F = -0.13) in *O. flocktoniae* population. This might be due to overdominance where heterozygous loci have an advantage over homozygous ones (Mitton, 1989). Heterozygote excess can be a result of a reduction in effective population size (Cornuet and Luikart, 1996), the number of individuals likely to contribute genes to the next generation. A reduction in the number of individuals had been seen in *O. flocktoniae* since 1997, with the highest decline of 37% observed between 2000 and 2001 (Mackay and Gross, 2001). The individuals sampled from populations were mostly adults that have survived the years of continuous decline.

Two rare pioneer plant species *Gentiana pneumonanthe* (a perennial herb) and *Helicteres brevispira* (a small tree) have F_{ST} values comparable to that of *O. flocktoniae* [0.19, 0.12 and 0.15, respectively (see Table 2.4)]. However there are not many pioneer species that have been studied in terms of their genetic diversity to be able to make significantly meaningful comparisons.

The over-all high genetic identity (mean I = 0.9677) and the existence of *O. flocktoniae* population pairs that are geographically distant and yet have very high genetic identity (e.g. ERA-110/140 [northwest] and MRW-080 [northeast], I = 0.9974; and ERA-110/140 [northwest] and FHR-GP [northeast], I = 0.9971) suggest a founding event—perhaps the same event that re-established populations of a presumed extinct species. This evidence might again be related to the excess of heterozygotes. These populations could be a result of that founding event where parents were each homozygote to a different allele

(e.g. *Calystegia collina*; Wolfe *et al.*, 2000). The reduced number of alleles per locus (averaged over populations, A= 1.32) may also be a result of that founding event (e.g. *Gentiana pneumonanthe*; Raijmann *et al.*, 1994). Multiple founding events could account for the recent populations. MRW-080, a population only discovered in 2001 was most identical to a 5-year old population ERA-110/140 (I=0.9974).

The rate of migration has not been measured in this study since there were no rare alleles found (Slatkin, 1985). Being a pioneer species, (re)colonisation is an expected trait of the species. However colonisation and migration are sometimes hard to differentiate (Wade and McCauley, 1988) especially in pioneer species with populations that are prone to extinction and recolonisation depending on disturbance opportunities and succession. Wade and McCauley (1988) contended that when possible sites for colonisation are within the boundaries of the metapopulation, then colonisation and migration are one and the same and that, agreeing with Wright's (1978) predictions, high genetic differentiation among these populations is expected. However the mean F_{ST} value and mean genetic identity of *O. flocktoniae* populations do not describe this degree of differentiation. The observed moderate degree of substructuring lies in between Wright's (1978) and Slatkin's (1985) conflicting predictions (page 29).

The phenogram (Fig. 2.7) of Nei's (1972) genetic identities shows that populations in a region are more identical to each other compared to those from other regions of Dorrigo (except for the new population MRW-080 which is genetically closest to ERA-110/140). If recruitment of new populations was due to seed flow by wind or by seed bank disturbance (e.g. seeds clinging onto vehicle tyres) a high genetic identity within neighbouring populations makes sense. Gene flow through pollinators could also explain high genetic identities between some neighbouring populations (especially in the northwestern region) that are not separated by thick rainforest vegetation or are not beyond pollinator travel range. However some geographically close populations like BBR-175 and BBR-250, and ERA-110/140 and ERA-095 were not found to be genetically similar pairs even though they (except ERA-100/140) were still genetically grouped with other populations from the same region. This might be due to sampling error; ERA-095 has only 3 individuals in the entire population compared to ERA-110/140 with a sample size of 26. BBR-175 also contains only 3 individuals, less than half of BBR-250's population size of 7.

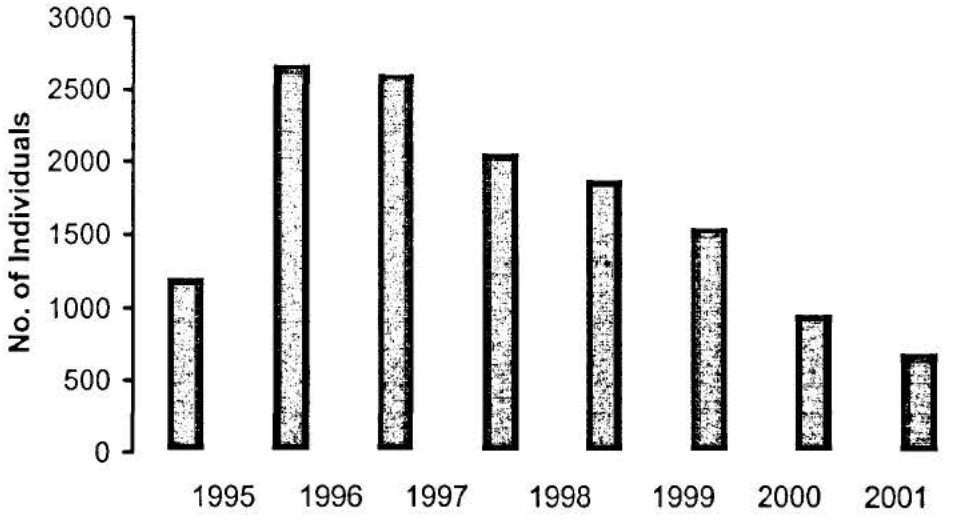

Fig. 2.18. Total number of individuals counted each year from 1995-2002 showing the decline of plant counts. (Source: Mackay and Gross, 2002).

Table 2.13. Population sizes of 14 *O. flocktoniae* from 1998-2001.

Population	Presence before 1998	1998	1999	2000	2001
ERA-110/140	present	180	156	139	173
ORC-030/060	present	15	2	11	39
EFD-030	absent	absent	13	24	14
SRW-000	present	249	212	133	34
ERA-095	present	6	3	3	3
SRW-055	absent	absent	absent	13	9
CRO-090/100	present	4	6	35	25
YRS-000/055	present	0	65	34	11
BBR-175	present	0	2	6	1
BBR-250	present	40	7	13	4
FHR-GP	present	285	73	33	4
MRR-340/370	present	39	22	12	4
ESF-340	absent	absent	115	117	78
MRW-080	absent	absent	absent	absent	26

Heterozygosity is often strongly correlated to population size (Nei and Graur, 1984) which was not seen among populations in *O. flocktoniae* where there was a lack of correlation between genetic diversity (H_e and H_o) and population size (r^2 = 2.5% and 2.7%, respectively; see Table 2.12).

Whilst a positive correlation had been observed in some insect pollinated herbaceous perennials (Raijmann *et al.*, 1994; Godt *et al.*, 1996; Kang and Chung, 2000) some studies had reported the absence of any positive relationship (Linhart and Premoli,

1993; Berge *et al.*, 1998; Kahmen and Poschlod, 2000). Berge *et al.* (1998) did not find a strong correlation in their study of three herbaceous species because of breeding system and gene flow. The outcrossing species in their study *Festuca ovina* (wind-pollinated) and *Lychnis viscaria* (insect-pollinated) had enough gene flow among their small populations to counteract genetic erosion (both species' F_{ST} values were close to 0.0) and the inbreeding species *Arabis thaliana*, (with an F_{ST} value closer to 1.0) showed high genetic diversity compared to Hamrick and Godt's (1989) averaged values (though lower than the two outcrossers). The mating system in *O. flocktoniae* has not been determined genetically using progeny testing and its effect to the lack of strong population substructure in the species could not be inferred [For example, self-incompatible species exhibit substantial gene movement compared to self-compatible ones; the populations of the former are usually less substructured with F_{ST} values closer to 0.0. See also Table 2.3 and Hamrick and Godt, 1989.]

Cruzan (2001) suggested that high levels of genetic variation in some small populations like that seen in FHR-GP, MRR-340/370, BBR-175 and BBR-250 may suggest recent reductions in population size. Data from *O. flocktoniae* surveys conducted almost yearly since 1984 do show population decline since 1996 (Table 2.13). ERA-095 a population that has been constantly small in the last 4 years (see Table 2.9) has the most reduced genetic diversity (P, A, A_e and H_e). Cruzan (2001) notes that large populations that have remained constant in size tend to have high levels of genetic diversity. ERA-110/140 showed this pattern.

The positive correlation between population age and genetic diversity (Table 2.12) specifically between A_e and between H_e may be due to the contribution made by persistent seed banks (see Chapter 3). *O. flocktoniae* seeds in the seed bank are viable up to four years with greatest seedling recovery occurring within one year of population extinction (Gross, unpublished data). Thus the higher genetic diversity found in older populations may be due to recruitment from their seed banks (this is discussed in the next chapter).

There was no population that resolved as the founding population. No rare alleles were found that would have helped in identifying a founder population (Slatkin, 1985) or estimate migration among populations. A suspected founder population FHR-GP had been dramatically reduced in size that rare alleles would be difficult to find. The FHR-GP population is located in a gravel pit centrally in the *O. flocktoniae* range (see Fig. 1.3). Road maintenance vehicles frequented this site for roadbase to maintain the roads for use by logging trucks. It was therefore hypothesised (Gross unpub data) that this site could be

a likely founder source. Historically, FHR-GP had a large population (285 plants in 1988). However a population size n = 4 is unlikely to give a full array of isozyme alleles that this population might have possessed in the past.

A temporal study that would have incorporated the genetic analyses of parent plants (pollen donors) and siblings was not included in the genetic investigation of *O. flocktoniae* populations thus determining the species' possible mating and breeding systems was not possible.

Summarily, the *O. flocktoniae* populations investigated genetically in this study showed similar and comparable degree of polymorphism and frequency of alleles as that of endemic species. They also showed moderate substructuring consistent with wind pollinated and wind dispersed species and displayed a high genetic identity pointing to the possibility that they all came from one founding population after being presumed extinct. A geographic pattern could be seen in the genetic hierarchical cluster of *O. flocktoniae* populations that suggests wind or other natural seed dispersal mechanism as agents of geneflow among populations; founding events facilitated anthropogenically could not be completely discounted, though. A positive correlation with age of populations that may be facilitated by seed banks and an excess of heterozygotes that may have arisen from repeated founding events, whether natural or human-induced, were also observed.

Chapter 3 – Patterns of Genetic Diversity of Seed Banks in Extinct and Extant *Olearia flocktoniae*

"A seed bank conserves genetic variability'. – H. G. Baker (1989).

3.1. Introduction.

The populations of the pioneer shrub *Olearia flocktoniae* have been in steady decline for the last five years and are predicted to be extinct within the next eight years under the current management regime (Mackay and Gross, 2001; see also Fig. 2.14). Gross (unpub data) studied *O. flocktoniae* seed banks via germination in the glass house and found that seeds remain viable for up to four years, the greatest seedling recovery though occurs within the first year after extinction of the above ground populations (Fig. 3.1).

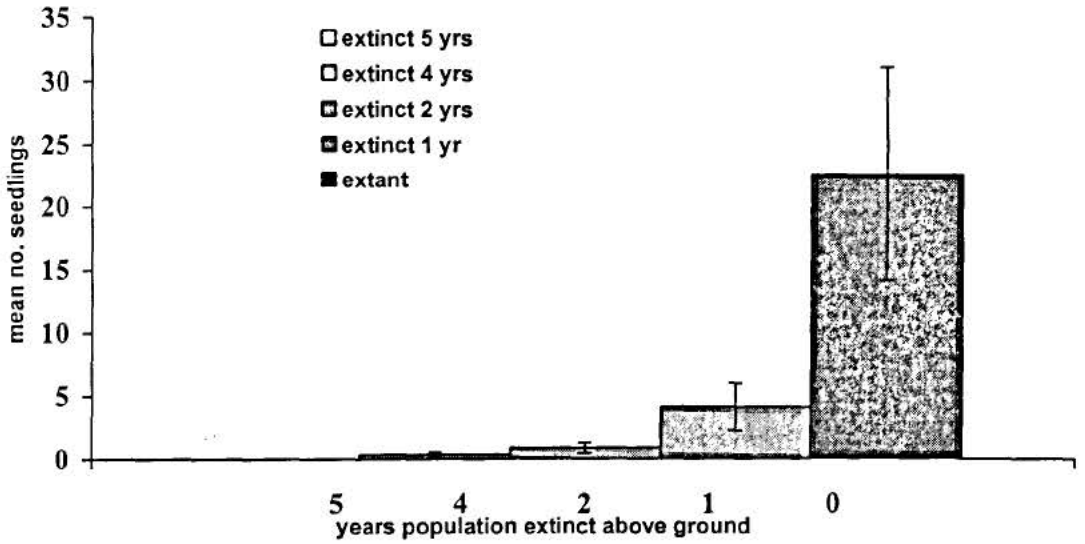

Fig 3.1 Mean number of seedlings germinated (±SE) from 10 extant populations and 3 seed bank populations extinct for 1 to 5 years. Source: Gross, unpub data.

Investigating the genetic variability and structure of seed bank populations and comparing them with above ground populations may assist in making conservation recommendations (i.e. which sites to disturb to create habitat).

3.1.1. Seed Bank and Species Survival.

A seed bank is a collection of ungerminated seeds capable of replacing aboveground plants when the latter die (Baker, 1989). Seed dormancy, thought to have evolved as a response to environmental variability by arresting seed growth and development (Evans and Cabin, 1995), makes it advantageous for seed banks to exist. Seed banks can be transient or persistent (Thomson and Grime, 1979); the former has seeds that germinate within a year or within a germination season after initial dispersal and the latter has seeds that remain viable in the soil for more than a year or more than one germination season (Simpson *et al.*, 1989; Walck *et al.*, 1996). The existence of seed banks is very crucial for the survival of plant species especially annuals where there is a great chance for adult individuals to die before reproducing. Persistent seed banks promote the survival of plant species in their environment in the absence of spatial immigration (Baskin and Baskin, 1998) thus providing temporal migration and adding to the age-structure of populations (Evans and Cabin, 1995). Their absence makes populations prone to local extinction (Baskin and Baskin, 1978; Kalisz and McPeek, 1993); their presence on the other hand enhances population growth rate under most environmental conditions thus decreasing the likelihood of extinction and increasing the time to extinction (Kalisz and McPeek, 1993). In annual species without persistent seed banks (e.g. *Floerkea proserpinacoides*), the production of a large seed output and/or having a higher seed survival from dispersal to emergence can compensate for this drawback (Houle *et al.*, 2001).

Persistent seed banks also affect the adaptive potential of plant populations because they accumulate seed genotypes produced over time (Cabin, 1996). This adaptive potential differs in annual and perennial plants. Seed banks in annuals generally contain genotypes that were "successful in good years" while that of perennials contain seeds from plants that have survived both the favourable and unfavourable times (Baker, 1989). After considering the initial abundance of seeds in a seed bank, seed germination under varying conditions may be subject to genetic control (McCue and Holsford, 1998) just as seed resistance or susceptibility to destruction in the seed bank is also dependent on the genotypes that seeds carry (Baker, 1989). Thus selection for post-germination traits may depend on seeds in the seed bank that germinate in varying conditions and times (Evans and Cabin, 1995).

3.1.2. Seed Bank and Population Size.

Effective population size, i.e. the number of individuals contributing to the next generation, describes the "true breeding" population and influences genetic variation within populations (Kimura, 1983). An on-going reduction in effective population size may result in the loss of genetic diversity brought about by genetic drift and would severely affect the adaptive potential of the population (Lande, 1994); through (a) inbreeding depression from consanguineous matings that result in the fixation of deleterious mutations (Lande, 1994); and (b) in increased selfing due to a reduction in pollinator activity as in *Scutellaria montana*, an endemic herbaceous perennial (Cruzan, 2001). Restricted gene flow creates subpopulations with small effective population size, genetic drift isolates them from each other and spatial substructuring ensues (Wright, 1969). Small populations' proclivity towards extinction is not only described by genetic population studies but also by field studies on population persistence. Ouborg's (1993) study with several vascular plant species in their natural populations revealed that relatively small and isolated populations became extinct in a 30- year study period. In studying populations of the rare bellflower, Eisto *et al.* (2000) found that the risk of losing fertile individuals increased with decreasing population size. The degree of total relative fitness including seed set (Morgan, 1999; Luijten *et al.*, 2000; Jacquemyn *et al.*, 2001), number of seeds per head (Morgan, 1999), production output or the number of seedlings produced per plant (Luijten *et al.*, 2000; Mavraganis and Eckert, 2001), seed mass (Jacquemyn *et al*, 2001), number of flowering stems and flower heads (Luijten *et al.*, 2000), and adult survival (Luijten *et al.*, 2000) can be positively correlated with population size. Some *Olearia flocktoniae* individuals have failed to set viable seeds even when outcross pollen was introduced and seed variability is as varied as 12-96% (Gross *et al.*, 1998).

Even though effective population size is usually positively correlated with genetic diversity (Ellstrand and Elam, 1993; Raijmann *et al.*, 1994; Godt *et al.*, 1996; Sun, 1996; Lammi *et al.*, 1999) negative correlations have been observed. Isolation, selective out-crossing of self-compatible populations, recent colonization events, reduced reproductive success of individuals not attributed to genetic erosion, absence of equilibrium in populations, populations varying in the amount of migration and seed dispersal by frugivores are among the reasons suggested (Shapcott, 1999; Williamson and Werth, 1999; Kahmen and Poschlod, 2000; Lutz *et al.*, 2000; Neel and Ellstrand, 2001; Podolsky, 2001).

In 14 natural populations of *Olearia flocktoniae* heterozygote excess due to repeated founding events or colonization by overdominant heterozygote individuals may be the cause of the absence of a positive correlation (Chapter 2).

Evans *et al.* (2000) attributed this negative correlation between census population size and population genetic statistics (%P, A_p, H_e, etc.) of a scrub annual to its seed banks. McCue and Holsford (1998) calculated 5-year estimates of the effective population size of *Clarkia springvillensis* populations using both census data of above ground populations and soil seeds. The seed banks showed N_e of 2-3 factors greater than above ground populations. They went on to investigate the potential contribution of seed banks for maintaining genetic diversity of the rare annual because it exhibited characteristics of susceptibility to genetic erosion (narrow distribution and small population size subject to fluctuation). They attributed the high genetic diversity in populations and low differentiation between populations of *C. springvillensis* to the buffering effects of its seed banks to small population size and bottleneck events. A higher genetic variability was seen in older *O. flocktoniae* populations (Chapter 2) that may be attributed to the existence of persistent seed banks in this species.

3.1.3. Genetic Parameters of Seed Banks in Relation to the Above Ground Populations.

A few studies had been conducted to compare seed bank and aboveground genetic diversity (Tonsor *et al.*, 1993; Cabin *et al.*, 1998; McCue and Holtsford, 1998; Mahy *et* al, 1999; Morris *et al.*, 2002; see also Table 3.1). The varying results may be due to historical events and the types of mating systems of the different populations and species. The factors that contribute to a higher genetic diversity in seed banks include: (a) their existence as a multigenerational pool for above ground populations, (b) delayed germination or prolonged dormancy, (c) possession of strong germination barriers that could result in a wider range of seed age classes, and (d) the generation of new alleles from accumulated mutations (McCue and Holsford, 1998; Mahy *et al.*, 1999; Morris *et al..*, 2002). The lack of strong bottleneck events and a consistent reduction of effective population size or sexual generations since population founding (combined with limited gene flow and genetic drift) may have resulted in the lack of significant difference in genetic diversity in both seed bank and adult populations of a long-lived perennial *Calluna vulgaris* (Mahy *et al.*, 1999). The only published study that showed a significantly higher genetic diversity in surface plants compared to soil seeds is that of a short-lived perennial

Lesquerella fendleri (Cabin *et al.*, 1998). Selection is assumed to have caused this observation though it is unclear where in the life cycle of the plant this was occurring. An increase in differentiation among populations in the adult stage relative to the seed bank had been observed in some studies (Tonsor *et al.*, 1993; McCue and Holsford, 1998). Morris *et al.* (2002) in their stratified soil seed bank study found an increase in heterozygote deficiency in the top stratum compared to the lower. They attributed this to recent inbreeding events since the older soil contained larger numbers of private alleles. Higher levels of selfing and sib mating may lead to the random loss of some rare alleles. Consistent with the lack of differentiation between its seed bank and adult stages, no significant change in homozygosity/heterozygosity was found in *C. vulgaris* (Mahy *et al.*, 1999). In *L. fendleri* surface plants were significantly more heterozygous than the seed bank (Cabin *et al.*, 1998) just as seedlings and soil seeds were found to significantly differ genetically (Cabin, 1996).

Table 3.1 Comparison Between Seed Bank and Aboveground Genetic Diversity Measures (H, P and A) and F_{ST} Values. DNG = Data not given.

Species	Aboveground				Seed Bank				Reference
	F_{ST}	H	P	A	F_{ST}	H	P	A	
Astragalus bibullatus (Fabaceae)	DNG	0.063	0.25	1.4	DNG	0.156	0.50	1.97	Morris *et al.*, 2002.
Calluna vulgaris (Ericaceae)	0.008	0.137	0.50	2.3	0.006	0.128	0.50	2.64	Mahy *et al.*, 1999.
Clarkia springvillensis (Onagraceae)	0.045	0.26	DNG	DNG	0.008	0.35	DNG	DNG	McCue and Holtsford, 1998.
Lesquerella fendleri (Brassicaceae)	2x higher	Significantly higher	DNG	DNG	Half	Significantly lower	DNG	DNG	Cabin *et al.*, 1998.
Plantago lanceolata (Plantaginaceae)	0.12	0.5[a]	DNG	DNG	0.02	0.46[a]	DNG	DNG	Tonsor *et al.*, 1993.

[a] Measured using Weir and Cockerham's.

3.1.4. Aims of the Study.

This part of the research project was aimed at studying the genetic or allozyme diversity of seed bank populations of *O. flocktoniae* extinct and extant sites. The seedlings from C. L. Gross' seed bank germination trials in 2000 were utilized (see Fig.

3.1). These populations were also compared to their corresponding aboveground populations (Chapter 2.3. Results) in terms of genetic diversity and population substructure.

3.2. Materials and Methods.

3.2.1. Sampling.

3.2.1.1. Soil Sampling.

In 1999, in a separate experiment, C.L. Gross investigated the presence (if any) of a persistent seed bank in *Olearia flocktoniae*. Soil samples were placed in trays under a watering system. Seedlings from this study were used here. Plants at the sapling stage (3-6 mos old) were sampled and leaves 20 mm long were used for electrophoresis of isozymes.

Table 3.2. Seed bank population description used in the present study. Note that not all populations had adequate sample sizes for electrophoresis.

Population	Age of Population[a] (Years)	No. of Aboveground Plants (2000)	Type of Aboveground Population
FPR-350-450	2	1	Extant
SRW-000	4	133	Extant
ESF-350	3	89	Extant
CRO-090E	2	16	Extant
BPP-060	7	0	*Extinct*
COM-170	11	0	*Extinct*

[a]As of 2000.

3.2.1.2. Populations Sampled.

A sample size of 25 individuals per population was chosen in order to optimise the detection of alleles. To score an allele that occurs in 5% of the population, there has to be at least 20 individuals per population.

Only six seed banks from C.L.Gross' trial had 25 or more individuals; these were selected for genetic assay. BPP-060 and FPR-350/450 only had 14 and 22 plants, respectively, by the time allozyme study was initiated.

3.2.2. Electrophoresis.

The same procedures outlined in Chapter 2 Materials and Methods were used in this section. Fourteen working loci were used: ACP, ALD, GDH, GPI-1, G6PDH-1, G6PDH-2, G3PDH, ME, MDH, PRX-, PGD-1, PGD-2, PGM and SOD. Five of the above were polymorphic (ACP, GPI-1, MDH, PRX- and SOD). ACP was not visualised in adult population samples because of poor banding patterns caused by freezing samples at -70°C. Fresh samples were used in seed bank assays and ACP gave readable gels. IDH was not assayed in this study because of the unavailability of a reagent. GPI-2 was not scored in this part of the project because longer overnight runs were not performed initially. The seedlings were no longer available when the protocol was refined.

3.2.3 Statistical Analysis.

All of the statistical analysis outlined in Chapter 2 Materials and Methods were also performed in this chapter.

Three seed bank populations were compared with their corresponding adult population data (from Chapter 2). These were SRW-000, CRO-090E and ESF-340 seed bank and adult populations. These were compared using the same set of isozymes GPI-1, MDH, PRX- and SOD. Allele frequencies in each of the above locus were compared using G-test (Sokal and Rohlf, 1995) with $P < 0.05$.

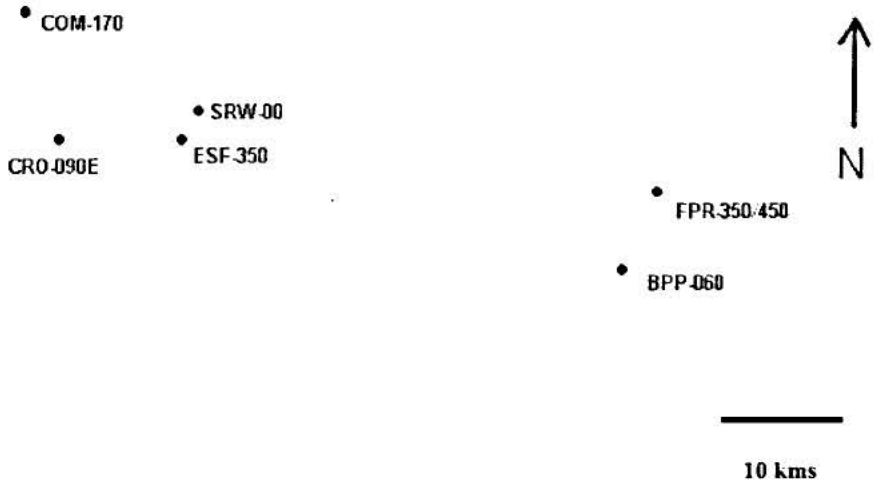

Fig.3.2. Map of the Dorrigo area showing the seed bank populations of *O. flocktoniae*. Source: www.wilmap.com.au/ausmaps.

3.3. Results

3.3.1. Genetic Diversity.

Five of fifteen isozymes studied were polymorphic in *O. flocktoniae* seed bank populations (33.3%). The proportion of polymorphic loci (P) within populations ranged from 0.267 to 0.333 with an average of 0.303 (SE±0.014). The number of alleles per locus ranged from 1.27 to 1.33 with a mean A=1.30 (SE±0.012). Observed heterozygosity (H_o) ranged between 0.075 and 0.179 with an average of 0.126 (SE±0.015). Expected heterozygosity (H_e) ranged from 0.090 to 0.160 and has an average of 0.118 (SE±0.010). The number of effective alleles (A_e) per population ranged from 1.10 to 1.19 (mean= 1.13; SE±0.014). All populations had a number of alleles per polymorphic locus A_p = 2.0. The mean observed heterozygosity was slightly higher than the mean expected heterozygosity giving a mean H_o/H_e of 1.07.

Table 3.3. Levels of allozyme variation within 6 seed bank populations of *Olearia flocktoniae*: n = sample size, P = proportion of polymorphic loci, A = number of alleles per locus, A_e = number of effective alleles, H_e = expected hereozygosity, H_o = observed heterozygosity and f = fixation index. [Extinct populations in *italics*].

Population	n	P	A	A_e	H_e	H_o	f
FPR-350	22	0.286	1.29	1.11	0.103	0.075	0.278
SRW-000	24	0.267	1.27	1.10	0.090	0.104	-0.158
ESF-350	25	0.333	1.33	1.19	0.160	0.179	-0.120
CRO-090	24	0.333	1.33	1.12	0.110	0.112	-0.015
BPP-060	*13*	*0.267*	*1.27*	*1.12*	*0.111*	*0.148*	*-0.387*
COM-170	*23*	*0.333*	*1.33*	*1.15*	*0.134*	*0.141*	*-0.051*
Mean (SE)		**0.303** (0.014)	**1.30** (0.012)	**1.13** (0.014)	**0.118** (0.010)	**0.126** (0.015)	**-0.076** (0.088)
endemics[a]		**0.29**	**1.41**		**0.10**		
widespread[b]		**0.43**	**1.72**		**0.16**		
Mean$_{adults}$[c]	**16**	**0.32**	**1.32**	**1.17**	**0.15**	**0.203**	**-0.340**

[a] Godt *et al.* (1996).
[b] Hamrick and Godt (1989).
[c] Results from Chapter 2.

ESF-350, an extant population possessed the most genetic diversity (P = 0.33, A = 1.33, A_e = 1.19, H_e = 0.16 and H_o = 0.179) in its seed bank. SRW-000, another extant

population, has the lowest over-all diversity figures (P = 0.267, A = 1.27, A_e = 1.10 and H_e = 0.09) for its seed bank. COM-170, an extinct population displayed the second most genetically diverse seed bank individuals.

3.3.2. Wright's F-statistic and Hardy-Weinberg Equilibrium.

The fixation index over all populations f [=(H_e-H_o)/ H_e] is slightly below zero (-0.076). The mean inbreeding coefficient of individuals within a population F_{IS} (-0.104) was significantly different from 0 thus showed departure from Hardy-Weinberg expectations in populations. The mean inbreeding coefficient for all populations F_{IT} (0.148) also showed considerable deviation from Hardy-Weinberg expectations over all populations. Genetic differentiation among populations was represented by F_{ST} = 0.217 indicating that 80% of the genetic diversity in this species is distributed among or common to all populations. Fisher's (1954) exact test (Table 3.5) showed that only 53.3% of individual loci (16 out of 30) were found to be in Hardy-Weinberg equilibrium at P≤0.05 or 60% (18 out of 30) at P≤0.01.

Table 3.4. F-statistics for five loci in six *O. flocktoniae* seed bank populations.

Locus	H_S	H_T	F_{IS}	F_{IT}	F_{ST}
PRX-	0.456	0.390	-0.155	0.188	0.298
ACP	0.351	0.321	-0.156	0.126	0.263
MDH	0.472	0.415	-0.346	0.183	0.393
SOD	0.471	0.124	0.719	0.740	0.075
GPI-1	0.484	0.732	-0.581	-0.496	0.054
mean (SD)	**0.447** (0.054)	**0.396** (0.220)	**-0.104** (0.492)	**0.148** (0.438)	**0.217** (0.147)
C.I. (upper)*			0.424	0.520	0.343
C.I. (lower)*			-0.480	-0.229	0.075
$Adult_{mean}$	**0.412**	**0.485**	**-0.344**	**-0.133**	**0.150**

*Bootstrapping over loci (at 95% confidence interval and 1000 repetitions).

Table 3.5. P-values for Fisher's (1954) exact test for Hardy-Weinberg equilibrium.

	PRX-	ACP	MDH	SOD	GPI-1
FPR-350/45	0.876*	0.000	0.371*	0.000	0.000
SRW-000	0.758*	0.000	0.780*	0.001	0.000
ESF-350	0.394*	0.614*	0.079*	0.000	0.030**
CRO-090E	0.081*	0.117*	0.406*	0.000	0.047**
BPP-060	0.665*	0.952*	0.000	0.972*	0.670*
COM-170	0.007	0.001	0.459*	0.000	0.135*

*P≤5%
**P≤1%

3.3.3. Nei's (1972) Genetic Distance and Identity.

Nei's (1972) genetic identity estimates between populations ranged from 0.9092 (between COM-170 and BPP-060) to 0.9950 (between CRO-090E and SRW-000) with an average value of 0.9596 (SD= 0.026). A distance of 0.0951 separated the most genetically distant population pair (COM-170 and BPP-060) and the closest pair (CRO-090E and SRW-000), by 0.0050. CRO-090E and SRW-000 are both from the northwest region of the metapopulation while COM-170 and BPP-060 (both extinct populations) are from the northwestern and southeastern region.

3.3.4. Regression Analyses.

Regression analyses investigating the relationship between age of populations since reappearance after presumed extinction after 1912 and P, H_e and A_e were not significant.

Table 3.6. Nei's original (1972) identity. Extant poulations: FPR, SRW, ESF & CRO. Extinct populations: BPP & COM.

Pop	FPR	SRW	ESF	CRO	BPP	COM
FPR	*****					
SRW	0.9857	*****				
ESF	0.9814	0.9755	*****			
CRO	0.9909	0.9950	0.9772	*****		
BPP	0.9505	0.9441	0.9777	0.9335	*****	
COM	0.9459	0.9188	0.9628	0.9459	0.9092	*****

Table 3.7. Nei's original (1972) distance. Extant poulations: FPR, SRW, ESF & CRO. Extinct populations: BPP & COM.

Pop	FPR	SRW	ESF	CRO	BPP	COM
FPR	*****					
SRW	0.0144	*****				
ESF	0.0188	0.0248	*****			
CRO	0.0092	0.0050	0.0231	*****		
BPP	0.0508	0.0575	0.0226	0.0688	*****	
COM	0.0556	0.0846	0.0379	0.0557	0.0951	*****

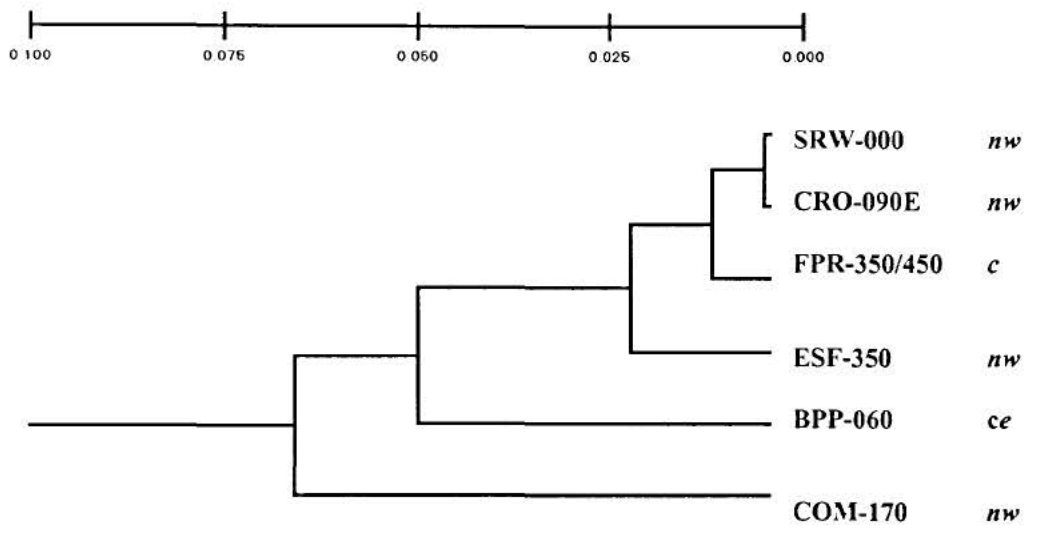

Fig. 3.3. Phenogram for the six *O. flocktoniae* seed bank populations based on Nei's (1972) genetic identity. *nw* = northwest; *c* = central and *ce* = central east in relation to Dorrigo district.

Table 3.8. Coefficient of determination (r^2), significance and standard error (SE) values from regression analyses (at 95% confidence interval).

	r^2	SE
Age v H_e	0.046ns	0.027
Age v H_o	0.140ns	0.038
Age v P	0.006ns	0.037
Age v A_e	0.030ns	0.036
Age v f	0.162ns	0.222

*Significant relationship between variables, ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

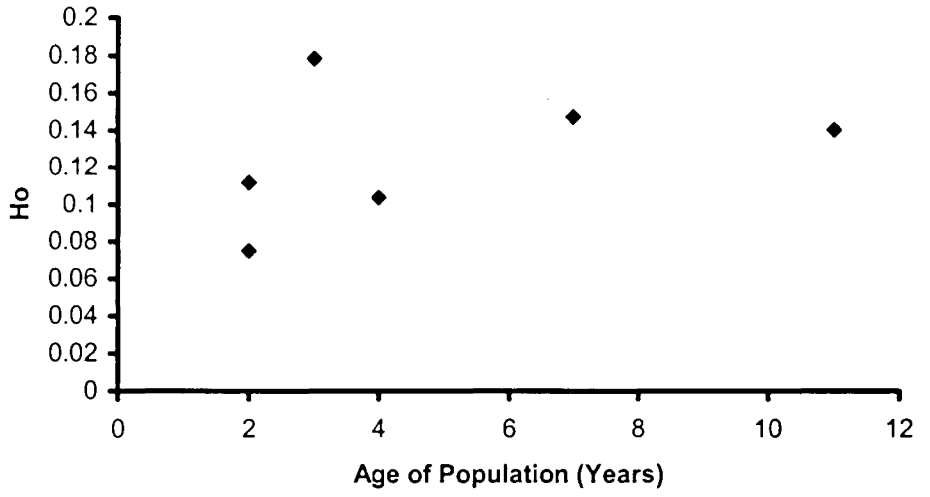

Fig. 3.3. Scatterplot between age and observed heterozygosity of seed bank and adult *O. flocktoniae* populations (r^2= 14.0%, p =0.46).

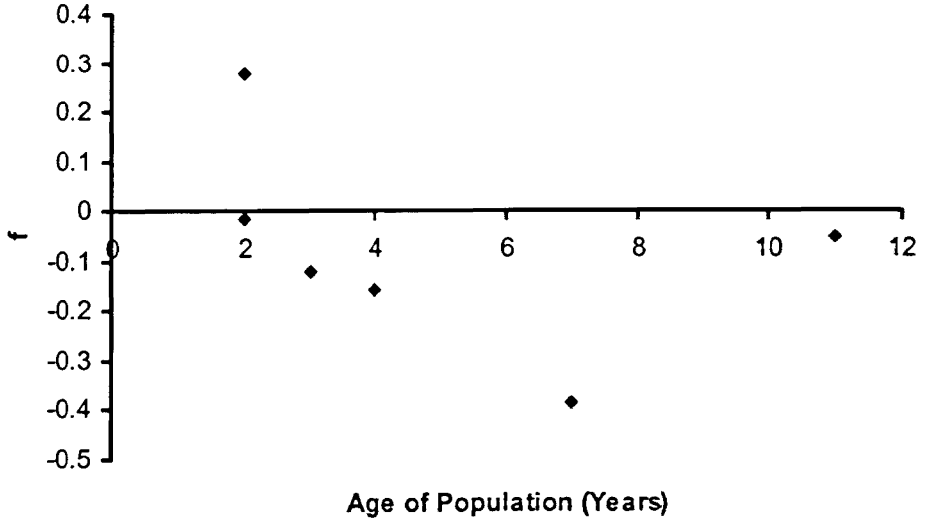

Fig. 3.5. Scatterplot between age and fixation index of seed bank and adult *O. flocktoniae* populations (r^2= 16.2%, p =0.43).

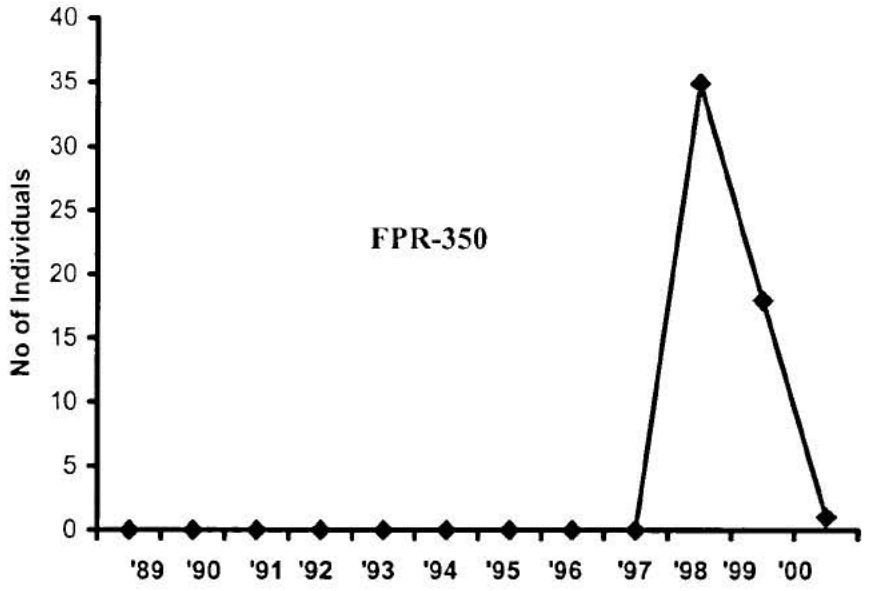

Fig. 3.6. Number of individuals in population FPR-350 from 1989 – 2000. Population founded in 1998 had 35 individuals. Data from Mackay and Gross (2001).

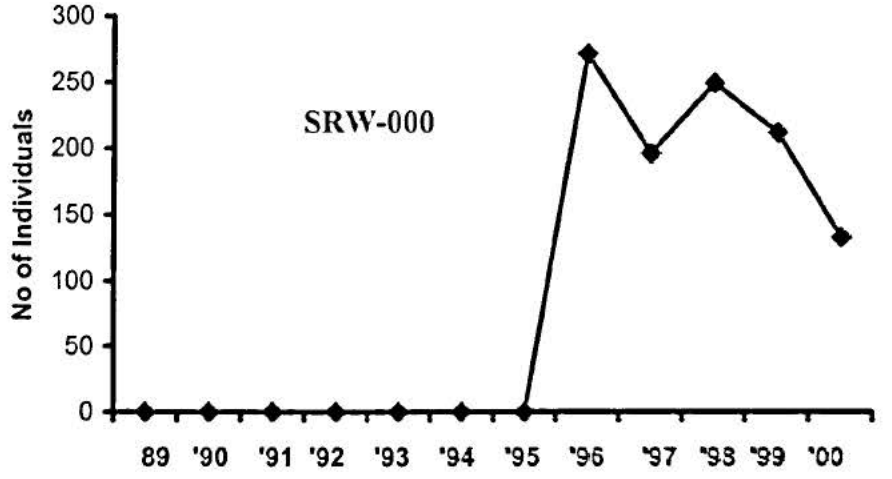

Fig. 3.7. Number of individuals in population SRW-000 from 1989 – 2000. Population founded in 1996 had 271 individuals. Data from Mackay and Gross (2001).

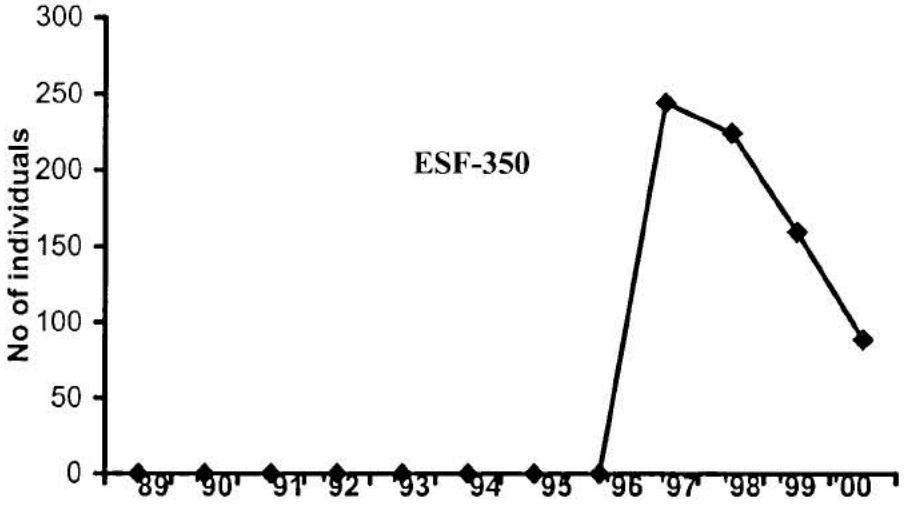

Fig. 3.8. Number of individuals in population ESF-350 from 1989 – 2000. Population founded in 1997 with 245 individuals. Data from Mackay and Gross (2001).

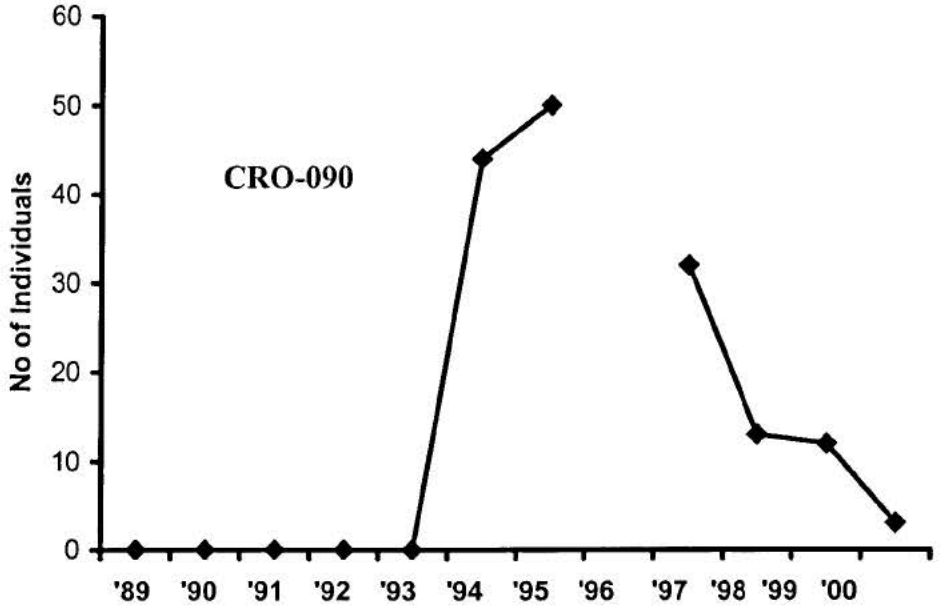

Fig. 3.9. Number of individuals in population CRO-090 from 1989 – 2000. Population founded in 1994 had 44 individuals. (No data for 1996.) Data from Mackay and Gross (2001).

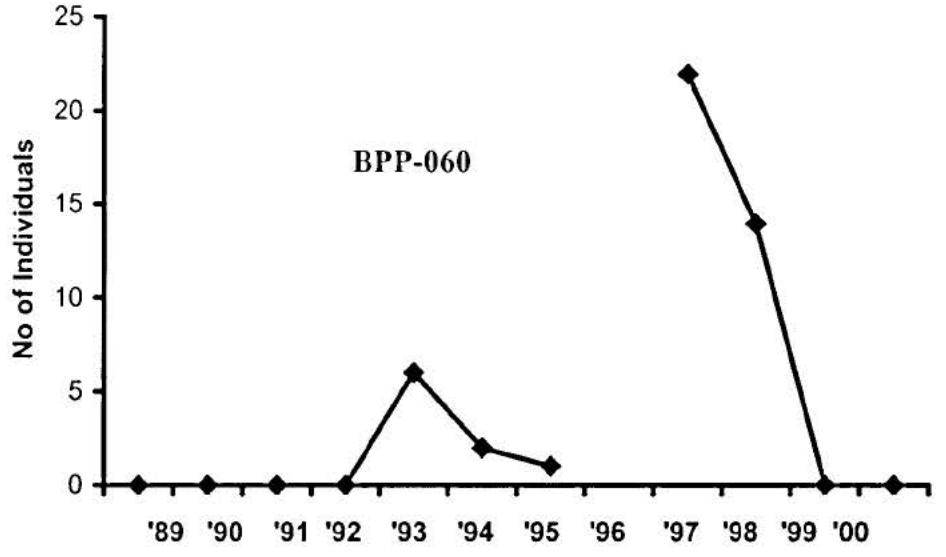

Fig. 3.10. Number of individuals in population BPP-060 from 1989 – 2000. Population founded in 1993 had 6 individuals. Year of extinction: 1999. (No data for 1996.) Data from Mackay and Gross (2001).

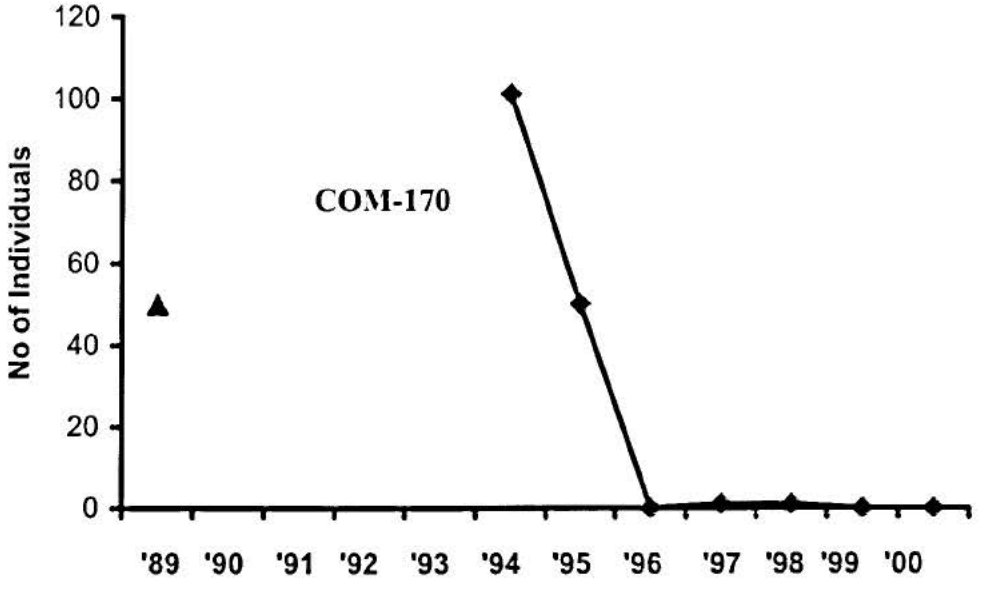

Fig. 3.11. Number of individuals in population COM-170 from 1989 – 2000. Population founded in 1989 with *abundant* (▲) number of individuals. (No data between 1990-1993). Year of first extinction: 1996. One individual found in 1997-98. Year of recent extinction: 1999. Data from Mackay and Gross (2001).

3.3.5. Seed Bank and Aboveground Population Comparison.

Allele frequencies at three out of four isozyme loci of seed bank and above ground populations differed significantly ($P<0.05$). An analysis of variance between means of seed bank and above ground population genetic diversity measures P, A, A_e, H_e and H_o including fixation index f showed a significant differences at $P<0.05$.

Table 3.9. Levels of allozyme variation comparing 3 adult and 3 seed bank populations of *Olearia flocktoniae*: n = sample size, P = proportion of polymorphic loci, A = number of alleles per locus, A_e = number of effective alleles, H_e = expected hereozygosity, H_o = observed heterozygosity and f = fixation index.

Population	n	P	A	A_e	H_e	H_o	f
SRW- seed bank	25	0.286	1.29	1.12	0.096	0.111	-0.158
SRW- adult	23	0.214	1.21	1.10	0.092	0.132	-0.451
CRO- seed bank	25	0.286	1.29	1.12	0.110	0.112	-0.013
CRO- adult	25	0.286	1.29	1.15	0.133	0.200	-0.515
ESF- seed bank	25	0.286	1.29	1.16	0.138	0.151	-0.101
ESF- adult	24	0.385	1.38	1.20	0.164	0.288	-0.793

Table 3.10. Allele frequencies of seed bank and adult populations of SRW-000, CRO-090 and ESF-340 at four loci and their P-values from G-tests.

Locus	Stage	Allele 1	Allele 2	P-value
GPI-1 ns	Seed bank	0.6212	0.3788	0.99ns
	Adult	0.6098	0.3904	
MDH*	Seed bank	0.2698	0.7302	0.81ns
	Adult	0.4859	0.5141	
PRX-*	Seed bank	0.6884	0.3116	0.57ns
	Adult	0.1712	0.8288	
SOD*	Seed bank	0.7174	0.2826	0.75ns
	Adult	0.4247	0.5753	

*Significant relationship between variables, ns = not significant, * $p< 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4. Discussion

3.4.1 Seed Bank Genetic Diversity and Substructure.

The six seed bank populations of *Olearia flocktonia* displayed degrees of polymorphism and allele frequencies comparable to endemic species (Table 33.) The most genetically diverse population, ESF-350, in terms of P, A, A_e, H_e and H_o is an extant population that is 3 years old (as of 2000) [Table 3.3]. The oldest (4 yo) extant population SRW-000 showed both the lowest A_e and H_o (Table 3.3). Populations BPP-060 and COM-170 had been extinct for two years and first sighted 7 and 11 years ago, respectively. COM-170 displayed the second most genetically diverse individuals. It had undergone two extinction events: in 1996 and 1999. There was only 1 individual in years 1997-98 (Mackay and Gross, 2001).

Grouping all extant and extinct populations separately showed no significant differences in averages in terms of P (0.304 and 0.300, respectively), A (1.305 and 1.300, respectively) and A_e (1.130 and 1.135, respectively). The combined extinct populations showed greater H_e and H_o values over extant populations (average H_e = 0.122 and 0.116, respectively and average H_o = 0.144 and 0.118, respectively). However, the extant populations showed almost no heterozygote excess (f= -0.004) compared to extinct ones (f= -0.219). A bias caused by sampling error could be in place considering that there were only 2 extinct seed bank populations sampled compared to 4 extant ones. Otherwise this shows that the above ground populations that existed prior to extinction might have reached a plateau in levels of heterozygosity, followed by a decline. Although fitness components are usually related to the level of heterozygosity, this pattern apparently does not continue indefinitely (reviewed by Mitton, 1989).

Almost 80% of the genetic diversity in the six *O. flocktoniae* seed bank populations was distributed among populations. This substructuring is more pronounced than the above ground populations investigated in Chapter 2 where 85% diversity was shared among the 14 studied populations (F_{ST}= 0.15). This may be due to the existence of more homozygous individuals [as indicated by a positive F_{IT} (0.148)] in the seed bank. H_o for seed bank populations is less than that of aboveground populations: 0.13 and 0.20, respectively. The higher F_{ST} (0.22) in the seed bank denotes more substructuring among these populations and as they progress to the next life-stage, they display less substructuring. Clearly, seeds in *O. flocktoniae* seed banks were less heterozygous than

the individuals that survive through to the juvenile and adult stages (most of the samples in Chapter 2 were adults the rest were subadults). A selective pressure must be operating whereby heterozygous individuals survive to subadult and adult stages.

The high allozyme heterozygosity or genetic diversity of the extinct seed banks in this study suggests that genetic diversity could be recaptured from extinct populations

Similar to the aboveground populations the seed bank populations had high genetic identities. The most genetically identical populations CRO-090E and SRW-000 (I = 0.9950) are both from the northwest region of the metapopulation while the least genetically identical populations COM-170 and BPP-060 (I = 0.9092, both extinct populations) are from the opposite directions of the metapopulation. However, the second most identical populations CRO-090 and FPR-350 (0.9909) are not from the same area while the second least identical populations COM-170 and SRW-000 (I = 0.9188) are both from the northwestern side of the metapopulation. This pattern shows that there is no distinct identity among neighbouring populations but a more coherent one throughout the metapopulation.

3.4.2. Comparing Genetic Diversity Between Seed Bank and Aboveground Populations Using the Same Set of Isozyme Loci.

Aboveground and seed bank populations were significantly different from each other ($P<0.05$) when the means of P, A, A_e, H_e, H_o and f (Table 3.9) were compared using analysis of variance (Table 3.10). All aboveground populations had higher H_o and two out of three had higher H_e compared to seed bank populations. Grouped fixation indices were lower in aboveground (-0.586) than seed bank (-0.091) populations, indicating an increasing degree of heterozygote excess from seed bank to adult. This had been observed in other seed bank genetic studies (Tonsor *et al.*, 1993; Cabin *et al.*, 1998; McCue and Holtsford, 1998). This same observation is also consistent when comparing the above ground (Chapter 2) populations' mean F_{IT} value with that of the six extant and extinct seed bank populations' mean F_{IT} value (-0.133 and 0.148, respectively), though both are separate investigations using different aged tissue and sets of isozyme loci.

Heterozygote excess can be maintained in populations when both homozygotes are less fit than the heterozygote genotype. This phenomenon, also called overdominance, allows a heterozygote (e.g. Hh) to display a phenotype (any fitness trait) greater than either homozygotes (e.g. HH or hh). The "associative overdominanace" hypothesis (Ohta, 1971) considers allozymes as neutral indicators responsible for the positive correlation found in

many organisms including plants (reviewed by Bierne *et al.*, 2000) between heterozygosity and fitness-related traits. This may be due to allozyme loci being frequently linked with deleterious recessive alleles (Butlin and Tregenza, 1998). The increase in frequency of heterozygotes from seed to adult may be due to inbreeding depression in early stages of an organism's life history due to lethal mutations (Husband and Schemske, 1996). Two sources of lethal or deleterious mutations are homozygous recessives and dominant lethals that do not usually reach reproductive age to pass on the mutation. Assuming the existence of overdominance at neutral loci due to a bottleneck event(s) (see Chapter 2.4. Discussion) and that some lethal mutations in *O. flocktoniae* are recessive, a lethal trait *l* and a wildtype allele *L* are carried by heterozygotes *(Ll)*. After the first generation half of the offspring are "normal" heterozygotes *(Ll)*, a quarter carry the normal homozygous wildtype *(LL)* and the other quarter carry the recessive lethal *(ll)* which might have survived embryonic stage (seed formation), hence were found in the seed bank assay, but did not survive seedling or juvenile stages. The aboveground populations therefore would consist of one-third wildtype homozygotes and two-thirds heterozygotes. If some allozyme and lethal trait loci are linked this would explain the increased heterozygote excess observed in aboveground populations of *O. flocktoniae* compared to its seed bank populations.

One of the limitations in using different age classes in genetic assays is the selective expression of isozymes (Weeden and Wendel, 1989). For example, only seed bank samples gave resolvable acid phosphatase (ACP) bands (see Fig. 2.3). ACP therefore was not included in the comparative genetic study of aboveground and seed bank populations thus reducing the sample size of isozymes used.

3.4.3. Conclusion.

O. flocktoniae seed banks contain more genetic richness in terms of the number of loci that are polymorphic, the degree of heterozygosity and the number of alleles found in those loci than above ground plants. The existence of seed banks in this endemic species certainly assist to maintain above ground genetic diversity as seen in Chapter 2 where older populations tend to have more genetic diversity (see Chapter 2.3.4 and Table 2.8) than younger ones that have not accumulated seeds in their seed banks. The geographic and genetic relationships seen among seed populations are similar to above ground populations (where a region can host both genetically similar and genetically distant populations).

Extinct aboveground populations are worth reviving. Their demise is most probably not caused by genetic depauperacy because, as seen in this study, the second most genetically diverse seed bank population came from an extinct above ground population (COM-170). Heterozygote excess due to overdominance is a reality in this metapopulation and whether populations become extinct because they reach their plateau followed by decline in fitness due to excessive levels of heterozygosity, or whether they do because of some other phenomenon, the only way to overcome final extinction is by human intervention. Allowing populations to expand in order for drift and mutations to increase their adaptive potential may be the only course of action.

Chapter 4 - Conclusions and Recommendations

"One goal of conservation biology is to preserve genetic diversity. Another goal should be to preserve evolutionary processes." - J.C. Avise (1994).

The 14 above ground *Olearia flocktoniae* populations as well as 6 seed bank populations assayed genetically using allozyme loci were comparable to Hamrick *et al.*'s (1996) figures for endemic species except for the above ground populations' level of expected heterozygosity which was more identical to a widespread species. I have concluded that this discrepancy is due to the increasing level of heterozygosity observed from seed bank to above ground plants. This phenomenon had been observed in three out of five genetic comparison studies involving other species between seed bank and aboveground plants while another study showed no significant differences between the two life histories (Table 3.1). From these genetic investigations (both seed bank and aboveground), it can be conjectured that the *O. flocktoniae* metapopulation in Dorrigo NSW could be a product of a founding event that took place before the species' rediscovery in the early 1980's. The excess of heterozygotes in the metapopulation indicates that the founding parents may be both homozygous to certain fitness traits linked to allozyme loci; "overdominance" has caused the heterozygote excess and this would also explains the moderate substructuring observed among aboveground populations, the F_{ST} value for which is closer to Hamrick & Godt's (1989) averaged values for widespread plant species than to that of endemic ones [see also Table 2.3]. It is also surmised that repeated founding events ensued since the initial event. These later events can also be considered migration or colonisation events from one local population to a new one. This assumption is supported by high genetic identities between some populations from opposite sides of the metapopulation and low genetic identities between some neighbouring populations. This over-all high genetic identity had been observed in another narrow endemic *Erigeron parishii*. Neel and Ellstrand (2001) speculated that the fragmented populations of this species have not reached sufficient isolation to allow genetic drift or selection to result in differentiated populations. The difference however is that *E. parishii* may not have experienced recent founding events or that its populations are not derived from a founding event but were once continuous before mining activities disrupted them. An endangered

endemic Hawaiian species *Brighamia insignis* have populations that are declining rapidly due to exotic species, natural calamities and goats (Gemmil *et al.*, 1998). It has a comparable degree of polymorphism and frequency of alleles (0.27 and 1.4, respectively) as *O. flocktoniae*. However the populations of the former are less heterozygous (H_e = 0.021) and hence had a positive fixation index (0.158) denoting homozygote excess. When a population experiences a reduction of its effective size, it generally develops an excess of heterozygotes (Cornuet & Luikart, 1996). Another endemic species *Jepsonia malvifolia* has populations with high genetic identities (mean I = 0.975) and a comparable H_e (0.158) to *O. flocktonia*. No significant relation between genetic and geographic distances were found and its populations were more substructured (Helenurm, 2001); its outcrossed mating system estimated from the fixation index denotes heterozygote excess.

The genetic diversity in aboveground populations of *Oleaarie flocktoniae* is assisted by the existence of persistent seed banks in this species. Older aboveground populations have significantly higher genetic diversity than younger ones. Extinct aboveground populations should be revived from the seed bank within the first two years of extinction. Even without genetic diversity data, extinct populations should be revived with the knowledge that individuals can be recovered from the seed banks especially within the first year of extinction (Gross, unpub data). The available genetic data gathered through allozyme analysis however allowed the researcher to make assumptions as to the historical events that had likely occurred since the species' presumed extinction. A genetic study to directly determine the species' mating system might shed more insight into its present status. The degree of genetic differentiation among populations as indicated by F_{ST} (or G_{ST}) suggests that the species may be outcrossing or partly outcrossing and partly selfing [see also Table 2.3].

Despite the lack of a positive relationship between N and H_o, it is recommended that all of the remaining populations in their natural environment should be given the opportunity to expand in size. Larger population sizes will allow the metapopulation to accrue novel alleles through genetic drift and mutation. The increasing heterozygote excess seen from seed bank to adult stage implies reduced fitness as life stages in the species progresses. Homozygote individuals either stay in the seed bank or survive only until early stages of plant life. An infusion of new alleles would indeed increase *O. flocktoniae*'s evolutionary potential. The higher level of heterozygosity observed in the two extinct seed banks, despite sampling bias (there were four extant seed banks sampled) may suggest transient fitness benefits derived from being a heterozygous population. This

wearing off of fitness traits when a population has reached a certain level of heterozygosity may seal its fate towards extinction. However, I still believe that allowing populations to expand and thrive longer are their only chance of increasing their adaptive traits.

Table 4.1. Population sizes of *Olearia flocktoniae* populations during genetic assay (2000) and at present (2002). Adapted from Mackay & Gross, 2001 and 2002.

Population	Location	Population Size During Genetic Studies	Population Size in 2002	Percentage Change
ERA-110/140	E 460156 N6665478	139 plants	27	-80.6%
ORC-030/060	E 449032 N 6663070	39[a]	30	-23.1%
EFD-030	E 455900 N 6662903	24	8	-66.7%
SRW-000/015	E 456825 N 6665760	133	64	-51.9%
ERA-095	E 460420 N 6665373	3	1	-66.7&
SRW-055	E 456450 N 6665750	13	2	-84.6%
CRO-090/100	E 448850 N 6663550	35	14	-60.0%
YRS-000/055	E 457119 N 6665853	34	11	-67.6%
BBR-175	E 478200 N 6654240	6	0	-100%
BBR-250	E 477637 N6654115	13	0	-100%
FHR-GP	E 483542 N 6656490	4[a]	0	-100%
MRR-340/370	E 475478 N 6660037	4[a]	1	-75.0%
ESF-340	E 454250 N 6663750	78[a]	36	-53.8%
MRW-080	E 478150 N 6657350	26[a]	49	+188.5%
Total number of plants		**551**	**243**	**-55.9% (average)**

[a]From 2001 field survey.

Table 4.1 shows an average 55.9% reduction in the population sizes of all aboveground populations involved in the study (2000 or 2001) compared to 2002 population sizes as determined through a recent survey (Mackay & Gross, 2002). This year, there is an overall 27% decrease of *O. flocktoniae* individuals in their natural habitat

(Mackay & Gross, 2002). The trend seen in Fig 2.18 is predicted to continue until the species' aboveground populations are extinct (Mackay & Gross, 2001). This cycle of extinction-recolonisation may exist due to the seed banks. However depletion of the genetic reservoir of the populations and the species itself is still very likely if the populations are not allowed to increase in size in order for mutations and genetic drift to occur. As with *Erigeron parishii*, a threatened endemic with a history of fragmentation (Neel & Ellstrand, 2001), it is difficult to prioritise any specific population(s) for conservation or for reserve protection.

If feasible, however, maintaining all the aboveground populations is recommended. ORC-030/063 and SRW-055 were the only aboveground populations that had positive fixation indices suggesting homozygote excess. These two populations may be a valuable source of homozygote alleles. ERA-110/140 with $H_o = 0.306$ and ESF-340 with $H_o = 0.27$ (Chapter 2) are two large populations with high degrees of heterozygosity. [Another large population, SRW-000 may only have $H_o = 0.141$, however this value is still considerable when compared to Hamrick & Godt's (1989) compilation (Table 2.3)]. It is in the average number of alleles (A) and effective alleles (A_e), 1.32 and 1.18, respectively, that the *O. flocktoniae* populations are found wanting. All smaller populations except ERA-095 had higher A_e compared to the rest of studied populations (see Table 2.5); this makes them valuable as well.

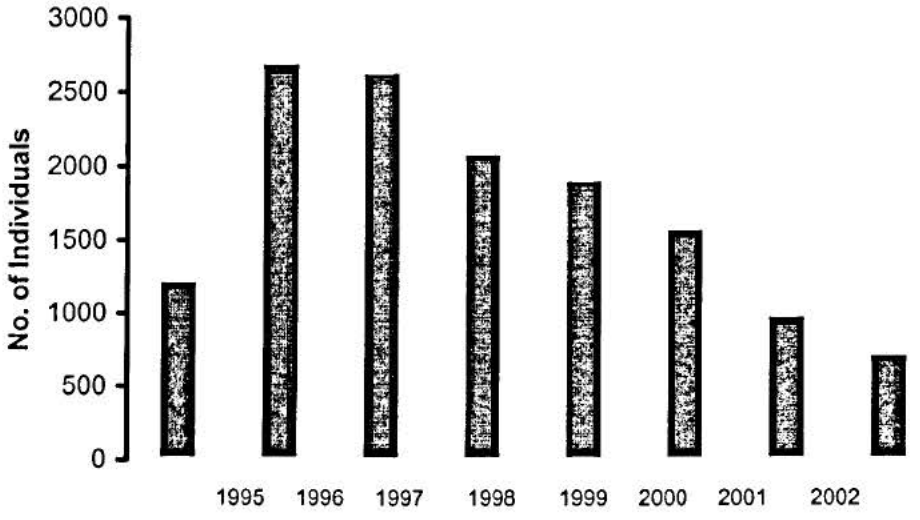

Fig. 2.18. Total number of individuals counted each year from 1995-2002 showing the decline of plant counts. Source: Mackay & Gross, 2002.

Applying a disturbance regime to recently extinct aboveground populations like BBR-175, BBR-250 and FHR-GP (Table 4.1) is also recommended. BBR-175 and FHR-GP had the highest degrees of expected heterozygosity (H_e = 0.21) and observed heterozygosity (H_o = 0.31), respectively. FHR-GP also happens to be the oldest population in the study and its seed bank may contain rare alleles (which were not detected in the aboveground genetic study, having a population size in 2001 of 3).

Some populations sampled in Chapter 2 were very small in size (e.g. ERA-095 with N = 3). Such populations were included in order to acquire a wide demographic representation necessary in comparing sample size effects to genetic heterozygosity. In other studies of endemic taxa "small" population size probably means 50 individuals. Unfortunately in *O. flocktoniae* a number of populations in their natural habitat have a population of 1 (Mackay & Gross, 2001). This type of sampling creates an error that makes interpretation of results difficult. However there was sufficient number of isozymes sampled for electrophoresis. To begin with, plant isozymes used in this type of study are limited compared to what is available for animal isozyme studies. Nonetheless 15 scorable isozymes are ample for statistical interpretation.

Olearia flocktoniae or the Dorrigo Daisy is a naturally rare species (Gross & Mackay, 1998). Apart from protecting the populations from threatening factors such as exotic vegetation, damage from vehicles, deliberate destruction for grazing purposes and untimely roadside slashing (before seed-set) [Gross & Mackay, 1998], providing the species suitable habitats with pioneer conditions should be part of its recovery plan. Genetic assays should be routinely conducted in this species. A range of different life stages can also be genetically analysed. Aboveground and seed bank genetic analysis should be conducted at least every five years. A genetic assay of other *Olearia* species, whether rare or common, may also prove useful in understanding *O. flocktoniae*.

Conclusively, the interpretation of genetic data from these studies could only be deemed useful and meaningful if the demographic data of the populations under investigation are also considered, especially when the former are not very similar to compiled published available data for plant species of the same status.

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Appendix I. Staining Gels.

Aspartate aminotransferase (AAT) E.C. 2.6.1.1 (Wendel & Weeden, 1989)

AAT substrate solution*	50 ml
Fast Blue BB salt	50 mg

*AAT substrate solution:

H_2O	800 ml
α-Ketoglutaric acid	292 mg
L- Aspartic acid	1.07 g
PVP- 40	4.00 g
EDTA, Na_2 salt	400 mg
Sodium phosphate, dibasic	11.36 g

(This solution can be refrigerated for up to three weeks without noticeable loss of activity.)

Procedure: Add Fast Blue BB to substrate solution and incubate at room temperature in the dark, until blue bands appear. Rinse and fix.

Acid phosphatase (ACP) E. C. 3.1.3.2 (Wendel & Weeden, 1989)

50 mM Na-acetate buffer, pH 5.0	50 ml
Na-α-napthyl acid phosphate	50 mg
$MgCl_2$	50 mg
Fast Garnet GBC salt	50 mg

Procedure: Dissolve reagents on buffer and pour on gel. Incubate in the dark until desired staining intensity has occurred. Rinse, and store in water or fix.

Alcohol dehydrogenase (ADH). E.C. 1.1.1.1 (Wendel & Weeden, 1989)

50mM Tris-HCl, pH 8.0	50 ml
NAD	10 mg
Ethanol	0.2 ml
NBT or MTT	10 mg
PMS	2 mg

Procedure: Combine ingredients and pour over gel. Incubate until bands are optimally developed. Rinse and store in water (if NBT is used) or fixative.

Aldolase (ALD). E.C. 4.1.2.13 (Wendel & Weeden, 1989)

50mM Tris-HCl, pH 8.0	50 ml
NAD	10 mg
Arsenic acid, Na salt	75 mg
Fructose-1,6-diphosphate, Na salt	200 mg
Glyceraldehyde-3-phosphate dehydrogenase	100 units
NBT or MTT	10 mg
PMS	2 mg

Procedure: Combine ingredients and pour over gel. Incubate until bands are optimally developed. Rinse and store in water (if NBT is used) or fixative.

Catalase (CAT) E.C. 1.11.1.6 (Wendel & Weeden, 1992)

Hydrogen peroxide, 0.01 %	50 ml
H_2O	50 ml
Ferric chloride	500 mg
Potassium ferricyanide	500 mg

Procedure: Pour H_2O_2 on gel slice and leave for 5 minutes . Meanwhile, mix remaining ingredients. Pour off peroxide and add stain solution. Swirl gently until bands are developed. Catalase activity is revealed as achromatic zones on a green background. Rinse three or four times and store in water.

Diaphorase (DIA) or NAD(P)H dehydrogenase E. C. 1.6.99 (Acquaah, 1992)

200 mM Tris-HCl, pH 8.0	50 ml
Menadione	20 mg
NADH	10 mg
MTT	10 mg

Procedure: Incubate in the dark for 30 minutes. Rinse and fix.

Glucose-6-phosphate dehydrogenase (G6PDH). E.C. 1.1.1.49
(Wendel & Weeden, 1989)

50mM Tris-HCl, pH 8.0	50 ml
NADP	5 mg
$MgCl_2$	50 mg
Glucose-6-phosphate, Na_2-salt	50 mg
NBT or MTT	10 mg
PMS	2 mg

Procedure: Mix ingredients and pour over gel. Incubate until blue bands have appeared. Rinse and store in water (if NBT is used) or fixative.

Glucose-6-phosphate isomerase (GPI). E.C. 5.3.1.9
(Wendel & Weeden, 1989)

50mM Tris-HCl, pH 8.0	50 ml
NAD	10 mg
Fructose-6-phosphate, Na_2-salt	20 mg
Glucose-6-phosphate dehydrogenase (NAD)	20 U
MTT or NBT	10 mg
PMS	2 mg

Procedure: Combine ingredients and pour over gel. Incubate until

blue bands are optimally developed. Rinse with water and fix.

Glutamate dehydrogenase (GDH). E.C. 1.4.1.2 (Wendel & Weeden, 1989)

50mM Tris-HCl, pH 8.0	50 ml
NAD	10 mg
$CaCl_2$	50 mg
L-Glutamate, Na salt	200 mg
MTT or NBT	10 mg
PMS	2 mg

Procedure: Combine ingredients and pour over gel. Incubate until blue bands are optimally developed. Rinse with water and fix.

Glyceraldehyde-3-phosphate dehydrodenase (G3PDH). E.C. 1.2.1.12 (Wendel & Weeden, 1989)

50mM Tris-HCl, pH 8.0	50 ml
NAD	10 mg
Arsenic acid, Na salt	75 mg
Fructose-1,6-diphosphate	100 mg
Aldolase	50 units
NDT or MTT	10 mg
PMS	2 mg

Procedure: Mix ingredients and pour over gel. Incubate until blue bands have appeared. Rinse and store in water (if NBT is used) or fixative.

Isocitrate dehydrogenase (IDH). E.C. 1.1.1.42 (NADP form) (Wendel & Weeden, 1989)

50 mM Tris-HCl, pH 8.0	50 ml
$MgCl_2$	50 mg
DL- Isocitric acid, Na_3 salt	50 mg
NADP	50 mg
MTT	10 mg
PMS	2 mg

Procedure: Combine ingredients and pour over gel. Incubate until blue bands appear. Rinse and fix.

Malate dehydrogenase (MDH). E.C. 1.1.1.37 (Wendel & Weeden, 1989)

50mM Tris-HCl pH 8.5	50 ml
NAD	10 mg
Malic acid	150 mg
NBT or MTT	10 mg
PMS	2 mg

Procedure: Malic acid is usually added as a neutralised (with NaOH)

aqueous solution. Ingredients are combined and poured over gel. Incubate until blue bands appear. Rinse and store in water (if NBT is used) or fixative.

Malic enzyme (ME). E. C. 1.1.1.40 (Wendel & Weeden, 1989)

50 mM Tris-HCl, pH 8.0	50 ml
$MgCl_2$	50 mg
NADP	5 mg
Malic acid	150 mg
MTT	10 mg
PMS	2 mg

Procedure: Malic acid is usually added as a neutralised (with NaOH) aqueous solution. Ingredients are combined and poured over gel. Incubate until blue bands appear. Rinse and store in water (if NBT is used) or fixative.

Peroxidase (PRX). E. C. 1.11.1.7 (Wendel & Weeden, 1989)

50 mM Na-acetate buffer, pH 5.0	50 ml
$CaCl_2$	50 mg
Hydrogen peroxide, 3%	0.25 ml
3-Amino-9-ethylcarbazole	25 mg
N,N-Dimethylformamide	2 ml

Procedure: Dissolve 3-amino-9-ethylcarbazole in the N,N-dimethylformamide. Add along with the remaining ingredients to buffer and pour over gel. Incubate at room temperature until red bands appear.

Phosphoglucomutase (PGM). E.C. 5.4.2.2 (Wendel & Weeden, 1989)

50mM Tris-HCl, pH 8.5	50 ml
$MgCl_2$	50 mg
Glucose-1-phosphate, Na_2-salt	150 mg
NAD	10 mg
Glucose-6-phosphate dehydrogenase (NAD)	20 units
MTT	10 mg
PMS	2 mg

Procedure: Combine ingredients and pour over gel. Incubate until blue bands are optimally developed. Rinse well and fix.

Phosphogluconate dehydrogenase (PGD). E.C. 1.1.1.44
(Wendel & Weeden, 1989)

50mM Tris-HCl, pH 8.0	50 ml
$MgCl_2$	50 mg
6-Phosphogluconic acid, (Na or Ba salt)	20 mg
NADP	5 mg
NBT or MTT	10 mg
PMS	2 mg

Procedure: Combine ingredients and pour over gel. Incubate until blue bands appear. Rinse and store in water (if NBT is used) or fixative.

Superoxide dismutase (SOD). E.C. 1.15.1.1

Bands viewed in gels stained for GPI.

Xanthine dehydrogenase (XDH). E.C. 1.1.1.204 (Murphy *et al.*, 1996)

200 mM Tris-HCl, pH 8.0	5 ml
100 mM KOH	5 ml
Hypoxanthine	200 mg
Stir above solution for at least 10 mins, then add:	
200 mM Tris-HCl, pH 8.0	40 ml
NADH	20 mg
NAD	10 mg
NBT	5 mg
PMS	2 mg

Procedure: Adjust pH to 8.0 if necessary. Cover gel slice with stain solution making sure that any undissolved hypoxanthine covers the gel slice. Incubate at 37°C in the dark.

Appendix II. Regression Analyses (Chapter 2).

logN v P

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: P
Independent variable: logN

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	0.276566	0.0524436	5.2736	0.0002
Slope	0.0323314	0.0369642	0.874667	0.3989

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.00373737	1	0.00373737	0.77	0.3989
Residual	0.0586221	12	0.00488517		
Total (Corr.)	0.0623594	13			

Correlation Coefficient = 0.244812
R-squared = 5.99327 percent
R-squared (adjusted for d.f.) = -1.84063 percent
Standard Error of Est. = 0.069894
Mean absolute error = 0.0545941
Durbin-Watson statistic = 0.837613 (P=0.0056)
Lag 1 residual autocorrelation = 0.578407

logN v A

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: A
Independent variable: logN

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	1.27657	0.0524436	24.3417	0.0000
Slope	0.0323314	0.0369642	0.874667	0.3989

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value

Model	0.00373737	1	0.00373737	0.77	0.3989
Residual	0.0586221	12	0.00488517		
Total (Corr.)	0.0623594	13			

Correlation Coefficient = 0.244812
R-squared = 5.99327 percent
R-squared (adjusted for d.f.) = -1.84063 percent
Standard Error of Est. = 0.069894
Mean absolute error = 0.0545941
Durbin-Watson statistic = 0.837613 (P=0.0056)
Lag 1 residual autocorrelation = 0.578407

logN v A_e
Regression Analysis - Linear model: Y = a + b*X

Dependent variable: Ae
Independent variable: logN

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	1.18449	0.0407455	29.0704	0.0000
Slope	-0.00661675	0.0287189	-0.230397	0.8217

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.000156533	1	0.000156533	0.05	0.8217
Residual	0.0353863	12	0.00294886		
Total (Corr.)	0.0355429	13			

Correlation Coefficient = -0.0663632
R-squared = 0.440407 percent
R-squared (adjusted for d.f.) = -7.85623 percent
Standard Error of Est. = 0.0543034
Mean absolute error = 0.0411544
Durbin-Watson statistic = 0.621999 (P=0.0008)
Lag 1 residual autocorrelation = 0.65376

logN v H_e

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: He
Independent variable: logN

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	0.15119	0.0298583	5.06358	0.0003
Slope	-0.00186755	0.0210453	-0.0887398	0.9308

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.0000124699	1	0.0000124699	0.01	0.9308
Residual	0.0190024	12	0.00158353		
Total (Corr.)	0.0190149	13			

Correlation Coefficient = -0.0256086
R-squared = 0.0655799 percent
R-squared (adjusted for d.f.) = -8.26229 percent
Standard Error of Est. = 0.0397936
Mean absolute error = 0.0292083
Durbin-Watson statistic = 0.65412 (P=0.0011)
Lag 1 residual autocorrelation = 0.646149

logN v H_o

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: Ho
Independent variable: logN

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	0.197839	0.0619811	3.19193	0.0077
Slope	0.00416224	0.0436866	0.095275	0.9257

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value

Model	0.0000619401	1	0.0000619401	0.01	0.9257
Residual	0.0818833	12	0.00682361		
Total (Corr.)	0.0819452	13			

Correlation Coefficient = 0.0274931
R-squared = 0.0755871 percent
R-squared (adjusted for d.f.) = -8.25145 percent
Standard Error of Est. = 0.0826051
Mean absolute error = 0.0689223
Durbin-Watson statistic = 0.896156 (P=0.0086)
Lag 1 residual autocorrelation = 0.487219

logN v *f*

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: f
Independent variable: logN

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	-0.423324	0.262421	-1.61315	0.1327
Slope	0.0304167	0.184964	0.164446	0.8721

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.00330781	1	0.00330781	0.03	0.8721
Residual	1.46782	12	0.122319		
Total (Corr.)	1.47113	13			

Correlation Coefficient = 0.0474181
R-squared = 0.224848 percent
R-squared (adjusted for d.f.) = -8.08975 percent
Standard Error of Est. = 0.349741
Mean absolute error = 0.268928
Durbin-Watson statistic = 2.03714 (P=0.4606)
Lag 1 residual autocorrelation = -0.0829395

Appendix III. Regression Analyses (Chapter 3).

Age of Population v H_o

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: Ho
Independent variable: age

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	0.107615	0.0280343	3.8387	0.0185
Slope	0.00390716	0.00481968	0.810669	0.4630

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.000959208	1	0.000959208	0.66	0.4630
Residual	0.00583829	4	0.00145957		
Total (Corr.)	0.0067975	5			

Correlation Coefficient = 0.375649
R-squared = 14.1112 percent
R-squared (adjusted for d.f.) = -7.36101 percent
Standard Error of Est. = 0.0382044
Mean absolute error = 0.0242325
Durbin-Watson statistic = 1.95931 (P=0.2977)
Lag 1 residual autocorrelation = -0.127523

Age of Population v f

Regression Analysis - Linear model: Y = a + b*X

Dependent variable: f
Independent variable: age

Parameter	Estimate	Standard Error	T Statistic	P-Value
Intercept	0.0434615	0.162838	0.2669	0.8027
Slope	-0.0246127	0.0279952	-0.879177	0.4290

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Model	0.0380636	1	0.0380636	0.77	0.4290
Residual	0.196978	4	0.0492445		
Total (Corr.)	0.235042	5			

Correlation Coefficient = -0.402423
R-squared = 16.1944 percent
R-squared (adjusted for d.f.) = -4.75698 percent
Standard Error of Est. = 0.221911
Mean absolute error = 0.153347
Durbin-Watson statistic = 2.06598 (P=0.2856)
Lag 1 residual autocorrelation = -0.316261