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Application of microsatellite markers for population genetics of three New Zealand *Corybas* taxa (Orchidaceae)

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Abstract

Many New Zealand *Corybas* orchid species are endangered due to habitat destruction caused by changes in land use. Conservation efforts are needed to secure survival of those endangered species. Unfortunately, there are very few studies on these orchids and several aspects of their ecology, biology, and reproductive biology remain unknown. It is difficult to develop efficient conservation plans for those species without this information. This study describes the first genetic investigation in *Corybas* species and provides insight to their reproductive methods. Genetic diversity assessment was conducted in population(s) of three *Corybas* taxa, and the results were used to infer their genetic structure and reproductive methods. Pollination experiments and pollinator observations were carried out in one species, *Corybas carsei,* to understand the pollination strategies in this endangered species.

Twelve previously developed microsatellite markers were used to genotype 37 *Corybas* “Remutaka” individuals collected from one population; these detected a medium level of genetic variation within population. No correlation between genetic and geographic distances were found. The genetic assessment results indicated that self-pollination, cross-pollination, and asexual reproduction likely occur in this population.

As the readily available *Corybas* microsatellite markers were not transferable to *C. carsei*, novel microsatellite markers were developed using next-generation sequencing. Eleven markers were used to genotype 29 *C. carsei* individuals sampled from the single remaining *C. carsei* population. With the finding of only monomorphic loci, no, or possibly extremely low, genetic variation was detected within the population and, hence, reproductive methods employed by the *C. carsei* population could not be inferred. Conversely, 11 microsatellite markers used to genotype 67 *Corybas macranthus* individuals from three populations detected low to medium genetic variation within populations, and a low degree of genetic differentiation between populations. The results of within-population genetic assessment indicated that cross-pollination, self-pollination, and clonal propagation occurred.

Pollination experiments in *C. carsei* showed that this orchid is self-compatible and autonomous self-pollination may occur. Its putative pollinator, flies, approaching the flower was captured on camera. No pollination events were observed and the species of

those flies were not identifiable. Overall, the findings contribute to our understanding of New Zealand *Corybas* orchids and offer future directions for their conservation.

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List of Abbreviations

%P	percent polymorphic loci
ΔK	delta K
A	number of alleles per locus
AFLPs	amplified fragment length polymorphisms
BC	Butterfly Creek
bp	base pair
BVF	Bridal Veil Falls
CASS	cheap as size standard
cpDNA	chloroplast DNA
CTAB	cetyltrimethylammonium bromide
ddRAD	double digest restriction-site associated DNA
DoC	Department of Conservation
F_{IS}	Wright's fixation coefficient
F_{ST}	measure of population differentiation
GBS	genotyping-by-sequencing
H_E	expected heterozygosity
H_O	observed heterozygosity
HWE	Hardy-Weinberg equilibrium
ITS	internal transcribed spacer
MCMC	Markov Chain Monte Carlo
MLGs	multi-locus genotypes
mtDNA	mitochondrial DNA
MY	million years
MYA	million years ago
nDNA	nuclear DNA
NE	number of effective alleles
NeSI	New Zealand eScience Infrastructure
P/O	pollen to ovule
PCoA	principal coordinates analysis
QEII	Queen Elizabeth II
R^2	coefficient of determination
RAPDs	random amplified polymorphic DNA

SEM	scanning electron microscope
SNPs	single nucleotide polymorphisms
TMP	Te Mata Peak
UV	ultraviolet
VNTRs	variable number of tandem repeats

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Chapter 1: Introduction

1.1 The Orchidaceae family and the genus *Corybas* in New Zealand

Orchidaceae is one of the largest families of flowering plants, consisting of 736 genera and approximately 28,000 species (Christenhusz & Byng, 2016; The Plant List, 2013). Orchids can be found in almost every continent, apart from Antarctica, and they occupy a wide range of habitats with the most abundance in tropical and subtropical regions (Chase, 2005; Govaerts *et al.*, 2007). The genus *Corybas* belongs to the orchid subtribe Acianthinae (Orchidaceae: Orchidoieae: Diurideae), which contains about 135 species (Lyon, 2014). Acianthinae consists of four other genera, *Acianthus*, *Cyrtostylis*, *Stigmatodactylus* and *Townsonia*; with *Corybas* being the largest genus in this subtribe (Chase *et al.*, 2003; Govaerts *et al.*, 2007). There are about 120 species of *Corybas* that can be found in the Himalayas, southern China, the Malay Archipelago, New Guinea, Australia, New Zealand, and Polynesia; all members of *Corybas* are exclusively terrestrial (Jones *et al.*, 2002; Lyon, 2014).

The Orchidaceae represent approximately 17.4% of the monocot species in New Zealand and over 70% of species are endemic (de Lange *et al.*, 2017; Lehnebach *et al.*, 2005). New Zealand orchids comprise 34 genera with over 100 species (Dawson *et al.*, 2007). Although the number of endemic species is high, many genera also occur in the South Pacific islands and Australia, and only six genera are endemic to New Zealand (Johns & Molloy, 1983; Molloy & Dawson, 1990). *Corybas* along with *Pterostylis*, *Thelymitra* and *Caladenia* are the most speciose genera in the country, including 26, 15, 13, and 9 formal and informal taxa, respectively (Lehnebach, 2002). *Corybas* species are the most widely distributed terrestrial orchids, frequently occurring in humid and dark habitats from lowland to alpine areas (Lehnebach *et al.*, 2016). Currently, there are 22 accepted species of *Corybas* in New Zealand (Schönberger *et al.*, 2020). Terrestrial orchids are considered to be the most threatened group in New Zealand, with habitat destruction due to changes in landscape use and over-collection being the main factors affecting their survival; some *Corybas* species such as *Corybas dienemus* and *Corybas carsei* are among the most threatened groups (de Lange *et al.*, 2004; Lehnebach *et al.*, 2005). In their reappraisal report of the conservation status of the indigenous New Zealand vascular plants de Lange *et al.* (2017) listed six species of orchids as “At risk”, two species “Nationally vulnerable”, two species “Nationally endangered”, and ten

species "Nationally critical". Additionally, three taxa within the nationally critical category are taxonomically indetermined.

Apart from their diverse floral structure, orchids are also known to have highly specialised pollination syndromes (Khasim *et al.*, 2020). Specialised pollinators are attracted by specifically modified floral structures, suitable to the particular pollinator, which signal resources such as food or mates (Gaskett *et al.*, 2014). Pollination is regarded as a mutualistic relationship, where both parties gain fitness benefits: the plant gets pollinated and the pollinator often gains food-related rewards (Morris *et al.*, 2010). However, about one-third of all orchid species attract pollinators without providing rewards, this is known as deceptive pollination (Schiestl, 2005). Instead, these orchids mimic signals that either indicate food resources (food deceptive pollination); potential mates (sexual deceptive pollination); or brood substrates (brood-site deceptive pollination). In addition to reproduction by pollination, many orchid species are also capable of clonal reproduction from lateral roots (Peakall & James, 1989).

1.2 Putative interactions between New Zealand *Corybas* and their pollinators

New Zealand *Corybas* have highly modified floral structures. The enlarged dorsal sepal and labellum form a trap-like structure; the labellum shows various modifications in different species; and the flowers are often marked with red or purple colouration (Figure 1.1). A unique feature of *Corybas* is that the species possess a pair of open auricles at the base of the labellum (Figure 1.2). These modified floral structures are believed to play a role in facilitating insect pollination (Lehnebach *et al.*, 2016; Lyon, 2014). New Zealand lacks certain common pollinator groups such as large social bees (indigenous bees are small and are often solitary), but has a diverse group of flies and birds that act as effective pollinators (Newstrom & Robertson, 2005). Preliminary field observations suggested that New Zealand *Corybas* are pollinated by brood-site deceptive pollination, which occurs when plants lure female insects, such as Coleoptera (beetles) and Diptera (true flies), to oviposit into the flowers by mimicking odours that are produced by oviposition substrates, for example, decaying organic matter, carrion, and fungi (Jürgens *et al.*, 2006; Urru *et al.*, 2011). Unlike the other members within *Acianthinae*, *Corybas* lacks nectar-secreting

tissues (Lyon, 2014), instead, these orchids attract female fungus gnats to oviposit into the flowers by imitating the fungus brood site (Pridgeon *et al.*, 2001).

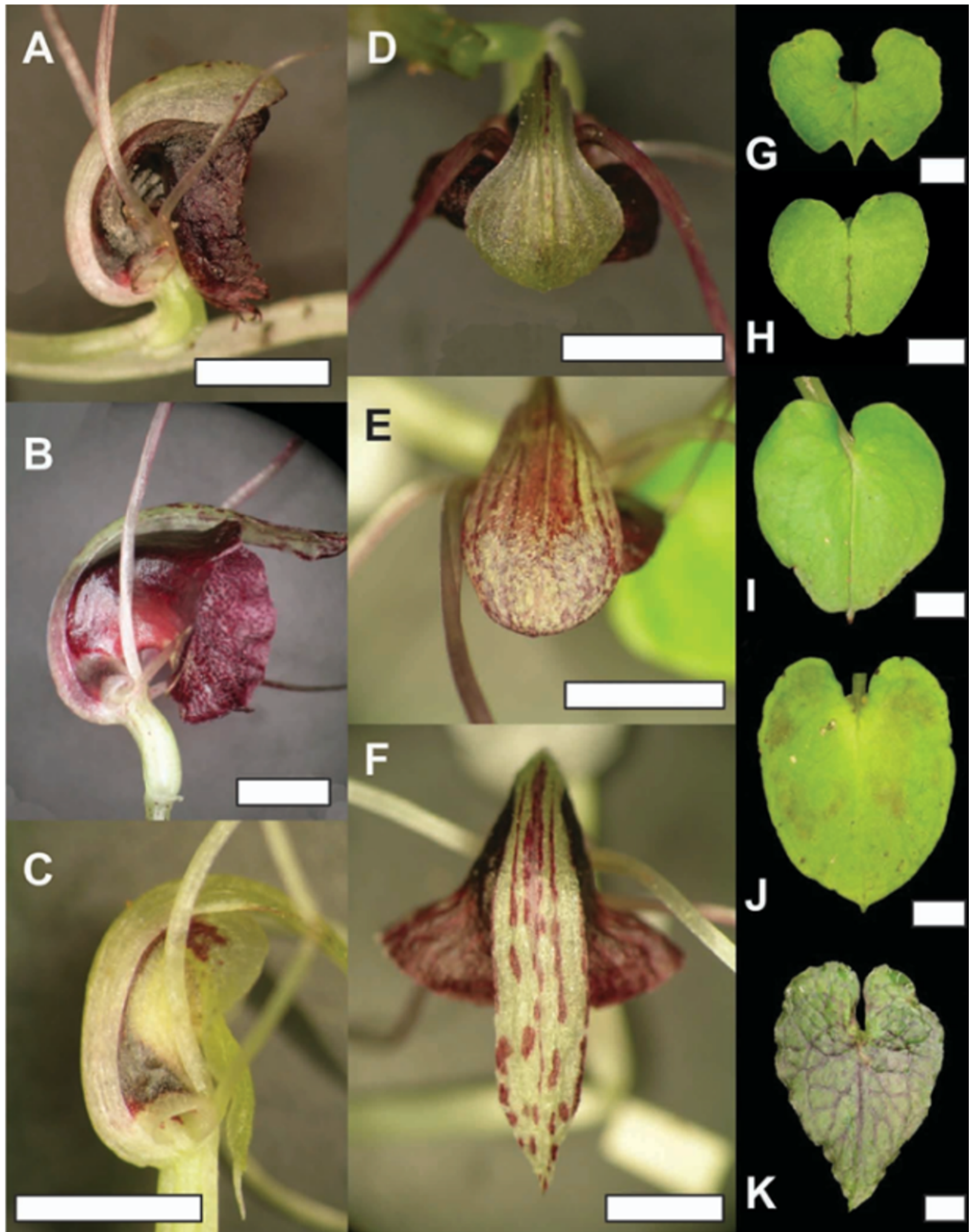

Figure 1.1 Morphological diversity of flowers and leaves in New Zealand spider orchids (*Corybas*). Labellum (A: *C. hypogaeus*, B: *C. macranthus*, C: *C. papa*); dorsal sepal (D: *C. hypogaeus*, E: *C. confusus*, F: *C. papillosus*); leaf (G: *C. hypogaeus*, H: “trotters”, I: *C. confusus*, J: *C. orbiculatus*, K: *C. acuminatus*). Scale bar = 5 mm. Image from Lehnebach *et al.* (2016).

Figure 1.2 *Corybas carsei* flower. (A) *C. carsei* plant in its habitat; (B) ventral view of a *C. carsei* flower, the flower is secured on top of a piece of playdough for photo taking. White arrows indicate auricles in both panels. Scale bar = 5 mm. Photos by Carlos Lehnebach.

Fungus gnats have been observed carrying the orchid's pollinia after visiting a flower, and flies' eggs have been discovered in the labellum (Figure 1.3; Lehnebach, 2014; Scanlen, 2006). A study on the volatile compound production in New Zealand *Corybas* showed that these orchids produce a mixture of chemical compounds such as fatty acid derivates, monoterpene, sesquiterpene, diterpene, benzenoid, and steroids (Lehnebach & Zeller, 2015). Among those compounds found in *Corybas*, the discovery of 3-Octanol provided evidence supporting brood-site deceptive pollination. Three-Octanol is usually produced by fungi. This suggests that the orchids are mimicking the smell of fungi to attract fungus gnats. Fatty acid derivatives and alkanes were also present. Alkane molecules are known to be used for communication in insects, however, alkane molecules in sexually deceptive pollinated bee orchids (*Ophrys*) are used to attract male bees to visit the flower. The combination of 3-Octanol and alkanes in *Corybas* orchids indicates that both female and male fungus gnats are attracted to the flowers, which is contradictory to the definition of brood-site deceptive pollination (Lehnebach & Zeller, 2015). Nonetheless, a study on *Corybas cheesemanii* could not identify any specific pollinators,

nor show evidence of brood-site deceptive pollination. The researchers suspected that the pollinators were either absent or rare in their study site (Kelly *et al.*, 2013).

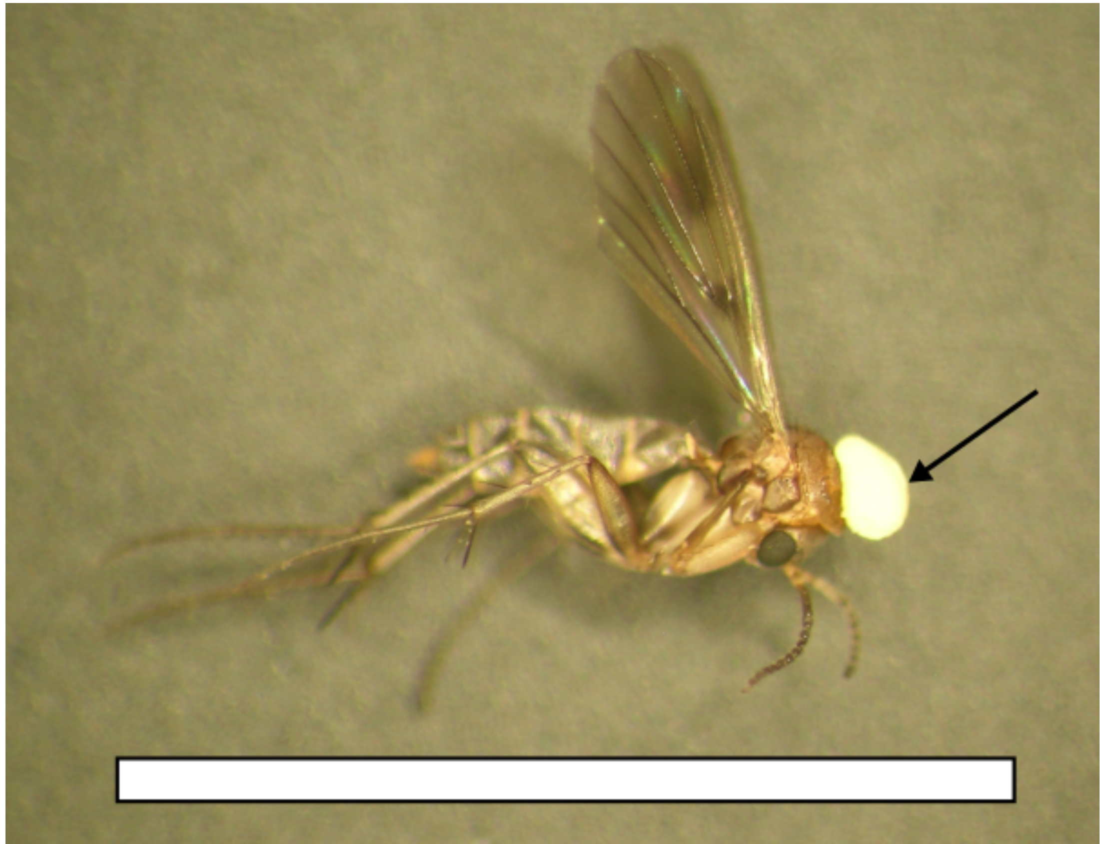

Figure 1.3 A fungus gnat (unknown species) with *Corybas* pollinia; black arrow indicates *Corybas* pollinia. Scale bar = 5 mm. Photo by Carlos Lehnebach.

In fact, brood-site deceptive pollination has been reported to attract more generalist pollinators, because the orchids are not just mimicking the mushroom brood sites where females oviposit, but also rendezvous sites for reproducing fungus gnats (Kelly *et al.*, 2013). Another study investigating the spectral reflectance and volatile odours of *C. cheesemanii* did not find evidence supporting the production of generalised fungal scent compounds. However, the results showed that these orchids may produce visual cues to attract pollinators. The dorsal sepal of *C. cheesemanii* strongly reflected ultraviolet (UV), which creates a strong contrast to the surrounding forest floor (Kelly & Gaskett, 2014). Flies have innate preferences for shorter wavelengths, and UV wavelengths are known to induce wavelength-specific fixed behaviours in insects such as phototaxis and other behaviours that are associated with foraging and reproduction (Goldsmith, 1994; Yamaguchi *et al.*, 2010). Hence, the authors proposed that in addition

to deceptive pollination, *C. cheesemanii* orchids also use visual cues to attract both sexes of fungus gnats to the visit the flowers by exploiting the flies' visual biases towards shorter wavelengths (Kelly & Gaskett, 2014).

Cryptic speciation has been suspected in New Zealand *Corybas* (Lehnebach *et al.*, 2016). Cryptic species are those that have similar morphological features and are often classified under the same species name (Bickford *et al.*, 2007). Cryptic orchid species are likely to arise from the accumulation of mutations in the genes that contribute to odour attractant production (Schiestl & Ayasse, 2002). Due to the high level of species-specificity between an orchid and its pollinator, the orchid needs to produce a specific amount and specific types of active compounds to trigger certain behaviours from flower visitors in order to achieve pollination (Ayasse *et al.*, 2000; Schiestl & Ayasse, 2002). For example, in sexually deceptive pollinated Drakaeinae orchids, the orchids mimic species-specific semiochemicals produced by female thynnine wasps, which are used by the females to attract mates and induce male sexual behaviour. Pollination is achieved when the males attempt to mate with different flowers (Bower & Brown, 2009; Jersáková *et al.*, 2006). While these orchids also attract a range of flower visitors, they only induce mating behaviour in male thynnine wasps (Bower, 1996; Bower & Brown, 2009). Alteration in odour production has the potential to lead to changes in pollinator behaviour, because the odours each orchid produces are crucial to their reproduction (i.e., pollinator attraction), and mutations that alter odour attractant production may lead to a pollinator switch. This can then lead to rapid speciation and result in morphologically indistinguishable, but genetically differentiated species (Schiestl & Ayasse, 2002).

Although, brood-site deceptive pollination is less species-specific, the different compounds produced by spider orchids are believed to attract female fungus gnats and induce oviposition in specific species (Bänziger *et al.*, 2012; Kelly *et al.*, 2013). However, several studies on *Corybas cheesmanii* pollination (Kelly & Gaskett, 2014; Kelly *et al.*, 2013) and on chemical compound production by *Corybas* orchids (Lehnebach & Zeller, 2015) did not find evidence supporting mycetophilid brood-site deception, and no pollinator had been identified. The insufficient knowledge on pollinator identity and on the active compounds produced by orchids and compounds used by their pollinators, combined with the unresolved taxonomy of New Zealand *Corybas* have led to several obstacles. Firstly, it is not clear how many different species of *Corybas* there are. Secondly, it is uncertain how many insect species are pollinating the *Corybas* orchids.

Finally, whether the orchids are mimicking the odour produced by a specific fungus to attract a specific species or just the general mushroom odour to attract a wide range of fungus gnat is also unknown (Lehnebach & Zeller, 2015).

1.3 The pollination system of New Zealand *Corybas* and cryptic species

New Zealand has been isolated from other landmasses since approximately 80 million years ago after it broke away from Gondwana resulting in two origins of its biota - lineages that originated in Gondwana and others that arrived in New Zealand from overseas via dispersal (Gibbs, 2006). The orchid subtribe Acianthinae is thought to have originated in Australia around 27 MY and dispersed to New Zealand through long distance dispersal (Lyon, 2014). Molecular studies estimated that the *Corybas* genus has a crown age of 15MY, and its lineages dispersed to New Zealand from Australia in eight separate dispersal events. *Corybas oblongus* was the first to arrive around 12.5MYA, followed by *Corybas cryptanthus* 9MYA, followed by *Corybas rivularis* and related species 8MYA, then followed by four more long distance dispersal events in the last 1MY (Lyon, 2014). The remote habitats provided the new arrivals with novel niches, less competitive conditions, and new resources that promoted rapid speciation giving rise to multiple species within different lineages (Losos & Ricklefs, 2009). Studies have suggested that the New Zealand flora has a low level of self-incompatibility and low rates of dependence on pollinators compared to the global average (Newstrom & Robertson, 2005). These ideas are supported by "Baker's Rule" that colonisations resulting from long-distance dispersal favour breeding systems that do not require biparental reproduction (Baker, 1967; Newstrom & Robertson, 2005). Despite the fact that orchids usually have a specialised pollination system, self-pollination in New Zealand orchids is prominent (Lehnebach *et al.*, 2005). Kelly *et al.* (2013) and Jones *et al.* (1981) showed that *Corybas cheesemanii* and *Thelymitra longifolia* (Lehnebach *et al.*, 2005) had a mixed-pollination strategy, utilising both insect pollination and autonomous self-pollination. Furthermore, New Zealand *Corybas* orchids have also been reported to have a mixed-reproductive system that employs both outcrossing and clonal propagation (Clements *et al.*, 2007). The clones are reproduced vegetatively from lateral roots (Clements *et al.*, 2007; Peakall & James, 1989). Every year, the plant produces a new replacement tuber for the following season before the old tuber withers and dies. Sometimes, up to five or more offshoot tubers away from the parent might be formed at

the end of the lateral roots (Figure 1.4; St George & McCrae, 1990). A mixed-pollination strategy is thought to be an adaptation when pollinator numbers are low or when the orchid populations are isolated, as self-pollination ensures the survival of the species (Bernhardt & Edens-Meier, 2010). Accordingly, a mixed-reproductive system is also believed to be an adaptation to the rapidly changing environment. While clonal propagation ensures the persistence of the species, shifts to outcrossing buffers the effect of a lack of genetic diversity which may result in the population becoming vulnerable to extinction (Hegland & Rydgren, 2016).

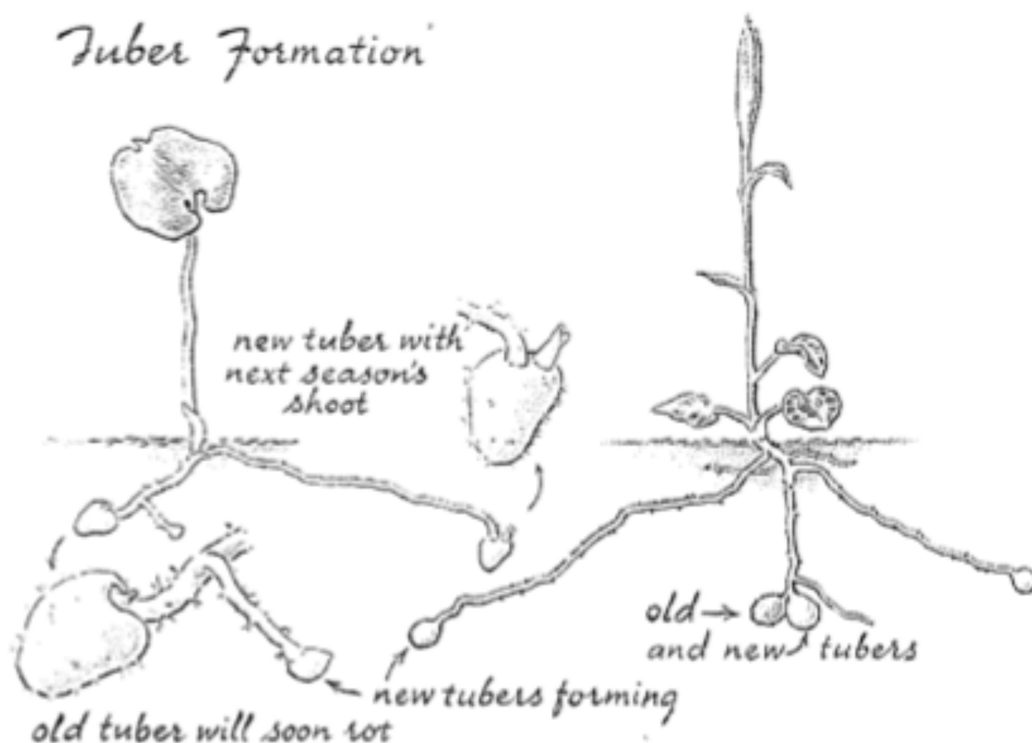

Figure 1.4 A diagram showing tuber formation in *Corybas* (left) and *Pterostylis* (right). Image from St George and McCrae (1990).

Cryptic species occur in New Zealand *Corybas*. For example, several taxa with diverse floral structures are suspected to be classified under *Corybas trilobus* (Lehnebach *et al.*, 2016). However, these morphologically distinct taxa are genetically indistinguishable in their internal transcribed spacer (ITS) and plastid DNA sequences (Bower & Brown, 2009; Lehnebach *et al.*, 2016). Difficulties in identifying species based on morphology is not limited to *Corybas*. Before the development of molecular biology, many descriptions of new orchid genera were heavily based on morphology (Chase *et al.*,

2015). As molecular technology advances, researchers suggested that both morphological and genomic data should be taken into consideration when describing new taxa. Whilst morphological data can be used for statistical tests for phenotypic disparity between cryptic and non-cryptic species, comprehensive genomic data can be used to build a robust phylogeny within lineages that will aid in categorising cryptic species (Chase *et al.*, 2015; Struck *et al.*, 2018; Surveswaran *et al.*, 2018). Plants are presumed to show adaptations to the most effective pollinator, particularly those with specialised pollination systems (van der Niet *et al.*, 2014) The unresolved taxonomic status of the *Corybas* lineages imposes difficulties on attempts to understand their pollination systems. For instance, it is uncertain which features are the result of typical evolutionary processes and which are the result of pollination adaptation (Joffard *et al.*, 2020).

A study by Lehnebach *et al.* (2016) that used ITS and chloroplast markers (*trnL* intron, *trnL-F* spacer, and *psbJ-petA* spacer) to investigate phylogenetic relationships and diversification among 11 New Zealand *Corybas* orchid taxa reported that multiple accessions with distinct morphological features were not distinguishable in their ITS nor plastid DNA sequences. For example, two unnamed orchids within the *C. trilobus* aggregate, *C.* "Remutaka" (refer to section 2.2 Introduction, Chapter 2 for name and authority) and *Corybas.* "trotters", are morphologically distinct from each other and from a named species, *C. hypogaeus*. However, their ITS and plastid sequences are indistinguishable from each other. The authors proposed that the low level of genetic divergence found in *Corybas* orchids is likely a result of recent diversification (Lehnebach *et al.*, 2016; Lyon, 2014). Low genetic divergence in New Zealand *Corybas* species has hindered taxonomic decisions and the unresolved taxonomy has impacted their conservation. Some of the undescribed orchids have been listed in the list of Threatened and Uncommon Plants of New Zealand and are in need of conservation (de Lange *et al.*, 2017). By utilising more rapidly evolving markers such as microsatellite markers may be able to capture more genetic variation to resolve insufficient detection of phylogenetic signals (Zimmer & Wen, 2015).

1.4 Reproductive strategies, genetic variation, and conservation

Many studies have shown that genetic diversity is positively correlated with the survival and persistence of a species (Reed & Frankham, 2003); it is important for individual-level

fitness, providing adaptation to environmental changes, and increasing ecosystem stability and resilience (Abbot *et al.*, 2011; Hughes *et al.*, 2008; Leimu *et al.*, 2006). Accordingly, a population that is experiencing loss of genetic diversity is of conservation concern as it becomes increasingly susceptible to extinction (Spielman *et al.*, 2004). In plants, life history characteristics such as life form, reproductive strategies, taxonomic status, and population size have profound impacts on the genetic structure and genetic partitioning within and among populations (Hamrick, 1983; Loveless & Hamrick, 1984). Due to their sessile nature, plants rely on pollen transport by biotic and/or abiotic vectors, such as animals and wind, which results in cross-pollination (Hamrick *et al.*, 1991). Sexual reproduction generates genetic variation through recombination, and cross-pollination accounts for gene flow through pollen dispersal (Solé *et al.*, 2004). These mechanisms create genetic variation within the population on which natural selection can act. Thus, population fitness is increased as individuals can respond and adapt to the continuously changing environment (Solé *et al.*, 2004).

Deceptive pollination is a fascinating topic in orchid pollination ecology. Studies have shown that on average, deceptive orchids produce lower fruit set compared to rewarding species (Neiland & Wilcock, 1998; Tremblay *et al.*, 2005). Moreover, many orchids have a low level of self-incompatibility, therefore self-pollination can significantly reduce population fitness due to reduced quality and quantity of seed sets resulting from inbreeding depression (Johnson *et al.*, 2004; Peakall & Beattie, 1996). Despite the fact that deceptive orchids produce less fruit set, deceptive pollination is believed to be a mechanism that promotes outcrossing. Dressler (1981) proposed the *outcrossing hypothesis*: deceptive pollination reduces pollen deposition between flowers of the same inflorescence (geitonogamous pollination) and increases the distance of pollen flow. Because in a deceptive pollination system, pollinators tend to visit fewer flowers on the same inflorescence and travel longer distances before visiting another flower after being defrauded. Field experiments have confirmed that when visiting deceptive species, pollinators probe fewer flowers in close proximity and depart quickly to a further location (Ayasse *et al.*, 2000). As a result, pollen flow and pollen exportation rates increase compared to rewarding orchids (Johnson *et al.*, 2004). Empirical data also showed that deceptive orchids have higher seed quality than rewarding species which increases the life-time reproductive merit of deceptive orchid lineages (Peakall & Beattie, 1996; Sonkoly *et al.*, 2016).

Conversely, clonal propagation produces genetically identical individuals, reducing the population's stress resistance due to the lack of genetic variation to provide adaptation (Després *et al.*, 2002; Hamrick & Godt, 1990; Peakall & Beattie, 1991). Furthermore, in species that are self-compatible, clonality within a population further reduces genetic diversity by inbreeding as the pollen being deposited is from genetically identical individuals (Angeloni *et al.*, 2011). Although clonal propagation appears to have a negative impact on the population, it is believed to be an adaptation to the unfavourable conditions such as low temperature and low light environment, as it ensures the persistence of the species (van Groenendael *et al.*, 1997). Nonetheless, populations with limited or reduced genetic variation are of conservation concern, as they become more susceptible to genetic drift and extinction (Chung *et al.*, 2004). New Zealand *Corybas* orchids are reported to have a mixed-reproductive system, they are capable of reproducing vegetatively from propagation through underground lateral roots and to reproduce sexually from brood-site deceptive pollination. In addition, autonomous self-pollination may also occur (Lehnebach, 2014; Peakall & James, 1989). These different reproductive methods may have a profound impact on a population's genetic structure and genetic variation (Loveless & Hamrick, 1984).

The ability of natural populations to respond to climate and environmental changes depends on the presence of genetic variation within the population (Loveless & Hamrick, 1984). Being able to conserve genetic diversity within populations will enable *in situ* evolution of adaptations that benefit the long-term persistence of a species (Bailey *et al.*, 2009). New Zealand terrestrial orchids have been reported to be one of the most threatened groups, for instance compared to epiphytic orchids, with 16 described species and 3 taxonomically unresolved taxa being listed on the threatened and uncommon New Zealand plants list (de Lange *et al.*, 2017). Due to the species-specific pollination system, a decrease in the number of pollinators will also hamper the possibility of successful pollen deposition (Bernhardt & Edens-Meier, 2010). Because New Zealand flora show a high level of self-compatibility, inbreeding depression may happen if pollinators are scarce or become extinct (Van Etten *et al.*, 2015). Therefore, maintaining pollinator populations at an adequate level is important for the persistence of a species and for the maintenance of the genetic diversity within orchid populations as pollinators promote outcrossing (Fay, 2018).

Endangered species usually comprise small and fragmented populations, and these small populations are of more conservation concern. In many rewarding and deceptive orchids, genetic variation is positively correlated with population size (Gijbels *et al.*, 2015). Reproduction between related individuals is more likely to occur, and there is a higher probability for self-pollination due to failure to attract pollinators and pollen limitation in small populations (Hens *et al.*, 2017). As a result, heterozygosity within populations may decrease, and consequently an increase in the expression of deleterious recessive alleles, which may cause reduced fitness of individuals within these small populations (Reed & Frankham, 2003; Young *et al.*, 1996). Even though it is difficult to capture the long-term effects of low genetic variation on populations, there is evidence of the impacts of inbreeding in plant reproduction. In an endangered orchid species, *Platanthera praeclara*, outcrossed flowers produced a higher percentage of seeds with an embryo and a greater proportion of seeds had a larger embryo compared to self-pollinated flowers (Travers *et al.*, 2018). Self-pollination in *P. leucophaea*, a sister species of *P. praeclara*, also resulted in a reduction in seed mass and absence of large embryos (Wallace, 2003).

Unfortunately, it is difficult to develop efficient conservation programmes for some *Corybas* species due to their unresolved taxonomy and the unclear orchid-pollinator relationships. A better understanding of a population's genetic structure provides insights to the reproductive strategies employed by the plants, and this information will help develop more efficient conservation plans to ensure the persistence and to increase population size of the target species (Johnson, 2010).

1.5 Genetic Markers for Assessing Genetic Variation

Genetic markers have been widely applied to investigations of population, conservation, and evolutionary biology (Sunnucks, 2000). Fundamentally, there are two different classes of genetic markers: protein variants (allozymes) and DNA-based markers. Allozymes were the first molecular markers developed for assessing genetic variation. However nowadays, many of them have been replaced by DNA markers. This is because DNA markers survey DNA variation directly from the DNA sequence, rather than relying on differences in the electrophoretic mobility of proteins that are encoded by the DNA in order to infer genetic variation (Schlötterer, 2004). DNA-based markers can be further

categorised into three classes: nuclear DNA (nDNA), mitochondrial DNA (mtDNA), and chloroplast DNA (cpDNA) (Wan *et al.*, 2004). mtDNA is a haploid, maternally inherited genome. Due to the differences in its inheritance and ploidy, for every copy of mtDNA being passed down to the next generation, there are about four copies of nDNA being passed on. Therefore, mtDNA markers only have one-quarter of the genetic effective population size of nuclear markers (Ballard & Whitlock, 2004). Furthermore, although plant mtDNA has high rates of duplication, rearrangement, and recombination (Palmer & Herbon, 1988), its rate of evolution is the slowest compared to nDNA and cpDNA (Wolfe *et al.*, 1987). This leads to a lack of resolution for population genetic studies (Zhang & Hewitt, 2003). Similar to mtDNA, the chloroplast genome is also maternally inherited, and is the smallest genome (120-170 kb) compared to mitochondrial (200-2,500 kb) and nuclear genomes (60 Mbp-150,000 Mbp) (Patwardhan *et al.*, 2014). cpDNA is assumed to be conserved, it has low rates of rearrangement and nucleotide substitutions, which results in an insufficient amount of information for determining relationships between closely related taxa (Palmer *et al.*, 1988). nDNA markers such as amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNA (RAPDs), single nucleotide polymorphisms (SNPs), and variable number of tandem repeats (VNTRs: minisatellites and microsatellites) are biparentally inherited. VNTRs have a faster rate of evolution and can be used to reconstruct population history and identify contemporary gene flow between fragmented subpopulations (Wan *et al.*, 2004).

Microsatellites are short tandem nucleotide repeats that are widely distributed in eukaryotic genomes. Microsatellite markers typically consist of several 1-6 bp repeats, each repeat can be comprised of mono-, di-, tri, or tetra- tandemly repeating nucleotide units, and are highly polymorphic due to high mutation rates (Ashley & Dow, 1994). These markers are flanked by unique, non-repetitive DNA sequences, the flaking regions, and these regions are used to identify a specific microsatellite locus (Selkoe & Toonen, 2006). High-standing allelic diversity accompanied with co-dominance in microsatellite markers enables them to detect genetic variation among closely related species, to identify and track parentage and relatedness between interacting individuals, and to differentiate between heterozygosity and homozygosity (Sunnucks, 2000). Microsatellites are often found in non-coding regions of the genome, which means that they are neutral markers and variation in them is not the result of natural selection. Moreover, the shorter lengths of microsatellite loci allow the use of degraded or small amounts of DNA, which make

them ideal genetic markers for studies on endangered species as genetic material might be limited (Queller *et al.*, 1993). Although it is more time and cost intensive to develop microsatellite markers for non-model species, their high sensitivity, high resolution, and high polymorphism can provide comprehensive information on the genetic structure of populations and on species delimitation (Duminil & Di Michele, 2009).

1.6 Focus of this research

The aim of this study is to use microsatellite markers to assess the genetic composition and genetic variation (the observed and expected heterozygosity) of *Corybas* populations, to examine the extent of clonal propagation within a population, and to infer the reproductive methods employed by the population based on the genetic variation within the population.

1.7 Study group

Corybas carsei, also known as the swamp helmet orchid, is the most threatened terrestrial orchid in New Zealand. This orchid used to occur in several locations across the North Island (Clarkson *et al.*, 1993). However, the number of populations of this species drastically reduced to a single small population over the past 100 years due to habitat destruction. Today, this species is represented by a single population with about 300 individuals found in the Whangamarino Wetland, Waikato (Department of Conservation, 2019). Certain life history traits of *Corybas,* including clonal propagation, self-pollination, and self-compatibility are associated with low heterozygosity in populations (Hamrick *et al.*, 1979; Loveless & Hamrick, 1984). Although, it is unknown if *C. carsei* shares these life history traits that have been observed in other New Zealand *Corybas*, we expect to find low genetic diversity within *C. carsei*. Additionally, a reduction in population size may also cause decrease in allelic variation by eliminating alleles that occur at low frequencies (Lowe *et al.*, 2005). On the other hand, an unnamed taxon, *C.* "Remutaka" is part of the *C. trilobus* aggregate that occurs in the lower part of the North Island of New Zealand. Although this entity has a much larger population size compared to *C. carsei*, we still expect to find low genetic diversity within *C.* "Remutaka" due to the life history traits of *Corybas* that are correlated with low genetic variation in populations. With the sensitivity of microsatellite markers, genetic diversity within and among populations will

be assessed which will provide information of the genetic composition and genetic variation of these populations. The results will help understand the reproductive method of *Corybas* orchids, thus more efficient management plans can be developed for target groups.

Objectives

1. To assess genetic composition, genetic variation, and the extent of clonality within a population of *C.* "Remutaka", a member of the *Corybas trilobus* aggregate.
2. To assess the genetic composition and genetic variation within the single remaining population of *Corybas carsei*, and to infer the reproductive methods employed by this population based on the genetic variation within the population.
3. To investigate the pollination biology of *C. carsei* and to identify its pollinators.

The aforementioned objectives will be addressed in the following two chapters. Chapter 2 consists of the population genetic study of *C.* "Remutaka". Chapter 3 investigates the genetic variation, reproductive strategies, autogamy, self-incompatibility, and pollinator identity of *C. carsei*. Lastly, Chapter 4 summarises the results of the previous chapters and provides a conclusion to this study.

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Chapter 2: A population genetics study in *Corybas* "Remutaka", a taxonomically undetermined orchid in the *Corybas trilobus* aggregate

2.1 Abstract

Life history traits in plants, such as pollination strategies and reproductive methods, greatly impact a population's genetic variation. Cross-pollination generates genetic diversity within a population through gene flow and recombination, it in turn provides genetic potential for the population to respond to a changing environment. Conversely, self-pollination reduces genetic variation and ultimately leading to inbreeding depression. Consequently, the fitness of the population decreases as it is less adaptive to the environment. Clonal structure within a self-compatible population may further reduce genetic variation through self-pollination as the pollen being deposited is from genetically identical individuals. A number of preliminary observations suggested that New Zealand *Corybas* orchids have a mixed reproductive system—they can reproduce asexually from underground tubers and sexually from deceptive pollination mediated by insects (likely fungus gnats). Whether this pollination system promotes geitonogamous self-pollination or cross-pollination is unknown.

Twelve microsatellite markers were used to examine the genetic composition, genetic variation, and the extent of clonality within a taxonomically uncertain orchid population *Corybas* "Remutaka" found at a QEII Covenant, Wellington, New Zealand. A medium level of genetic variation was detected (mean $H_E = 0.573$) within population. The population as a whole showed homozygote excess ($F_{IS} = 0.314$), suggesting that inbreeding occurs. However, heterozygosity varied across the 12 loci tested, likely caused by cross-pollination. Therefore, it is inferred that both self-pollination and cross-pollination occurred. Clonality assessment indicated the presence of clonal individuals within this population, suggesting that asexual reproduction also occurs. The population showed no correlation between genetic distance and geographic distance, this likely resulted from gene flow assisted by pollinators and seed distribution within the population. However, genetic structure was present among clonal individuals. This study provides insight to genetic variation and reproductive methods in *C.* "Remutaka" and has suggested relevant future studies.

2.2 Introduction

Whilst sexual reproduction is thought to be a more prominent reproductive method in nature, about 80% of angiosperms are able to reproduce asexually by clonal growth (Klimes, 1997), and both methods often occur simultaneously (Barrett, 2015). Clonal growth or clonality is the vegetative propagation of offspring that are genetically identical to each other and to the parent plant (Gaudeul *et al.*, 2019).

It has been suggested that asexual reproduction has some ecological and evolutionary advantages for plants. For instance, this mode of reproduction avoids the cost of flowering, pollinator attraction (e.g., nectar production), and fruiting. It also ensures the persistence of a species when the germination rates of the seeds and the survival rates of seedlings are low. Further, it allows reproduction independent of pollinator activities; this is especially important for self-incompatible plant species (Vallejo-Marín *et al.*, 2010). In agreement with the assumption that clonality is an adaptive feature in unfavourable conditions such as in cold, nutrient-poor, and/or low light environments that limit sexual reproduction (van Groenendael *et al.*, 1997), many herbs growing in understory habitats in temperate forests exhibit clonal propagation (Lin *et al.*, 2016). Furthermore, in species that are capable of clonal propagation, peripheral populations that are exposed to suboptimal environmental conditions can reproduce by vegetative propagation more frequently than populations that are located in the centre of their distribution (Dorken & Eckert, 2001; Silvertown, 2008). Conversely, clonal reproduction may impact plant fitness within a population depending on the distribution and extent of clonal structure. In self-compatible species, significant clonal structure may promote geitonogamous self-pollination (pollination between flowers of the same individual) as the pollen being deposited is from genetically identical individuals (Angeloni *et al.*, 2011). As a result, the individual fitness of progeny resulting from self-pollination may be reduced due to inbreeding depression (Vallejo-Marín *et al.*, 2010).

Additionally, different reproductive methods also affect the genetic variation and genetic composition within and/or between populations (Loveless & Hamrick, 1984). Sexual reproduction in plants relies on pollen transport by either biotic or abiotic vectors such as animals and wind to transfer pollen between different individuals; this process is known as cross-pollination (Hamrick *et al.*, 1991). Cross-pollination promotes gene flow and enables recombination between variable copies of a chromosome, thus sexual reproduction is a mechanism that creates genetic variation within and among populations

(Solé *et al.*, 2004). On the other hand, asexual reproduction produces genetically identical individuals, therefore no genetic variation is generated. Moreover, inbreeding, including self-fertilisation in self-compatible species and reproduction between close relatives, may further reduce genetic variation (Chung *et al.*, 2004). Nevertheless, populations that are experiencing reduction in genetic variation or with limited genetic variation are of conservation concern, because genetic variation is responsible for the persistence and resilience of a species as it provides adaptions that allow the populations to respond to environmental changes (Solé *et al.*, 2004).

Members within the genus *Corybas* in New Zealand are known as helmet orchids or spider orchids, which refers to some species with helmet-like flowers or with leg-like elongated lateral sepals and petals, respectively (New Zealand Plant Conservation Network, 2021). A mixed reproductive method, both asexual and sexual reproduction, has been reported in New Zealand *Corybas* orchids (Clements *et al.*, 2007; Lehnebach & Zeller, 2015). It has been hypothesised that sexual reproduction is achieved through a pollination strategy known as brood-site deception, where the flowers attract gravid female fungus gnats by producing fungus odours to mimic the insect brood sites, and the flowers are then pollinated when the females deposit eggs into different flowers (Lehnebach & Zeller, 2015; Urru *et al.*, 2011). The interactions between *Corybas* orchids and their pollinators are thought to be highly specialised, in which the orchids produce species-specific odours to attract specific species of fungus gnats from the genus *Mycetophila* (Mycetophilidae; Lehnebach & Zeller, 2015). Fly eggs have been observed in the midrib of the labellum in different *Corybas* species (Scanlen, 2006), and fungus gnats have been spotted carrying pollen after visiting the flowers (Kelly, 2012; Lehnebach & Zeller, 2015). However, the identity of the fungus gnat species pollinating *Corybas* have not been identified. Preliminary studies showed that some *Corybas* orchids are self-compatible and autogamous self-pollination may occur (Kelly *et al.*, 2013). On the other hand, floral structure such as the rostellum in other *Corybas* taxa prevents autogamous self-pollination. Nonetheless, self-pollination could still happen when a pollinator deposits pollen onto the stigma of the same flower (LEHNEBACH personal communication, 2021).

Asexual reproduction in *Corybas* is attained through clonal propagation from lateral roots (Clements *et al.*, 2007). Apart from proliferating through seed dispersal, many terrestrial orchids also have an annual growth cycle from an underground tuber

(Debeljak *et al.*, 2002; Dutra *et al.*, 2008; St George & McCrae, 1990). At the beginning of each growth cycle, a stem emerges from the underground tuber and grows to the soil surface where leaves are formed; in the case of *Corybas*, generally one leaf is formed (Jane *et al.*, 2017). After the leaves start to photosynthesise, the plant produces a new replacement tuber before flowering, and the old tuber dies at the end of the annual cycle, leaving the new tuber for the following season (St George & McCrae, 1990). Some species are known as colony-formers, in particular some members of *Aporostylis*, *Corybas, Cyrtostylis, Microtis*, and *Pterostylis* produce up to five or more new tubers (St George & McCrae, 1990); the new tubers are formed at the end of lateral roots that are running away from the parent plant (Figure 2.1; St George & McCrae, 1990).

Figure 2.1 Morphology of the root system in *Corybas* "Remutaka". White arrows indicate new replacement tubers formed at the end of lateral roots; black arrow indicates old tuber. Scale bar = 5 mm; Image form Carlos Lehnebach.

In recent years, habitat destruction due to changes in landscape use is known to be one of the main factors that impacts the survival of New Zealand terrestrial orchids (Lehnebach *et al.*, 2005). *Corybas* orchids are among the most threatened members within the Orchidaceae family in New Zealand and are in need of conservation (de Lange *et al.*,

2017). Conservation of biodiversity is not only important at the species level, but also is crucial at the genetic level as it provides a population with the ability to respond to selection and persist in their environment (Kalinowski, 2004). Genetic structure of a population reflects the exchange of genetic material, or gene flow, within and/or between populations, and it has major impacts on a population's genetic composition and genetic variation (Balloux & Lugon-Moulin, 2002). Therefore, understanding gene flow and its effects on a population can aid the development of efficient conservation programmes. For example, a population's genetic composition and genetic variation provide insight into how the population reproduces and indicates populations that might be given priority for conservation (Moritz, 2002). Then conservation projects can be designed accordingly to maximise reproductive success and preserve genetic variation within populations to ensure the persistence of target species. Unfortunately, there are several *Corybas* species and potentially undescribed species with small populations, and their genetic composition and genetic variation are unknown. In fact, studies focused on *Corybas* population genetics and reproductive biology are scarce and the knowledge on these topics is poor (Lyon, 2014).

Microsatellite markers are a frequently used tool in population genetic studies due to several advantages over other marker types. Firstly, nuclear microsatellite loci have faster mutation rates compared to chloroplast sequences or nuclear ribosomal spacer sequences (Takahashi *et al.*, 2011) and have a larger number of alleles compared to allozymes (Gao *et al.*, 2002), and can provide high resolution results enabling them to distinguish closely related individuals and/or species. Secondly, microsatellite loci are neutral and co-dominant molecular markers that allow evaluation of gene flow, population structure, and calculations of population genetic parameters such as F-statistics (Selkoe & Toonen, 2006; Semagn *et al.*, 2006).

In this study, polymorphic microsatellite markers developed previously (van Etten *et al.*, 2018) were used to assess (1) genetic composition and genetic variation within a taxonomically undetermined orchid, *Corybas aff. trilobus* (CHR 537604; Remutaka) population at a QEII (Queen Elizabeth II) Covenant near Wellington and (2) the extent of clonality within the population. The results were used to infer the reproductive strategies employed by this population and to provide information for future conservation management of *Corybas* orchids in New Zealand.

2.3 Materials and methods

2.3.1 Study taxon

The taxon under study is *Corybas aff. trilobus* (CHR 537604; Remutaka), from now onwards *Corybas* "Remutaka"; it is believed to be a single, as yet unnamed species among a few undescribed entities within the *Corybas trilobus* aggregate (Lehnebach *et al.*, 2016). Other undescribed entities within this aggregate include *Corybas* "pygmy", *Corybas* "rewanui", and *Corybas* "trotters" (Jane *et al.*, 2017). *C. trilobus* is widespread and occupies a wide range of habitats from coastal to subalpine areas on both the North and South Island of New Zealand (de Lange, 2021). The *C.* "Remutaka" population investigated in this study occurs in a QEII Covenant near Wellington, New Zealand, inhabiting dry areas under beech trees (Fuscospora species; Jane *et al.*, 2017). An individual plant of a *Corybas* species typically consists of an underground tuber, a single leaf, and mostly produces a single flower (Jane *et al.*, 2017; Lyon, 2014). *C.* "Remutaka" flowers between June and October, their flowers have a red-streaked dorsal sepal with a small point, a dark crimson labellum with a green midlobe, and the flower usually appears below the leaf (Jane *et al.*, 2017). *Corybas* species have been observed occurring in discontinuous patches, each patch with numerous individuals (Clements *et al.*, 2007; Elder & Moore, 1973); these colonies are likely maintained through clonal propagation from replacement tubers (Duguid, n.d.).

2.3.2 Sample collection

To investigate genetic variation and the extent of clonality within this *C.* "Remutaka" population, three different sampling methods were employed. The first method aimed to estimate genetic variation within the population; the second and the third method were intended to investigate the extent of clonality within the population at different scales. All samples were collected on 30th October 2015, at QEII Covenant, Eastbourne, New Zealand (-41.302371, 174.898845). The specimens were stored in silica gel until DNA extraction.

2.3.2.1 Sampling for investigating genetic variation within the population

To estimate the genetic variation within this *C.* "Remutaka" population, 38 samples as detailed below were collected along a transect, approximately 1.15 km (from now onward this transect is denoted as the "main transect"; Figure 2.2) across the Covenant to represent the population. The first sample was taken at GPS waypoint 1179 and the last sample at waypoint 1219. Each sample was collected at a ten-metre interval and was labelled with its corresponding GPS waypoint as sample ID. At each point, a sample consisted of three leaves collected from plants in close proximity, and each leaf was considered to be from different individual plants (i.e., different genotypes). Therefore, individual DNA extractions were made from a single leaf per sample. The extra leaves served as back-up material in case the initial extractions failed. Two large gaps were identified in the distribution of the orchids along the track: one of the gaps overlapped with a change in dominant overstory species, from black beach (*Fuscospora solandri*) to silver fern (*Cyathea dealbata*); the second gap co-occurred with drier soil. When the plants were not found at the nearest 10-metre interval, the next closest plants on the trail were sampled. A leaf specimen from each of the 38 samples was used for population genetic analysis and the extracted DNA samples were labelled with their corresponding sample ID. GPS waypoints 1204–1206 were part of the clonality investigation, see description below.

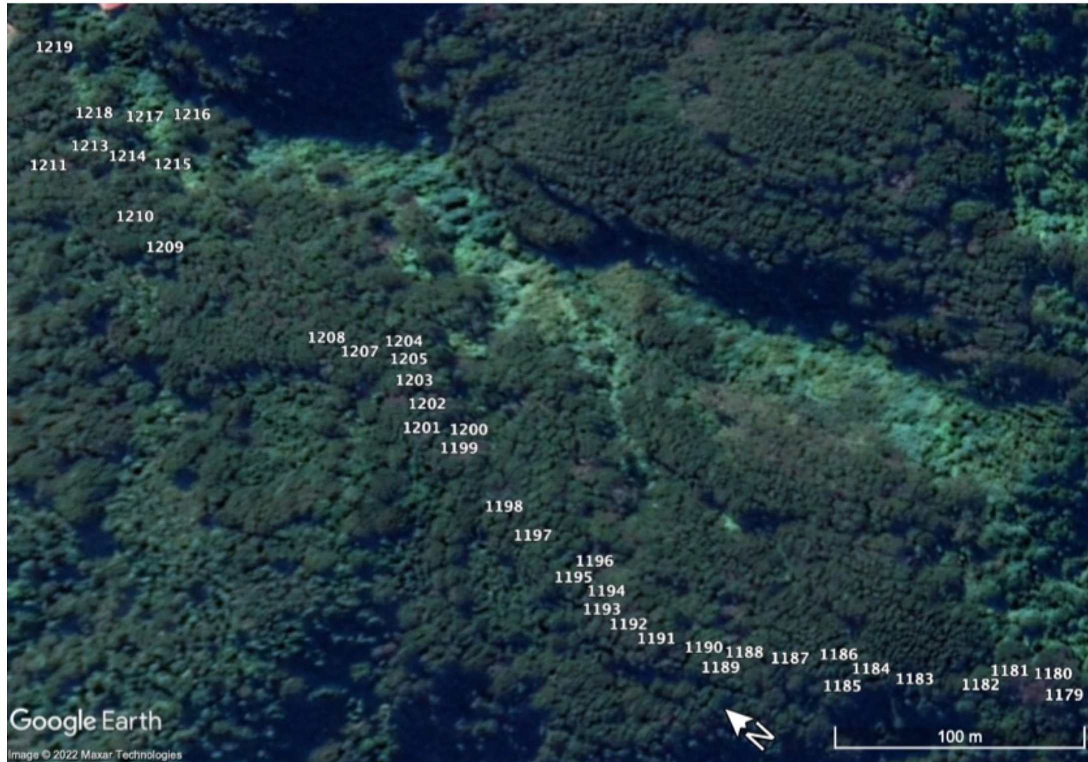

Figure 2.2 Distribution of *C.* "Remutaka" samples collected along the main transect at a QEII Covenant for genetic analyses in this study (not all samples are visible on the map).

2.3.2.2 Sampling for investigating the extent of clonality

Two different sampling methods were used to assess the extent of clonality in this *C.* "Remutaka" population:

1. Sampling along a small transect

   The first sample was collected at waypoint 1205. Waypoint 1205 was taken at the beginning of a three-metre linear transect, extending roughly from the south to the north. A cluster of about five leaves were sampled every 20 cm; 14 samples were collected along this small transect. The samples were labelled 1205-0 to 1205-300. The number after waypoint 1205 corresponded with the distance from the transect origin in centimetres. For example, 1205-0 meant that the sample was collected at zero cm from waypoint 1205 and 1250-300 indicated that the sample was collected 300 cm from waypoint 1205. There was a discontinuous distribution of the plants between 200 cm to 280 cm along the small transect, so that the next two samples after 200 cm (1205-200) were collected at 230 cm (1205-230) then

at 280 cm (1205-280). Again, each leaf within a sample was assumed to be from a different individual plant. Hence, individual DNA extractions were made from a single leaf per sample. A leaf from each of the 14 samples was used in this study, the DNA samples were again labelled with their corresponding sample ID.

2. Extensive sampling in close proximity

   Three dense clusters of leaves were sampled extensively at waypoint 1206; this data set was also referred to as the "microsite population". The three clusters were located approximately 10 cm from each other and were separated by roots of overstory vegetation. The three cluster samples were labelled as 1206-1, 1206-2, and 1206-3; each sample contained approximately 10 to 15 leaves from each cluster. Eight specimens from each cluster were used in this study. Once more, the leaves within any one cluster were considered to be different plants, thus DNA samples were extracted individually. DNA from eight leaves from each of the clusters was extracted and the DNA samples labelled as 1206-1-1 to 1206-1-8, 1206-2-1 to 1206-2-8, and 1206-3-1 to 1206-3-8.

2.3.3 DNA extraction and genotyping

Genomic DNA was extracted from silica gel-dried leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987). The resulting DNA was eluted in 100 µL of TE buffer. All DNA samples were run on a 1% agarose gel at 75 volts for 35 minutes to assess DNA quality.

Thirty-eight individuals sampled along the main transect across the Covenant and 14 individuals sampled along the small transect at GPS waypoint 1205 were screened at 12 microsatellite loci (Corybas-07, Corybas-12, Corybas-16, Corybas-19, Corybas-23, Corybas-24, Corybas-28, Corybas-32, Corybas-36, Corybas-44, Corybas-45, and Corybas-48); and 24 individuals sampled at GPS waypoint 1206 were screened at 11 microsatellite loci (Corybas-07, Corybas-12, Corybas-16, Corybas-19, Corybas-23, Corybas-24, Corybas-28, Corybas-32, Corybas-44, Corybas-45, and Corybas-48) using polymerase chain reaction (PCR; see van Etten *et al.*, 2018 for details of microsatellite markers used in this study). PCR amplification was performed in a volume of 10 µL with 1× buffer (Solis BioDyne, Tartu, Estonia), 2.5 nM of $MgCl_2$, 100 µM of dNTP mix, 0.02 µM of forward primer, 0.45 µM of reversed primer, 0.45 µM of M13 primer (labelled with FAM, VIC, or NED), 0.5 U of FirePol Taq polymerase (Solis BioDyne, Estonia), 5–

50 ng of DNA, and 3.7 µL of water. The PCR programme had an initial denaturation of 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds; 53°C for 40 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. Amplification at each locus was assessed by running the PCR products on a 1.5% agarose gel at 75 volts for 90 minutes (van Etten *et al.*, 2018).

The PCR products (0.7–1.25 µL, depending on their strength) from two to three loci, each labelled with different fluorescent dyes (FAM, VIC, and NED) were pooled for analysis. Loci of distinguishable sizes were pooled and added to 1 µL of CASS ladder (Symonds & Lloyd, 2004) and 9 µL of Hi-Di formamide (Applied Biosystems, Carlsbad, California, USA) for fragment sizing on an ABI 3730 Genetic Analyzer (Applied Biosystems) at Massey Genome Service at Massey University, Palmerston North, New Zealand. Alleles were visualised and sized using GeneMapper (version 5.0; Applied Biosystems).

2.3.4 Data analysis

2.3.4.1 Assessing genetic variation and genetic structure within the population (main transect data)

GenAlEx (version 6.503; Peakall & Smouse, 2012) was used to assess genetic diversity within the population by assessing each microsatellite locus for the percentage of polymorphic loci (P), number of alleles per locus (A), number of effective alleles (N_E). The observed heterozygosity (H_O), expected heterozygosity (H_E), and F-statistics (FIS, inbreeding coefficient; Wright, 1950) were also calculated in GenAlEx according to the methods by Weir and Cockerham (1984). The presence of null alleles was assessed in MICRO-CHECKER (version 2.2.3; van Oosterhout *et al.*, 2004). One individual (1219) with data missing for more than six microsatellite markers was excluded from the data set.

The relatedness of individuals within the site was assessed in the programme STRUCTURE (Pritchard *et al.*, 2000), and an appropriate format of the data set was exported from GenAlEx for analyses in STRUCTURE. STRUCTURE uses a Bayesian clustering method to infer population genetic structure from multi-locus genotype data. This method assumes a model that consists of *K* populations (*K* may be any number), and each of these populations is characterised by a set of allele frequencies at each locus that conform to Hardy-Weinberg expectations. An individual's genotypes are assigned to

different populations, also known as genetic clusters (Pritchard *et al.*, 2000). STRUCTURE (version 2.3.3) was used to assess population genetic structure along the main transect. Gene flow was assumed to occur within the population as facilitated by fungus gnats. Therefore, two priors (an admixture model and correlated allele frequencies) along with the following parameters were used for STRUCTURE analyses to determine the most likely number of genetic clusters: 12 iterations of each K value (K = 1–6) with a burn-in of 100,000 generations and a 1million Markov Chain Monte Carlo (MCMC) iterations of data collection. The best fit K value was assessed from the rate of change in posterior probability between successive values of K (ΔK) following the method of Evanno *et al.* (2005). The results of the main transect data from STRUCTURE were imported into STRUCTURE HARVESTER (Earl & von Holdt, 2012), where the Evanno test was performed and the mean posterior probability (Ln$P(K)$) was plotted. The results from STRUCTURE were also imported to CLUMPAK (beta version; Kopelman *et al.*, 2015), to find the best alignment of the inferred clusters across the range of K values from multiple runs at each K value.

A genetic distance matrix based on Prevosti's genetic distance (Prevosti *et al.*, 1975) was calculated in R (version 4.0.3; R Core Team, 2013) using R package, poppr (version 2.8.7; Kamvar *et al.*, 2014). A principal coordinates analysis (PCoA) was performed, and the first two axes were plotted to visualise genetic distance between individuals. The correlation between genetic distances and geographic distances among different individuals within the population was investigated using a Mantel test (Mantel, 1967) with default permutation (permutation = 999) in the R package ade4 (version 1.7-16), based on the Prevosti's genetic distance matrix. The same genetic distance matrix was further formatted into a Nexus format file in R using the R package phangorn (version 2.5.5; Schliep, 2011), for creating a population NeighborNet in SPLITSTREE (Huson, 1998). SPLITSTREE4 (version 4.17.1) was used to create a population NeighborNet to show the relationships among individuals within the population. Additionally, a combined genetic distance matrix containing both the main transect, small transect, and microsite individuals was also generated for plotting a PCoA graph and creating a population NeighborNet to illustrate the relationship among individuals within the three different data sets.

2.3.4.2 Assessing for clonality (small transect and microsite samples)

Because the small transect and the microsite data set represent different sampling methods to assess the extent of clonality within this *C.* "Remutaka" population, these two data sets were analysed separately.

The Monte Carlo procedure in the R package RClone (version 1.0.3; Bailleul *et al.*, 2016) is a procedure used to discriminate all multi-locus genotypes (MLGs) and to identify identical MLGs within data sets. Marker Corybas-36 had amplifying issues when genotyping microsite individuals where the negative control was always contaminated despite using new PCR components, including new primers. The cause of this contamination was not solved. As such, locus Corybas-36 was excluded from the microsite data set.

In the small transect and microsite data set, some markers did not amplify well due to low DNA concentration (Table 2.2). RClone does not support missing data and are considered as new alleles if included (Bailleul *et al.*, 2016). Therefore, identical MLGs within the two data sets were also observed manually complementing those that had been identified by RClone. Manual observation omitted missing data at each locus. Individuals that were manually identified to have the same MLG as those that had been detected by RClone were assumed to belong to the same genetic clusters. The "MLG_tab" function in RClone returned a table with one row per MLG and multiple columns when multiple samples were detected to have the same MLG. A PCoA graph and a NeighborNet were generated for both the small transect and the microsite data set to visualise the genetic distances and to illustrate the relationships among different individuals within these two data sets (refer to section 2.3.3.1 for the methods used to generate PCoA graph and NeighborNet).

2.4 Results

2.4.1 Genetic variation within the *C.* "Remutaka" population

Thirty-seven samples collected the main transect at the Covenant were genotyped at 12 microsatellite loci. Amplification success for the 12 markers ranged from 91.89–100% across individuals (Table 2.1). All 12 markers genotyped for the main transect individuals were polymorphic; the number of alleles per locus ranged from two (Corybas-28) to 17

(Corybas-48) with an average of 7.08 alleles per locus (Table 2.1). There was a high frequency allele at loci Corybas-28, Corybas-19, Corybas-12, Corybas-24, Corybas-07, and Corybas-45, these high frequency alleles had an occurrence ≥50%. Notably, the frequency of allele 203 was 97.1% at locus Corybas-28; the frequency of allele 265 was 91.7% at locus Corybas-19; and the frequency of allele 311 was 80% at locus Corybas-12 (Figure 2.3 & Figure 2.4). There were no high frequency alleles at loci Corybas-23, Corybas-32, Corybas-44, Corybas-16, Corybas-36, and Corybas-48, however there were few alleles that occurred more often than the others at these loci (Figure 2.3 & Figure 2.4).

The F_{IS} (Wright, 1950) calculated for each locus within the population showed positive deviation ($F_{IS} > 0$) from Hardy-Weinberg equilibrium (HWE; $F_{IS} = 0$) in nine of the 12 loci genotyped, ranged 0.034–1.000, which indicated homozygote excess. Locus Corybas-19 showed the highest homozygote excess, $F_{IS} = 1.000$. Although four different alleles were observed at this locus, all individuals were homozygous ($H_O = 0.000$), and $H_E = 0.157$. Negative deviation ($F_{IS} < 0$) from HWE was detected at three loci, Corybas-28, Corybas-07, and Corybas-48, ranged from -0.029 to -0.070, which indicated heterozygote excess. Locus Corybas-48 showed the highest heterozygote excess, $F_{IS} = -0.070$, and $H_O = 0.919$ which was greater than $H_E = 0.859$. The average $H_O = 0.402$ of the population was lower than the average $H_E = 0.573$. On the whole, this population showed a positive deviation from HWE, with a mean $F_{IS} = 0.314$, indicating homozygote excess (Table 2.1).

Table 2.1 Characteristics of 12 microsatellite loci for 37 samples of *C.* "Remutaka" collected along the main transect at a QEII Covenant near Wellington.

Locus	A	Size range (bp)	H_O	H_E	F_{IS}	% amplification
Corybas-07	5	305-334	0.600	0.564	-0.063	94.59
Corybas-12	3	311-316	0.314	0.325	0.034	94.59
Corybas-16	5	267-275	0.568	0.674	0.158	100.00
Corybas-19	4	244-267	0.000	0.157	1.000	97.30
Corybas-23	5	319-357	0.162	0.600	0.730	100.00
Corybas-24	8	235-271	0.235	0.624	0.623	91.89
Corybas-28	2	196-203	0.057	0.056	-0.029	94.59
Corybas-32	13	164-189	0.622	0.837	0.258	100.00
Corybas-36	5	146-152	0.250	0.740	0.662	97.30
Corybas-44	11	217-239	0.541	0.746	0.276	100.00
Corybas-45	7	262-285	0.556	0.688	0.193	97.30
Corybas-48	17	145-259	0.919	0.859	-0.070	100.00
Mean	7.083		0.402	0.573	0.314	97.30
SE	1.288		0.079	0.075	0.103	
%P	100.00%					

A = number of alleles per locus; size range (bp) = range of allele size at each locus; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = estimates of Wright's inbreeding coefficient for all microsatellite markers; % amplification = percentage of amplification at each locus.

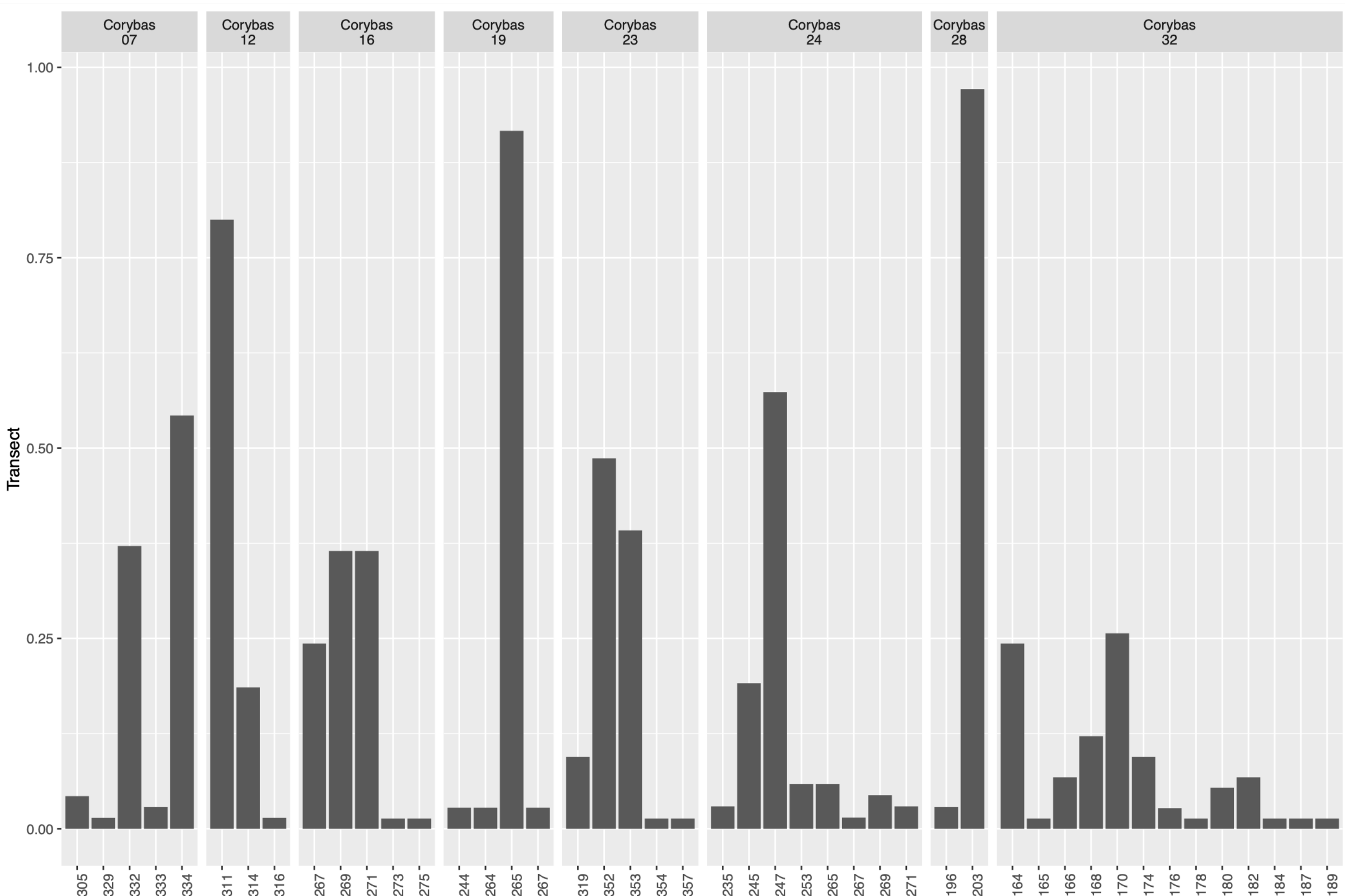

Figure 2.3 The number of alleles and their estimated frequencies at each locus within the *C.* "Remutaka" main transect data set collected at the QEII Covenant (the first eight of the 12 markers used in the main transect data set). The x-axis shows the alleles detected at each locus; the y-axis shows the frequencies of each allele.

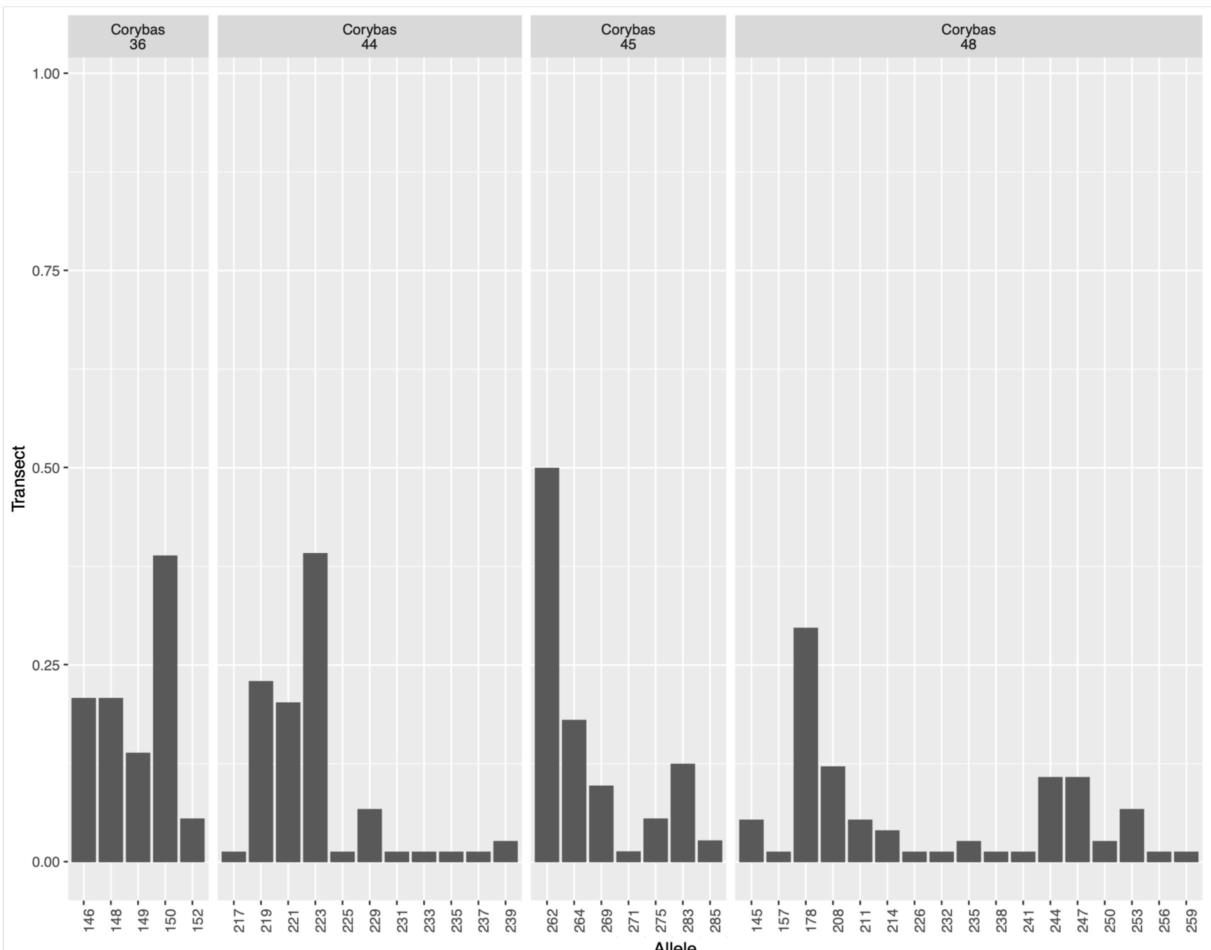

Figure 2.4 The number of alleles and their estimated frequencies at each locus within the *C.* "Remutaka" main transect data set collected at the QEII Covenant (the remaining four of the 12 markers used in the main transect data set). The x-axis shows the alleles detected at each locus; the y-axis shows the frequencies of each allele.

2.4.2 Null alleles

MICRO-CHECKER detected that null alleles might be present at seven loci: Corybas-19, Corybas-23, Corybas-24, Corybas-32, Corybas-36, Corybas-44, and Corybas-45, indicated by the homozygote excess at most allele size classes within those loci. There was no evidence for large allele dropout at any loci.

2.4.3 Genetic structure within the *C.* "Remutaka" population

The K value with the best fit to the data was determined using the ΔK method (Figure 2.5) following Evanno *et al.* (2005) and the mean posterior probability (LnP(K)) was plotted (Figure 2.6). The ΔK method for determining the most likely value of K showed that K = 2 was the best fit and this result was supported by CLUMPAK results (Figure 2.7); and in the posterior probability plot the highest values were seen at K = 1 and K =2 (Figure 2.6). In the CLUMPAK alignment of different K values, the number of colours corresponded to the number of different genetic clusters at each K. For instance, at K = 2, all individuals were assigned to two genetic clusters represented by orange and blue; at K = 3, all individuals were assigned to three genetic clusters represented by dark purple, orange, and blue (Figure 2.7). Figure 2.7 showed that at K = 2, all individuals showed an admixed genotype where orange and blue each made up about 50% of their genotype. This was seen across all K values (K = 1–6), each colour equally made up the genotype of the samples. The even split of all samples suggested the best fit was actually K = 1 and no genetic structure could be identified.

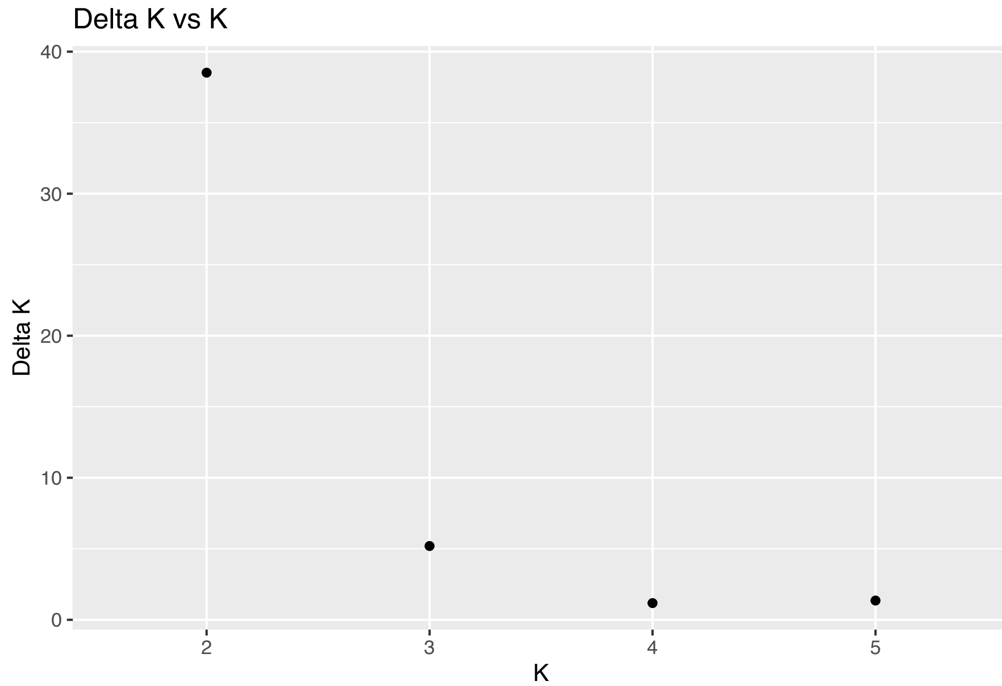

Figure 2.5 Plot of ΔK vs K for STRUCTURE results following Evanno *et al.* (2005) based on 12 iterations for each K value.

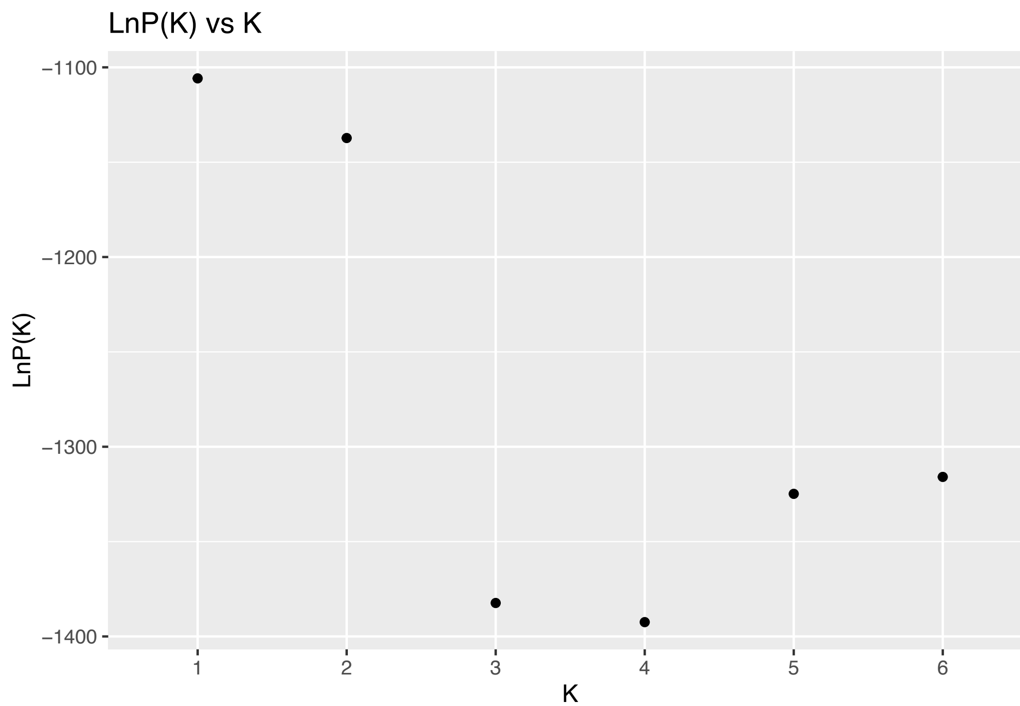

Figure 2.6 Plot of mean posterior probability (LnP(K)) values per cluster (K) based on 12 iterations per K from STRUCTURE analyses (Pritchard *et al.*, 2000).

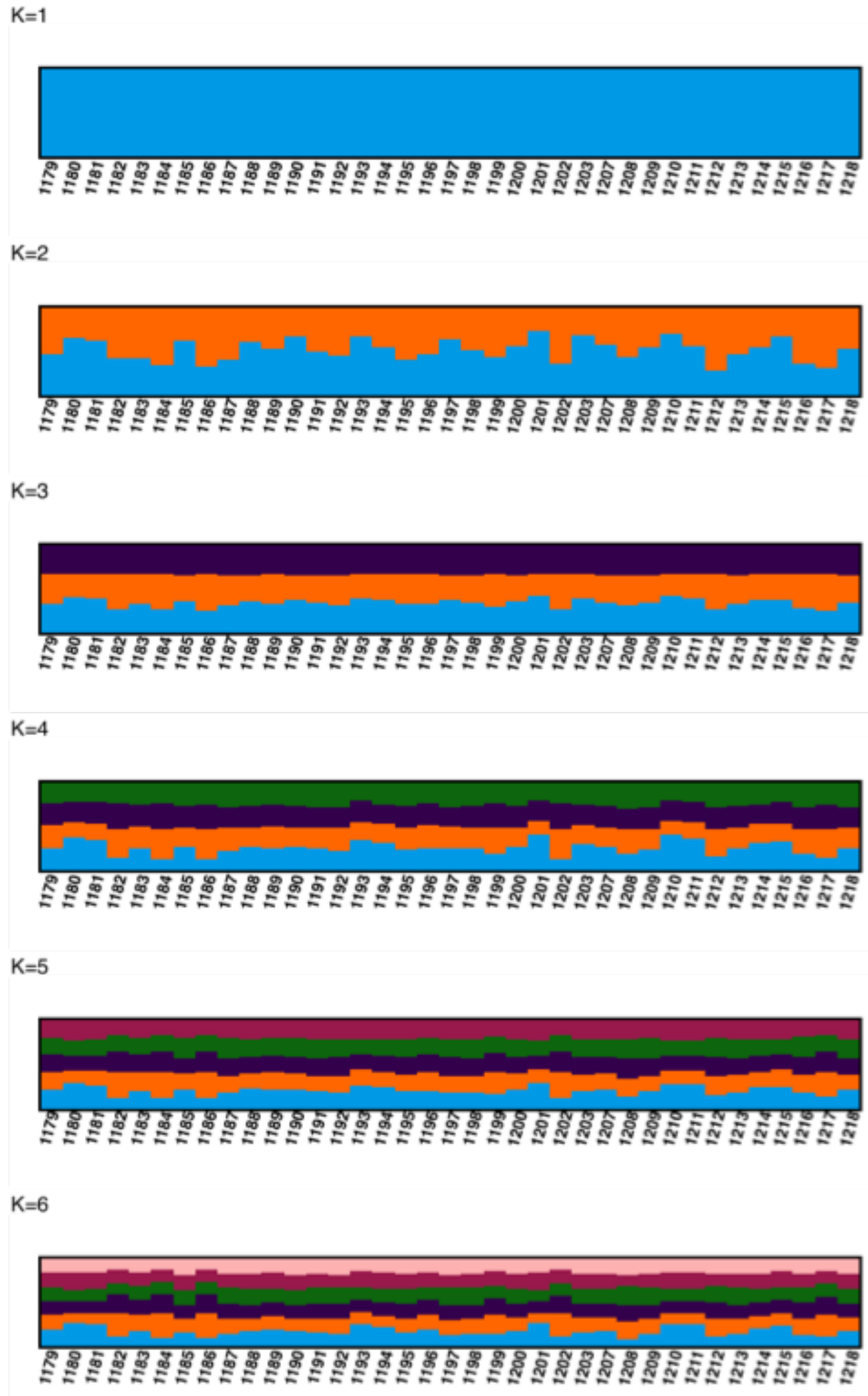

Figure 2.7 CLUMPAK output based on STRUCTURE assigment of 37 individuals within the maintransect data set for each *K* value (*K* = 1–6). Each column within a *K* value represents an individual collected along the transect (from left to rignt: 1179–1218). The proportion of membership in a genetic cluster within in each individual is denoted by different colours; *K* = 2 is the best likelihood fit according to the Evanno method (Evanno *et al.*, 2005).

The PCoA graph of the main transect data (Figure 2.8) gave an indication of the genetic distances between different individuals within the *C.* "Remutaka" population at the Covenant. The more genetically similar the individuals were, the closer they would be located to each other on the PCoA graph, and genetically identical individuals would be overlapping. The PCoA graph did not show overlapping individuals, suggesting that no genetically identical individuals were present in the main transect data set. A Mantel test showed that genetic distances between individuals did not significantly correlate with their geographic distances (R^2 = -0.0416, *p*-value = 0.772); that is, individuals that were located closer to each other on the main transect were not more genetically similar compared to those that were further apart. The PCoA graph (Figure 2.8) agreed with the Mantel test; the majority of individuals appeared to be randomly scattered. Nonetheless, a few individuals collected from neighbouring GPS waypoints appeared closer to each other on the PCoA graph in comparison to those that were located further away. For example, individual 1194 and 1195 were slightly overlapping; individual 1211, 1213, and 1214 formed a small cluster; and individual 1216 was located close to sample 1217. However, individual 1212 did not cluster with 1211, 1213, and 1214. Similarly, individual 1215 did not cluster with 1216 and 1217. No correlation was found between the geographical distance and genetic distances among members within this site. In addition, samples 1197, 1201, and 1203 appeared to be more genetically distinct as they were located the furthest from the rest of the samples on the PCoA graph.

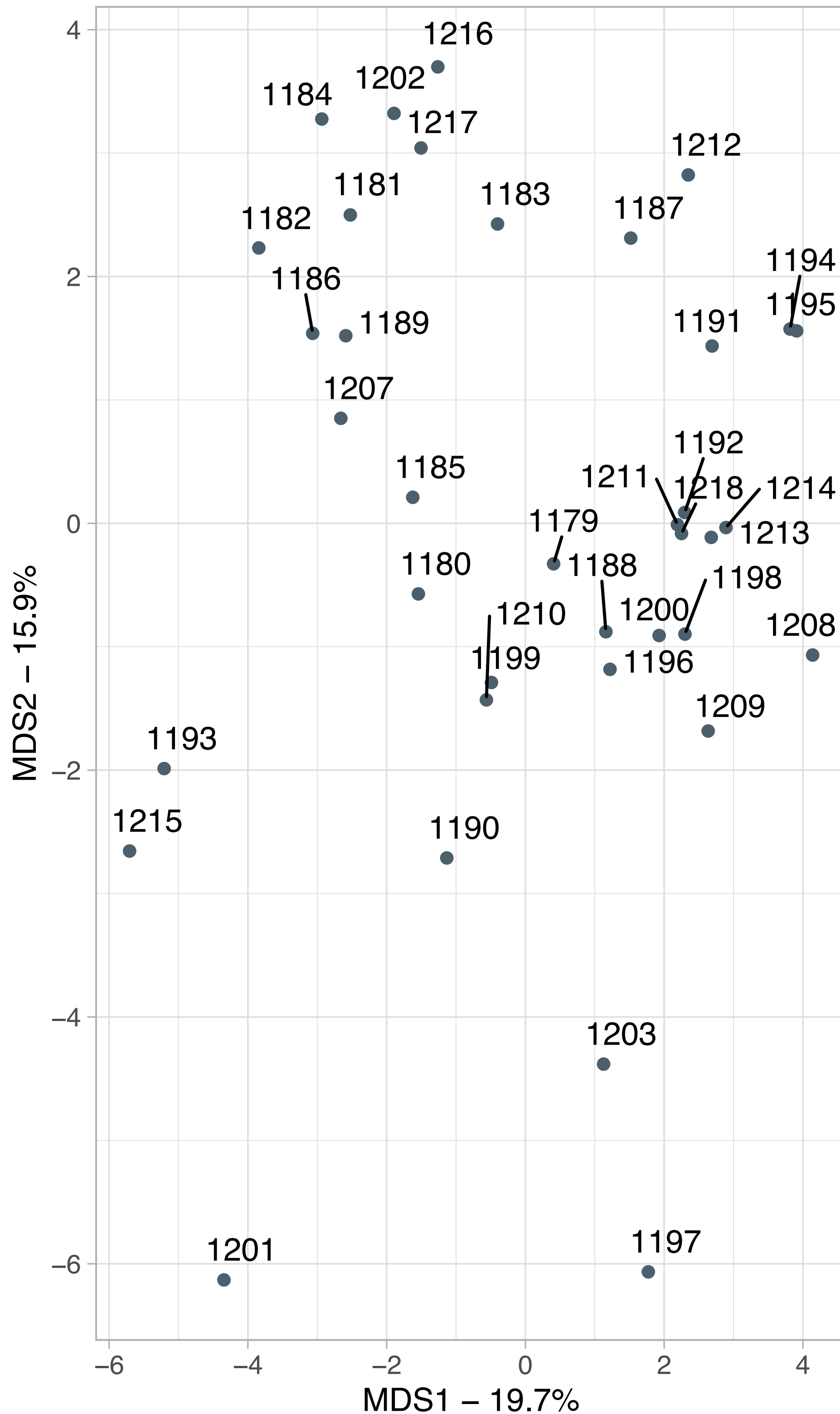

Figure 2.8 Principal coordinate analysis (PCoA) of Prevosti's genetic distances among the 37 *C*. "Remutaka" samples collected along the main transect across the QEII Covenant. The first two axes explain 19.7% and 15.9% of total variation, respectively.

The NeighborNet of the main transect data showed a reticular pattern; no splits were able to partition individuals within the data into distinct groups (Figure 2.9). The reticular pattern in the NeighborNet of the main transect data was supported by the CLUMPAK alignments (Figure 2.7) of STRUCTURE results in which all individuals within the population had an admixed genotype and no genetic cluster was identified.

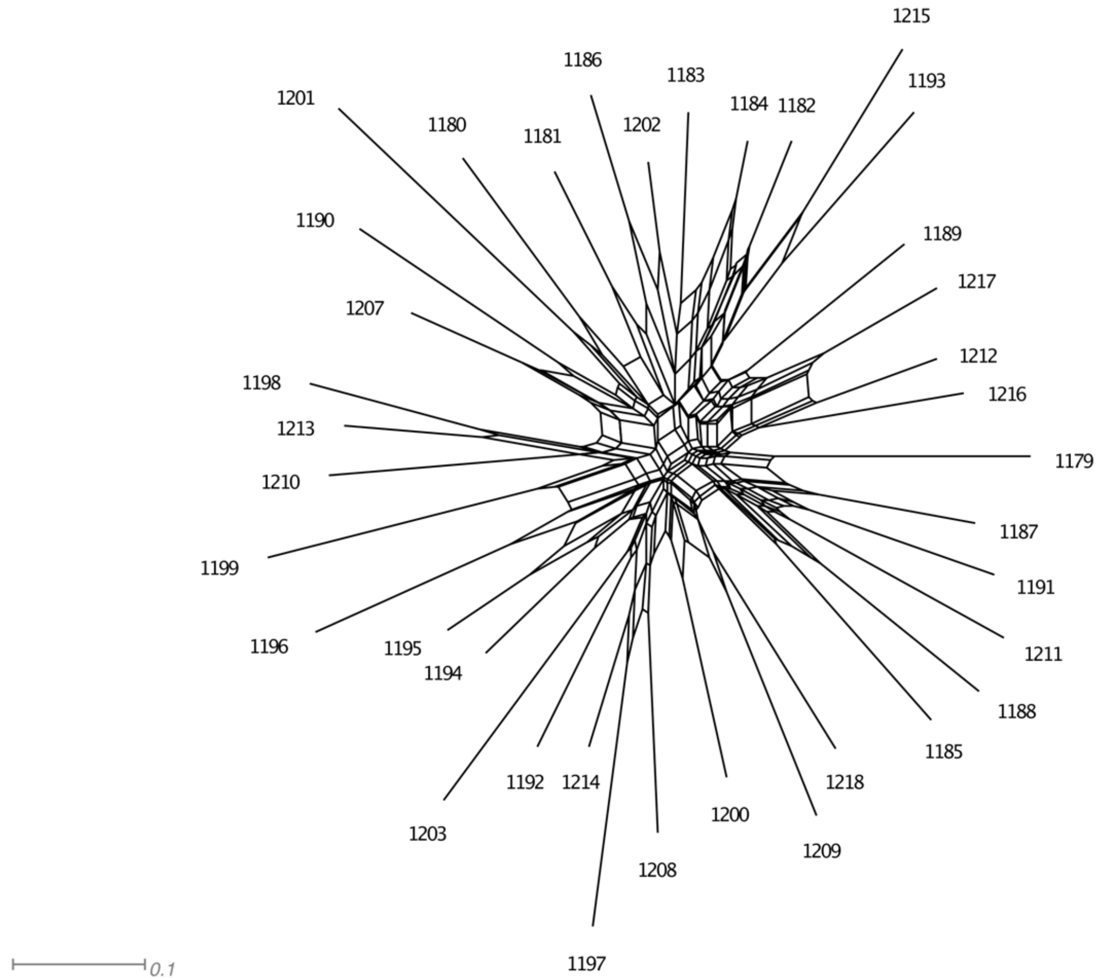

Figure 2.9 NeighborNet generated in SPLITSTREE4 based on Prevosti's distances between all individuals of *C.* "Remutaka" sampled along the main transect at the QEII Covenant. The numbers on the graph correspond to sample ID of each individual.

2.4.4 Clone structure

Twelve microsatellite loci were genotyped for 14 individuals within the small transect data set, and 11 loci were genotyped for 24 individuals within the microsite data set. A total of 42 alleles were observed from the small transect individuals, and a total of 34 alleles were observed from the microsite individuals (Table 2.2). Success rate of marker

amplification ranged 50–100% across small transect individuals; marker amplification success rate ranged 66.67–100% across microsite individuals (Table 2.2). The boxplot output of the Monte Carlo analysis in RClone showed that the set of 12 loci detected 14 MLGs in the small transect data set (Figure 2.10), and the set of 11 loci detected 19 MLGs in the microsite data set (Figure 2.11).

Table 2.2 The number of allele(s) per locus and percentage amplification per locus for 14 individuals of *C.* "Remutaka" collected along the small transect and 24 individuals collected at the microsite.

	Small transect		**Microsite**	
Locus	**A**	**% amplification**	**A**	**% amplification**
Corybas-19	1	50.00	1	79.17
Corybas-23	2	100.00	3	100.00
Corybas-28	2	92.86	1	100.00
Corybas-07	2	100.00	2	100.00
Corybas-12	2	100.00	1	100.00
Corybas-24	4	78.57	7	75.00
Corybas-32	6	100.00	5	100.00
Corybas-44	4	92.86	3	91.67
Corybas-45	6	92.86	3	100.00
Corybas-16	3	92.86	4	66.67
Corybas-36	3	100.00	-	-
Corybas-48	7	92.86	4	100.00
Mean	3.50	91.07	3.09	92.05
Total	42.00		34.00	

A = number of alleles per locus; % amplification = percentage of amplification at each locus

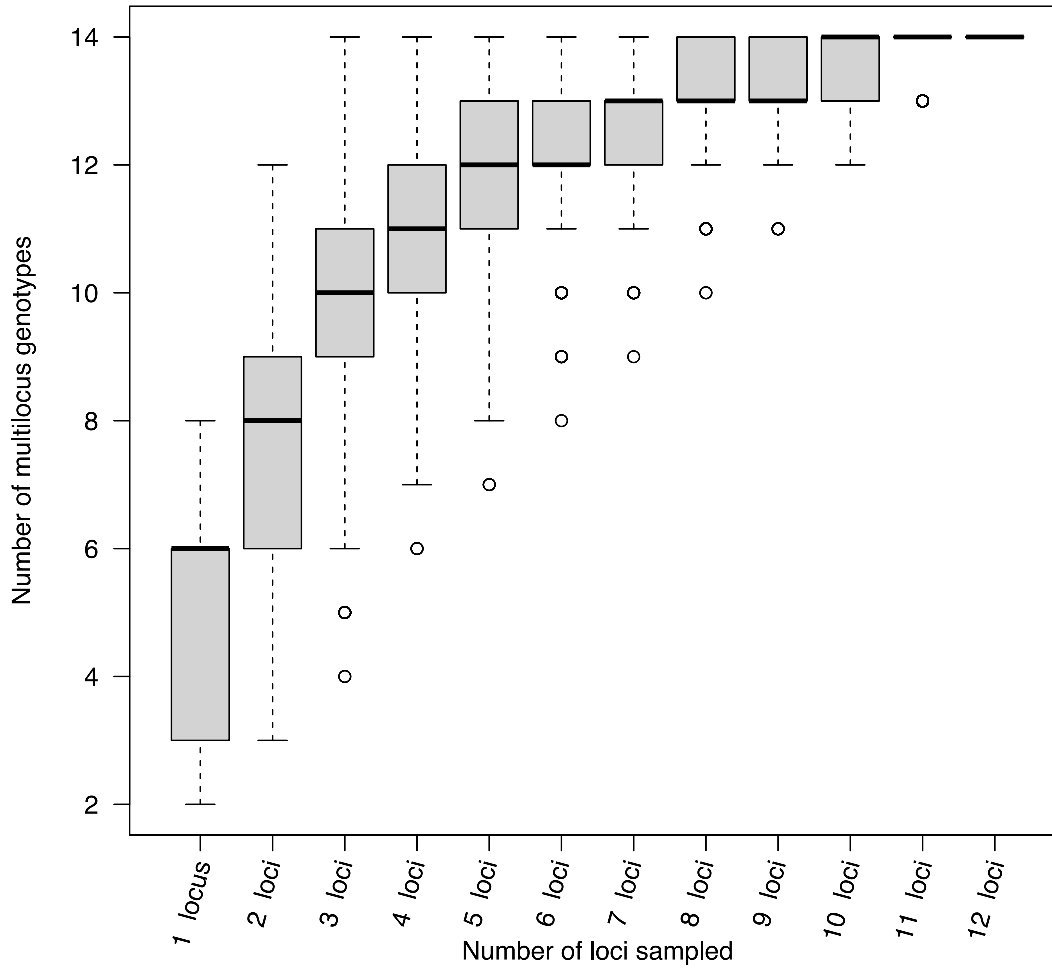

Figure 2.10 Genotype accumulation curve indicating the genotypic resolution of 12 microsatellite loci for all 14 samples in *C.* "Remutaka" small transect data set. The lower and upper bars indicate the minimum and maximum number of MLG(s), respectively; the central line indicates the mean number of MLGs detected, circles represent outliers.

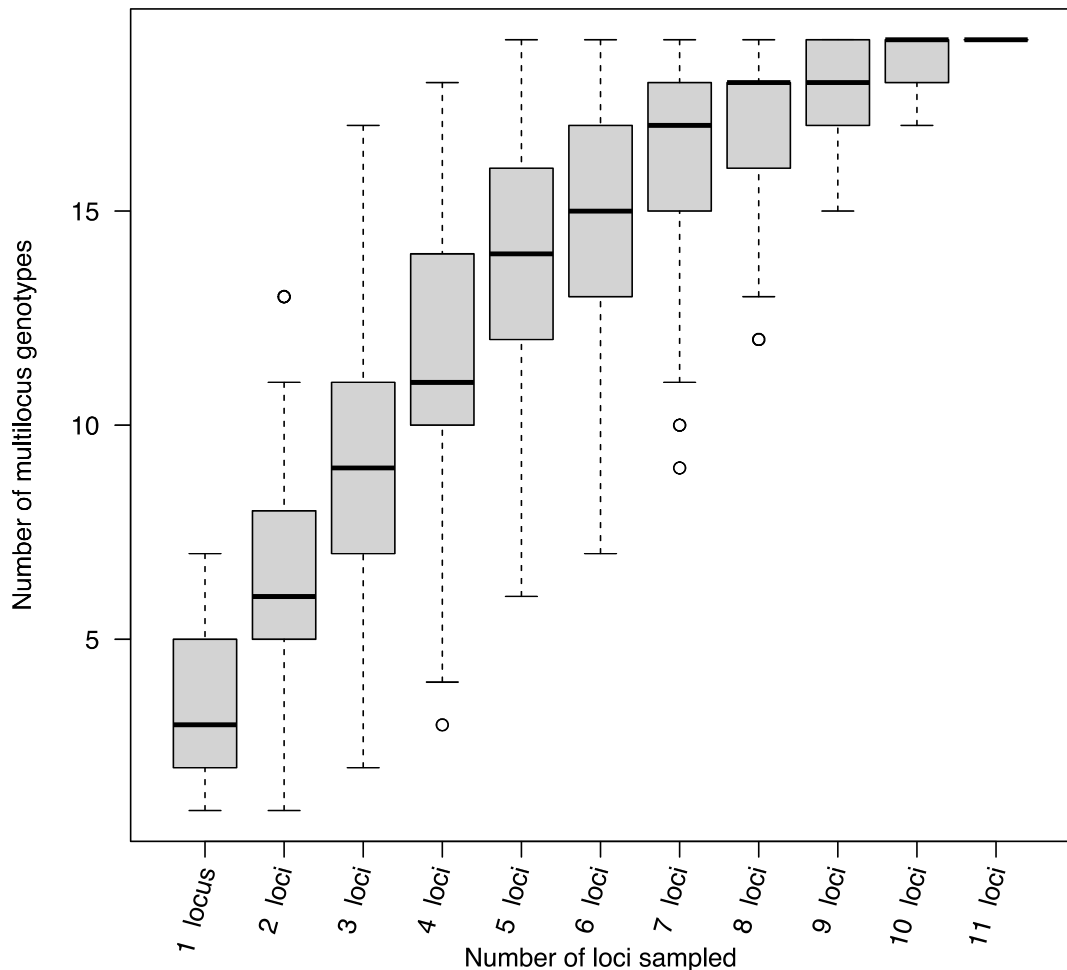

Figure 2.11 Genotype accumulation curve indicating the genotypic resolution of 11 microsatellite loci for all 24 samples in *C.* "Remutaka" microsite data set. The lower and upper bars indicate the minimum and maximum number of MGL(s), respectively; the central line indicates the mean number of MLGs detected; circles represent

In the small transect, RClone identified 14 distinct MLGs (Figure 2.10) and no genetic cluster among the 14 individuals sampled. In comparison, manual observation identified a total of 11 distinct MLGs and two genetic clusters within those 11 MLGs, these two clusters are (1) 1205-120, 1205-160, and 1205-180; and (2) 1205-280 and 1205-300 (Table 2.3). Individuals within the small transect data set were sampled at a 20 cm interval along the small transect. In the first genetic cluster, 1205-160 and 1205-180 were located 20 cm apart, and 1205-120 was located 40 cm away from 1205-160 with 1205-140 in the middle separating them. Individual 1205-140 is not a part of any genetic cluster, it has a unique MLG. In the second genetic cluster, 1205-280 was located 20 cm away from 1205-300 (Table 2.3).

In the microsite data set, a total of 19 distinct MLGs were detected among the 24 individuals by RClone. RClone also identified four clusters of individuals with identical genotypes among these 19 MLGs, these genetic clusters are (1) 1206-1-3 and 1206-1-4; (2) 1206-1-5 and 1206-2-3; (3) 1206-2-1, 1206-2-8, and 1206-3-3; and (4) 1206-2-5 and 1206-3-8. Individuals within the microsite data set were collected extensively from three closely located clusters of leaves (1206-1, 1206-2, and 1206-3), each cluster of leaves was approximately10 cm apart from each other. Within those four genetic clusters identified, one individual from 1206-1 clusters with one individual from 1206-2; some individuals from 1206-2 cluster with individuals from 1206-3; no individuals from 1206-1 clustered with individuals from 1206-3 (Table 2.3). Conversely, manual observation suggested that individuals in genetic cluster (4), 1206-2-5 and 1206-3-8, along with individual 1206-1-2, 1206-1-7, and 1206-2-7 shared the same MLG as individuals within genetic cluster (1), and these individuals could be considered as belonging to the same genetic cluster. Therefore, according to manual observation, the following three genetic clusters were identified: (1) 1206-3-4 and 1206-1-4; (2) 1206-1-5, 1206-2-3, 1206-2-5, 1206-3-8, 1206-1-2, 1206-1-7, and 1206-2-7; and (3) 1206-2-1, 1206-2-8, and 1206-3-3. Manual observation identified a higher number of individuals with the same MLG than RClone in the microsite data set.

According to the small transect and microsite clonality assessment results, the maximum distance between two individuals with identical genotypes was 40 cm, between 1205-120 and 1205-160. Clones were also found at an interval of 20 cm along the small transect and at approximately 10 cm or less at the microsite.

Table 2.3 The 11 distinct MLGs and 2 genetic clusters identified at the small transect by manual observation; and the 19 distinct MLGs and 4 genetic clusters identified at the microsite using RClone.

Small transect		Microsite	
Dis. MLGs	**Genetically identical individuals and genetic clusters**	**Dis. MLGs**	**Genetically identical individuals and genetic clusters**
1	1205-0	1	1206-1-1
2	1205-20	2	1206-1-2
3	1205-40	3	1206-1-3, 1206-1-4
4	1205-60	4	1206-1-5, 1206-2-3
5	1205-80	5	1206-1-6
6	1205-100	6	1206-1-7
7	1205-120, 1205-160, 1205-180	7	1206-1-8
8	1205-140	8	1206-2-1, 1206-2-8, 1206-3-3
9	1205-200	9	1206-2-2
10	1205-230	10	1206-2-4
11	1205-280, 1205-300	11	1206-2-5, 1206-3-8
		12	1206-2-6
		13	1206-2-7
		14	1206-3-1
		15	1206-3-2
		16	1206-3-4
		17	1206-3-5
		18	1206-3-6
		19	1206-3-7

Dis. MLGs = distinct MLGs

The small transect PCoA graph did not show an obvious relationship between genetic profile and geographic distances among individuals collected along the small transect—individuals that were collected closer together on the small transect did not necessarily appear closer on the PCoA (Figure 2.12). Although some individuals located closer together on the small transect were also located close to each other on the PCoA graph, such as individual 1205-140 was located close to 1206-160 and 1208-180, and 1205-40 was close to 1205-60, many individuals seemed to be randomly scattered. This association was similarly observed in the main transect data set, where no correlation between genetic and geographic distances was observed among samples (Figure 2.8). The small transect PCoA graph (Figure 2.12) showed that individual 1205-160 overlapped with 1205-180, which indicated that they are genetically identical. On the contrary,

manual observation of clones found that individual 1205-120 also had an identical MLG to 1205-160, and 1205-180; individual 1205-280 shared the same MLG with 1205-300. However, on the PCoA graph (Figure 2.12), individual 1205-120 did not overlap with 1205-160 and 1205-180, nor did 1205-280 overlap with 1205-300.

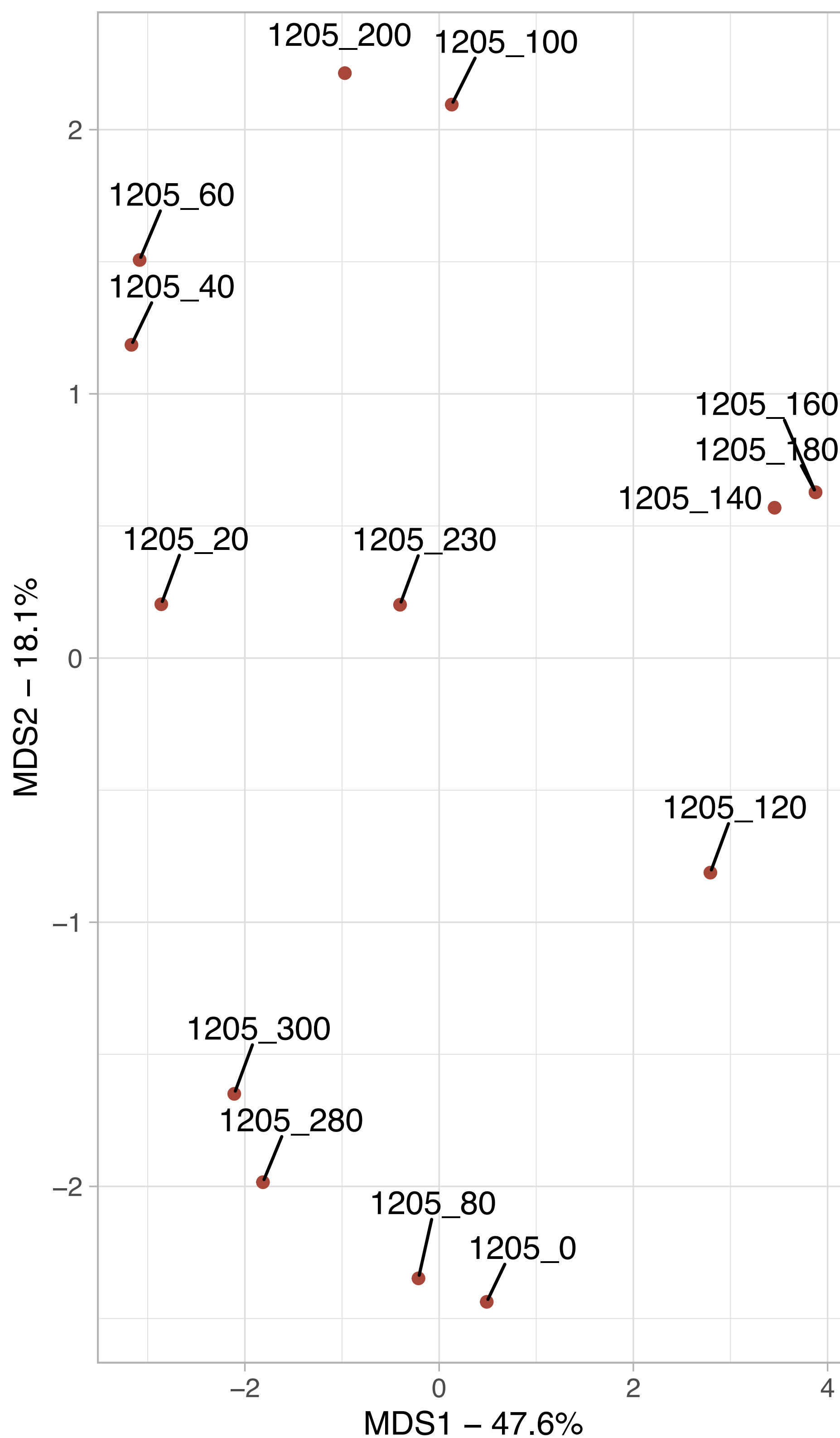

Figure 2.12 Principal coordinate analysis (PCoA) of Prevosti's genetic distances among the 14 *C.* "Remutaka" samples collected along a small transect at the QEII Covenant. The first two axes explain 47.6% and 18.1% of total variation, respectively.

The NeighborNet of the small transect showed that there is a main split separating the small transect individuals into two groups: (1) 1205-0, 1205,120, 1205-160, 1205-180, and 1205-140; and (2) 1205-230, 1205-200, 1205-100, 1205-20, 1205-60, 1205-40, 1205-300, 1205-280, and 1206-0 (Figure 2.13). One of the two genetic clusters identified by manual observation containing 1205-120, 1205-160, and 1205-180 was found in group (1). Individual 1205-160 and 1205-180 appeared on the same node, however 1205-120 was located on a shorter branch. The second genetic cluster consisted of 1205-280 and 1205-300 was found in group (2). Even though manual observation suggested that these two individuals were clones, they did not appear on the same node on the NeighborNet. This pattern was also observed on the small transect PCoA graph (Figure 2.12), where manually identified clones did not overlap. Whilst no obvious relationship was observed between the genetic and geographical distances, some individuals from neighbouring GPS ways points indeed clustered together on the NeighborNet (Figure 2.13). In particular, 1205-120, 1205-140, 1205-160, and 1205-180 clustered, and 1205-20, 1205-40, and 1205-60 were grouped together. Furthermore, all the clones that had been identified were individuals that were physically located close to each other on the small transect.

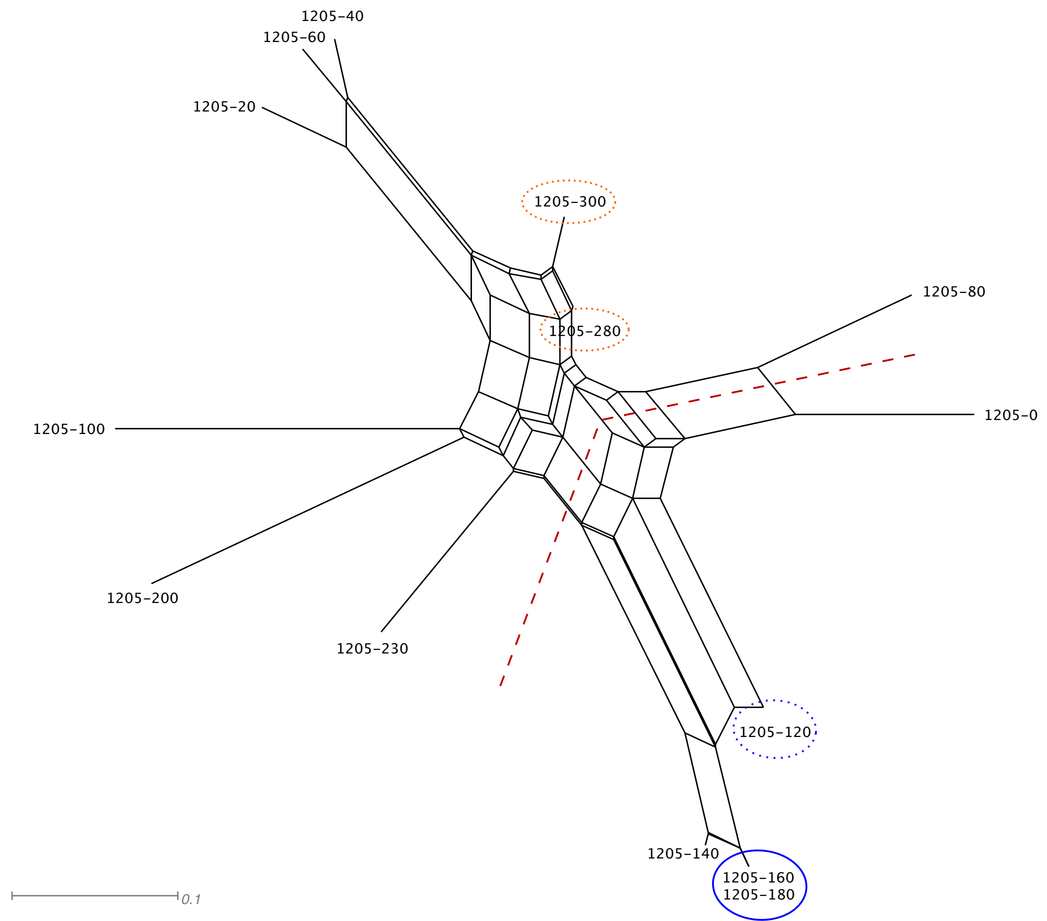

Figure 2.13 NeighborNet generated in SPLITSTREE4 based on Prevosti's genetic distance between all individuals sampled along a small transect at the QEII Covenant. The two genetic clusters (individuals with identical MLG) identified by manual observation are indicated by blue and orange circles, respectively; solid lined circle indicates individuals with identical MLG; dotted lined circles indicate individuals that were considered having the same MLG by manual observation, in which missing data were omitted. The dashed red line indicates the split that separates individuals into two main groups. The numbers on the graph correspond to sample ID of each individual.

The first axis of the microsite PCoA explains a large proportion, 58.3%, of the variation, and the second axis explains 16.9% of the variation (Figure 2.14). However, both axes failed to distinguish individuals collected from the three different clusters of leaves, no obvious clustering by sampling localities was observed. Individuals did not appear to be more closely related to those that were within the same cluster of leaves. Furthermore, four data points on the PCoA graph showed overlapping individuals, this indicated that these individuals were genetically identical. Clonality detection in RClone also suggested that these overlapping individuals shared the same MLG and were assigned into the same genetic clusters (Table 2.3). One outlier belonging to leaf cluster

1206-3 was seen on the PCoA graph, it appeared to be more genetically different compared with other individuals from the microsite.

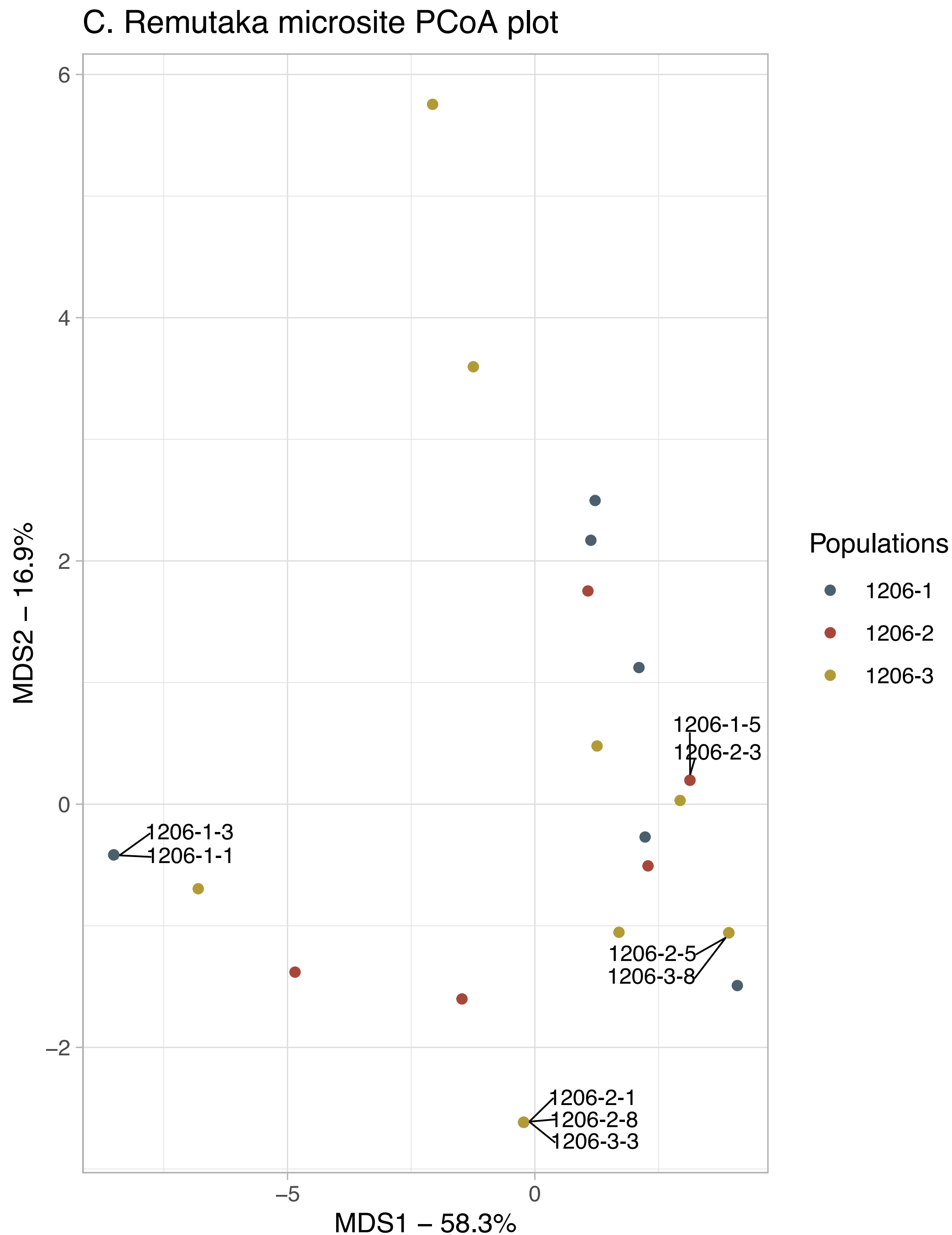

Figure 2.14 Principal coordinate analysis (PCoA) of Prevosti's genetic distances among the 24 *C.* "Remutaka" samples collected at a microsite at the QEII Covenant. The first two axes explain 58.3% and 16.9% of total variation, respectively.

The microsite NeighborNet (Figure 2.15) showed a similar pattern observed in its PCoA graph (Figure 2.12), where the partition of individuals was not based on their

physical location at the microsite. Two genetic clusters (indicated by orange and green circles) were positioned nearer to each other on one side of the NeighborNet in comparison to the two genetic clusters on the other side. Manual observation of identical MLG suggested that the two closely located genetic clusters shared the same MLG and could be considered being the same genetic cluster. Manual observation further proposed three individuals, 1206-1-1, 1206-1-7, and 1206-2-7, that were positioned near to the two closely located genetic clusters could also be considered having the same MLG, therefore belonging to the same genetic cluster.

Figure 2.15 NeighborNet generated in SPLITSTREE4 based on Prevosti's genetic distance between all individuals sampled at a microsite at the QEII Covenant. The four genetic clusters (individuals with identical MLG) identified by RClone are indicated by orange, green, blue, and yellow solid lined circles, respectively; dotted lined circles indicate individuals considered having the same MLG observed manually, which missing data are omitted, and are assumed to belong to the same genetic cluster with matching colour. The numbers of the graph correspond to sample ID of each individual.

The NeighborNet containing individuals collected along the main transect, the small transect, and microsite also showed a reticulate pattern; at the first glace no genetic structure was found (Figure 2.16). However, many individuals from 1206 were grouped

together on the NeighborNet suggesting that they were more genetically similar. Therefore, it was inferred that genetic structure was present at the microsite where a higher number of genetically identical individuals were observed.

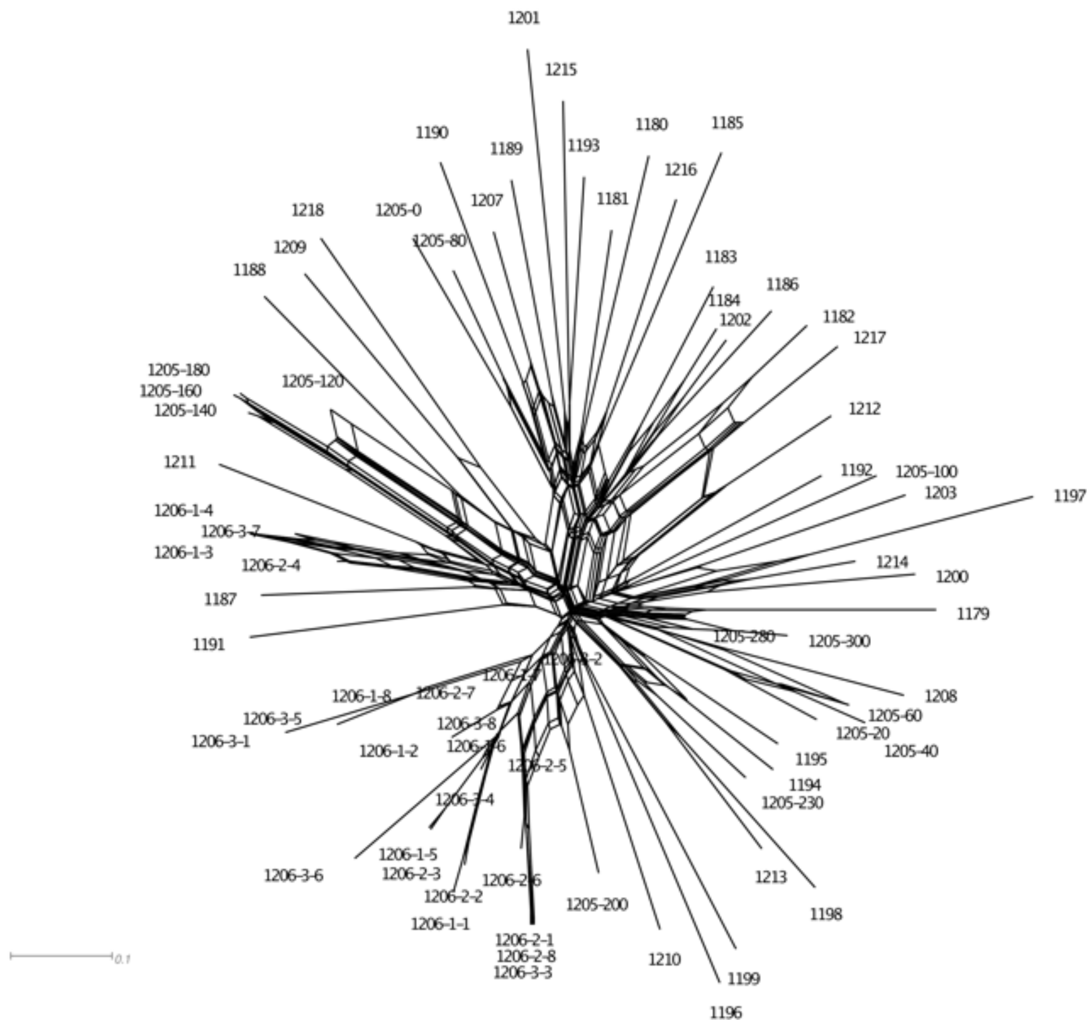

Figure 2.16 NeighborNet generated in SPLITSTREE4 based on Prevosti's distances between individuals sampled along the main transect, the small transect, and at a microsite at the QEII Covenant. The numbers on the graph correspond to sample ID of each individual.

2.5 Discussion

2.5.1 Within population genetic variation

Twelve microsatellite markers were used to genotype 37 *C.* "Remutaka" samples collected along the main transect at the QEII Covenant to assess the genetic diversity within this population. Based on microsatellite data, a medium level of genetic variation

(mean H_E = 0.573) was found. On the whole, this *C.* "Remutaka" showed homozygote excess in general (mean F_{IS} = 0.314). Wright's fixation index (F_{IS}) or inbreeding coefficient, measures the allelic frequency differentiation within population (Wright, 1950, 1969). A positive deviation from HWE, like the mean F_{IS} of the Covenant *C.* "Remutaka" population, can indicate that inbreeding occurs within the population (Wong & Sun, 1999).

In comparison to other endemic and/or endangered orchid species, the mean F_{IS} of this *C.* "Remutaka" population was higher than *Caladenia huegelii* (F_{IS} = 0.22), an endangered orchid endemic to Australia (Swarts *et al.*, 2009), and was similar to *Jumellea fragrans* (F_{IS} = 0.303), a threatened orchid that is endemic to the Mascarene archipelago (Blambert *et al.*, 2016). Threatened species usually comprise small populations, where the probabilities of self-pollination and mating with related individuals are higher than in larger populations (Hens *et al.*, 2017). Moreover, clonality assessment suggested that clones were present within the population. Clonal structure within populations may also increase inbreeding rate as pollen flow between genetically identical individuals is equivalent to self-pollination (Angeloni *et al.*, 2011), and consequently contributes to homozygote excess found in this population (Young *et al.*, 1996).

Although the population showed a positive deviation from HWE, the observed and expected heterozygosity varied across the 12 polymorphic loci tested. Gene flow increases the likelihood of polymorphism in populations (Balloux & Lugon-Moulin, 2002) and heterozygotic variation observed at different loci suggested that sexual recruitment has taken place (Forrest *et al.*, 2004). Therefore, both outcrossing and self-pollination are likely to occur in this population. However, preliminary observations suggested that autonomous self-pollination does not occur in *C.* "Remutaka", and this orchid cannot produce seeds if the pollinator is not involved (LEHNEBACH personal communication, 2021). The positive F_{IS} (Table 2.1) observed in this population could be a result of pollination between genetically identical individuals (refer to section 2.4.4.2 Clonal genetic structure), and/or self-pollination may occur when a pollinator deposits pollen onto the stigma of the same flower when moving within the flower (LEHNEBACH personal communication, 2021).

Life history and ecological variables such as population substructure, reproductive mode, pollination systems, and changing environments have profound effects on the genetic variation of a plant population (Hamrick *et al.*, 1979). New Zealand

Corybas are predicted to have a specialised pollination system; the orchids produce visual cues and odours to mimic oviposition substrates to lure female fungus gnats to visit the flowers for pollination (Kelly & Gaskett, 2014; Kelly *et al.*, 2013; Lehnebach & Zeller, 2015). Highly specialised orchids have been reported to be visited by fewer pollinator species (Tremblay, 1992); in some extreme cases, the orchids only rely on a single species as pollinators (Phillips *et al.*, 2015). Species with specialised pollination systems may experience demographic declines and become vulnerable to extinction due to pollination failure (Johnson, 2010).

In contrast, the *outcrossing hypothesis* suggests that the specialised deceptive pollination system of orchids promotes cross-pollination (Dressler, 1981). In the *outcrossing hypothesis,* pollinators visit fewer flowers on the same inflorescence and travel a further distance after being deceived, as a result, increasing pollen flow and pollen exportation (Ayasse *et al.*, 2000; Johnson *et al.*, 2004; Peakall & Beattie, 1996). The presence of clonality within populations and self-compatibility in *Corybas* orchids can explain variabilities in heterozygosity across the 12 microsatellite loci used to assess genetic variation within the QEII Covenant population. Outcrossing within the population is facilitated by pollinators, which enables gene flow within populations and the maintenance of polymorphisms (Table 2.1). Conversely, pollination between genetically identical individuals and/or pollinator facilitated self-pollination may lead to inbreeding. The quality of pollination service is important for maintaining genetic variation within a population or within a species (Waser *et al.*, 1996).

Genetic variation within this population could be affected by the distance between genetically similar individuals and clonal structure within the population. Short distances between genetically similar individuals may lead to biparental inbreeding, which is the crossing between relatives (Uyenoyama, 1993). Whereas, clonal structure within a population can increase inbreeding rate through self-pollination as pollen deposition between genetically identical individuals is more likely to occur (Angeloni *et al.*, 2011). Similar to self-pollination, biparental inbreeding also decreases genetic variation within a population leading to mates becoming more genetically similar to each other (Griffin & Eckert, 2003). The difference between autogamous self-pollination and biparental inbreeding is that in autogamous self-pollination, pollen being deposited is from the same individual, biparental inbreeding occurs when the pollen being deposited is from closely related individuals (Uyenoyama, 1993). Biparental inbreeding affects the cost and

benefits between outcrossing and self-pollination (Griffin & Eckert, 2003). Because autogamous self-pollination serves as a mechanism to ensure species survival and lower the cost of outcrossing, biparental inbreeding is essentially outcrossing, but pollen deposition is from closely related relatives (Griffin & Eckert, 2003). In contrast, although clonal structure within a population may lead to an increase in inbreeding rate and subsequently cause reductions in fitness, clonal propagation helps preserve genetic diversity in the short term (Wolf *et al.*, 2000).

2.5.2 Genetic structure within the population

The Evanno test (Evanno *et al.*, 2005) results of population structure suggested that two genetic clusters ($K = 2$; Figure 2.5) were identified within this *C.* "Remutaka" population. Despite this, all individuals collected on the main transect had an admixed genotype regardless of different K values as illustrated by the CLUMPAK results (Figure 2.7). This suggested that no genetic structure was found among the main transect individuals and the highest peak $K = 1$ was observed in the mean posterior probability plot (Figure 2.6). If genetic structure was present within the population, the expectation is that certain individuals would have stronger affinities to one of two genetic clusters, unless there was very recent admixture between two previously distinct groups. The reticular pattern seen in the NeighborNets of the main transect data set (Figure 2.9) also support the CLUMPAK results that no genetic structure was identified in the main transect data set. However, the NeighborNet of the combined data sets showed that genetic structure was present among the microsite individuals where the number of clonal individuals were high (Figure 2.16).

A Mantel test of the correlation between the genetic and geographic distance showed a slight negative result (R^2 = -0.0416). A negative correlation indicates that an increase in genetic distance between individuals is related to a decrease in geographic distance. However, the R^2 was close zero and the p-value = 0.772 was not significant. R^2 close to zero suggested that there was no correlation between genetic and geographic distances of individuals within the population, and a non-significant p-value implied that the correlation observed between genetic and geographical distances was random. The Mantel test results were in agreement with the CLUMPACK results, NeighborNets, and the main transect PCoA graph that no genetic structure was observed within population.

The lack of correlation between genetic and geographic distances within population revealed that the entire population is interbreeding. Gene flow occurs within the population allowing exchange of genetic material between different individuals, which subsequently prevents the formation of genetic structure (Mills & Allendorf, 1996). Gene flow could occur through outcrossing promoted by pollinators when they transport pollen among the members of this *C.* "Remutaka" population. Another feature of *Corybas* species that might contribute to gene flow within the population is their wind-dispersed seeds. Like most orchids, *Corybas* species have microscopic seeds. The peduncle, which bears the capsule at its end, undergoes elongation to release seeds above the forest floor (Lyon, 2014). These small seeds can be carried away by the wind and establish further from the parent plant (Phillips *et al.*, 2012). Consequently, resulting in the lack of correlation between genetic and geographic distances. In short, there appear to be no significant barriers to gene flow at this location.

2.5.3 Null alleles

MICRO-CHECKER detected the presence of null alleles at seven out of the 12 microsatellite loci used to genotype the main transect individuals. Null alleles are alleles that fail to amplify during PCR due to mutations in the primer binding site that prevents amplification (Pemberton *et al.*, 1995). In diploid individuals, null alleles at a given locus may appear as missing data when both alleles are null alleles; or it can appear as a homozygous locus when one of the alleles is a null allele (Callen *et al.*, 1993; Wagner *et al.*, 2006). As such, null alleles are not scored, subsequently causing genotyping errors in data sets that lead to overestimates of heterozygote deficit and deviation from HWE (Dakin & Avise, 2004). Although the presence of null alleles causes overestimates of heterozygote deficit, excluding loci that had null alleles would remove a large part of resolution and explanatory power. Therefore, all loci were included in this study.

Additionally, population structure, inbreeding, and selection at a locus can also result in decreased genetic variation and deviation from HWE (Dakin & Avise, 2004). Clonal propagation and autonomous self-pollination are known to occur in *Corybas* orchids, moreover some of these orchids are self-compatible (Clements *et al.*, 2007; Kelly *et al.*, 2013). Self-compatibility might contribute to the observed homozygote excess at some loci. On the other hand, Carlsson (2008) argued that null alleles had only moderate

effects on accuracy in Bayesian assignment analysis such as STRUCTURE even in populations with very high null-allele frequencies. Therefore, loci that were potentially affected by null alleles should not impede STRUCTURE assignment of the individuals within this *C.* “Remutaka” population.

Moreover, the small transect and microsite data set (Table 2.2) contained a larger amount of missing data in comparison to the main transect data set (Table 2.1). For example locus Corybas-19 and locus Corybas-16 had an amplification rate of 50.00% and 66.67% in the small transect and microsite data set, respectively. Whereas in the main transect data set, the lowest amplification rate observed was 94.59%. These missing data might be caused by poor DNA quality due to DNA degradation over time rather than null alleles (Weiß *et al.*, 2016). The *C.* “Remutaka” samples were collected in 2015 and had been silica-gel dried and stored in silica gel until the time of DNA extractions in 2019-2020. The small transect and microsite samples were genotyped using a set of microsatellite markers that were developed under the same parameters and conditions as those that were used to genotype the main transect samples and *Corybas macranthus* samples in Chapter 3 (refer to Section 3.3, Chapter 3). The size of individuals’ leaves collected from the main transect were larger than individual leaves from the small transect and microsite. Less leaf material was available for a single DNA extraction of the small transect and microsite samples. It is likely that the smaller leaves were produced by young replacement tubers from vegetative propagation (LEHNEBACH personal communication, 2021). Due to *Corybas* orchid morphology where a single plant generally consists of a single leaf (Lyon, 2014), it was not possible to combine two DNA extractions into one DNA sample to increase the quantity of DNA in the small transect and microsite DNA samples. The main transect samples (Table 2.1) and *C. macranthus* samples (refer to Table 3.3, Chapter 3) both had a higher PCR amplification rate than *C.* “Remutaka” small transect and microsite samples (Table 2.2). Therefore, the missing data in *C.* “Remutaka” data set were likely caused by DNA degradation rather than null alleles.

Identification of clonal individuals relies on assessing identical MLGs, therefore the presence of missing data (either due to DNA degradation or null alleles, or both) may hinder clonality assessment. Rclone, which was used to perform clonality analyses, does not yet support missing data (Bailleul *et al.*, 2016), and identification of clones in the microsite data set was carried out manually. Resampling new *C.* “Remutaka” materials from those locations at the Covenant for DNA extractions may increase the accuracy of

clonality assessment. Although the missing data present in the small transect and microsite data set were likely caused by poor DNA quality, Honnay and Bossuyt (2005) proposed that somatic mutations in individuals from the same clonal lineage (individuals that originated from the same sexual reproductive event) may contribute to the variations in their MLG. Nonetheless, it is necessary to sequence the entire microsatellite region and both primer binding sites in all individuals with missing data in order to identify null alleles, because the missing data can be caused by null alleles and poor DNA quality that led to low PCR amplification rates (Carlsson, 2008).

2.5.4 Clonality within the population

Individuals with identical MLG were detected within the population indicating that clonal propagation has taken place. Results of clonality assessment revealed that clones occurred close to each other in their habitat, the maximum distance detected between two genetically identical individuals was 40 cm. More individuals with the same MLG were detected at the microsite where samples were collected intensively from three dense leaf clusters, and the distance separating these clusters was 10 cm. Clonal propagation in *Corybas* orchids is known to occur through replacement tubers formed at the tip of lateral roots that are growing away from the parent plant (St George & McCrae, 1990); lateral roots can extend to certain distance away from the parent plant (Figure 2.1). This offers a plausible explanation as to why some *C.* "Remutaka" plants are seen in condensed clusters of leaves and clones are found in close proximity.

The two sets of microsatellite markers used to assess clonality in the small transect and microsite data set have been shown to provide enough resolution to distinguish unique MLGs (Figure 2.10 and Figure 2.11). However, discrepancies in clone detection by statistical software (i.e., R packages RClone and poppr) and manual observation were seen in the PCoA graph (Figure 2.12 and Figure 2.14) and NeighborNet (Figure 2.13 and Figure 2.15). The PCoA graphs and the NeighborNets were constructed based on Prevosti's genetic distance matrix generated in R using poppr, genetically identical individuals will overlap in the PCoA graph and will be partitioned on the same node on the NeighborNet. In contrast, some of the manually identified clone clusters in the small transect and microsite data set (Table 2.3) did not overlap on the PCoA graphs nor were they on the same node on the NeighborNets. These mismatches resulted from missing

data present within data sets due low PCR amplification rate at some loci (Table 2.2). Manual observation ignored missing data and only compared alleles successfully amplified, whereas missing data were taken into account in the statistical software.

2.6 Future directions

This study is the first population genetic investigation in New Zealand *Corybas* orchids. Further studies could focus on different *Corybas* populations at a wider geographic range in order to study the correlation between genetic variation and life history traits (i.e., reproductive methods, pollination strategies, and population size, etc), and the effects of habitat loss on genetic diversity and genetic differentiation within and among populations.

Pollinator species of *Corybas* orchids have yet to be identified. Pollinators play a crucial role in recruitment and maintaining self-sustaining populations (Phillips *et al.*, 2020). Pollinator identifications are needed for New Zealand *Corybas* orchids. Field observations carried out by either manual observation and/or utilising camera systems accompanied with insect trapping can be used to investigate insects that visit the flowers and to identify true pollinators (Howard *et al.*, 2021; Kelly *et al.*, 2013; Lehnebach, 2002), but pollination events are uncommon (e.g. Chapter 3) so this is a difficult task. Genetic assessments of a population however can help uncover indirectly the role of pollinators promoting outcrossing and also contributing to self-pollination.

2.7 Conclusion

This study used microsatellite markers to investigate the genetic composition, genetic variation, and the extent of clonality within a population of a taxonomically uncertain New Zealand terrestrial orchid, *C.* “Remutaka”. The results suggested that this population has a medium level of genetic variation (mean $H_E = 0.573$). As a whole, this population showed homozygote excess, which indicated inbreeding or selection against heterozygotes occurs. However, the 12 loci used to assess genetic diversity within the population showed varying degrees of heterozygote and homozygote excess, which suggested that outcrossing also occurs. Thus, outcrossing and inbreeding is assumed to occur within population. Cross-pollination is likely facilitated by pollinators, and inbreeding could result from pollen deposition between genetically identical individuals

as clonal structure is present within population. Inbreeding through self-pollination is also possible when the pollinator deposit pollen to the stigma of the same flower while moving within the flower. Further, no correlation between genetic distance and geographic distance were found within the population. This implied that the entire population was largely interbreeding; pollinators may facilitate gene flow within a population, in turn preventing the formation of genetic structure. Additionally, the wind-distributed seeds of *Corybas* orchids are adapted for long-distance distribution, which may also contribute to the lack of genetic structure within the population as the seeds are distributed randomly. Clonality assessment showed that clones were present, which means that this population also reproduces from clonal propagation. Clones generally occur close to each other, the distance between two genetically identical individuals was found to range from ≤10 cm to 40 cm. The presence of clonal structure may further increase inbreeding by increasing the occurrence of pollination between genetically identical individuals. New Zealand *Corybas* orchids face conservation concerns as there are many known from a single location and some already are of conservation concern (refer to Chapter 3). Further studies focused on population genetics and plant-pollinator interactions are needed to gather comprehensive information for conservation. The finding of this study provides preliminary context for the knowledge of genetic composition, genetic variation, and clonality in *Corybas* and, also provides information that allows us to infer the behaviour of pollinators which not often observed. Altogether, these studies may contribute to further research into understanding New Zealand orchids and support their conservation.

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Chapter 3: A population genetics study in *Corybas carsei* and *Corybas macranthus*, and pollination studies of *C. carsei*

3.1 Abstract

The swamp helmet orchid (*Corybas carsei*) is one of the most threatened terrestrial orchids in New Zealand. It was once thought to be extinct in New Zealand until its rediscovery at the Whangamarino Wetland in 1983. Today, Whangamarino Wetland is the only known location of this orchid, hosting a single population of ~350 individuals. Active management such as periodic controlled burning is carried out within orchid management sites to maintain a suitable habitat for *C. carsei*. Genetic diversity is important for the long-term survival of a species as it provides adaptation for the population to respond to the changing environment. Here, 11 microsatellite markers were developed and used to assess the genetic diversity within this single remaining *C. carsei* population. The 11 microsatellite markers failed to detect any genetic variation among the 29 individuals sampled from this population. All 11 loci genotyped were monomorphic, suggesting all individuals within the population are likely genetically identical, or extremely low genetic variation may be present within the population. In contrast, a set of 11 microsatellite markers used to genotype 67 individuals of a common and widespread species, *Corybas macranthus*, collected from three populations across the North Island, detected low to medium genetic variation within populations (mean H_E = 0.291). Across the three *C. macranthu* populations, a low number of alleles were found, each population on average had 3.727 alleles per locus. A low F_{IS} (0.104) and a low degree of genetic differentiation between populations (mean F_{ST} = 0.119) were detected. F_{IS} showing homozygote excess ($F_{IS} > 0$) indicated inbreeding, but heterozygosity varied across the 11 loci tested, which was likely caused by cross-pollination. Therefore, it is inferred that the *C. macranthus* populations have a mixed-pollination system in which both self-pollination and cross-pollination were employed. In addition, genetic structure was detected between and within populations. Clonality assessment indicated the presence of clonal individuals within all three *C. macranthus* populations, suggesting that asexual reproduction occurs. Pollination experiments in *C. carsei* showed that this orchid is self-compatible and autonomous self-pollination may occur. Fruit capsules observed in the population could result from both outcrossing and self-pollination. A camera set up in the field to film floral visitors failed to identify pollinators of *C. carsei*. However,

footage of its putative pollinator, flies, were captured approaching the flower though they did not enter. The species of flies that approached the flower could not be identified from the footage. The finding of this study provides preliminary context for the knowledge of genetic variation, pollination strategy, and pollinator identification in *C. carsei*, which may contribute to further research into understanding this species and its conservation.

3.2 Introduction

Wetlands are located at the junction between land and water, particularly where land meets estuaries, lakes, rivers, and streams (Johnson & Gerbeaux, 2004). Wetlands are characterised by a water table near or at the ground surface, oxygen-poor soils, and the dominance of plants that are adapted to wetland environments (Warner & Rubec, 1997). Wetlands are vital to maintaining healthy ecosystems and contribute almost 50% in the world's ecosystem services (Barbier *et al.*, 1997). For example, wetlands provide habitats for indigenous fauna and flora, regulate atmospheric gases, and lock up carbon (Millennium Ecosystem Assessment, 2005). They also provide protection against floods and tsunamis by storing excess stormwater and slowly releasing it to groundwater systems; they also filter sediments and pollutants in turn maintaining water quality and supply (Barbier *et al.*, 1997; Millennium Ecosystem Assessment, 2005).

Despite their important role in sustaining healthy ecosystems, approximately 50% of wetland areas globally have been drained and converted to other land uses to support the growing human population (Zedler & Kercher, 2005). The extent of land conversion varies among regions, ranging from minor losses in boreal countries, to 53% in USA, 60% in China, and in some parts of Europe wetland loss is over 90% (Mitsch & Gosselink, 2000). The rate and extent of wetland loss in New Zealand have been considered as among the highest in the world (Mitsch & Gosselink, 2000). With over 90% of the original wetland areas lost and only 1% of New Zealand mass estimated to be covered by palustrine wetlands, wetlands are a highly threatened ecosystem in the country (Ausseil *et al.*, 2011).

New Zealand experienced the most significant reduction of wetlands during the main period of European settlement in the mid 1800s (Duncan & Young, 2000; Myers *et al.*, 2013). More than two-thirds of indigenous land cover, including forested wetlands and open swampland, was converted to exotic forests, settlements, and agricultural lands

(McGlone, 2009). More recent loss of wetlands occurred during 1920–1980 when the government started to subsidise for agricultural land and forestry clearance in the 1970s (Taylor & Smith, 1997). Government subsidies have been gradually removed since 1984, however surveys of changes in wetlands in New Zealand showed that loss of wetlands is still ongoing, particularly smaller wetlands that are on private land (Ministry for the Enviroment & Stats NZ, 2021; Myers *et al.*, 2013). Many drained wetlands had high wildlife values, alterations in land uses were one of the main factors that contributed to biodiversity decline (Myers *et al.*, 2013). For instance, the swamp helmet orchid, *Corybas carsei*, is one of the many species affected by land modification. The number of populations of this orchid drastically declined due to loss of suitable habitats.

C. carsei is one of the 22 accepted species within the genus *Corybas* in New Zealand (Schönberger *et al.*, 2020). *C. carsei* plants are diminutive, only 10–30 mm tall when flowering. An individual orchid consists of an underground tuber, a single heart-shaped leaf and produces a solitary flower. *C. carsei* flowers from August to November with summer dormancy (New Zealand Plant Conservation Network, 2021). Each flower consists of a dorsal sepal, a labellum, two lateral sepals, and two petals. The dorsal sepal and labellum are maroon-purple in colour with white longitudinal stripes. Many *Corybas* species have filiform lateral sepals and petals, but the lateral sepals and petals in *C. carsei* are greatly reduced and are white in colour (Jane *et al.*, 2017). Similar to other New Zealand *Corybas* orchids, *C. carsei* is perennial. At the end of each growing season, the old tuber produces new replacement tubers before it dies back. The new tubers remain dormant until the beginning of the next growing season (Clarkson *et al.*, 1993; Figure 3.1). However, the longevity of an individual plant and of the clonal groups are not well known. *C. carsei* has very specific habitat requirements—it is restricted to restiad bogs and is known to grow among open vegetation of bog sedges (*Schoenus* sp.: Cyperaceae) and wire rush (*Empodisma* sp.: Restionceae; Jane *et al.*, 2017; New Zealand Plant Conservation Network, 2021).

Figure 3.1 A *C. carsei* plant (the orange arrow indicates the old tuber, the blue arrow indicates the new replacement tuber, the black arrows indicate lateral roots); scale bar = 1 cm; photo from Carlos Lehnebach.

C. carsei was described in 1911 upon its discovery at Lake Tāngonge, Northland, New Zealand (Cheeseman, 1911). Historically, this species had been recorded at four different locations on the North Island of New Zealand (Lake Tāngonge between Kaitāia and Ahipara (Clarkson *et al.*, 1993), a bog near Tauhei, Moanatuatua Bog in Ōhaupō, and at Whangamarino Wetland (Clarkson *et al.*, 1993)), all locations had experienced extensive drainage and conversion to agricultural and horticultural lands (McKenzie *et al.*, 2002). Although the site at Moanatuatua Bog where the orchids occurred was designated as a scientific reserve, this effort was not enough to sustain the orchid population. Wetland drainage caused the water table to drop over time and vegetation shifted towards woody manuka (*Leptospermum scoparium*; Clarkson *et al.*, 1993). Records showed that the orchid population at Moanatuatua Bog started to decrease in the 1960s, and they were last seen there in 1974 (Clarkson *et al.*, 1993). *C. carsei* was believed to be extinct in New Zealand until its rediscovery at Whangamarino Wetland in 1983 (Clarkson *et al.*, 1993; Department of Conservation, 2019). A group of botanists unexpectedly encountered a small population of *C. carsei* when they were visiting the

Whangamarino Wetland (Department of Conservation, 2019). Today, Whangamarino Wetland is the only known location of *C. carsei*, with a single population of approximately 350 plants, making *C. carsei* one of the most threatened terrestrial orchids in New Zealand. The current conservation status of this orchid is nationally critical (de Lange *et al.*, 2017).

Whangamarino Wetland is the second largest peat bog and swamp complex on the North Island (Department of Conservation, 2021). It is one of the three nationally important wetlands managed under the Arawai Kākāriki national wetland restoration programme, and is regarded as being of international importance under the Ramsar Convention (Department of Conservation, 2014). This wetland occupies 7290 hectares, in which 5138 hectares have been administered as a reserve and priority site for the conservation of *C. carsei* by the Department of Conservation (DoC). *C. carsei* historically occurred at four sub-sites located in the southern portion of the Te Raeo peat bog at Whangamarino Wetland (Department of Conservation, 2014), but now plants are only found in one of the sub-sites. Even though at present *C. carsei* only occurs in one of the sub-sites, active management is carried out in all sub-sites. Within each sub-site, a polygon-shaped area ranging from 105–224 m^2 is designated as an orchid management area. Active management, such as controlled burning, is carried out within these demarcated areas. As of 2017, the orchid sites were dominated by *Schoenus* (Cyperaceae) and *Empodisma* (Restionaceae) vegetation (Department of Conservation, 2018). *C. carsei* has been observed on several substrates such as bare ground, liverwort (*Goebelobryum unguiculatum*), and algal mats (*Nostoc* species). A number of species were recorded associating with the orchids within the orchid sites. For example, wire rush (*Empodisma robustum*), bog Schoenus (*Schoenus brevifolius*), tamingi (*Epacris pauciflora),* tangle fern (*Gleichinia dicarpa*), liverwort (*Goebelobryum unguiculatum*), slender club moss (*Lycopodiella lateralis*), bog clubmoss (*Lycopodiella serpentina*) and *Nostoc* species (Department of Conservation, 2018).

Upon its rediscovery in 1983, this *C. carsei* population was estimated to consist of hundreds of individuals, but other than that, the history and the extent of this population was unknown (de Lange & Clarkson, n.d.; Department of Conservation, 2018). Te Raeo Bog is not an ideal habitat for *C. carsei*, as its vegetation structure, trophic level, trophic succession, and nature of peat deposits are different from other locations where *C. carsei* used to occur (Department of Conservation, 2018). The acidity in Whangamarino

Wetland is not high enough to maintain open areas among taller vegetation in the centre of the bog for the orchids to inhibit (de Lange & Clarkson, n.d.). In the 20th century, fire frequently occurred in Te Raeo Bog caused by land clearance and steam engine sparks from a railway on the western edge of the bog (Clarkson & Stanway, 1994). *C. carsei* likely established at Whangamarino Wetland from seed dispersal following the fire disturbances, which created open areas among the otherwise thick bog vegetation (Brandon, 2006). Today, with legal protection of the wetland, fire no longer occurs (Clarkson & Stanway, 1994), and this miniature orchid will be out competed by the dense bog vegetation without disturbance to maintain open areas. The number of individual orchids has been shown to decrease with an increase in sedge growth and a reduced light intensity (de Lange & Clarkson, n.d.).

The development of a management plan for the conservation of *C. carsei* was initiated in 1991, which aimed to study the orchid's ecology and the effects of disturbance on the orchid population (Clarkson *et al.*, 1993; Department of Conservation, 2018). Disturbance was imitated in two ways: vegetation clearance using scrub bars (Clarkson *et al.*, 1993) and fire (Norton & de Lange, 2003). Both scrub bar clearance and fire disturbance increased vegetative reproduction in *C. carsei*, the density of orchids (the number of leaves) reappearing within management sites increased by four times after disturbance (Clarkson *et al.*, 1993; Norton & de Lange, 2003). Although, there was no evidence of scrub bar cleaning increasing the orchid's flowering and fruiting rate. On the other hand, Norton and de Lange (2003) found that in additional to increasing vegetative reproduction, fire also induced sexual reproduction in *C. carsei*. Whilst the above ground portion of the orchids was destroyed during the fire, the underground tuber survived. The orchids reappeared in higher density one year post-burning and had a peak density in the second year. However, the number of orchids declined from the next four following years and were no longer visible in the sixth year. Nonetheless, fire increased flowering rate from 1–4 years after burning, approximately 30% of the orchids flowered compared to the naturally low flowering rate of 1% (Clarkson *et al.*, 1993; de Lange & Clarkson, n.d.). The number of seed set also increased after burning with a peak observed in the third year (Norton & de Lange, 2003). At Whangamarino Wetland, no fruit capsules were observed until experimental burning was carried out (Department of Conservation, 2018; Norton & de Lange, 2003). Based on the results, Norton and de Lange (2003) suggested that fire

disturbance was beneficial and proposed a five-year rotation of controlled burning for the management of *C. carsei*.

Additionally, *C. carsei* is sometimes considered to be the same species as the Australian swamp pelican-orchid, *Corybas fordhamii* (Department of Conservation, 2019; Threatened Species Section, 2021), although further studies are required to resolve the unclear taxonomic status of these two orchids (New Zealand Plant Conservation Network, 2021). Nevertheless, similarly to *C. carsei*, fire disturbance has been recorded to stimulate flowering and increase flowering rate in *C. fordhamii*, as well as other *Corybas* species (Gordes & Gordes, 1992; Jones, 2006). At present, periodic controlled burning is carried out within the four orchid management sites at Whangamarino Wetland for the removal of dense vegetation to maintain a suitable habitat and to promote sexual reproduction in *C. carsei* (Department of Conservation, 2019). Scrub bar removal of vegetation is utilised between the scheduled controlled burns to improve habitat conditions for the orchids (Department of Conservation, 2018). Monitoring of the Whangamarino Wetland population was conducted by DoC in all management sites annually to record the number of plants, the number of flowers, and the number of fruits. This orchid used to occur at all four management sites, however as of 2020 and 2021, when our field work was carried out, these orchids were only found in management site 2.

Despite a management plan being in place for the conservation of *C. carsei*, many aspects of the orchid's reproductive biology remain unknown. Field observations suggested that asexual reproduction is predominant in *C. carsei*, though sexual reproduction also occurs (Clarkson *et al.*, 1993; de Lange & Clarkson, n.d.). Like other *Corybas* orchids, asexual reproduction in *C. carsei* is known to occur from vegetative growth from replacement tubers, and sexual reproduction results from insect mediated pollination most likely by fungus gnats (Department of Conservation, 2018). However, pollinators of *C. carsei* have not been observed (Department of Conservation, 2018). Furthermore, *C. carsei* is speculated to be self-compatible, therefore self-pollination may also occur (de Lange, 2017 personal communication to DoC). As a result, capsules observed in the field could be the result of both self-pollination and cross-pollination. Flowering rate and fruiting rate are low in this *C. carsei* population, for example in the years of 2018 and 2019, a total of nine fruits had been reported from the site (Department of Conservation, 2018). and the persistence of a population relies on successful reproduction and recruitment of new individuals (Aguilar *et al.*, 2006; Neiland & Wilcock,

1998). Moreover, genetic variation within a population is important for the long-term survival of a species as it provides adaptative potential for the organisms to respond to a changing environment (Ellstrand & Elam, 1993).

Different reproductive methods affect a population's genetic composition, genetic variation, and genetic structure (Loveless & Hamrick, 1984). For example, asexual reproduction produces genetically identical individuals within a population where clonal structures are seen. On the other hand, sexual reproduction results from cross-pollination between genetically distinct individuals, which shuffles genetic variation through recombination (Vrijenhoek, 1990). Conversely, self-pollination (inbreeding) can reduce genetic variation (Chung *et al.*, 2004). Further, clonal structure within a self-compatible population may lead to geitonogamous self-pollination—pollination between flowers of the same individual, as the pollen deposited between different flowers is from genetically identical individuals (Angeloni *et al.*, 2011). As a result, genetic variation within the population reduces as self-pollination increases. Moreover, genetic diversity can also decrease through genetic drift that leads to allele fixation in the population. Understanding the distribution of genetic variation and reproductive biology within this single remaining population of *C. carsei* is crucial for the development of its management plan and to ensure survival.

In this study, microsatellite markers were developed for *C. carsei* and were used to assess genetic variation within this single remaining population. As a comparison, a set of 11 microsatellite markers developed by van Etten *et al.* (2018) were used to assess genetic variation and genetic structure in three populations of a common New Zealand *Corybas* orchid, *Corybas macranthus*. The genetic results of the *C. carsei* population were compared with the genetic results of the three *C. macranthus* populations to explore potential differences in genetic variation and structure between a restricted and a widespread species. Pollination experiments also were carried out in *C. carsei* to investigate self-incompatibility and pollination strategies within the species. The number of pollen grains produced by the pollinia were counted to estimate the amount of pollen grains produced by a single *C. carsei* flower. Lastly, a camera was set up in the field to film floral visitors and to help identify potential *C. carsei* pollinators.

3.3 Materials and Methods

3.3.1 Population genetic analysis of *C. carsei* and *C. macranthus*

3.3.1.1 Microsatellite marker development for *C. carsei*

In a preliminary investigation, five of the readily available *Corybas* microsatellite markers (Corybas-23, Corybas-28, Corybas-36, Corybas-44, and Corybas-48; van Etten *et al.*, 2018) were trialled on 10 *C. carsei* individuals, but no PCR products were visible for the majority of individuals tested. This indicated that these *Corybas* microsatellite markers were not transferable to *C. carsei*, therefore new markers were developed to carry out population genetic studies in *C. carsei* (*C. carsei* sample collection refer to section 3.3.1.3; DNA extraction refer to section 3.3.1.4)

One *C. carsei* individual was selected as the source of DNA for marker development. Genomic DNA was extracted from silica gel-dried leaf tissue using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) with a modification of the manufacturer's protocol (25 μL of Buffer AE was used for elution instead of 100 μL). A Qubit 2 Fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) reading indicated that the sample had a DNA concentration of 2.36 ng/L. A DNA library was prepared using the Illumina Nextera XT DNA Library Preparation Kit (Illumina, San Diego, California, USA). The indexed library was pooled with two other libraries of different plant taxa in equal concentration and sequenced on an Illumina MiSeq (Illumina) using 250-base-pair paired-end chemistry (Massey Genome Service, Palmerton North, New Zealand).

The Illumina MiSeq sequencing generated 3,141,089 reads with an average 250 base pair (bp) in length. A 0.01 quality cut-off was used to trim low-quality results from these sequences in DynamicTrim in SolexaQA (Cox *et al.*, 2010). The remaining sequences were assembled into contigs in SPAdes (version 3.15.0; Bankevich *et al.*, 2012) on the Mahuika high-performance computing cluster of New Zealand eScience Infrastructure (NeSI). STR detection (version 1.0.0; Fungtammasan *et al.*, 2015) on Galaxy (Afgan *et al.*, 2018) was used to identify dinucleotide repeats with criteria of a minimum number of seven and a maximum number of 12 uninterrupted repeats, and at least 50 bp surrounding the repeat regions on both sides. Primers were designed in Geneious (MacOS version 2021.1.1; Biomatters Ltd., Auckland, New Zealand) using Primer3 (Rozen & Skaletsky, 2000) with the following parameters: product size = 150–

320 with no mononucleotide repeats longer than 4 bp in the sequences surrounding the microsatellite region; a GC clamp at the 3′ end, GC clamp = 1; GC content = 20 (minimum)–50 (optimal)–80% (maximum); melting temperature (T_m) = 54 (minimum)–55 (optimal)–59°C (maximum); maximum T_m difference = 3°C; and maximum poly x = 5. An M13 sequence (CACGACGTTGTAAAACGAC) was added to the 5′ end of each forward primer (Boutin-Ganache *et al.*, 2001) and a PIG-tail sequence (GTTTCTT) was added to the 5′ end of each reverse primer to reduce non-templated addition of nucleotides (Brownstein *et al.*, 1996).

Twenty primer pairs were designed in Primer3 and manufactured by IDT (Singapore, Republic of Singapore). These 20 primer pairs were screened on seven individuals from the Whangamarino *C. carsei* population. PCR amplification was performed in a volume of 10 µL with 1× buffer (Solis BioDyne, Tartu, Estonia), 2.5 nM of $MgCl_2$, 100 µM of dNTP mix, 0.02 µM of forward primer, 0.45 µM of reversed primer, 0.45 µM of M13 primer (labelled with FAM, VIC, or NED), 0.5 U of FirePol Taq polymerase (Solis BioDyne, Estonia), 5-50 ng of DNA, and 3.7 µL of water. The PCR programme had an initial denaturation of 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds; 53°C for 40 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. Three microliters of PCR product with 2 µL of loading dye was loaded on 1.5% agarose gel and run at 75 volts for 90 minutes to assess amplification at each locus. PCR products (3 µL) for three loci labelled with different fluorescent dyes (either FAM, VIC, or NED) and of distinguishable sizes were co-loaded and added to 1 µL of CASS ladder (Symonds & Lloyd, 2004) and 9 µL of Hi-Di formamide (Applied Biosystems, Carlsbad, California, USA) for fragment sizing on an ABI 3730 Genetic Analyzer (Applied Biosystems) at Massey Genome Service at Massey University, Palmerston North, New Zealand. Alleles were visualised and scored using GeneMapper (version 5.0, Applied Biosystems). The observed heterozygosity (H_O) and expected heterozygosity (H_E) were determined using GenAlEx (version 6.503; Peakall & Smouse, 2012).

3.3.1.2 Study site

C. carsei sample collection and pollination experiments were carried out at the management Site 2, Whangamarino Wetland, Waikato, New Zealand (global positioning coordinates will not be reported here to protect the site). Access to the orchid sites is only

permitted when accompanied by DoC staff members; sample collection and pollination experiments were carried out under their supervision. Within management Site 2, an area of 224 m^2 (14 m x 16 m) was demarcated by marking the four corners with permanent posts. This actively managed area has been divided into 13 transects (1 m x 16 m), controlled burns are carried out by DoC in this marked area: each transect is burnt every second year and fire returns with a five-year rotation. From July to August 2019, controlled burns were carried out in Transects 0, 1, 4, 5, 8, 9, 12, and 13. From July to August 2021, controlled burns were conducted in Transects 2, 3, 6, 7, 10, and 11.

The majority of plants were distributed on Transect 1–5, very few individuals were found beyond Transect 5. In addition to these plants, a small cluster of plants, approximately 80 individuals, were recently discovered outside of the actively managed area. This small cluster of plants was found within an area ~ 0.25 m^2, and it was located about a metre away from the active management site. This small area was not burnt, but has a thinner overhead vegetation, as it is located on one side of the walking track that DoC staff members use to access the management site.

3.3.1.3 Sample collection for genetic assessment

Corybas carsei

A total of 41 individuals (leaf material) were sampled from the Whangamarino *C. carsei* population on 9th July 2020 as part of a research contract between DoC and the Museum of New Zealand Te Papa Tongarewa. In order to collect samples to represent the population's genetic profile, samples were collected from both the active management area (hereafter denoted as S2A) and the newly discovered cluster (hereafter denoted as S2B) at management site 2. Twenty-nine samples were collected from S2A. Samples from S2A were collected at a ~50 cm interval along all the transects; when plants were not found at 50 cm, the next closest plant was sampled. Thirteen samples were randomly collected from S2B. Individual samples were placed in separate coffee filters, S2A samples were labelled 01cc-A to 29cc-A; S2B samples were labelled 30cc-B to 41cc-B. All samples were dried and stored in silica gel until DNA extraction. Twenty-one samples form S2A and nine samples from S2B (30 samples in total) were randomly selected for the genetic assessment for this population.

Corybas macranthus

Samples of *C. macranthus* were collected from three locations on the North Island, New Zealand:

(1) Bridal Veil Falls, Waikato, New Zealand (-37.910286°, 174.895816°)
Samples were collected on 4th November 2020. The distribution of *C. macranthus* at Bridal Veil Falls was discontinuous, and the orchids occurred in four different patches, each patch was located ~1 m from each other. A total of 20 individuals were collected at a 10 cm spacing from all four patches, with five individuals randomly collected from each patch. When plants were not found at 10 cm, the closest plant was sampled. These individuals were labelled as 01-BVF-1 to 05-BVF-1, 06-BVF-2 to 10-BVF-2, 11-BVF-3 to 15-BVF-3, and 16-BVF-4 to 20-BVF-4. All samples collected were used for genetic analyses.

(2) Te Mata Peak, Hawke's Bay, New Zealand (-39.698300°, 176.901000°)
The distribution of this population was discontinuous at Te Mata Peak, and the orchids occurred in patches. Leaf material was collected from nine sub-sites, the minimum distance separating any two sub-sites was 3 m. A total of 73 samples were collected on 19th October 2020: seven individuals from Sub-site 1 labelled as TMP1-1 to TMP1-7; nine individuals from Sub-site 2 labelled as TMP2-1 to TMP2-9; nine individuals from Sub-site 3 labelled as TMP3-1 to TMP3-9; eight individuals from Sub-site 4 labelled as TMP4-1 to TMP4-8; six individuals from Sub-site 5 labelled as TMP5-1 to TMP5-6; nine individuals from Sub-site 6 labelled as TMP6-1 to TMP6-9; ten individuals from Sub-site 7 labelled as TMP7-1 to TMP7-10; 11 individuals from Sub-site 8 labelled as TMP8-1 to TMP8-11; and four individuals from Sub-site 9 labelled as TMP9-1 to TMP9-4. All samples were collected at a ~150 cm spacing, when plants were not found at 150 cm, the closest plant was sampled. Twenty-eight individuals were random selected for genetic assessment (three individuals from Sub-site 18, and four individuals from Sub-site 9).

(3) Butterfly Creek, Wellington, New Zealand (-41.308000°, 174.887000°)
A total of 19 individuals were collected on 20th July 2020. The Butterfly Creek population was relatively small compared to Bridal Veil Falls and Te Mata Peak population. This population occurred along the walking track, and it extended approximately 5m along the bank. There was no barrier at this location to prevent

vegetative propagation, and no gaps were observed in the distribution of this population. Samples were collected at a ~60 cm spacing, when plants were not found at 60 cm, the closest plant was sampled. Samples were labelled 1-BC to 19-BC. All samples were used for genetic analyses.

Like *C. carsei*, individual *C. macranthus* plants also produce a single leaf. Therefore, each sample consisted of one leaf from a different individual plant. Each individual leaf was stored in separate coffee filters. All samples were dried in silica gel and stored in silica gel until DNA extractions.

3.3.1.4 DNA extraction and genotyping

Genomic DNA was extracted from leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle & Doyle, 1987). The resulting DNA was then eluted in 50 µL of TE buffer. The DNA samples were then run on a 1% agarose gel at 75 volts for 35 minutes to assess DNA quality.

Thirty *C. carsei* individuals were screened at the 11 microsatellite loci developed for *C. carsei* (marker development results refer to section 3.3.1.1), and 67 *C. macranthus* samples were screened at 11 microsatellite loci developed by van Etten *et al.* (2018) (Corybas-06, Corybas-07, Corybas-12, Corybas-23, Corybas-27, Corybas-33, Corybas-41, Corybas-42, Corybas-46, Corybas-47, and Corybas-48) using PCR. PCR amplification was performed in a volume of 10 µL with 1× buffer (Solis BioDyne, Tartu, Estonia), 2.5 nM of $MgCl_2$, 100 µM of dNTP mix, 0.02 µM of forward primer, 0.45 µM of reverse primer, 0.45 µM of M13 primer (labelled with FAM, VIC, or NED), 0.5 U of FirePol Taq polymerase (Solis BioDyne, Estonia), 5-50 ng of DNA, and 3.7 µL of water. The PCR programme had an initial denaturation of 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds; 53°C for 40 seconds, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. PCR products (0.7–1.25 µL) for two to three loci labelled with different fluorescent dyes and of distinguishable sizes were co-loaded and added to 1 µL of CASS ladder (Symonds & Lloyd, 2004) and 9 µL of Hi-Di formamide (Applied Biosystems, Carlsbad, California, USA) for fragment sizing on an ABI 3730 Genetic Analyzer (Applied Biosystems) at Massey Genome Service at Massey University, Palmerston North, New Zealand. Alleles were visualised and scored using GeneMapper (version 5.0, Applied Biosystems).

3.3.1.5 Assessing genetic variation in *C. carsei* and *C. macranthus*

The presence of null alleles was assessed in MICRO-CHECKER (version 2.2.3; van Oosterhout *et al.*, 2004). One *C. carsei* individual (15cc-A) with missing data for more than six microsatellite markers was excluded from the data set. GenAlEx (version 6.503; Peakall & Smouse, 2012) was used to assess genetic diversity within and/or between *C. carsei* and *C. macranthus* population(s) by assessing each microsatellite locus for the percentage of polymorphic loci (P), number of alleles per locus (A), number of effective alleles (N_E). The observed heterozygosity (H_O), expected heterozygosity (H_E), and F-statistics (F_{IS} and F_{ST}; Wright, 1950) were also calculated in GenAlEx according to the methods by Weir and Cockerham (1984).

To illustrate the relationship between microsatellite marker characteristics and genetic variation found in the *C. carsei* population and the three *C. macranthus* populations, the number of uninterrupted repeats in microsatellite regions were plotted against the number of alleles found per locus and the expected heterozygosity for *C. carsei* and *C. macranthus* microsatellite markers used in this study. The plots were generated in R (version 4.0.3; R Core Team, 2013) using the R package, ggplot (Wickham, 2006).

3.3.1.6 Assessing genetic structure in *C. macranthus* populations

STRUCTURE (Pritchard *et al.*, 2000) was used to assess the distribution of genetic variation by identifying the most likely number of genetic clusters within and among the three populations. An appropriate format of the data set was exported from GenAlEx for analyses in STRUCTURE. STRUCTURE uses a Bayesian clustering method to infer population genetic structure in multilocus genotype data. This method assumes a model that consists of *K* populations (*K* may be any number), and each of these populations is characterised by a set of allele frequencies at each locus. Individuals in the data set are assigned to different populations (also known as genetic clusters) based on their genotypes (Pritchard *et al.*, 2000). STRUCTURE (version 2.3.3) was used to assess the allele frequencies at the 11 microsatellite loci genotyped in the three *C. macranthus* populations. Gene flow was assumed to occur within the population through outcrossing, facilitated by pollinators, most likely fungus gnats (Lehnebach & Zeller, 2015; Scanlen, 2006). Therefore, two priors: an admixture model and correlated allele frequencies, along

with the following parameters were used for STRUCTURE analyses to determine the most likely number of genetic clusters: 12 iterations of each *K* value (1–15*K*) were run with a burn-in of 100,000 generations and 1 million MCMC iterations of data collection.

The best fit *K* value was assessed from the rate of change in posterior probability between successive values of *K* (Δ*K*) following the method of Evanno *et al.* (2005). The results of STRUCTURE analyses were imported into an online programme, STRUCTURE HARVESTER (Earl & von Holdt, 2012), where the Evanno test was performed and the mean posterior probability (Ln*P*(*K*)) was plotted. The results from STRUCTURE were also imported into CLUMPAK (beta version; Kopelman *et al.*, 2015) to find the best alignment of the inferred clusters across the range of *K* values from multiple runs at each *K* value. A plot containing the whole range of *K* values was then produced.

A genetic distance matrix containing the three *C. macranthus* populations was calculated based on Prevosti's genetic distance (Prevosti *et al.*, 1975) in R (version 4.0.3; R Core Team, 2013) using the R package poppr (version 2.8.7; Kamvar *et al.*, 2014). A PCoA for the three populations was performed, and the first two axes were plotted to visualise genetic distance between individuals. The same genetic distance matrix was further formatted into a Nexus format file in R using the R package phangorn (version 2.5.5; Schliep, 2011), to import into SPLITSTREE (Huson, 1998). SPLITSTREE4 (version 4.17.1) was used to create a population NeighborNet to show the relationships among the three populations of *C. macranthus*.

The presence of genetically identical individuals was assessed in the R package RClone (version 1.0.3; Bailleul *et al.*, 2016). Individuals with identical MLG were assigned into genetic clusters. Then the "MLG_tab" function returned a table with one row per MLG and multiple columns when multiple samples were detected to have the same MLG.

3.3.2 Pollination experiments in *C. carsei*

To investigate self-incompatibility and pollination strategies within this population, four hand-pollination treatments were performed. Prior to anthesis, 16 individuals were covered with an enclosure made from hydroponic pots with the perforations covered by a fine net. This enclosure excluded pollinator visitation and therefore interference with our pollination experiment. The four hand-pollination treatments were:

(1) Autogamous self-pollination (AS)— five flowers were enclosed before anthesis until senescence, thereby excluding any pollinators. This treatment tested whether fruit-set developed by autonomous self-pollination.
(2) Self-compatibility (S)—six flowers were enclosed until the stigma was receptive. All pollinia were removed and the flowers were hand-pollinated with their own pollinarium. The flowers were enclosed again until the fruit capsule was formed. This treatment was also repeated in 2021 where 16 flowers were hand pollinated. This treatment tested self-compatibility in *C. carsei*.
(3) Cross-pollination (C)—same as the previous treatment, but five flowers were first emasculated and then hand-pollinated with pollen from another individual.
(4) Natural pollination (N)—29 individuals showing floral a bud at various developmental stages were tagged and allowed to develop fruit capsule under natural conditions of pollination.

3.3.3 Pollinator observations

Flower visitor activity was recorded by both direct observations and video recordings (Sony Handycam HDR-XR 160). Two observers working separately during two days of field work from 11:30 to13:30 on 22nd September and 23rd September 2020. Observations were carried out in a 10-minute observation period followed by a 10-minute break, yielding approximately one hour of observation. A camera was set up on 22nd September 2020 from 12:00 to 18:00 and on 25th October 2021 from 10:00 to 14:00 to record potential visitors to one *C. carsei* flower (Figure 3.2).

Figure 3.2 A camera set up in the field to film floral visitor activities in *C. carsei* flowers. White arrows indicate tagged individuals for natural fruit set and the yellow arrow indicates an individual enclosed in a hydroponic pot for pollination experiments.

3.3.4 Pollen count in *C. carsei*

The *C. carsei* pollinia remaining from hand-pollination treatments was used for estimating the number of pollen grains produced by a flower of *C. carsei*. The pollinia were stored in an Eppendorf tube in a refrigerator until the time of pollen count. The pollinia were suspended in 50 µL of stain a modified Alexander's solution (stain solution was prepared and pollen grains were stained according to Peterson *et al.* (2010)) inside the Eppendorf tube, then a glass rod was used to crush the pollinia to release the pollen grains. The glass rod was flushed twice, each flushed using 50 µL of the same stain, all solution was collected in the Eppendorf tube. The pollen grains were suspended in a final volume of 150 µL of the stain solution. The stain solution was loaded on to both chambers of a Neubauer haemocytometer; the solution was pipetted a few times to re-suspend pollen grains before loading onto the haemocytometer. Pollen grains present on all nine large squares on a chamber of the haemocytometer were counted; pollen grains that

touched the top and left boundaries were counted, but grains that touched the right and bottom boundaries were ignored. The number of viable and unviable pollen grains was also recorded. Artifacts such as debris that appeared blurry or did not have a defined shape were excluded from the count. After each count, the haemocytometer and cover glass were rinsed under running water, sprayed with 70% ethanol, and dried using Kimwipes. Pollen counts were repeated five times. The total number of viable pollen grains produced by a flower was calculated by:

$$\frac{total\ volume\ of\ stain\ solution}{volume\ of\ a\ square\ on\ haemocytometer} \times average\ No.viable\ pollen\ per\ square$$

3.3.5 Floral characteristics of *C. carsei* and *C. macranthus*

A *C. carsei* flower collected at Whangamarino was kept fresh in a refrigerator and photographed under the dissecting microscope in the laboratory before scanning electron microscope (SEM) imaging. The floral microstructures of *C. carsei* and *C. macranthus* were examined using a SEM (FEI Quanta 200 environmental scanning electron microscope) at the Manawatu Microscope and Imaging Centre. The dorsal sepal of a *C. carsei* flower was separated from the labellum, then the column was separated from the labellum. The dorsal sepal of a *C. macranthus* flower was removed from the labellum, then the labellum as dissected along the midrib to separate the labellum into two halves. Each part of the two dissected flowers was treated as a single sample for SEM; one dorsal sepal, one labellum, and one column from *C. carsei*; one dorsal sepal, two halves of labellum, and one column from *C. macranthus* (seven samples in total).

Plant material was fixed in FAA fixative overnight and then dehydrated through an ethanol series. Afterwards, the samples were freeze-dried in liquid CO_2 and 100% ethanol. Once dried, the samples were placed on a piece of double-sided tape mounted on stainless steel stubs and sputter coated with gold prior to visualisation under SEM. Electron microscopy analysis was conducted using black scatter imaging and the images were collected through a Dual segment BSE detector. The SEM was operated in the high vacuum mode, and the samples were observed under an accelerating voltage 20 KeV and spot size 3.5 and size 4.

3.4 Results

3.4.1 Floral characteristics

C. carsei flowers are zygomorphic; each flower consists of a dorsal sepal, a labellum, one sepal and one petal on either side of the labellum (Figure 3.3A). The dorsal sepal covers the labellum, together forming a tube-like structure with the column located at the posterior end (Figure 3.3A). The column consists of an anther cap covering the pollinia; a rostellum is present underneath the pollinia, and the stigma is located beneath the rostellum (Figure 3.3D). Pollinators are presumed to enter the flower from the opening formed by the dorsal sepal and labellum (Figure 3.3C). A pair of open auricles are located at the base of the labellum (Figure 3.3B). Hair-like protrusions, known as calli, were observed around the tip and along the midline on the labellum, and these structures are oriented towards the column where the pollinia are located (Figure 3.4). Like *C. carsei*, *C. macranthus* also have hair-like protrusions covering the interior of the labellum (Figure 3.5A & 3.5B). The *C. macranthus* column is also located at the posterior end of the flower and has similar structures to the *C. carsei* column—an anther cap covers the pollinia and the rostellum under the pollinia and the stigma is located under the rostellum (Figure 3.5C & 3.5D).

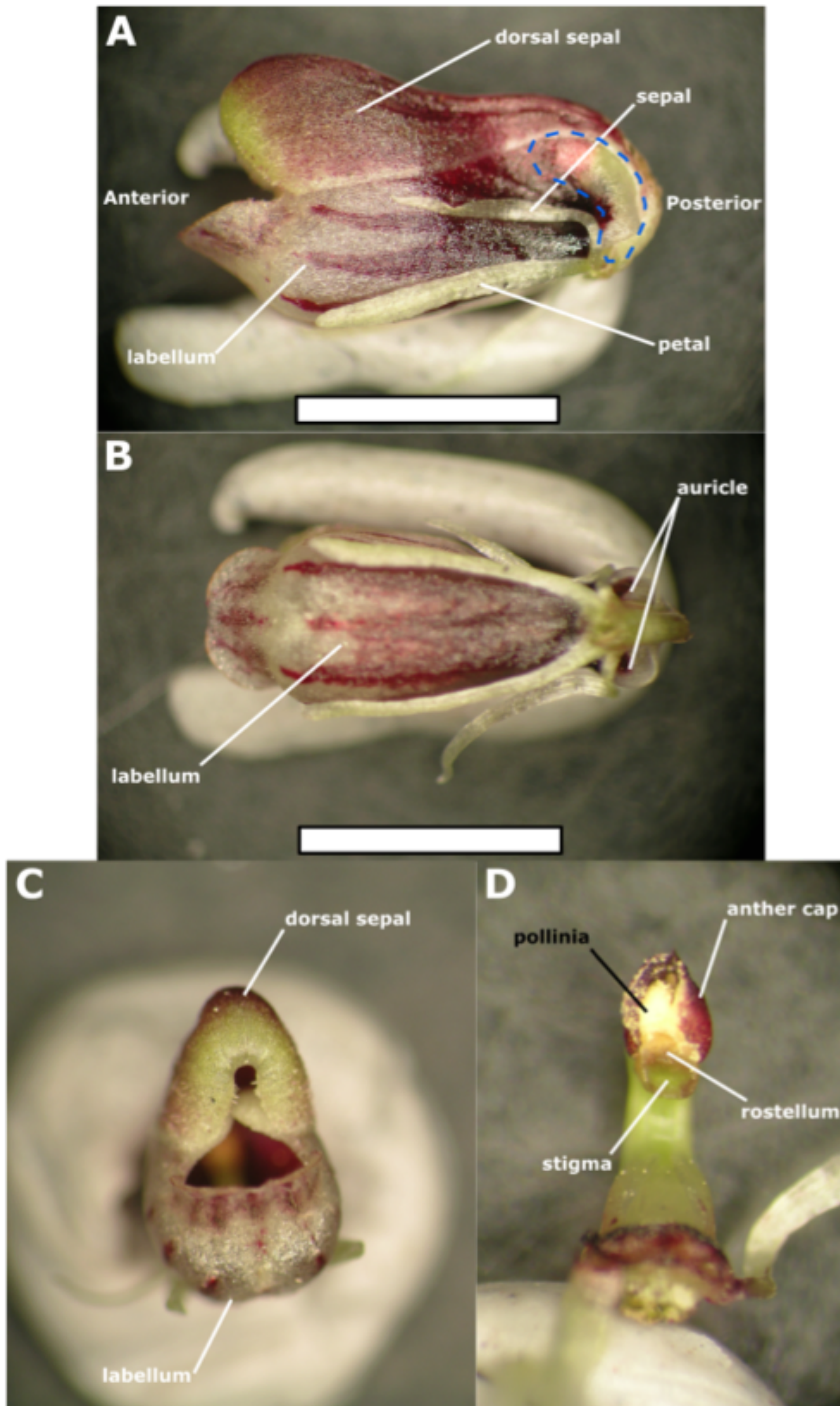

Figure 3.3 Lateral and ventral view of a *C. carsei* flower, the flower was placed on top of a play dough. A: lateral view of a *C. carsei* flower, blue broken-line indicates the position of column; B: ventral view of a *C. carsei* flower; C: anterior view of a *C. carsei* flower; D: anterior view of a *C. carsei* column. Scale bar = 5 mm.

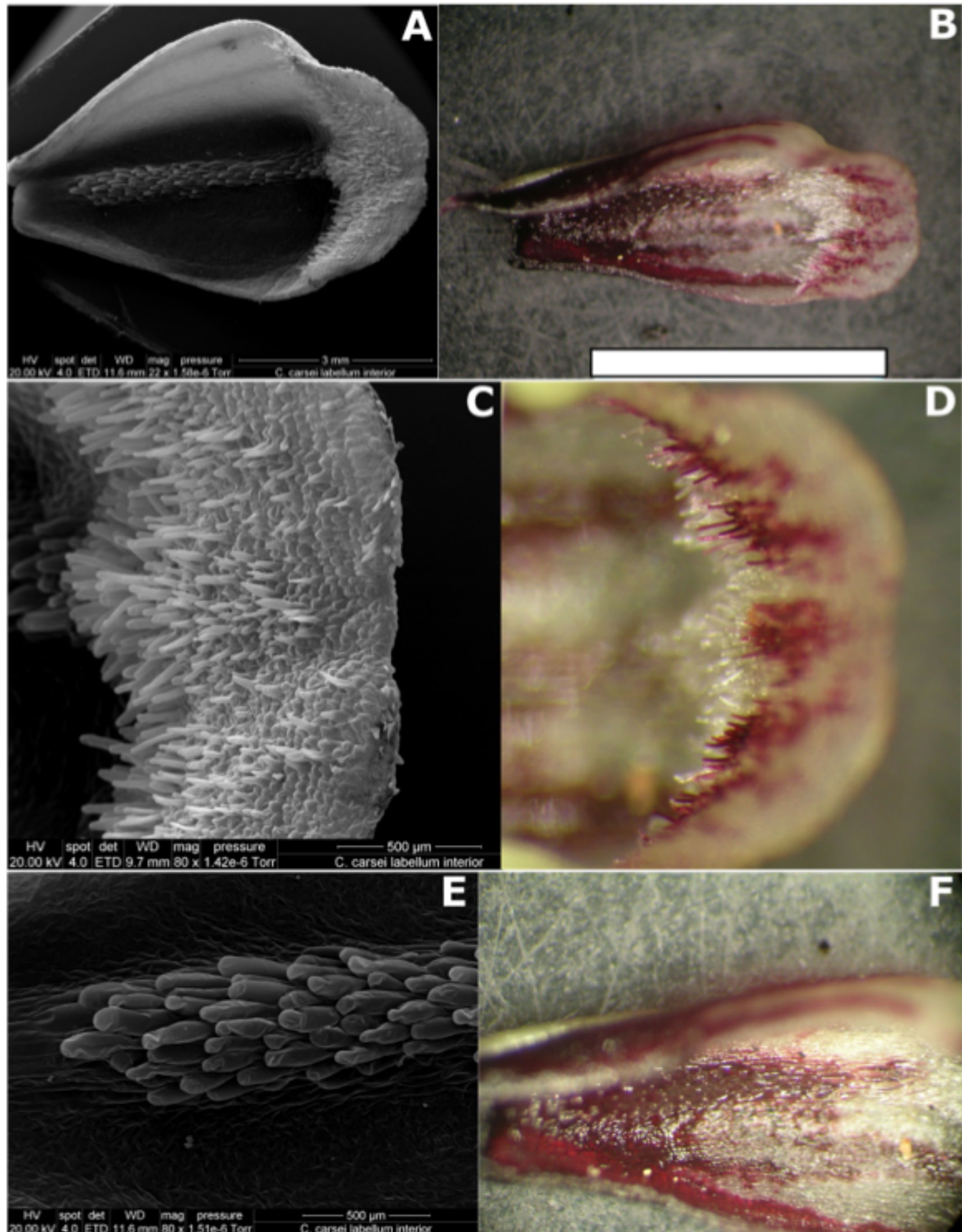

Figure 3.4 Interior structure of a *C. carsei* labellum. Hair-like protrusions are present around the tip and along the mid-line of the labellum; these protrusions are oriented towards the back of the flower (towards the column) where the pollinia and stigma are located. A: SEM showing interior structures in the labellum; B: dorsal view of the labellum, scale bar = 5 mm; C: SEM close-up showing hair-like protrusions on the tip of labellum; D: dorsal view close-up of the tip of the labellum; E: SEM close-up showing hair-like structures along the mid-line of the labellum; F: dorsal view close-up of the hair-like structures along the mid-line of the labellum.

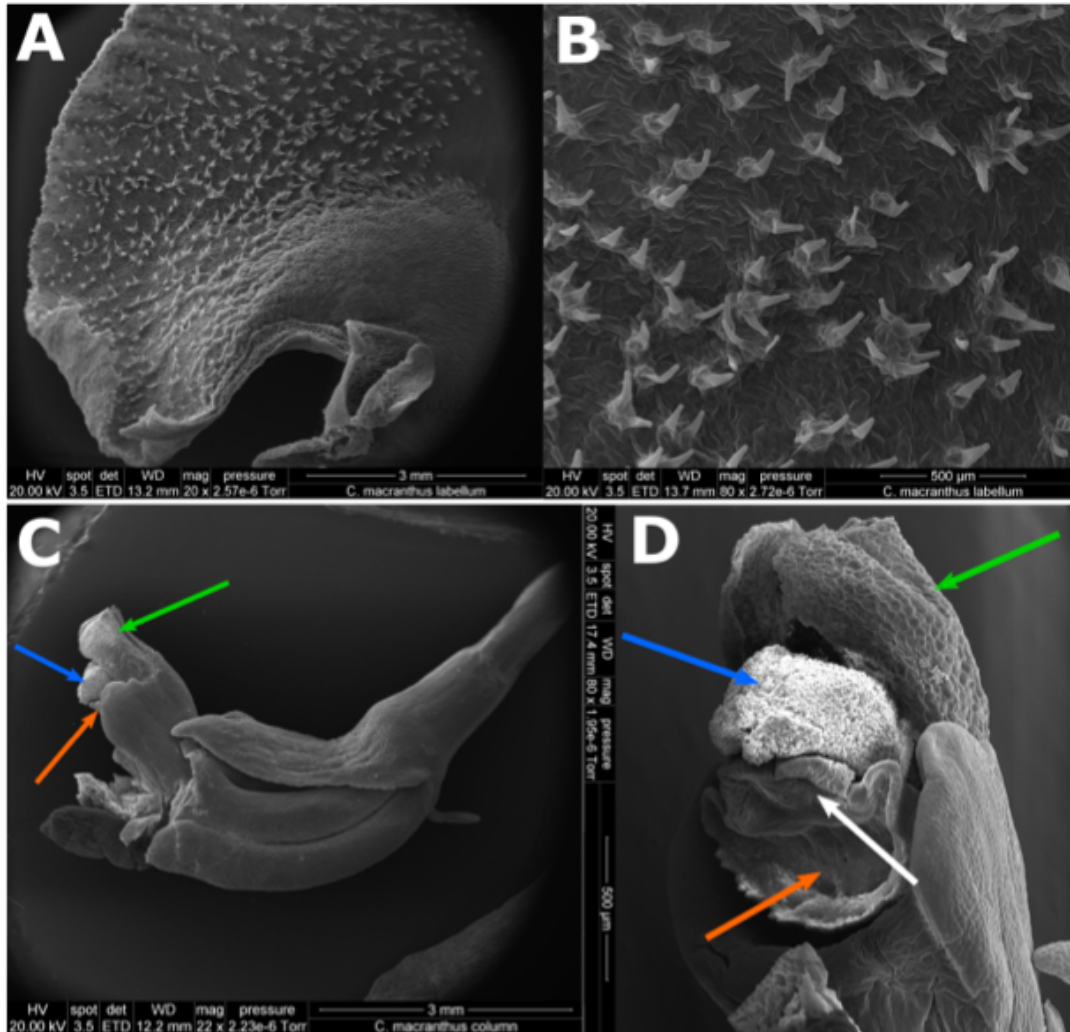

Figure 3.5 SEM showing the interior structure of a *C. macranthus* labellum and structure of the column. A: lateral view of the interior of the labellum, hair-like protrusions are oriented towards the back of the flower where the column is located; B: zoomed-in view of the hair-like protrusions of the labellum; C: lateral view of the column, green arrow indicates anther cap, blue arrow indicates pollinia, and orange arrow indicates stigma; D: zoomed-in anterior view of the anther cap (indicated by the green arrow), pollinia (indicated by the blue arrow), rostellum (indicated by the white arrow), and stigma (indicated by the orange arrow).

3.4.2 Microsatellite marker development results

Of the 20 primer pairs tested, six loci did not amplify, three loci amplified inconsistently and produced uninterpretable results. Eleven loci successfully amplified across all seven individuals; however all 11 loci were monomorphic (Table 3.1). Amplification rate was 100% across all loci; only one allele was detected per locus, and the observed heterozygosity (H_O) and expected heterozygosity (H_E) were therefore both zero at all loci.

Those 11 markers that successfully amplified were selected for genetic assessment in the *C. carsei* population.

Table 3.1 Characteristics of 11 microsatellite loci developed for *C. carsei*.

Locus	Primer sequences (5'-3')	Repeat motif	A	Allele size (bp)	% ampilification
C. carsei-012	F: CCAGTCCCATCAAACTAATTG R: AATAGCAAGTGCCTCACTG	(AT)7	1	335	100%
C. carsei-067	F: GGATGGTTTATTGTAGGCATG R: TTGGTTTGAGGAAGACGTG	(TC)7	1	186	100%
C. carsei-085	F: TTCTTCCTGGCCCCTTTC R: AGAATATAGAGACCTAGCGAAG	(AT)8	1	206	100%
C. carsei-115	F: CTTCATCAGCAGCTTTGTTC R: ACCCTTAGGTCAGCCAAG	(TC)7	1	226	100%
C. carsei-122	F: CAGTTCCCCAATTGCTCC R: TGTGATCACCACCTAACTTG	(AG)7	1	233	100%
C. carsei-150	F: CAGGTTTCTGTATGCACATG R: AAGCGGAAGTAGGAGAGC	(TG)8	1	244	100%
C. carsei-236	F: AATGTGATGTCTAGGCACC R: TGTCAAAGGAGGAAGTGATG	(CT)9	1	264	100%
C. carsei-356	F: CAGATGCATGTTTCGGAAC R: GGATCAACTCATATAGGGCC	(GA)7	1	271	100%
C. carsei-477	F: CACACATACACACAACCAC R: ACATATTGTGCAAGGGCTAG	(GT)11	1	286	100%
C. carsei-488	F: TTCTCTATGCTAGGGGCTTC R: GTAGGAGAGAGAGAGAGGG	(CT)8	1	294	100%
C. carsei-592	F: GTAGGTCAAATCGCAAACAG R: TCCTCTCCTCTCTAAGCTG	(CT)7	1	314	100%

3.4.3 Genetic variation within *C. carsei* and three *C. macranthus* populations

Eleven microsatellite markers were genotyped in 29 *C. carsei* individuals. The success rate of marker amplification ranged from 82.76–100.00% across individuals (Table 3.2). A total of 11 alleles were observed from the 11 markers within the Whangamarino population. Because the observed and expected heterozygosity were both zero, F_{IS} could not be calculated. With the finding of only monomorphic loci, no genetic variation was detected within the population.

Table 3.2 Characteristics of 11 microsatellite loci for 29 *C. carsei* individuals at the Whangamarino Wetland.

Locus	A	Allele size (bp)	H_O	H_E	F_{IS}	% amplification
C. carsei-012	1	225	0	0	N/A	82.76
C. carsei-067	1	186	0	0	N/A	100.00
C. carsei-085	1	206	0	0	N/A	100.00
C. carsei-115	1	226	0	0	N/A	100.00
C. carsei-122	1	233	0	0	N/A	100.00
C. carsei-150	1	244	0	0	N/A	100.00
C. carsei-236	1	264	0	0	N/A	100.00
C. carsei-356	1	271	0	0	N/A	100.00
C. carsei-477	1	286	0	0	N/A	89.66
C. carsei-488	1	294	0	0	N/A	100.00
C. carsei-592	1	314	0	0	N/A	100.00
Mean	1		0	0	N/A	97.49
SE			0	0	N/A	
%P	0%					

A = number of alleles per locus; allele size (bp) = allele size at each locus; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = estimates of Wright's inbreeding coefficient for all microsatellite markers; % amplification = percentage of amplification at each locus; N/A = observed and expected heterozygote are both zero, therefore F_{IS} cannot be calculated.

Eleven microsatellite markers were genotyped in 67 *C. macranthus* individuals from three populations. Marker amplification rate ranged from 92.54% to 100.00% with an average of 97.29%. The number of alleles per locus ranged from one to 6.667, with an average of 2.455. Marker Corybas-23 had the lowest number of alleles per locus and marker Corybas-48 had the highest number per locus (Table 3.3). The average number of alleles per locus within-populations was 2.455 (ranged from 1.455 to 3.364), with the number of effective alleles per locus averaging at 1.810 (ranged from 1.400 to 2.119; Table 3.4). For both the number of alleles per locus (A) and the number of effective alleles per locus (N_E), the Te Mata Peak population had the highest value and the Butterfly Creek population represented the lowest (Table 3.4).

Polymorphic loci per population (%P) calculated for the three *C. macranthus* populations ranged from 36.36–90.91%, with an average of 69.70%, all three populations were polymorphic (Table 3.4). The Butterfly Creek population had the lowest %P = 36.36%, while the Te Mata Peak population had the highest value %P = 90.91%. The range of the observed heterozygosity was 0.163–0.335, and the mean value was 0.271; the highest H_O was observed at Bridal Veil Falls population and the lowest was at Butterfly Creek. The H_E ranged from 0.183 to 0.354 averaging at 0.291. Again, the Bridal Veil Falls population had the highest H_E and Butterfly Creek population had the lowest value. Across all populations, H_E was greater than the H_O. Overall, these three *C. macranthus* populations had a low to medium level of H_O and H_E (Table 3.4). F_{IS} of the three populations ranged from 0.065 to 0.119. The Bridal Veil Falls population had a F_{IS} value very close to zero, F_{IS} = 0.065. The Butterfly Creek and the Te Mata Peak population had a F_{IS} = 0.119 and F_{IS} = 0.103, respectively, showing a positive deviation from HWE (Table 3.4). Mean F_{ST} across the 11 markers genotyped for all populations was 0.119, showing a low level of differentiation between populations (Table 3.3).

Table 3.3 Characteristics of 11 microsatellite loci for 67 samples of *C. macranthus*

Locus	**A**	**Size range (bp)**	**H_O**	**H_E**	**F_{ST}**	**% amplification**
Corybas-06	3.000	293-334	0.656	0.640	0.072	98.51
Corybas-07	2.000	332-334	0.396	0.360	0.235	95.52
Corybas-12	2.333	311-320	0.181	0.157	0.062	100.00
Corybas-23	1.000	352	0.000	0.000	N/A	100.00
Corybas-27	2.000	236-238	0.210	0.356	0.081	97.01
Corybas-33	1.667	161-165	0.041	0.143	0.132	97.01
Corybas-41	2.333	150-154	0.211	0.251	0.073	98.51
Corybas-42	1.667	321-325	0.071	0.066	0.055	98.51
Corybas-46	2.000	183-186	0.150	0.167	0.136	94.03
Corybas-47	2.333	319-325	0.457	0.502	0.054	92.54
Corybas-48	6.667	190-256	0.602	0.562	0.293	98.51
Mean	2.455		0.271	0.291	0.119	97.29

A = average number of alleles per locus; size range (bp) = range of allele size at each locus; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS}/F_{ST} = estimates of Wright's fixation index for all microsatellite markers; % amplification = percentage of amplification at each locus; N/A = observed and expected heterozygosity are both zero and therefore F_{IS} and F_{ST} cannot be calculated.

Table 3.4 Population information and genetic diversity estimates for three populations of *C. macranthus.*

Pop	**Location**	**N**	**A**	**N_E**	**%P**	**H_O**	**H_E**	**F_{IS}**
BC	Butterfly Creek	19	1.455	1.400	36.36%	0.163	0.183	0.119
BVF	Bridal Veil Falls	20	2.545	1.911	81.82%	0.335	0.354	0.065
TMP	Te Mata Peak	28	3.364	2.119	90.91%	0.314	0.336	0.131
Mean		22	2.455	1.810	69.70%	0.271	0.291	

Pop = different populations of *C. macranthus*; N = sample size genotyped and used in this study; A = number of alleles per locus; N_E = number of effective alleles per locus; %P = percentage of polymorphic loci; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = Wright's fixation index.

Figure 3.6 shows scatter plots between the number of uninterrupted repeats against the number of alleles per locus number, and Figure 3.7 showed H_E plotted against the number of uninterrupted repeats in all three *C. macranthus* populations, the *C.* "Remutaka" (refer to Table 2.1, Chapter 2) and the *C. carsei* population. A positive trend was found between the number of uninterrupted repeats and the number of alleles per locus, as well as between the number of uninterrupted repeats and H_E in *C.* "Remutaka" and *C. macranthus* populations. The coefficient of determination (R^2) for the number of uninterrupted repeats against the number of alleles per locus was 0.11 in the *C.* "Remutaka" population, 0.26 in *C. macranthus* populations, and it was not applicable in the *C. carsei* population (Figure 3.6). R^2 for the number of uninterrupted repeats against H_E was 0.09 in the *C.* "Remutaka" population, 0.21 in the *C. macranthus* populations; again, it was not applicable in the *C. carsei* population as no genetic variation was detected in the *C. carsei* population (Figure 3.7).

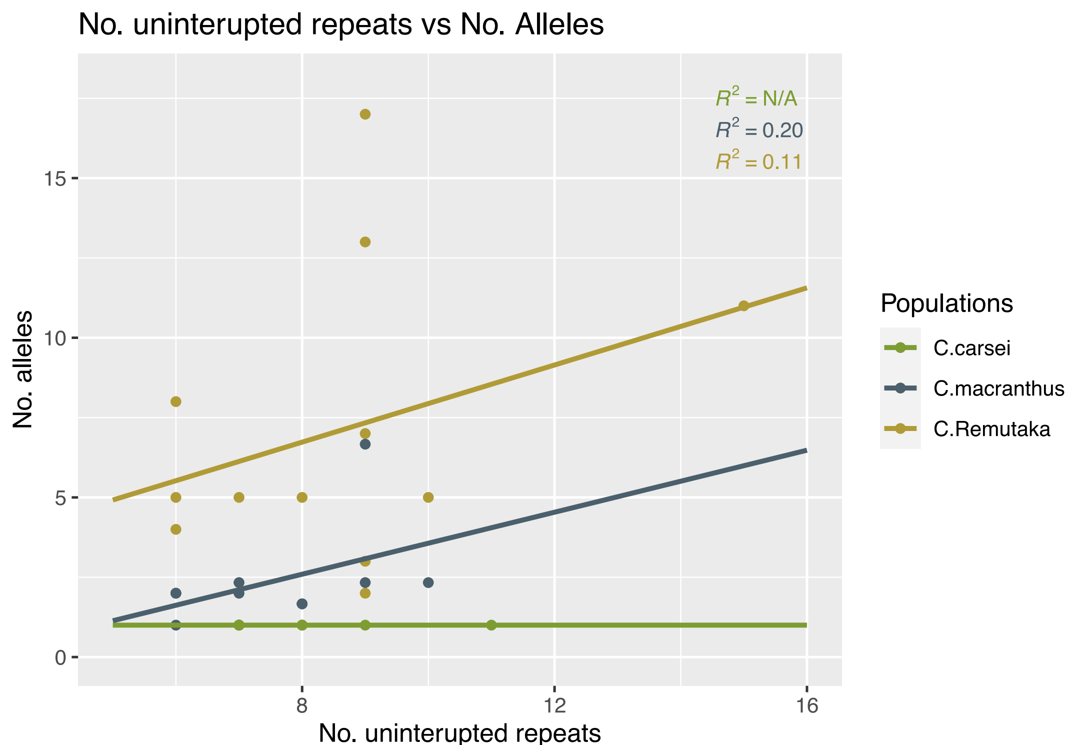

Figure 3.6 The number of uninterrupted repeats at microsatellite regions plotted against the number of alleles per locus in the *C. carsei*, *C. macranthus*, and *C.* "Remutaka" population(s).

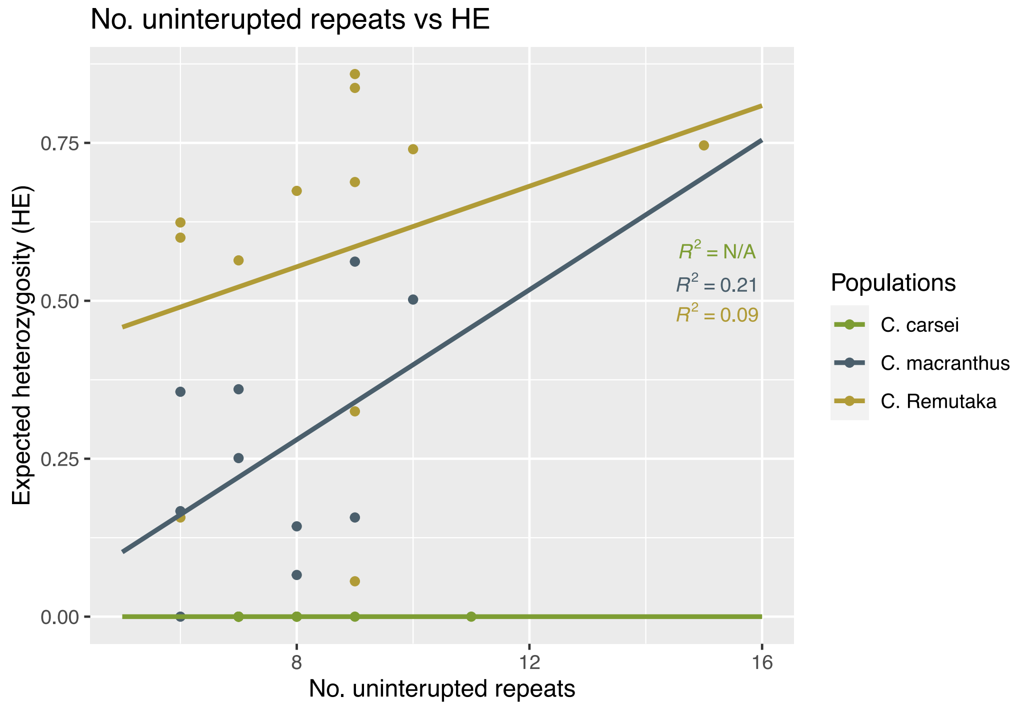

Figure 3.7 The number of uninterrupted repeats at microsatellite regions plotted against the expected heterozygosity in the *C. carsei*, *C. macranthus*, and *C.* "Remutaka" population(s).

3.4.4 Null Alleles

MICRO-CHECKER suggested that null alleles might be present at locus Corybas-27 in the Butterfly Creek population, at locus Corybas-33 in the Bridal Veil Falls population, and at locus Corybas-46 in the Te Mata Peak population as indicated by the homozygote excess at most alleles size classes within those loci. There was no evidence for large allele dropout at any locus.

3.4.5 Genetic structure in three *C. macranthus* populations

C. macranthus individuals were assigned into genetic clusters (*K* = 1-15) by STRUCTURE (Pritchard *et al.*, 2000), and the *K* value with the best fit to the data was determined by the rate of change in posterior probability between successive values of *K* (Δ*K*) following Evanno *et al.* (2005). The Δ*K* method revealed that *K*=2 was the best fit to the data (Figure 3.8). The CLUMPAK alignment of different *K* values showed that at *K* = 2 and *K* = 3, the majority of the Bridal Veil Falls individuals clustered with the Te Mata Peak individuals, and a small number of individuals from Bridal Veil Falls clustered with the Butterfly

Creek individuals forming two genetic clusters (Figure 10). No changes were observed in this clustering pattern regardless to the number of K (from K = 2–15; Figure 3.10). At peak was observed at K = 4 when the mean posterior probability calculated for the 12 iteration per cluster (K) from structure was plotted against the number of K (Figure 3.9). This was reflected by the CLUMPAK result (Figure 3.10) where a few individuals were assigned to a genetic cluster represented by green at K = 4, indicating the presence of genetic structure in the Bridal Veil Falls population. Again, this clustering pattern did not change regardless of the change in K (from K = 4-15). From $K \geq 3$, some individuals from the Butterfly Creek and Bridal Veil Falls population did not show a mixed genotype, which were represented by a single colour in each column. This was due to the presence of genetically identical individuals within these two populations (Table 3.5). Although, there was no obvious association between geographic distance and genetic clustering.

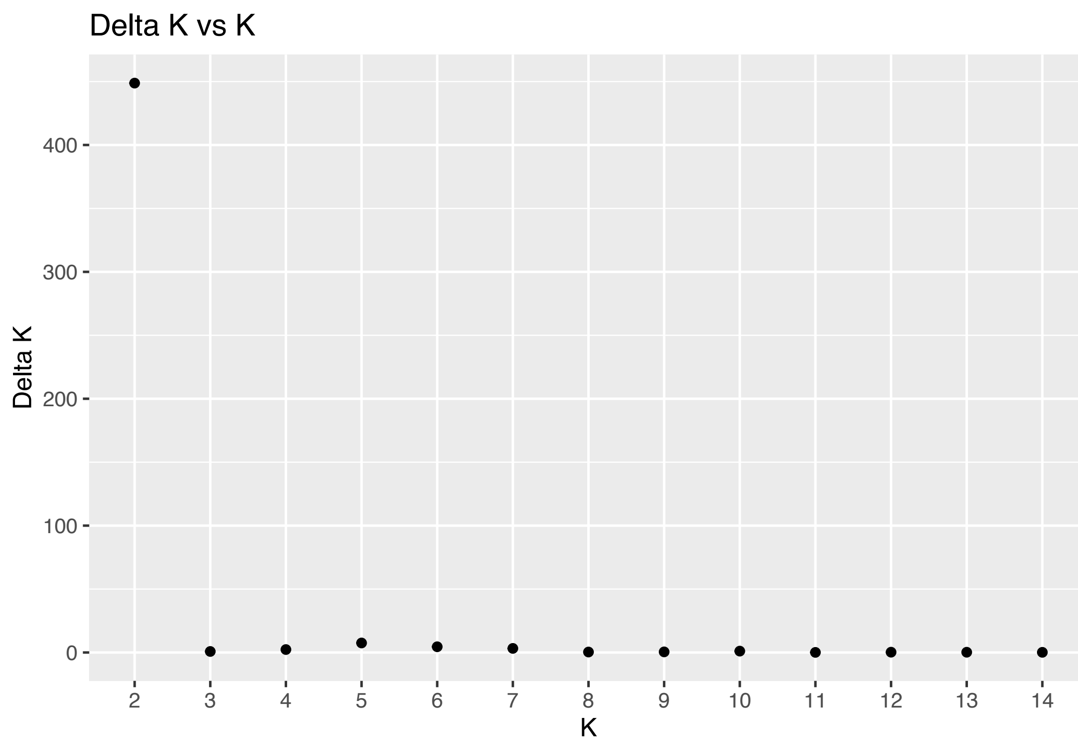

Figure 3.8 Plot of ΔK vs K for STRUCTURE results following Evanno et al. (2005) based on 12 iterations for each K value.

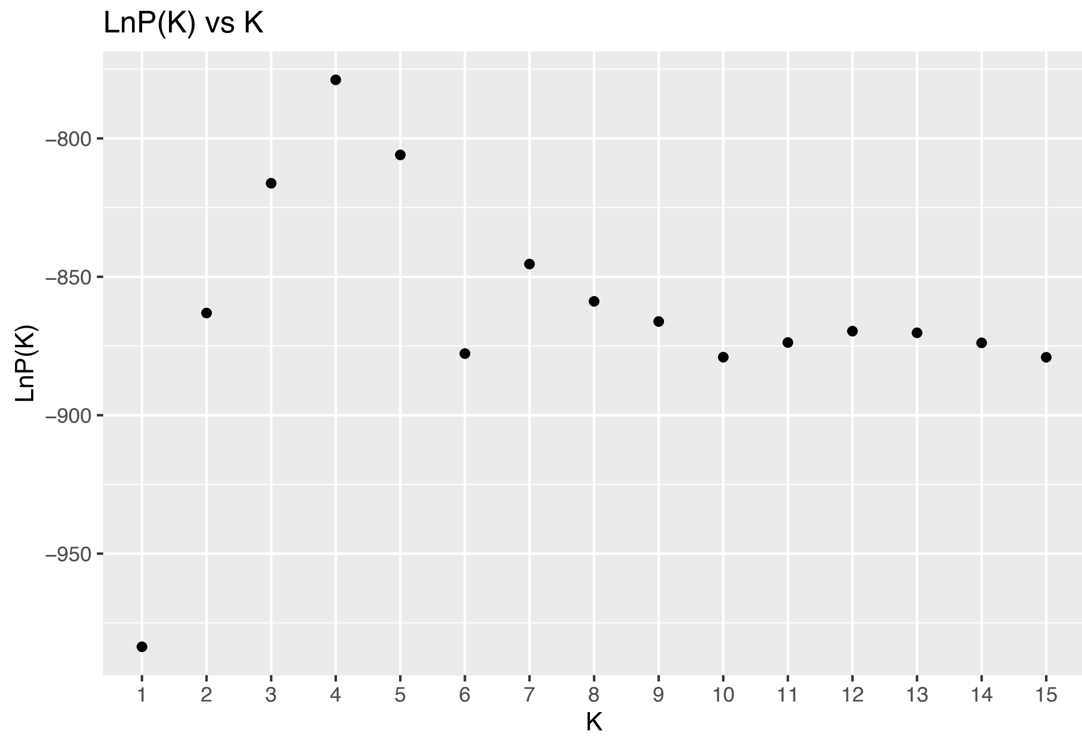

Figure 3.9 Plot of mean posterior probability (LnP(*K*)) values per cluster (*K*) based on 12 iterations per K from STRUCTURE analyses (Pritchard et al., 2000).

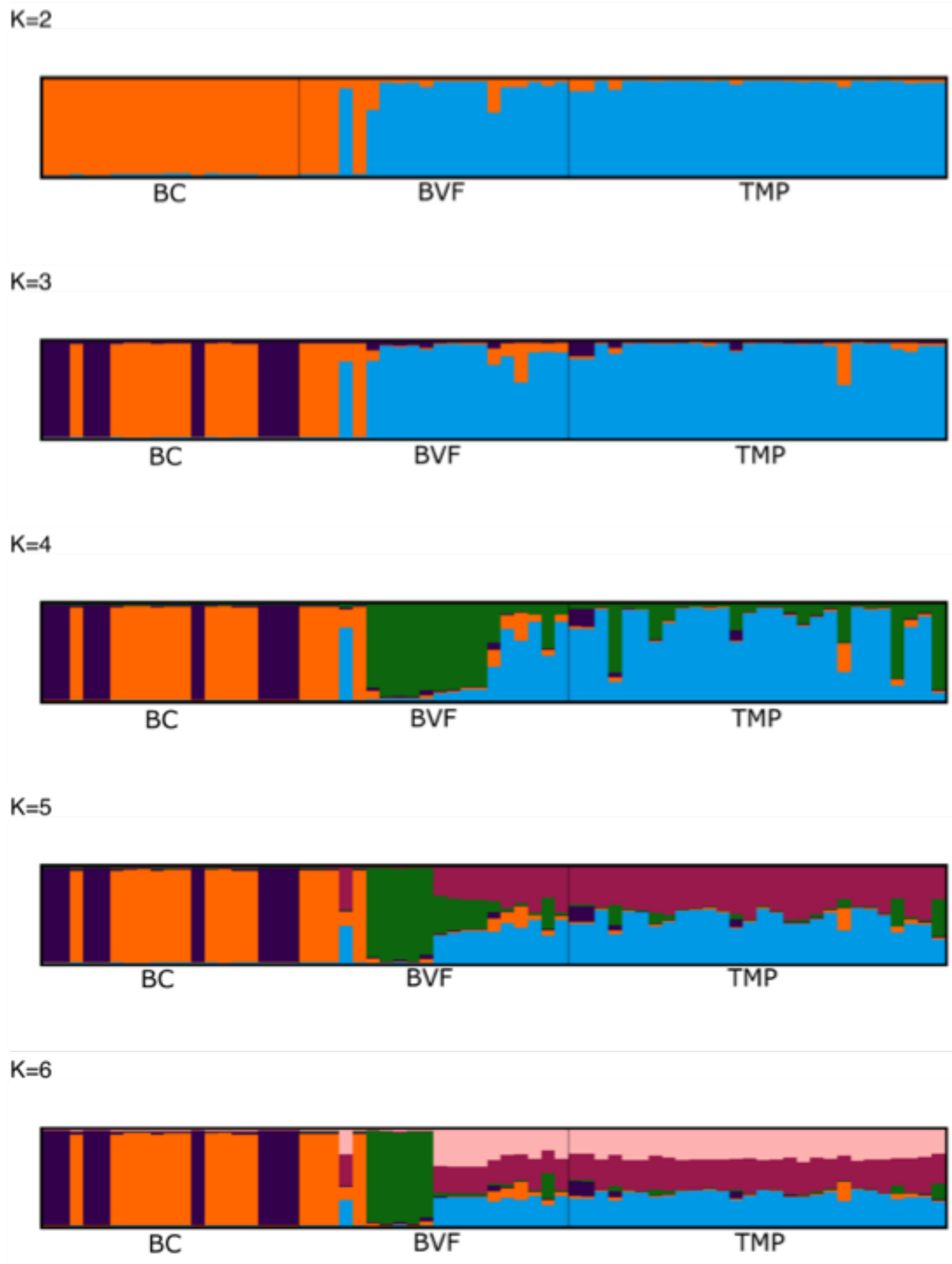

Figure 3.10 CLUMPAK output on STRUCTURE assignment of 67 *C. macranthus* individuals from three different populations (Butterfly Creek (BC), Bridal Veil Falls (BVF), Te Mata Peak (TMP)) for each *K* value (*K* = 1-15).) Each column within a *K* value represents an individual collected from the three populations, and the proportion of membership in a genetic cluster within each individual is denoted by different colour(s) within each column. *K* = 2 is the best likelihood fit. *K* =1, and *K* = 7-15 are not reported here.

RClone found 47 unique MLGs among the 67 *C. macranthus* individuals from three different populations. Within these 47 MLGs, nine MLGs were found in multiple individuals across all three populations. In each of the nine MLGs, all individuals were identical at the 11 microsatellite loci genotyped, suggesting the possible existence of genetically identical individuals (Table 3.5). Genetically identical individuals were found in four MLGs in the Butterfly Creek population; four MLGs in the Bridal Veil Falls population, and one MLG in the Te Mata Peak population (Table 3.5). There was no obvious association between the samples' physical location and genetic similarity at Butterfly Creek. However, genetically identical individuals found in the Bridal Veil Falls population were physically close to each other in the field. Bridal Veil Falls samples were collected from four sub-sites, each sub-site located approximately 1m away from each other. All clones found in Bridal Veil Falls population were within their own sub-site, none was found between sub-sites (Table 3.5). This was also observed at the Te Mata Peak population. Te Mata Peak samples were collected from nine sub-sites, and the only two genetically identical individuals were found within sub-site 1 (Table 3.5).

Table 3.5 Nine MLGs were identified in multiple individuals among the 67 *C. macranthus* individuals from three different populations, all individuals were identical at the 11 microsatellite loci genotyped within each MLG.

MLGs	1	2	3	4	5	6	7	8	9
	BC-01	BC-03	BC-07	BC-10	BVF-01	BVF-07	BVF-13	BVF-18	TMP1-1
	BC-02	BC-06	BC-08	BC-11	BVF-02	BVF-09	BVF-14	BVF-20	TPM1-5
	BC-04	BC-15	BC-14	BC-13	BVF-03				
	BC-05	BC-16			BVF-05				
	BC-17								
	BC-18								
	BC-19								

MLGs = multilocus genotypes; BC = Butterfly Creek, BVF = Bridal Veil Falls, TMP = Te Mata Peak.

The first and second axes of the PCoA graph (Figure 3.11) explained 39.7% and 23.6% of total variation, respectively. However, the first two axes were not able distinguish the Bridal Veil Falls and Te Mata Peak population; the second axis was able to separate the Butterfly Creek population from Bridal Veil Falls and Te Mata Peak population. Although, one data point from Bridal Veil Falls was still included in the Butterfly Creek cluster. This clustering pattern was in congruence with the STRUCTURE analysis where the majority of the Bridal Veil Falls clustered with the Te Mata Peak individuals, and a small number of the Bridal Veil Falls individuals clustered with the Butterfly Creek individuals (Figure 3.10).

The PCoA graph (Figure 3.11) gave an indication of the genetic distances among different *C. macranthus* individuals from the three populations on the North Island. On the graph, individuals that were more genetically similar, the closer they would be located to each other, and genetically identical individuals would be overlapping. Nineteen individuals from Butterfly Creek were genotyped, however only six data points were seen on the PCoA graph. Similarly, not all individuals from the Bridal Veil population were seen on the PCoA graph. This suggested that some data points overlapped and therefore genetically identical individuals exist within these populations, which reflected the RClone assessment on identical MLGs (Table 3.5).

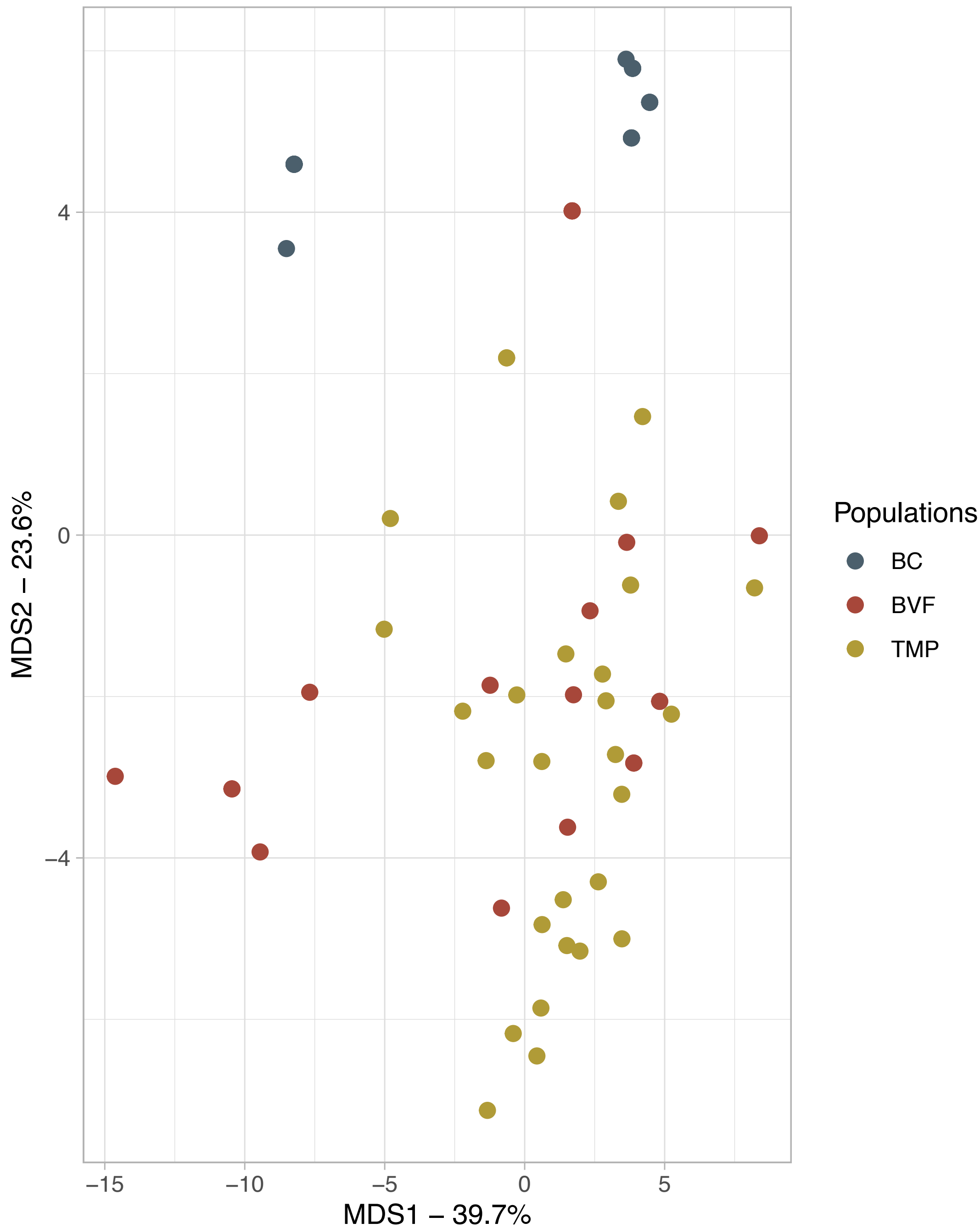

Figure 3.11 Principal coordinate analysis (PCoA) of Prevosti's genetic distances among the 67 *C. macranthus* individuals collected from three different populations, Butterfly Creek (BC), Bridal Veil Falls (BVF), and Te Mata Peak (TMP). The first two axes explain 39.7% and 23.6% of total variation, respectively.

The NeighborNet of *C. macranthus* populations overall showed a reticular pattern, no splits were able to partition individuals into distinct groups based on their geographical location (Figure 3.12). However, genetic structures were observed between and within

population. One of the branches contained only individuals from Butterfly Creek, while the rest of the individuals from Butterfly Creek were integrated with Bridal Veil Falls individuals. A branch contained only Bridal Veil Falls individuals was observed; these individuals were also seen in the CLUMPAK results at K = 4 represented by the green columns (Figure 3.10).

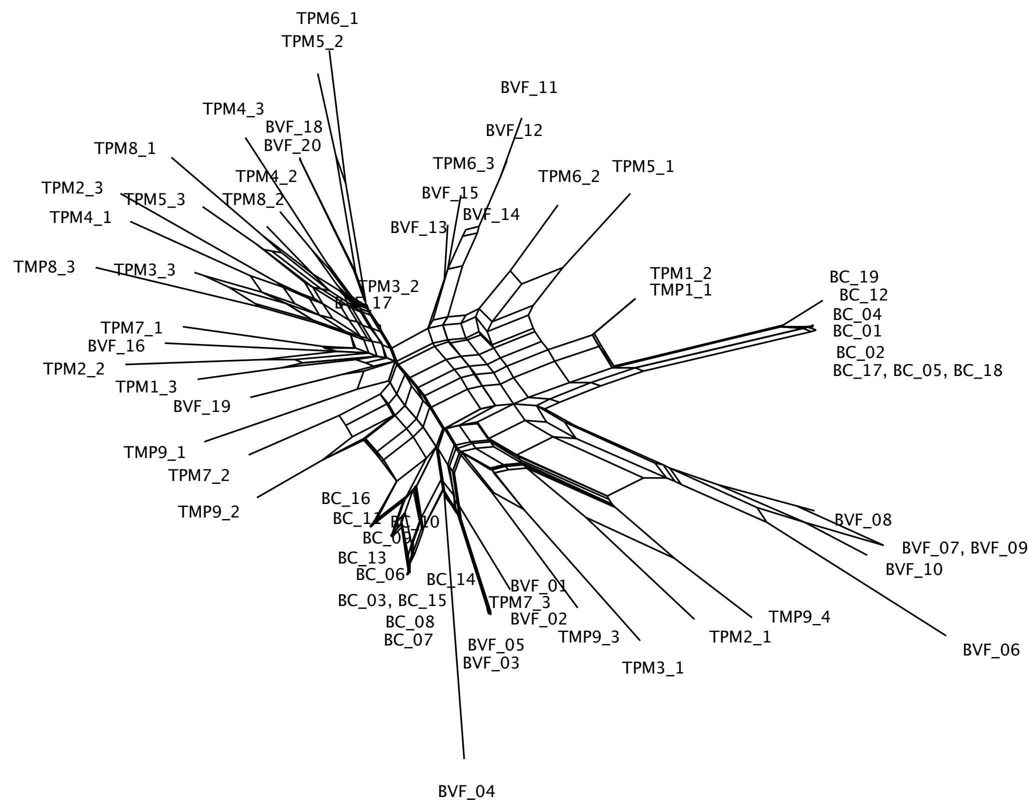

Figure 3.12 NeighborNet generated in SPLITSTREE4 based on Prevosti's genetic distance between all 67 *C. macranthus* individuals sampled from three different populations (Butterfly Creek (BC), Bridal Veil Falls (BVF), and Te Mata Peak (TMP)). Sample ID of each individual is displayed on the ends of branches.

3.4.6 Pollination treatments and pollen count in *C. carsei*

Of the six hand self-pollinated flowers, three flowers produced a fruit. A fruit capsule was observed from two out of the five flowers included in the autonomous self-pollination. These results were similar to hand cross-pollination in which three out of five flowers produced a capsule after pollination treatment. A total of 29 individuals were tagged for natural pollination, and only three produced a fruit (approximately 10%), showing low natural fruiting rate (Table 3.6). There are about 21,666.67 number of pollen grains in the

pollinia of *C. carsei* flower sampled. The amount of viable pollen grains produced was 99.99% (i.e. 21,166 pollen grains, Appendix 1).

Table 3.6 Number of fruit-set after pollination treatments in *C. carsei.*

	N	S	C	AS
C. carsei	29 (3)	5 (3)	5 (3)	5 (2)

N = natural fruit-set; S = hand self-pollination; C = hand cross-pollination; AS = autonomous self-pollination. Within each treatment, the number before parentheses indicates the number of flowers (individuals) used in each treatment, and the number of fruit set after each treatment in parentheses; * = hand self-pollination was performed in six individuals, however one of the individuals could not be found in the field when collecting fruits.

3.4.7 Floral visitors

A total of 10 hours of video and one hour of manual observation were obtained during field work on *C. carsei*. No visitors were observed during direct observation. The camera set up in the field captured footage of flies approaching the flower. The camera filmed five occasions where a fly landed near the flower (Figure 3.13), these instances were filmed at 15:00 to 17:00. However, none of the flies actually entered the flower. In all cases, the fly landed near the flower and stayed for a few seconds (maximum ~1 minute) before flying away. Although, five visitations from a fly were filmed, it was impossible to identify what species of flies approached the flower from the footage.

Figure 3.13 Potential floral visitors captured on camera, flies are indicated by orange circles.

3.5 Discussion

3.5.1 Genetic variation within *C. carsei* and *C. macranthus*

Eleven microsatellite markers genotyped in 29 *C. carsei* individuals from the only remaining population failed to detect any genetic variation within this population. All individuals are homozygous for one allele across each of the 11 markers (Table 3.2). It is not possible to infer what reproductive methods are employed by this population without any genetic diversity within the population. This population showed an exceptionally low value of genetic variation in comparison to other endangered orchids. For example, a Australian terrestrial orchid, *Phaius australis* H_E = 0.171 (Simmons *et al.*, 2018); and an epiphytic orchid occur in Southeast China and northern Vietnam, *Bulbophyllum bicolor* H_E = 0.266 (Hu *et al.*, 2017). In contrast to *C. carsei*, *C. macranthus* is a common and widespread species, it occurs throughout the North Island and upper portion of the South Island (Jane *et al.*, 2017). A low to medium level of genetic variation (H_E ranged 0.183-0.336, mean H_E = 0.291) was found across all three populations (Table 3.4). Another case known to have very low genetic variation was the endangered Wollemi pine (*Wollemia nobilis*). A study by Peakall *et al.* (2003) used 20 microsatellite markers, 11 allozyme loci, and more than 800 AFLP loci to assess the genetic variation among the 10 remaining trees in south-eastern Australia but failed to detect any polymorphism at all genetic

markers screened. Clonality and small population size were some of the contributing factors to the low genetic diversity in this species.

Studies showed that the number of alleles per locus, H_E, and the percentage of polymorphic loci are strongly correlated with population size, especially in rare species (Leimu *et al.*, 2006). *C. carsei* underwent a drastic decline in numbers, with a single small population remaining today (Department of Conservation, 2019). The lack of genetic variation observed in this population could be the outcome of multiple causes. Genetic diversity could be lost through a founder effect when the population established at Whangamarino Wetland from seed dispersal, and/or through a bottleneck when the species was rapidly reduced in number and populations disappeared (Young *et al.*, 1996). Moreover, small populations are more susceptible to inbreeding as the probability to mate with a related individual increases, resulting in a higher level of homozygosity (Reed & Frankham, 2003). It has been speculated that *C. carsei* is self-compatible and self-pollination may occur; results from hand-pollination experiments supported this speculation, fruit capsules were obtained from hand self-pollination (Table 3.6). In a population where self-compatibility is possible, genetic purging may help reduce the effects of inbreeding, because natural selection would act against recessive deleterious alleles, therefore reducing the negative effects of inbreeding depression in small populations (Busch, 2005). The absence of heterozygosity within this *C. carsei* population might be a result of genetic purging; beneficial alleles may have been fixed across the genome. Further, random genetic drift also seems to have a stronger effect in small populations leading to fixation of alleles (Wollstein & Stephan, 2014). In this study 11 microsatellite markers were screened which covered a limited subset of the genome. Other approaches such as genotyping-by-sequencing (GBS) or double digest restriction-site associated DNA (ddRAD) that are able to screen polymorphisms across more of the genome may increase the possibility of detecting genetic variation in this population; however, microsatellite tend to be more variable than SNP-based markers due to their elevated mutation rate.

Technical limitations can also constrain the detection of genetic diversity at microsatellite loci. However, it is unlikely that technical limitation is the reason for the lack of detectable variation across 11 loci. Microsatellite markers used to genotype *C. carsei* individuals were developed under the same criteria (refer to section 3.3.1.1) as the markers used to genotype *C. macranthus* and *C.* "Remutaka" individuals (van Etten *et al.*,

2018), and genetic variation was detected in the *C.* "Remutaka" population (Table 2.1, Chapter 2) and all three *C. macranthus* populations (Table 3.3 & Table 3.4). The amount of variation at microsatellite regions is known to associate with the properties of microsatellite markers, such as motif length, repeat frequency, and motif types (Merritt *et al.*, 2015). Motif length or simply motif is the nucleotide composition of the repeated sequence, such as mono-, di-, and trinucleotide repeats, etc. (Abdelkrim *et al.*, 2009). Dinucleotide repeats show higher numbers of alleles per locus, H_O, and H_E compared with other motifs, and the number of alleles detected per locus are strongly correlated with H_O and H_E (Merritt *et al.*, 2015). Repeat frequency refers to the number of times a given motif appears, it is also positively correlated with the number of alleles per locus, H_O and H_E (Merritt *et al.*, 2015; Scribner & Pearce, 2000). When comparing different motif types, perfect motifs exhibit a significantly higher number of alleles per locus and H_E than imperfect motifs, though no significant difference is observed in H_O (Merritt *et al.*, 2015). Perfect motifs are those that do not contain any intervening non-repeat nucleotide sequence between motifs; imperfect motifs are those that contain intervening non-repeat nucleotide sequence in between two or more motifs (Bhargava & Fuentes, 2010).

All microsatellite markers used to genotype *C. carsei* individuals are perfect dinucleotide motifs, and the number of motifs within each marker varies from seven to10 (Table 3.1). Microsatellite markers used to genotype *C. macranthus* and *C.* "Remutaka" individuals consisted of perfect di- and trinucleotide motifs, with the number of motif repeats ranging from six to 15 (van Etten *et al.*, 2018). In Figure 3.6, the number of alleles per locus is plotted against the number of uninterrupted repeats (number of perfect motifs); and in Figure 3.7, H_E is plotted against the number of uninterrupted repeats for all three *C. macranthus* populations, the *C.* "Remutaka" population, and the *C. carsei* population. Even though the R^2 values calculated in both plots were low, a positive trend was observed between the number of alleles per locus, H_E, and the number of uninterrupted repeats in *C. macranthus* and *C.* "Remutaka", but not in *C. carsei*. Therefore, it is not likely that the methods of marker development nor the properties of the microsatellite markers restricted the detection of genetic variation within this *C. carsei* population. Instead, no detectable genetic variation would appear to be a biological feature of *C. carsei* in New Zealand.

3.5.2 Genetic structure within *C. macranthus* populations

Genetic structures were observed within and between the three *C. macranthus* populations. CLUMPAK alignment of STRUCTURE results and the Evanno method (Evanno *et al.*, 2005) identified two genetic clusters between populations: the Butterfly Creek population with some Bridal Veil Falls individuals; and the majority of the Bridal Veil Falls individuals with the Te Mata Peak population (Figure 3.9 and Figure 3.10). On the other hand, within population genetic structure was observed in the Butterfly Creek and Bridal Veil Falls population, but not in the Te Mata Peak population (Table 3.5, Figure 3.9 and Figure 3.12).

The first and the second axis of the PCoA graph (Figure 3.11) failed to completely separate Butterfly Creek individuals from Bridal Veil Falls and Te Mata Peak individuals. The second axis was seemingly able to separate the Butterfly Creek population from the other two populations. However, a data point from the Bridal Veil Falls population was mixed with data points of the Butterfly Creek population. Furthermore, the NeighborNet (Figure 3.12) built based on a Prevosti's genetic matrix showed an overall reticulate pattern where individuals from the three population were mingled. This indicated that the population genetic structure did not correspond to the actual location of the three populations. A low value of $F_{ST} = 0.119$ suggested a low degree of differentiation between populations, which reflected the pattern seen in the PCoA graph (figure 3.11) and the NeighborNet (Figure 3.12). Low differentiation between populations implied that gene flow between populations occurred (Mills & Allendorf, 1996). *Corybas* orchids have very small seeds that are adapted for long distance dispersal through wind (Lyon, 2014). Seed dispersal between populations might contribute to gene flow among population which led to low differentiation observed between populations.

Nonetheless, genetically identical individuals were detected in all three populations suggesting the presence of clonal structure within each population (Table 3.5). Asexual reproduction is known to occur in *Corybas* species (St George & McCrae, 1990). The orchid can produce up to five replacement tubers via lateral roots in each growing season, resulting in clusters of genetically identical individuals (Clements *et al.*, 2007; St George & McCrae, 1990). No relationship was observed between genetic similarity and physical distances in genetically identical individuals collected from Butterfly Creek. Conversely, clones from the Bridal Veil Falls and Te Mata Peak population occurred close to each other in their habitat (Table 3.5). Butterfly Creek individuals occurred along

the banks of the walking track. No obstacles were present to prevent the growth of lateral roots for asexual reproduction and no gaps were observed in the distribution of plants. In contrast, individuals from Bridal Veil Falls and Te Mata Peak had a discontinuous distribution, and samples were collected from different sub-sites. All genetically identical individuals observed from these two populations were collected within the same sub-site, no clones were observed between sub-sites.

3.5.3 Null alleles

Null alleles were detected at one locus from all three *C. macranthus* populations: at locus Corybas-27 in the Butterfly Creek population, at locus Corybas-33 in the Bridal Veil Falls population, and at locus Corybas-46 in the Te Mata Peak population. The presence of null alleles was indicated by the homozygote excess at most alleles size classes within these loci. However, these loci were included in the genetic assessments of *C. macranthus* populations in this study. The presence of null alleles may lead to overestimates of heterozygote deficit, though inbreeding, and selection at a locus can also result in homozygote excess and consequently deviation from HWE (Dakin & Avise, 2004). *Corybas* species are self-compatible, and self-pollination is known to occur in some species of this genus (Clements *et al.*, 2007; Kelly *et al.*, 2013). The observed homozygote excess at those loci might result from inbreeding which led to MICRO-CHECKER to attribute the heterozygote deficit to a potential for null alleles in those loci. Conversely, some argued that null alleles had moderate effects on accuracy in Bayesian assignment analysis such as STRUCTURE even in populations with very high null-allele frequencies (Carlsson, 2008). Therefore, loci that were potentially affected by null alleles should not impede the STRUCTURE assignment of the individuals within the *C. macranthus* populations.

3.5.4 Pollination, floral structures, pollen count, and floral visitors in *C. carsei*

Four pollination treatments, natural pollination, hand self-pollination, hand cross-pollination and autonomous self-pollination were carried out to investigate self-incompatibility and pollination strategy in *C. carsei*. Fruits were obtained by hand cross-pollination, hand self-pollination and autonomous pollination (Table 3.6). These data suggested that *C. carsei* is self-compatible and self-pollination may occur, which is in

agreement with previous speculations (Department of Conservation, 2018). Autonomous self-pollination in *C. carsei* might occur when the pollinia collapse onto its own stigma when the flower deteriorates as it ages (de Lange per. comm. in Department of Conservation, 2018), but due to the small number or replicates in this study, this could not be confirmed here. An alternative explanation to the fruits formed by autonomous self-pollination could be caused by pollinators breaching the enclosure and pollinating the flowers. Self-compatibility is not unusual in New Zealand and it seems to be a generalised feature in the New Zealand flora, and it likely arise from the long isolation of the islands and an unspecialised pollinating fauna (Godley, 1979; Newstrom & Robertson, 2005). Both condition would impose selection pressure on the establishment of self-incompatible taxa (Webb & Kelly, 1993). A study by Lehnebach (2002) also found self-compatibility in other New Zealand terrestrial orchids such as *Thelymitra longifolia*, *Pterostylis alobula*, and *Pterostylis patens*.

Field observation and filming of floral visitors failed to identify pollinators of *C. carsei*. However, the camera captured footage of small flies approaching the flower, though none of them entered the flower (Figure 3.13). It is likely a longer period of observation may be able to identify the pollinator of this orchid, but currently this is not possible as there is limited accessibility to the orchid site due to a desire to reduce damage by trampling . Previous studies and field observations showed that flowering and fruiting rate were low in *C. carsei* populations (Brandon, 2006; Clarkson *et al.*, 1993; de Lange & Clarkson, n.d.; Department of Conservation, 2018; Norton & de Lange, 2003). Monitoring data of the Whangamarino Wetland *C. carsei* population showed that from 9th August 2018 to 21st November 2019, only 61 flowers and nine fruit capsules were observed during this period (unpublished report by DoC). In this study, three out of the 29 (10.34%) flowers included in the natural pollination treatment produced a fruit, also showing a low fruiting rate (Table 3.6). And in 2021 only two fruits were observed at the site. On average, fruit set in nonrewarding orchids is approximately 20.7% (Jacquemyn & Brys, 2015; Smithson, 2006). Low fruiting rate in this *C. carsei* population could be due to limited pollinator visitations (Smithson, 2006), but also to damage by herbivory. During both flowering seasons browsing damage was observed on leaves and on some of the developing floral buds and stems holding the capsules. Beside *C. carsei*, other *Corybas* species also exhibits a low proportion of flowering individuals and deceptive orchids on average have lower fruiting rates than rewarding species (Hatch, 1947;

Smithson, 2006; Tremblay *et al.*, 2005). Therefore, low flowering and fruiting rate could be a natural trait of *C. carsei*. Nonetheless, more pollinator observations and pollination treatments are needed to validate these conclusions.

Orchids have the highest seed production per fruit in flowering plants. This is a special trait due to adaptation of the orchid pollen grains which allows fertilisation of a large number of ovules at once possible (Nazarov & Gerlach, 1997). Pollen grains in orchids are organised into a cohesive mass known as pollinia (Dressler, 1993). This compacted structure ensures large pollen loads are deposited onto a stigma from a single visit of a pollinator, which increases the number of ovules being fertilised in a flower (Johnson & Edwards, 2000). The estimated number of pollen grains contained in each pollinium varies greatly from ~5,000 to ~4,000,000 (Johnson & Edwards, 2000). The number of pollen grains counted in *C. carsei* pollinia was estimated to be ~21,666 (Appendix 1). Since only one samples was available for pollen count, this result is not final and there could be some variation. Again, more samples are needed to produce a more accurate number and conclusion.

Pollen to ovule ratios (P/O) have been considered as a good indication of a plant's reproductive strategy (Cruden, 1977). Cruden proposed that P/O ratios reflect the likelihood of sufficient pollen grains reaching a stigma to achieve maximum seed production (Cruden, 1977). Therefore, the more efficient the pollen transportation, the lower the P/O. Thus, cleistogamous flowers would have the lowest P/O ratios, P/O ratios in autogamous flowers would be higher than in cleistogamous flowers, and xenogamous flowers would have the highest P/O ratios (Cruden, 1977). P/O ratios can be an alternate method for evaluating pollination strategy in *C. carsei*. However, in orchids where the pollen grains are aggregated in to pollinia, P/O ratios do not follow Cruden's principle and are generally lower than those observed in cleistogamous flowers (Cruden, 1977). P/O ratios in orchids are likely to be related to the mode in which pollinia are removed and pollinator efficiency (Neiland & Wilcock, 1995).

C. carsei and other *Corybas* species are members within the subfamily Orchidoideae, and taxa within this subfamily are known to have sectile pollinia (Johnson & Edwards, 2000; Lyon, 2014). In sectile pollinia, pollen grains are group into tetrads and coalesce into subunits called massulae which are often fragile and break apart easily (Johnson & Edwards, 2000). Pollination in orchids with sectile pollinia occurs when chunks of pollen or massulae attach to the stigma and break away from the rest of the

pollinium (Johnson & Edwards, 2000). P/O ratios of 19:1 was found in orchids with sectile pollinia which is the highest when compared with other pollinia types (Johnson & Edwards, 2000). P/O ratios are predicted to be higher in orchids that are visited by Diptera than those visited by Hymenoptera (Neiland & Wilcock, 1995). *Corybas* orchids are believed to be pollinated by fungus gnats (Lehnebach & Zeller, 2015; Scanlen, 2006). Studies suggested sectile pollinia are an adaptive feature in orchids pollinated by Diptera as the smaller dipteran could not support the weight of the pollinia in flight (Bernhardt, 1995). If only a portion of the pollinia is removed, a greater pollen count would be highly beneficial for allowing the removed portion of the pollinia to fertilise more ovules (Lehnebach, 2002). Unfortunately, fruits were not collected for ovule count to minimise damage on the number of seeds and loss of new recruitments. Thus, ovule count was unable to be carried out in this study.

Despite autonomous self-pollination is suggested to occur in *C. carsei*, its floral structure does not facilitate self-pollination (de Lange per. comm. in Department of Conservation, 2018). This is confirmed by the microscope and SEM observations made in this study. Figure 3.3D showed that the stigma of *C. carsei* is located underneath the pollinia, separated by a rostellum which makes it difficult for the pollen to fall onto the stigma. A similar structure is also observed in *C. macranthus*, in which the stigma is also located underneath the pollinia and separated by the rostellum (Figure 3.5D). The rostellum is a secretory tissue in the orchid gynostemium, it secrets a sticky adhesive substance that attaches the pollinia on the pollinator. However, despite this physical barrier, the sectile pollinia in *Corybas* orchids might still contribute to self-pollination as pollen grains are able to break apart from the pollinia (Johnson & Edwards, 2000). Figure 3.3D showed that lumps of pollen grains already separated from the pollinia and were scattered on the anther cap and on the body of the column. Although, the scattered pollen grains could be due to handling of the sample while taking photos under the dissecting microscope. On the contrary, floral characteristics in *Corybas* orchids are known to facilitate cross-pollination (Kelly & Gaskett, 2014; Lehnebach & Zeller, 2015). Besides producing fungus-mimicking volatiles to attract pollinators (olfactory cues), visual signals are also used to attract pollinators in *Corybas* orchids (Barnfather, 2019; Kelly & Gaskett, 2014). A study by Kelly and Gaskett (2014) showed that *Corybas cheesemanii* flowers strongly reflected all visible wavelengths especially in the red region (650–700 nm), yellow-green (500–600 nm), and UV (~320 nm) spectra. These orchids may attract

pollinators by exploiting fungus gnats' visual bias and innate attraction for yellow and UV light (Agee & Patterson, 1983; Stukenberg *et al.*, 2018). Scent or visual clues were not investigated in *C. carsei* and this should be the focus of future studies.

Another unique floral trait of *Corybas* species that is believed to be associated with sapromyophily is the presence of spurs or a pair of open auricles at the base of the labellum (Lyon, 2014). Sapromyophily refers to a pollination syndrome where the plants are pollinated by saprophilous flies, the plants attract pollinators by producing volatile compounds to mimic odours of decaying organic matter that signals food resource or oviposition sites (Jürgens *et al.*, 2006; Jürgens *et al.*, 2013; Stensmyr *et al.*, 2002; Vereecken & McNeil, 2010). Although, the function of auricles is still unclear, it has been suggested they may promote the release of fungus-mimicking volatiles (Jones, 1971), and serve as a window to direct pollinator movements (Faegri & Van Der Pijl, 1979). Many *Corybas* species have hair-like protrusions in the labellum tube known as calli that also are presumed to direct pollinator movements (Figure 3.4 and Figure 3.5; Lyon, 2014). Calli in *C. carsei* are present around the inner surface of the tip of the labellum and along the midline leading towards the back of flower where the column is located (Figure 3.3 & Figure 3.4). Calli are believed to be forcing pollinator movement towards the column (Lyon, 2014), and the auricles provide light source for which many insects display positive phototaxis—moving towards light (Park & Lee, 2017), as well as serve an exit for the pollinators (Lehnebach personal communication 2020). Furthermore, filiform later sepals and petals, as those present in many *Corybas* taxa, are known to associate with fly pollination (Faegri & Van Der Pijl, 1979; Vogel & Martens, 2000). These filiform structure may serve as osmophores, visual cues, or tactile guides (Lyon, 2014). However, the filiform lateral petals and sepals in *C. carsei* are greatly reduced (Jane *et al.*, 2017), and are probably not associated with these functions in this species.

3.5.5 Conservation of *C. carsei*

Habitat modification, destruction, and fragmentation are known to be some of the main threats to New Zealand terrestrial orchids (Lehnebach *et al.*, 2005). Habitat destruction and fragmentation subject plant populations to reduced population size, increased isolation, and reduced gene flow. Fragmented populations are susceptible to loss of allele richness and rare alleles via a genetic bottleneck and random genetic drift due to a

decreased population size (Blambert *et al.*, 2016; Nei *et al.*, 1975; Young *et al.*, 1996). *C. carsei* is greatly affect by habitat destruction. Herbaria records show this orchid was found in several other wetlands, but the number of populations has rapidly decreased due to loss of habitats; today, only a single population of ~300 plants remains at the Whangamarino Wetland (Department of Conservation, 2019). Furthermore, the 11 microsatellite markers used to genotype this population failed to detect genetic variation among *C. carsei* individuals (Table 3.2). Loss of most of the populations in this species might be a major contributor to the lack of genetic variation observed here. The lack of genetic variation might also result from potential founder effects from the establishment of a small number of individuals initially. This is of concern because species with low genetic variation are particularly prone to extinction as the evolutionary potential of species to adapt to the changing environment decreases (Ellstrand & Elam, 1993).

The plant-pollinator interactions and mycorrhizal fungi association in Orchidaceae are critical to orchid survival (Reiter *et al.*, 2016). Habitat modifications not only disturb the orchids, but also their pollinators and the mycorrhizal fungi that the orchids are associated with (Jacquemyn *et al.*, 2016; Kleijn *et al.*, 2015). Empirical studies have demonstrated the links between declines in plant communities with declines in pollinator communities due to anthropogenically modified landscapes (Anderson *et al.*, 2011; Biesmeijer *et al.*, 2006). *Corybas* orchids are predicted to have a specialised orchid-pollinator relationship (Lehnebach & Zeller, 2015), a reduction in pollinator efficiency and abundance would have a major impact on sustaining genetic resilience and reproductive success in the plants they pollinate. However, since genetic variation is lacking within this *C. carsei* population, cross-pollination between genetically identical individuals is unlikely to generate genetic diversity within population (Griffin & Eckert, 2003). Future research and long-term monitoring are needed to understand the effects of manual cross-pollination within this population.

All orchid species have life stages that depend partially or fully on orchid mycorrhizal fungi for essential resources, such as relying on mycorrhizal fungi to provide nutrition for germination and transition from protocorm to vegetative green leaf stage (McCormick *et al.*, 2018; Stöckel *et al.*, 2014; Whigham *et al.*, 2008). *C. carsei* also relies on the associations with mycorrhizal fungi for its establishment and persistence (McCormick *et al.*, 2012; Phillips *et al.*, 2020). A study on the effects of wildfire on soil fungal community found that fire disturbance affects soil fungal community by altering

pH level in the soil (Day *et al.*, 2019). The orchid sites at Whangamarino Wetland are actively managed by periodic controlled burning (Department of Conservation, 2018). This raises concern to how fire disturbance might affect the arbuscular mycorrhza fungal community and entomofauna at Whangamarino Wetland, and what effect might it have on the orchid-mycorrhizal and orchid-pollinator associations.

3.6 Future directions

New Zealand *C. carsei* is considered morphologically very similar to the Australian *Corybas fordhamii*, and they are considered by some as synonymous (New Zealand Plant Conservation Network, 2021; Threatened Species Section, 2021). At the moment, the recovery efforts of *C. carsei* remain as a priority as the Australian *C. fordhamii* is also endangered (Threatened Species Section, 2021). Future studies should focus on resolving the uncertain taxonomic status between *C. carsei* and *C. fordhamii* to better partition efforts and resource in the recovery of these two taxa.

To better understand the pollination mechanism in *C. carsei*, conducting surveys of entomofauna in the orchid site during different developmental stages of the orchid, especially during the flower season, would provide information on the plant-pollinator interactions. Additionally, browsing damage was observed on leaves, and in flowering season, it is also seen on some of the developing floral buds, stems holding the capsules, and fruit capsules, which contributed to a low fruiting rate. Identifying the herbivores that caused the damage would be of great interest to reduce herbivory for preventing the loss of fruit sets. All management sites were managed using periodic controlled burning, these surveys may also provide information on the effects of fire disturbance on the entomofauna, and how fire affects the plant-pollinator interactions. Longer periods of floral visitor and pollinator observation is also needed to identify the pollinator of *C. carsei*. Like other New Zealand *Corybas* species, *C. carsei* is predicted to be pollinated through brood-site deceptive pollination by fungus gnats. Carrying out scent analyses of the flowers to investigate whether this orchid produces fungus-mimicking odours could help confirming this assumption. Furthermore, hand-pollination treatments and experiments conducted to estimate P/O ratios should be repeated with a larger sample size in order to provide a more accurate indication of the reproductive methods utilised by this orchid

3.7 Conclusion

In this study, 11 microsatellite markers were developed and used to investigate the genetic composition and genetic variation within the single remaining swamp helmet orchid (*C. carsei*) population in New Zealand. No genetic variation was detected within this population, suggesting that all individuals are possibly genetically identical, or extremely low genetic variation may be present within population.

Microsatellite results of three populations of a common and widespread New Zealand *Corybas* species, *C. macranthus*, showed low to medium genetic variation within population, and low levels of genetic differentiation between populations, indicating gene flow likely occurs among all three populations, or alternatively, could indicate a recent origin of these populations.. Furthermore, genetic structure was detected between and within the three *C. macranthus* populations, though the population genetic clusters did not match with the populations' physical location. The 11 loci used to assess genetic diversity within the three *C. macranthus* populations showed varying degrees of heterozygote and homozygote excess, therefore it was inferred that both outcrossing and self-pollination occur in *C. macranthus* populations. Clonality assessment found individuals that were identical at the 11 microsatellite loci screened within populations, suggesting the presence of clonal individuals and so *C. macranthus* also reproduce from clonal propagation.

Pollination experiments in *C. carsei* showed that this orchid is self-compatible and autonomous self-pollination may occur. Fruit capsules observed in the population could result from both outcrossing and self-pollination. Video footage of floral visitors failed to identify pollinators, however its putative pollinator, small flies, were captured approaching the flower though did not enter; these insects could not be identified from the footage. The findings of this study provides of the first assessment ever made on the genetic variation of this orchid and provides basic information on the pollination strategy and potential pollinators in *C. carsei*. They will contribute to further research into understanding this species, its managements and ultimately its conservation.

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Chapter 4: Conclusion

4.1 Introduction

New Zealand *Corybas* orchids, also known as spider orchids, are the most widely distributed terrestrial orchids in New Zealand (Lehnebach *et al.*, 2016). Some *Corybas* species are threatened due to habitat destruction caused by changes in land uses (de Lange *et al.*, 2004; Lehnebach *et al.*, 2005). Conservation efforts are needed in order to secure survival of those endangered species. However, there are very few studies on New Zealand *Corybas* species, and many aspects of their ecology, biology and reproductive biology remain unknown.

It has been suggested that both sexual and asexual reproduction are possible in *Corybas*. Preliminary studies and field observations suggested that sexual reproduction occurs through insect pollination, and the flowers are likely pollinated by fungus gnats (Lehnebach & Zeller, 2015; Scanlen, 2006). Further, previous studies suggested that some of these orchids attract pollinators by producing fungus-mimicking volatile compounds that signal oviposition sites, and the flowers are pollinated when female fungus gnats move between different flowers to deposit eggs (Lehnebach & Zeller, 2015; Scanlen, 2006). The plant-pollinator interaction in *Corybas* orchids is predicted to be species-specific; however, the pollinator species of most *Corybas* orchids have not been identified. On the other hand, asexual reproduction is achieved through vegetative propagation from lateral roots and replacement tubers (St George & McCrae, 1990). Besides cross-pollination, self-pollination is also presumed to occur in New Zealand *Corybas* orchids as they are self-compatible (Kelly *et al.*, 2013), but this has been confirmed in only one species (i.e. *C. cheesemanii*).

Different reproductive methods employed by a population may affect the genetic composition and genetic variation within the population. For example, vegetative propagation results in genetically identical progenies; cross-pollination produces genetic variation within a population through gene flow and recombination (Solé *et al.*, 2004). Conversely, self-pollination reduces genetic diversity and may lead to inbreeding depression (Peakall & Beattie, 1996). Moreover, clonal structure within a population may further increase inbreeding rate due to cross-pollination between genetically identical individuals. Genetic variation provides a population with adaptation to the changing environment, therefore being able to conserve genetic diversity within populations will

enable *in situ* evolution of adaptations that benefit the long-term persistence of a species (Bailey *et al.*, 2009). Furthermore, a better understanding of a population's genetic structure provides insights to the reproductive strategies employed by the plants, and this information will help develop more efficient conservation plans to ensure the persistence and to increase population size of the target species. In this study, a population genetic approach was used to:

4. Assess genetic composition, genetic variation, and the extent of clonality within a population of *Corybas* "Remutaka", a member of the *C. trilobus* aggregate, via microsatellite marker variation.
5. Assess the genetic composition and genetic variation via microsatellite markers within the single remaining population of *Corybas carsei*, and to infer the reproductive methods employed by this population based on the genetic variation within the population.
6. Investigate the pollination biology of *C. carsei* and to identify its pollinators.

4.2 Findings

This study is the first one in New Zealand and overseas to study the genetic diversity of *Corybas* orchids.

Objective 1:

Chapter 2 showed that the *C.* "Remutaka" population as a whole showed homozygote excess (inbreeding index greater than zero), but heterozygosity varied across the 12 loci tested. A positive inbreeding index suggested that inbreeding occurs, and the varying heterozygosity at different loci was likely caused by cross-pollination. Therefore, it is inferred that this population has a mixed-pollination system in which both self-pollination and cross-pollination were employed. In addition, no genetic structure or correlation between genetic distance and geographic distance were found except at a very localised scale. This possibly results from extensive gene flow assisted by pollinators and seed distribution within the population. Clonality assessment indicated the presence of near neighbour clonal individuals within this population, indicating that asexual reproduction also occurs. Clonal structure within the population may further increase

inbreeding rate as it increases the probability of pollen deposition between genetically identical individuals.

Objectives 2 and 3:

In Chapter 3, the genetic diversity of the single remaining population of *C. carsei*, New Zealand's most threatened orchid, and its pollination system were investigated. The readily available microsatellite markers for *Corybas* (van Etten *et al.*, 2018) were not transferable to *C. carsei*, therefore 11 new microsatellite markers were developed for the use of genetic assessment in this *C. carsei* population. The main findings were summarised in a previous chapter: A population genetic study in *Corybas carsei* and *Corybas macranthus*, and pollination studies in *C. carsei*.

Eleven newly developed microsatellite markers were genotyped in 29 *C. carsei* individuals. All individuals were homozygous for one allele across all 11 markers, which indicated the absence of genetic variation or extremely low genetic variation within population. There are several explanations to these results. For instance, genetic diversity could be lost through a founder effect when the population established at Whangamarino Wetland from seed dispersal, and/or through a bottleneck when the species was rapidly reduced in number and populations disappeared. Moreover, small populations are more susceptible to inbreeding as the probability to mate with a related individual increases, resulting in a higher level of homozygosity. In addition, the absence of heterozygosity within this *C. carsei* population might be a result of genetic purging; beneficial alleles were fixed across all 11 loci for greater fitness. Further, random genetic drift also seems to have a stronger effect in small populations leading to fixation of alleles. Unfortunately, it is not possible to infer what reproductive methods are employed by this population without the necessary genetic diversity being detected.

Pollination experiments in *C. carsei* showed that this orchid is self-compatible and autonomous self-pollination may occur. Autonomous self-pollination might occur when the pollinia collapses onto its own stigma when the flower deteriorates as it ages. However, microscopic examination of the floral structure of *C. carsei* suggests that this orchid does not facilitate self-pollination. Due to a small sample size and low reproducibility, it is necessary to repeat pollination treatments to confirm the occurrence of autonomous self-pollination in this orchid. Fruit capsules observed in the population

could result from both outcrossing and self-pollination. Field observation and filming of floral visitors failed to identify pollinators of *C. carsei*. However, the camera footage captured small flies approaching the flower, though none of them entered the flower. Whether these flies are involved in the pollination is unknown.

4.3 Limitations

On the methodological aspect, poor amplification rate of microsatellite loci can bias results. For instance, in the clonality assessment in *C.* "Remutaka" some loci did not amplify and this led to a high level of missing data; locus Corybas-19 had the lowest amplification rate (50%) in the small transect data set. Missing data could be due to poor-quality template DNA, null alleles, and human error in microsatellite scoring. However, it is necessary to sequence the entire microsatellite region and both primer binding sites in all individuals with missing data in order to identify whether the missing data were due to null alleles or not. Although the presence of null alleles causes overestimates of heterozygote deficit, excluding loci that had null alleles would remove a large part of resolution and explanatory power. Therefore, those loci were included in this study. Nonetheless, missing data could lead to an overestimate of genetically distinct individuals as the computer programme, RClone, used to carry out clonality assessment did not support missing data well and considered missing data as new alleles.

Working with threatened species is challenging, because plant material is naturally rare and thus, the sample size and destructive experiments have to be limited to minimise damage to the reproductive success of the population. As a result, the reproducibility of the experiments, and the accuracy and reliability of the results is affected. Furthermore, site accessibility can also be an issue. In this case, this orchid population is located in the middle of a wetland and access is kept to the minimum to prevent physical disturbance and introduction of weeds.

In this study the sample size for hand pollination treatments in *C. carsei* was small, only five to six individuals (i.e., flowers) were included in each treatment. Ideally, each treatment would be performed on at least 10 flowers. Flowering time in this *C. carsei* population was not synchronised and individuals were in different developmental stages at each visit: many remained in vegetative stage, and only a few individuals were in reproductive stage that were suitable to be included for hand pollination treatments.

Moreover, the pollinia from an individual was used in pollen count and no ovaries were available for the estimation of P/O ratios. These were left to ripen and the seeds used for seed germination experiments. Field observation and filming of floral visitors failed to identify any pollinators of *C. carsei*. It is likely a longer period of observation may be able to identify the pollinator of this orchid. However, currently this is not possible as there is limited accessibility to the orchid site due to a desire to reduce damage by tramping. Further, with the absence of genetic variation, it was impossible to infer the reproductive methods used by this population.

4.4 Future directions

One of the main aspects that future studies should address is resolving the uncertain taxonomic status between *C. carsei* and the Australian orchid, *Corybas fordhamii*. *C. carsei* is considered morphologically very similar to the endangered Australian *C. fordhamii*, and they are considered by some as synonymous (New Zealand Plant Conservation Network, 2021). Sorting this taxonomic uncertainty would aid the partition of efforts and better allocation of resources in the recovery of these two taxa (Lehnebach, 2014). Also, it would provide information on whether *C. fordhamii* represents a subset of genetic variation found in Australia or rather it is an entirely different species to *C. carsei*.

Secondly, to better understand the pollination mechanism in *C. carsei*, it is advisable to conduct surveys of entomofauna in the orchid site during different developmental stages of the orchid, especially during the flowering season. This would provide valuable information on the plant-pollinator interactions (e.g. pollinator dependence and abundance). Additionally, browsing damage was observed on leaves during 2020 and 2021, and in the flowering season. This kind of damage was also seen on some of the developing floral buds, stems holding the capsules, and fruit capsules, which contributed to a low fruiting rate. Identifying the herbivores that caused the damage would be of great interest to manage them and to prevent the loss of fruits. All management sites at the wetland were managed using periodic controlled burning, these surveys may also provide information on the effects of fire disturbance on the entomofauna, and how fire affects the plant-pollinator interactions. Longer periods of floral visitation and pollinator observation is also needed to identify the pollinator of *C. carsei* or at least confirm the presence of its pollinator at the wetland. Like other New

Zealand *Corybas* species, *C. carsei* is predicted to be pollinated through brood-site deceptive pollination by fungus gnats (Lehnebach & Zeller, 2015; Scanlen, 2006). Carrying out scent analyses of the flowers to investigate whether this orchid produces fungus-mimicking odours could help to confirm this assumption. Furthermore, experiments conducted to estimate P/O ratios should be repeated with a larger sample size to provide a more accurate indication of the reproductive methods utilised by this orchid (Cruden, 1977).

If resources and more plant materials are available, it might be worthwhile to use other approaches such as GBS or ddRAD, to assess genetic variation within this *C. carsei* population. GBS and ddRAD are able to screen polymorphisms throughout the entire genome rather than being limited to genotype specific loci like microsatellite markers (Narum *et al.*, 2013). This may increase the chance of detecting genetic diversity within this orchid population.

Finally, further studies could focus on the population genetic studies of different *Corybas* species at a wider geographic range to study the correlation between genetic variation and life history traits (i.e., reproductive methods, pollination strategies, and population size, etc), and the effects of habitat loss on genetic diversity and genetic differentiation within and among populations. These studies could help better understand how a population is reproducing and how demographic factors and evolutionary histories affect genetic variation in *Corybas* orchids. This would aid the conservation of populations and the survival of *Corybas* orchids.

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Appendix

Appendix 1 Pollen counts of *C. carsei* pollinia from a single plant using a haemocytometer.

Squares on haemocytometer	**1**		**2**		**3**		**4**		**5**		**6**		**7**		**8**		**9**		
Repeats	V	N	V	N	V	N	V	N	V	N	V	N	V	N	V	N	V	N	$\bar{X}_V$
1	9	0	16	0	13	0	9	0	5	0	14	0	9	2	14	2	13	0	11.33
2	10	1	17	1	19	1	10	1	23	1	20	1	14	3	13	3	17	2	15.89
3	11	0	17	0	17	0	18	1	20	1	14	0	16	2	13	1	13	0	15.44
4	13	0	10	0	19	2	11	0	13	1	16	1	14	1	16	1	24	1	15.11
5	16	1	14	2	13	1	15	0	15	0	12	1	14	1	12	0	16	1	14.11
Average																			14.38

Note: All nine large squares on the chambers of the haemocytometer were counted and the counts were repeated five times; V = viable pollen grains per large square; N = non-viable pollen grains per large square; $\bar{X}_V$ = average number of viable pollens per large square.