Taxonomy and Population Genetics of the Flightless Moth Genus, *Pringleophaga* in the Sub-Antarctic

by

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DECLARATION

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ABSTRACT

Sub-Antarctic Islands are of considerable conservation importance due to their high endemicity and unique ecosystems. Furthermore, the rich geological and glaciological histories of these islands provide a unique platform to study the biodiversity and biogeography of its biota. Sub-Antarctic islands are divided into three biogeographic regions; the South Indian Ocean Province includes the Prince Edward Islands, Îles Kerguelen, Îles Crozet, Heard Island and McDonald Island. One of the taxa that have long fascinated biogeographers and taxonomists alike is the flightless moth, genus *Pringleophaga*, which is endemic to the Kerguelen, Crozet and Prince Edward Islands. This study addressed three questions relating to the genus *Pringleophaga* at various spatial and evolutionary scales.

The original *Pringleophaga* species' descriptions include only minor morphological differences between *P. kerguelensis* and *P. marioni*, with *P. crozetensis* being more diverse. Anomalies characterize their geographic distributions with *P. kerguelensis* recognized from Îles Kerguelen and Prince Edward but not from nearby Marion Island. Sequence data from two mitochondrial and two nuclear genes were compared for specimens from across *Pringleophaga*'s range. Parsimony networks as well Bayesian topologies revealed three lineages which correspond to the species' morphological classification. Specimens from Îles Crozet (*P. crozetensis*) were not monophyletic. This may be because of misidentification of specimens or alternatively, highlight as yet undescribed diversity in the genus. Genetic data confirm the presence of *P. marioni*, but not *P. kerguelensis*, on Prince Edward Island.

Marion Island's rich glacial / volcanic history and complex geomorphology has been shown to affect the genetic structure of various arthropod species including springtails, mites and weevils. This study extends previous phylogeographic work by testing the spatial genetic structure in *P. marioni* in light of previous hypotheses including the recently described geological lineament. In addition to mitochondrial COI data, a species-specific microsatellite library was developed. Analyses of molecular variance indicated population differentiation across the lineament. Evidence for glacial refugia associated with the high elevated locality of Katedraalkrans was provided by high genetic diversity and little differentiation from other localities.

The final part of the study was aimed at providing possible explanations behind the high abundance of *P. marioni* in Wandering Albatross nests compared to other plant communities. Caterpillars gain a thermal advantage from an occupied nest; hence the designation of Albatrosses as thermal ecosystem engineers. Three hypotheses were investigated: 1) Caterpillars or eggs being inadvertently added into the nest during nest construction; 2) Caterpillars moving into nests and 3) Females moths preferentially ovipositing in nests. A genetic relatedness approach comparing individuals from nests with surrounding vegetation showed that caterpillars from vegetation were, on average, more related than those taken from nests. Given the expected genetic relatedness outcomes, moth seeking out nests is the most likely outcome. Importantly, genetic data add information to a diverse array of other information on caterpillar and moth preferences, physiology and ecology.

This study highlights the potential of genetic data to unravel various questions relating to species biogeography, especially when morphological variation is limited and complex; landscape genetics; and also aiding in our understanding of ecological processes.

OPSOMMING

Sub-Antarktiese Eilande is van merkwaardige belang vir natuurbewaring te wyte aan hulle hoë inheemsheid en unieke ekosisteme. Daarbenewens, die ryk geologiese en glasiasie geskiedenis van hierdie eilande skep ‘n unieke platform vir studies oor die biodiversiteit en biogeografie van hulle biota. Sub-Antarktiese Eilande word verdeel in drie biogeografiese gebiede; die Suid Indiese Oseaan Provinsie sluit die Prince Edward, Kerguelen, Crozet, Heard en McDonald Eilande in. Een van die taxa wat biogeograwe en taksonome al vir n geruime tyd fasineer is die vluglose mot, genus *Pringleophaga*, wat endemies is tot die Kerguelen, Crozet en Prince Edward Eilande. Hierdie studie ondersoek drie vrae rakende die *Pringleophaga* genus op verskeie ruimtelike en evolusionêre vlakke.

Die *Pringleophaga* spesies se beskrywings sluit in geringe morfologiese verskille tussen *P. kerguelensis* en *P. marioni*, met *P. crozetensis* meer divers. Anomalieë karakteriseer hulle geografiese verspeidings met *P. kerguelensis* teenwoordig op die Kerguelen eilandgroep en Prince Edward, maar nie op die naasliggende Marion Eiland nie. DNS volgorde data van twee mitochondriale en twee nukluêre gene is vergelyk tussen spesies van regoor *Pringleophaga* se verspreidingsgrense. Parsimoniese netwerke sowel as Bayesiaanse topologieë het drie afstammelinge onthul wat ooreenstem met die spesies se morfologiese klassifikasies. Individue van die Crozet eilandgroep (*P. crozetensis*) was nie monofileties nie. Dit kan toegeskryf word aan wanidentifisering van individue of alternatiewelik, beklemtoon die tans onbeskryfde diversiteit in die genus. Genetiese data bevestig die teenwoordigheid van *P. marioni*, maar nie *P. kerguelensis*, op die Prince Edward Eilande.

Marion Eiland se glasiale / vulkaniese geskiedenis en komplekse geomorfologie het getoon om die genetiese struktuur van verskeie geleedpotige spesies te affekteer. Hierdie studie brei uit op vorige filogeografiese werk deurdat die ruimtelike genetiese struktuur van *P. marioni* getoets word in die lig van vorige hipoteses, insluitende die onlangs beskryfde geologiese lineament. Benewens mitochondriale COI data, is n spesie-spesifieke mikrosatelliet biblioteek ontwikkel. Molekulêre analises dui op populasie differensiasie oor die lineament. Bewyse vir die hoogliggende Katedraalkrans lokaliteit as ’n moontlike glasiale skuiling is verskaf deur sy hoë genetiese diversiteit en minimale differensiasie van die ander lokaliteite.

Die finale afdeling was ten doel om moontlike verklarings te vind vir die hoë getalle *P. marioni* in Grootalbatros neste in vergelyking met ander plant-komplekse. Ruspes verkry n termiese voordeel binne ‘n besette nes; vandaar die benaming van albatrosse as termiese ekosisteem ingenieurs. Drie hipoteses word ondersoek: 1) Ruspes of eiers word onbewustelik tot neste toegedien tydens die bou van die nes; 2) Ruspes kruip in die neste in en 3) Motte lê eiers by voorkeur in neste. Genetiese verwantskap toetse wat individue van neste vergelyk met dié van omringende plantmateriaal, dui daarop dat individue vanuit plant-komplekse meer verwant is aan mekaar as individue vanuit neste. Gegewe die genetiese verwantskap resultate, is motte wat neste uitsoek die mees waarskynlike hipotese. Meer belangrik, die genetiese data dra by tot verskeie ander vorme van informasie rakende ruspe en mot voorkeure, hul fisiologie en ekologie.

Hierdie studie beklemtoon die potensiaal van genetiese data om verskeie vrae rondom spesies se biogeografie, veral wanneer morfologiese variasie beperk of kompleks is; landskap genetika; en ekologiese prosesse te ontrafel.

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LIST OF ABBREVIATIONS

a.s.l.	Above sea level
A_R	Allelic richness
BS	Bootstrap
Cos^2	Congruence with consensus
COI	Cytochrome c oxidase subunit I
COII	Cytochrome c oxidase subunit II
°C	Degrees Celcius
ddH_2O	Double distilled water
DyadML	Dyadic likelihood estimator
E	East
EF-1α	Elongation factor-1 alpha
FIASCO	Fast Isolation by AFLP of Sequences Containing Repeats
F	Forward
GPS	Global Positioning System
H_d	Haplotype / gene diversity
HWE	Hardy-Weinberg Equilibrium
H_E	Expected Heterozygosity
H_O	Observed Heterozygosity
IBD	Isolation-by-distance
K	Number of clusters
km	Kilometre
LGM	Last Glacial Maximum
LD	Linkage Disequilibrium
m	Metre
MCMC	Markov Chain Monte Carlo
µl	Microliter

Myr	Million years
Mya	Million years ago
min	Minutes
MCOA	Multivariate co-inertia analysis
π	Nucleotide diversity
N	Sample size
N_a	Number of alleles
N_h	Number of haplotypes
N_P	Number of private alleles
ng	Nanogram
N	North
PEPCK	Phosphoenolpyruvate carboxykinase
PCR	Polymerase Chain Reaction
PP	Posterior Probability
R	Reverse
sec	Seconds
S	South
SIP	South Indian Ocean Province
SAMOVA	Spatial Analysis of Molecular Variance
SD	Standard Deviation
Tv	Topological value
TrioML	Triadic likelihood estimator
Var	Variance
v	Version
W	West

CHAPTER 1

INTRODUCTION

1.1 The sub-Antarctic

The sub-Antarctic refers to the region in the Southern Ocean roughly between the sub-Tropical Front (30°S to 47°S, average of 43°S) to the North and the Antarctic Polar Front to the South (Deacon, 1960; Gressitt, 1970). A more regional classification of the sub-Antarctic by Lewis Smith (1984) was based on vegetation (Wace, 1965) and climatic (Holdgate, 1964) criteria. Six archipelagos are typically considered as part of the sub-Antarctic region and include the Prince Edward Islands, Îles Crozet, Îles Kerguelen, Heard and McDonald Islands, South Georgia and also Macquarie Island (see figure 1.1 below). Some of these islands are situated a few latitudes south of the Antarctic Polar Front (considered by oceanographers as the southern boundary of the sub-Antarctic (Smith, 2007; Lutjeharms & Ansorge, 2008).

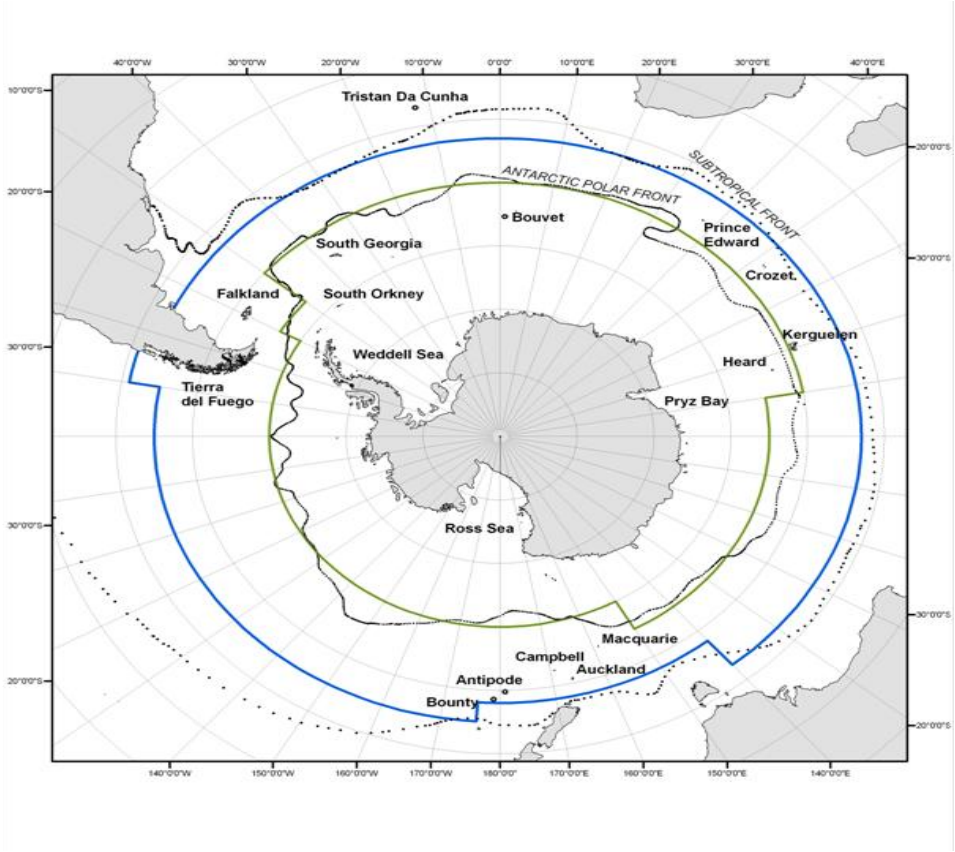

Figure 1.1: Map taken from De Broyer & Danis (2011) indicating the location of the sub-Tropical Front (in blue) and the Antarctic Polar Front (in green). The location of the islands are indicated.

The ecosystems on these sub-Antarctic islands are markedly different to those from the sub-Arctic mainly as a result of different water to land ratios (Smith, 1987). Whereas the sub-Arctic region comprises large continental masses (water to land ratio of approximately 1:1), the sub-Antarctic is covered by large bodies of ocean (water to land ratio of approximately 16:1) (Chown *et al.*, 2004). Sub-Antarctic islands are therefore considerably more isolated, often being separated from

continental or other landmasses by more than 1,500 km of water (Smith, 1987). As a result, the biota of these isolated Southern Ocean islands exhibits lower species diversity compared with most sub-Arctic areas mainly as a result of the slow immigration and colonization of species over such large distances (Chown *et al.*, 1998; Chown *et al.*, 2004). Not surprisingly therefore, the biogeographic patterns of taxa inhabiting sub-Antarctic islands are strongly linked with their vagility and mode of dispersal (i.e. their ability to disperse, actively or passively) (see Wace, 1960; Greve *et al.*, 2005). The ecosystems on most of these Southern Ocean islands are also relatively young compared to sub-Arctic systems and have varied origins with the majority of islands being volcanic (French & Smith, 1985; Smith, 1987; Bergstrom & Chown, 1999). As a result, the ecosystems are relatively simple and useful for studying ecological processes (Smith, 1993), but are nonetheless complex with regards to the effect that past glaciation and volcanic events as well as current climatic conditions have on shaping species' distributions.

1.2 South Indian Ocean Province (SIP) Islands

The sub-Antarctic has been divided into three biogeographic zones or provinces based on differences in climate and endemic terrestrial biota (Lewis Smith, 1984). This classification also translates to the geographic position of the islands and consists of the southern extents of the Indian, Atlantic and Pacific Oceans (Ansorge & Lutjeharms, 2007). The South Indian Ocean Province represents a distinct biogeographic unit with high endemicity within the province and includes the Prince Edward Islands, Îles Crozet, Îles Kerguelen, Heard Island and the McDonald Islands (Chown, 1989; Crafford, 1990a). These islands occur between latitudes of 45°S and 53°S which is in the region of the Antarctic Polar Frontal Zone. These islands share a similar hyper-oceanic climate, characterised by annual low temperatures and high precipitation as well as strong to gale-force winds year round (Wace, 1960; Van Zinderen Bakker *et al.*, 1971; French & Smith, 1985; Smith & Steenkamp, 1990; Bergstrom & Chown, 1999). Despite the fact that they are geographically isolated, of different ages and with distinct geological histories, they nonetheless share a number of endemic taxa with an insect fauna distinct from those found on other sub-Antarctic islands (Chown, 1990; Bergstrom & Chown, 1999). Îles Kerguelen is the oldest (~39 - 100 Myr) and largest island in the group and is often regarded as the source of biotas for the other islands (Nougier, 1972; Crafford, 1990a). The ages of the other islands in this group varies from as young as 0.3 Myr (Prince Edward) to ~0.5 Myr (Marion Island) for the Prince Edward Islands (Chevallier, 1986) and up to ~9 Myr for Îles Crozet (Nougier, 1972; Dosso & Murthy, 1980; Crafford, 1987; LeMasurier & Thomson, 1990; Frey *et al.*, 2003; Kumar *et al.*, 2003).

1.3 Biogeography of the Islands

The biogeography of islands in the Southern Ocean is controversial (Holdgate, 1960; Skottsberg, 1960; Abbott, 1974; Chown, 1990; Morrone, 1998; Van de Vijver & Beyens, 1999; Cox, 2001; Selmi & Boulinier, 2001). The description of biotas in the sub-Antarctic commenced in the 1800s, being dominated by hypotheses on the origins of the biotas and the biogeographic relationships among the islands (see e.g. Gressitt, 1970; Chown, 1990; Chown, 1994; Michaux & Leschen, 2005; Van der Putten *et al.*, 2010). Gressitt (1970) reviewed the biogeographic theories of authors such as Jeannel (1965), Brundin (1965) and Darlington (1965), but these were largely based on the incorrect premise that Îles Kerguelen and Îles Crozet had some kind of continental connection. Extensive studies on core samples from the Kerguelen plateau, collected by the Ocean Drilling Program, suggests that the Kerguelen islands have been formed by hotspot activity that started at least 115 mya (Frey *et al.*, 2003). Îles Crozet are also now known to be entirely of volcanic origin (Nougier, 1972; Dosso & Murthy, 1980; Crafford, 1987). Although Jeannel (1965) placed much emphasis on continental drift, he attributed much of the biota on the Southern Indian Ocean islands to trans-oceanic dispersal via wind and sea currents (see Gressitt, 1970).

Two opposing models have been proposed to account for the distribution of species at large spatial scales. The first model postulates rapid turnover of taxa with dispersal being the main driver (MacArthur & Wilson, 1963; Gressitt, 1967; MacArthur & Wilson, 1967). Secondly, with the confirmation of continental drift, taxa were suggested to be much older and mainly the result of vicariance (Jeannel, 1965; Wallwork, 1973; Rosen, 1978; Nelson & Platnick, 1981). However, vicariance from a once larger landmass is in conflict with the geological history of the Southern Ocean islands which are mainly volcanic (Beggs *et al.*, 1990; LeMasurier & Thomson, 1990; Wallace *et al.*, 2002). Although a vicariant origin of biotas for the majority of islands is therefore unlikely, it may be the case for certain smaller areas (Chown, 1994; Craig *et al.*, 2003). Several species are characterized by patterns that are not fully compatible with either of these proposed models (see e.g. De Queiroz, 2005; Heaney, 2007; Sanmartín *et al.*, 2007; Whittaker *et al.*, 2008). Evaluation of the nestedness among taxa highlighted the importance of dispersal and vagility in shaping distribution patterns. Specifically, species with poor dispersal capabilities such as arthropods show distribution patterns that are largely influenced by close lying source continents or large islands (Chown *et al.*, 1998; Morrone, 1998; Wright, 1998; Greve *et al.*, 2005).

Recent molecular genetic studies has significantly contributed to our understanding of the colonization and spatial distribution of Antarctic and/or sub-Antarctic species at large spatial scales

(see e.g. Grobler *et al.*, 2006; Stevens *et al.*, 2006; Leschen *et al.*, 2011; Mortimer *et al.*, 2011) as well as at more local (island or regional) scales (see e.g. Holderegger *et al.*, 2003; Mortimer & Jansen van Vuuren, 2007; Myburgh *et al.*, 2007; McGaughran *et al.*, 2008; Clarke *et al.*, 2009; Wilson *et al.*, 2009; Hardouin *et al.*, 2010; McGaughran *et al.*, 2010a; Born *et al.*, 2012; Mortimer *et al.*, 2012). A congruent finding to emerge from studies on terrestrial taxa at large spatial scales is that species are invariably and notably older than the Pleistocene, suggesting survival of glaciations in refugia (see e.g. Convey *et al*. 2008; Strugnell *et al*., 2012). At regional and local spatial scales genetic patterns are complex, driven by several factors including a heterogeneous landscape, various geomorphological and climatic histories as well as current climatic conditions.

1.4 The Prince Edward Islands

Marion Island (46°54'S, 37°45' E) and Prince Edward Island (46°38'S, 37°57'E) form the Prince Edward Islands approximately 2,300 km southeast of Cape Town, South Africa (Berry *et al.*, 1978; Chown & Language, 1994). The two islands are ~19 km apart, are of relatively recent volcanic origin (less than 500,000 years old) and represent the peaks of two closely associated shield volcanoes (Kable *et al.*, 1971; McDougall *et al.*, 2001; Chown & Froneman, 2008). Marion Island has a surface area of ~270 km^2 and the central mountainous region reaches a maximum height of 1,231 m a.s.l. (Smith, 1977; Crafford, 1990b; Lutjeharms & Ansorge, 2008). Prince Edward Island on the other hand is much smaller with a surface area of only ~45 km^2 and a maximum altitude of ~672 m a.s.l. (Crafford & Scholtz, 1987; Lutjeharms & Ansorge, 2008). These islands' climate is notably influenced by the surrounding ocean, which is evident from the air temperature being closely correlated to the sea surface temperature (Schulze, 1971; Smith & Steenkamp, 1990). For Marion Island, climatic data are available since the 1950s as it houses a South African meteorological station (Le Roux & McGeoch, 2008), and we can assume that similar more recent climatic conditions characterize nearby Prince Edward Island (Schulze, 1971). These islands are characterised by a low variability in daily and seasonal temperatures as well as high precipitation, high humidity and gale-force winds (Schulze, 1971; French & Smith, 1985; Smith & Steenkamp, 1990; Le Roux, 2008). Marion Island's history include eight volcanic episodes and at least five (possibly eight) glacial cycles, whereas Prince Edward seems to have remained unglaciated (McDougall *et al.*, 2001; Hall *et al.*, 2011). Two prominent topographical features of Marion Island include Long Ridge in the north and the Feldmark Plateau in the south (Chevallier, 1986; Mortimer *et al.*, 2012) which together form a lineament orientated along N26.5°E. These landforms are highly elevated grey lava plateaus that may have served as possible glacial refuge areas for plant and

animal taxa during the Last Glacial Maximum (LGM) (Chown & Froneman, 2008) but also form formidable barriers to movement (Mortimer *et al.*, 2012).

The biota of the Prince Edward Islands share commonality with other islands in the South Indian Ocean sector, such as with the Crozet and Kerguelen archipelagos (Gressitt, 1970; Greve *et al*., 2005; Shaw *et al*., 2010). Although some of the biogeography of the Prince Edward Islands appears superficially to be uncomplicated, links between the faunas of the islands in the Southern Ocean remain controversial (Chown *et al*., 1998; Morrone, 1998; Cox, 2001; Shaw *et al*., 2010) and insights benefit significantly from molecular systematic studies especially for terrestrial plants and invertebrates (Chown & Froneman, 2008; see e.g. Bartish *et al*., 2012). Initially, studies on sub-Antarctic biota was based on the morphological description of species (Jeannel, 1965; Gressitt, 1970) or shared species (see e.g. Wace, 1960). However, with the application of modern molecular techniques, specifically comparisons of DNA sequence data from specimens taken from across their distribution or in the least from several islands, traditional paradigms and beliefs are challenged (see Biogeographic section earlier for more detail); this is also true for taxa occurring on Marion Island (Grobler *et al.*, 2011a; Mortimer *et al.*, 2011).

It has become evident that complex genetic patterns characterize the majority of species on Marion Island (see e.g. Grobler *et al.*, 2006; Mortimer & Jansen van Vuuren, 2007; Myburgh *et al.*, 2007; McGaughran *et al.*, 2010a; Grobler *et al.*, 2011b; Mortimer *et al.*, 2012). The majority of these studies incorporated sequence data from mitochondrial DNA genes, and although a useful first step, it is clear that at smaller spatial scales, genetic patterns are equally complex and need further examination (see Born *et al.*, 2012). There is also very little information on the genetic patterns for species on Prince Edward Island with only a limited number of studies including representatives from this specially protected area (but see Grobler *et al.*, 2006; Grobler *et al.*, 2011b). It is clear that the addition of nuclear markers, specifically more variable microsatellite markers, may reveal population genetic processes hitherto only hinted at by a recent study on the keystone plant species *Azorella selago* (see Born *et al.*, 2012).

1.5 *Pringleophaga* of the Southern Ocean

The moth genus *Pringleophaga* was described by Enderlein in 1905 based on moths collected from the Kerguelen Islands by E. Vanhöffen (see Enderlein, 1905; Vari, 1971). The moth was classified under the Family Tineidae due to the high similarity of their mouthparts to other tineid moths. The first species to be described in this genus was given the name *Pringleophaga kerguelensis*

Enderlein. The description of this species was based on the morphology of both the immature (caterpillar) and mature (moth) stages of a few specimens collected from Îles Kerguelen. This particular species also occurs on Îles Crozet and was suggested to be present on Prince Edward Island but strangely enough, not on the nearby Marion Island (Vari, 1971). In 1905, Enderlein described a second species in the genus from a few caterpillars collected from Île de la Possession (Îles Crozet); *Pringleophaga crozetensis*. The third and last species in the genus, *Pringleophaga marioni*, was described by Viette (1968) from moths collected on Marion Island. This species, endemic to the Prince Edward Islands, was considered closely related to *P. kerguelensis* with only minor differences in the male genitalia. Vari (1971) also illustrated wing venation differences between the species that were not recorded in the initial description of *P. marioni*. *Pringleophaga kerguelensis* is also much larger than *P. marioni*, but the wing venation is the only true distinct characteristic that separates the two species (Crafford 1987).

At first glance the distribution and taxonomic affinities of some of these species may seem unexpected especially if we accept that Îles Kerguelen, as the oldest and largest island group in the SIP, is the source of the biota in this region (Crafford, 1990a). The Kerguelen archipelago is situated south-east of Marion Island and Îles Crozet (Gressitt, 1970; Crafford, 1990a) and therefore the suggested route of colonization runs against the prevailing wind (West wind drift) and ocean currents (Antarctic circumpolar current). Albatrosses and other seabirds frequently travel distances of thousand kilometres (Gartshore *et al.*, 1988; Keith *et al.*, 2002; Cooper & Weimerskirch, 2003) and may provide a mechanism for the transport of animals and / or eggs against the prevailing wind and current flow (Crafford, 1990a). Morphologically the moths from Îles Kerguelen differ only slightly from those on the Prince Edward Islands (Viette, 1968; Vari, 1971), which suggest that they may be recently diverged assuming that *Pringleophaga's* morphology are not constraint by evolution but could change under different local environmental conditions. The morphology of *P. crozetensis* is more different to that of *P. kerguelensis* / *P. marioni* which is somewhat unexpected given the likely route of colonization. Specifically, the direction of the islands (from east to west) is the Prince Edward Islands, Îles Crozet and then Îles Kerguelen.

Suggestions that *P. kerguelensis* is present on Prince Edward Island but not on Marion Island are curious since Marion and Prince Edward are separated by only 19 km, whereas Îles Kerguelen is 1,500 km from these islands. Whether this is indeed the case still remains unclear. However, several possible explanations may account for this anomalous distribution. For example, the house mouse (*Mus musculus domesticus*; see Jansen van Vuuren & Chown, 2007) has been present on Marion Island for several decades and has had a significant influence on local ecosystems and the food web

(Chown & Smith, 1993; Chown & Cooper, 1995; Huyser *et al.*, 2000; Angel & Cooper, 2006). These animals are known to selectively feed on *Pringleophaga* larvae with a preference for larger caterpillars (Crafford & Scholtz, 1987; Rowe-Rowe *et al.*, 1989; Crafford, 1990a; Chown & Smith, 1993; Van Aarde *et al.*, 2004). Since *P. kerguelensis* is the larger of the two species, mice might have preferentially preyed on *P. kerguelensis* and therefore this species may have gone extinct on Marion Island (Crafford & Scholtz, 1987). Alternatively, as mice are absent from Prince Edward Island, it is possible that size differences between Marion and Prince Edward can be accounted for by selective feeding of mice on Marion Island driving the average size of caterpillars downwards. Marion Island would therefore seem to lack species of marked size differences (Crafford, 1990a).

Pringleophaga marioni is an ecologically important species on the Prince Edward Islands. The adult moth stage is short-lived, and they do not feed. The females are rather sedentary, with the males being the active disperser in search of a mate. Adults mate shortly after their emergence (within a day or two) and females lay an average of 173 eggs within a few hours (Crafford, 1990a). The caterpillars that hatch are important detritivores on the island and together with other invertebrates are responsible for the decomposition of plant material whereby the mineralization rates of Sodium, Phosphorus and Potassium are increased (Smith & Steenkamp, 1992a; Smith & Steenkamp, 1992b; Smith & Steenkamp, 1993). The larvae prefer to live in nutrient rich soil and can often be found in plant complexes affected by animal manuring, otherwise known as biotic herbfield (Huntley, 1971; Gremmen, 1981; Smith & Steenkamp, 2001). They also occur in high abundances in the occupied or recently abandoned nests of Wandering Albatrosses (*Diomedea exulans*). Initially it was thought that the high caterpillar density in the nests may be related to a high nutrient content (Joly *et al.*, 1987), but it was shown that the nutrient content of the nest material is not significantly higher than those of the surrounding vegetation (Sinclair & Chown, 2006). It was suggested that the nests rather provide a thermal benefit for the caterpillars by elevating the temperature up to 5°C above that of the surrounding substrate (Sinclair & Chown, 2006). The Albatrosses are thereby indirectly protecting the caterpillars from sub-lethal low temperature stress and appear to be acting as thermal ecosystem engineers.

1.6 Hypotheses and aims of the study

1) The first aim of the study is to test the null hypothesis that each of the sub-Antarctic islands (Kerguelen, Crozet, Prince Edward and Marion) contains a distinct species of *Pringleophaga*. To address this, the validity of the three recognized species namely *P. crozetensis*, *P. kerguelensis* and *P. marioni*, will be investigated using genetic markers. The inclusion of animals from both Prince Edward and Marion Island may shed light on the presence of *P. marioni* and / or *P. kerguelensis* on the Prince Edward Islands.

2) Secondly, the null hypothesis that genetic variation on Marion Island is not geographically structured will be investigated. As an alternative hypothesis, structured genetic variation will be interpreted in the light of the variable climate, prominent geomorphological features as well as the known glacial and volcanic history of the island.

3) The final aim of the study is on a finer scale, where *P. marioni*'s tendency to be highly abundant in the nests of Wandering Albatrosses will be investigated from a genetic perspective. Several hypotheses are proposed to explain the high abundances in nests. In order to address these possibilities, the relatedness of *P. marioni* populations in nests and in plant communities on the eastern side of Marion Island will be compared.

CHAPTER 2

PHYLOGENY OF *PRINGLEOPHAGA* (LEPIDOPTERA: TINEIDAE)

2.1 Introduction

The evolution of insects in the sub-Antarctic has been a topic of interest for many years (Enderlein, 1909; Viette, 1948; Salmon & Bradley, 1956; Gressitt & Weber, 1959; Gressitt, 1970; Chown & Scholtz, 1989; Sinclair *et al.*, 2003). A general rule observed for insects in this region is a reduction in wing size or even total absence thereof (Gressitt, 1970). Also, the diversity of insect species on sub-Antarctic islands is greatly reduced compared to that observed on mainland with a limited number of species representing higher taxonomic groups, often even a single genus or species representing an entire family (Gressitt, 1970).

A case in hand concerns the tineid moth genus *Pringleophaga*, which is distributed amongst the islands in the South Indian Ocean Province namely the Prince Edward Islands, Îles Crozet and also Îles Kerguelen. These animals have greatly reduced wings and are considered flightless. Three species are recognized namely *Pringleophaga crozetensis* (confined to the Crozet Islands), *P. marioni* (reported only from Marion Island) and *P. kerguelensis*. The latter species present a somewhat anomalous case where it is relatively widespread, purportedly occurring on Îles Kerguelen, Îles Crozet as well as Prince Edward Island (Vari, 1971; Crafford, 1987).

Pringleophaga kerguelensis was described by Enderlein (1905) from moth specimens collected on Îles Kerguelen. His description was based on morphological characters of both the mature (moth) and immature (caterpillar) stages. A second *Pringleophaga* species, *P. crozetensis*, also described by Enderlein, occurs on Îles Crozet. Although the description of *P. crozetensis* is based only on the caterpillar stage, the morphology of the caterpillars of *P. crozetensis* deviates markedly from those of *P. kerguelensis* as described in the Deutsche Südpolar-Expedition by Enderlein in 1905. Figure 2.1 below illustrates *P. kerguelensis* and *P. crozetensis* moths obtained from web-based data presented at the Colloquium on sub-Antarctic ecosystems (CNFRA) in 1982 and 1987. No formal description of the mature (moth) stage of *P. crozetensis* could be found. Viette (1968) noticed minor differences in the male genitalia of moths collected on Marion Island from those collected on Kerguelen, and subsequently described a third species namely *P. marioni*. The distinction of *P. marioni* was supported by venation differences later described by Vári (1971) (see figure 2.2 below). *Pringleophaga kerguelensis* is also much larger than *P. marioni*, but the wing venation is the only distinct characteristic which separates the two species. Crafford (1987) reported that the caterpillars of *P. kerguelensis* and *P. marioni* are indistinguishable, except for the size difference of the mature caterpillar in its final instar phase.

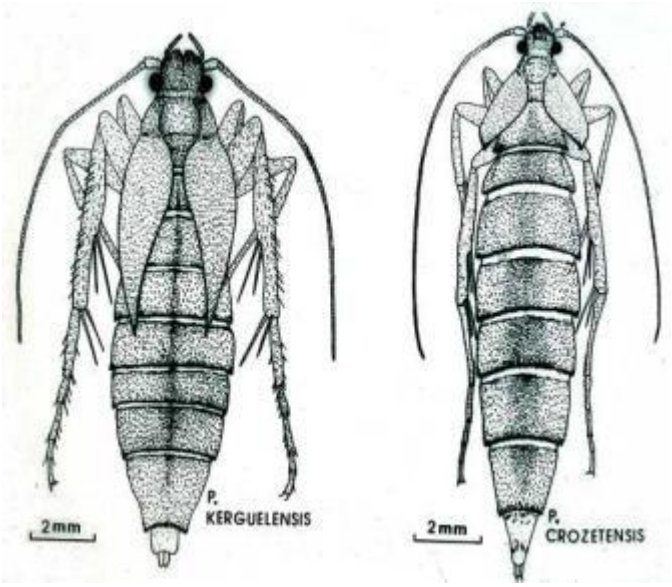

Figure 2.1: Illustrations of the moth stages of *P. kerguelensis* and *P. crozetensis* (taken from a website of the Colloquium on sub-Antarctic ecosystems (CNFRA) in 1982 and 1987, with drawings provided by Georges Chauvin (former lecturer at the University of Rennes 1)).

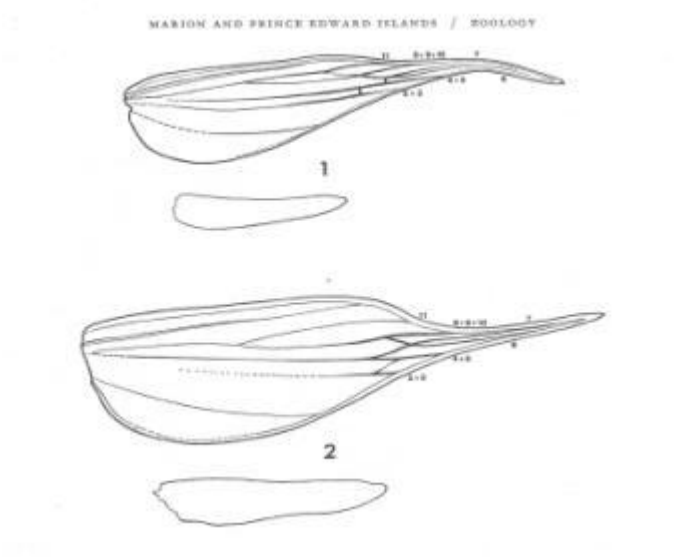

Figure 2.2: Wing venation of the forewing in 1: *Pringleophaga marioni* Viette; and 2: *Pringleophaga kerguelensis* Enderlein (figure taken from Vári (1971)).

According to Vári's report (1971) regarding the Lepidoptera on the Prince Edward Islands, both *P. kerguelensis* and *P. marioni* are present on Prince Edward Island, while only *P. marioni* is reported from Marion Island. The sympatric occurrence of *P. kerguelensis* and *P. marioni* on Prince Edward Island is also mentioned in a study on the quantitative differences between insect faunas on the Prince Edward Islands (Crafford & Scholtz, 1987). Following these authors, *P. kerguelensis* was the

main contributor to the higher biomass of Lepidoptera larvae on Prince Edward Island compared with Marion Island (Crafford & Scholtz, 1987). Given the close geographic proximity of these two islands and the distance between Prince Edward Island and Îles Kerguelen, it seems questionable that *P. kerguelensis* would occur on Prince Edward Island but not on Marion Island. Two possible explanations may account for the reported distribution of *P. kerguelensis*. First, if the presence of *P. kerguelensis* on Prince Edward Island but not on Marion Island is accepted, a possible explanation involves selective feeding of the house mouse (*Mus musculus domesticus*; see Jansen van Vuuren & Chown, 2007) on Marion Island on caterpillars of a larger size (Rowe-Rowe *et al.*, 1989; Crafford, 1990b; Chown & Smith, 1993; Van Aarde *et al.*, 2004). Since *P. kerguelensis* is the larger of the two species, mice on Marion Island might have caused the demise of this species (Crafford, 1990a). Prince Edward Island has remained mice-free which may provide a reason why *P. kerguelensis* could still be present on the island. Secondly, the recognition of these two species as separate entities is largely based on size differences (Vari, 1971) and it may therefore be possible that *P. kerguelensis* is in fact not present on the Prince Edward Islands, but that in the absence of mice, *P. marioni* caterpillars reach a larger size on Prince Edward than they do on Marion leading to the erroneous recognition of *P. kerguelensis.*

The objective here is to address the null hypothesis by using molecular tools to (a) test the validity of the three recognized species within the genus *Pringleophaga* and (b) to investigate whether *P. kerguelensis* is indeed present on Prince Edward Island. For this, a phylogenetic approach is followed. Sequences from four gene fragments (two nuclear and two mitochondrial) are compared for specimens collected from across the distribution of the genus (Îles Crozet, Îles Kerguelen and the Prince Edward Islands).

2.2 Materials and Methods

Pringleophaga collection

Caterpillars of various sizes were collected from several localities on the Prince Edward Islands during the 2008, 2009 and 2010 relief voyages. Specifically, five to six *Pringleophaga* individuals were included from six localities on both Marion and Prince Edward Island. The localities are evenly spaced across the islands to avoid any potential biases in distributions of species. Representative samples of *P. crozetensis* and *P. kerguelensis* were kindly donated by Prof. Marc Lebouvier (University of Rennes; France). These specimens were collected from Îles Crozet (Île de la Possession) and Îles Kerguelen (main island, Grande Terre) respectively. Collections on the Crozet and Kerguelen archipelago's were mostly made from localities around the research stations.

In total, 20 *P. kerguelensis* and 26 *P. crozetensis* individuals were included. Collection details and number of specimens for each species is reported in table 2.1.

Specimens were preserved in absolute ethanol and shipped to the Evolutionary Genomic Laboratory at the Stellenbosch University. Images of the caterpillar and moth stages (except for *P. marioni* from Prince Edward Island) are shown in figures 2.3 – 2.6. Note that discolouration of the specimens occur after being exposed to alcohol for prolonged time periods.

Table 2.1: *Pringleophaga* collected from the four islands on which the species occur.

Island	Locality	GPS coordinates		Year collected	Number of specimens
		Latitude	Longitude		
Marion	Archway Bay	-46.89697	37.88843	2008	5
	Trypot Beach	-46.8844	37.8674	2009	5
	Boulders Beach	-46.8775	37.8596	2009	5
	Prinsloomeer	-46.8447	37.7873	2008	5
	Mixed Pickle Cove	-46.8747	37.6355	2008	5
	Swartkop Point	-46.9106	37.6017	2008	5
Prince Edward	Notable Rock Outcrop	-46.6499	37.9546	2008	6
	Upland mire	-46.6444	37.9881	2008	3
	Golden Gate	-46.6462	37.9939	2008	5
	Van Zinderen Bakker Peak	-46.6280	37.9318	2008	5
	West of Platkop	-46.6385	37.9733	2008	5
	Wolkberg	-46.6346	37.9515	2008	6
Kerguelen	Port-aux-Francąis	-49.3495	70.2185	2009	20
Crozet	Île de la Possession	-46.4477	51.8229	2009	3
	Base	-46.4477	51.8229	2007	17
	Pointe Basse	-46.3631	51.7131	2010	6

Figure 2.3: *Pringleophaga marioni* from Marion Island: Light microscope images of (a) the dorsal view of a moth (alcohol preserved since 2010) & (b) the lateral view of a caterpillar (alcohol preserved since 2007). Images of a (c) living moth and (d) a caterpillar.

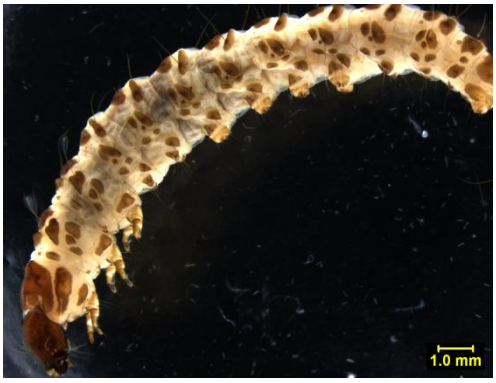

Figure 2.4: *Pringleophaga marioni* from Prince Edward Island: Light microscope image of the lateral view of a caterpillar (alcohol preserved since 2008). No moths were available from this island.

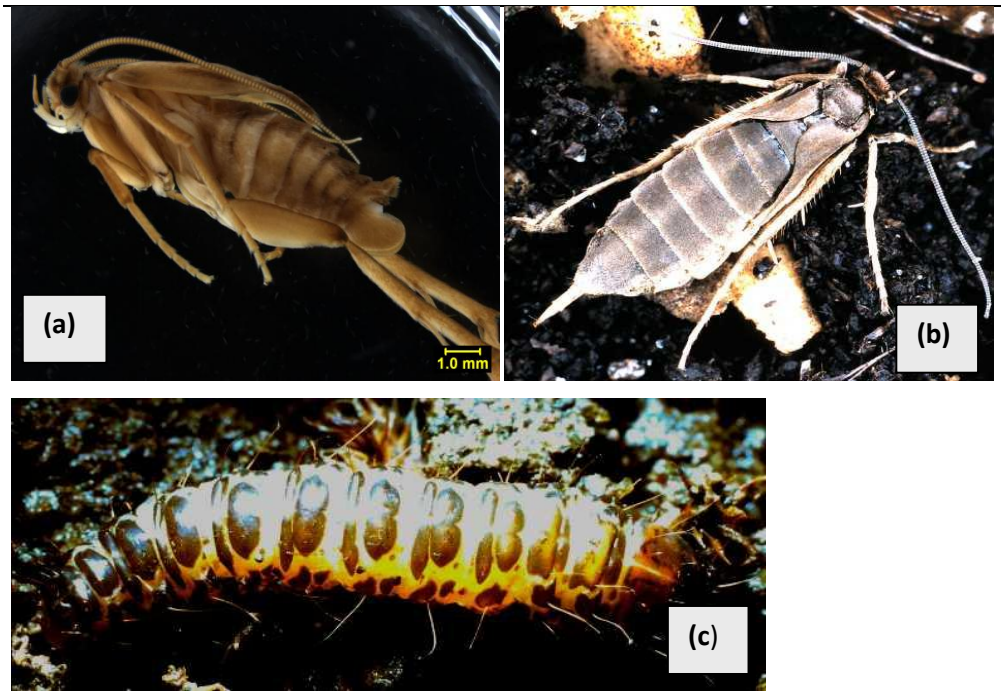

Figure 2.5: *Pringleophaga kerguelensis* from Îles Kerguelen: (a) Light microscope image of the lateral view of a moth (alcohol preserved since 2007); Images of a (b) living moth and (c) caterpillar (photos taken from the website of CNFRA 1982 & 1987, University of Rennes 1).

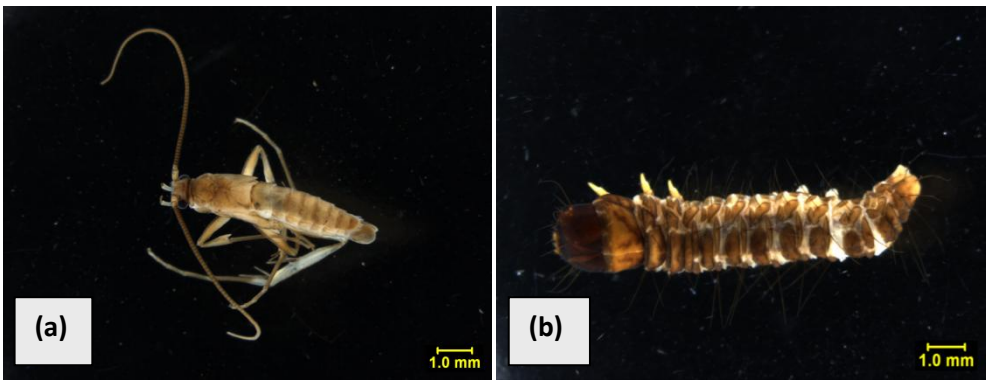

Figure 2.6: *Pringleophaga crozetensis* from Îles Crozet: Light microscope images of the dorsal view of (a) a moth and b) a caterpillar (alcohol preserved since 2010).

Molecular markers and outgroup selection

To obtain a reliable estimate of the phylogenetic relationships among *Pringleophaga* species, DNA sequences of two mitochondrial and two nuclear gene fragments were generated for all specimens. DNA fragments included are the mitochondrial cytochrome c oxidase subunit I (COI; 606bp) and subunit II (COII; 502bp) and the nuclear elongation factor-1 alpha (EF-1α; 307bp) and phosphoenolpyruvate carboxykinase (PEPCK; 448bp) genes. Three of these markers (COI, COII and EF-1α) have proven to be phylogenetically informative for a wide range of study specimens (see e.g. Foley *et al.*, 2007; Ekrem *et al.*, 2010) including several Lepidopteran species (see e.g.

Caterino *et al.*, 2001; Monteiro & Pierce, 2001; Sperling, 2003; Simonsen *et al.*, 2010). These selected gene fragments are also characterized by variable mutation rates which allows the resolution of relationships at different taxonomic levels (see e.g. Friedlander *et al.*, 1992; Brower & DeSalle, 1994; Mitchell *et al.*, 1997; Friedlander *et al.*, 1998). There is also a substantial comparable database available for Lepidopteran species for COI, COII and EF-1α which simplifies the selection of outgroup taxa for phylogenetic reconstruction. The second nuclear gene, PEPCK, was useful in a previous study to resolve Mesozoic age divergences in Lepidoptera (Friedlander *et al.*, 1996) as well as in phylogenetic studies of Diptera (Moulton, 2000), Coleoptera (Sota *et al.*, 2005) and Hymenoptera (Desjardins *et al.*, 2007).

Two outgroup taxa, *Sesamia nonagrioides* (Lepidoptera: Noctuidae*)* and *Glyphodes flavizonalis* (Lepidoptera: Crambidae*)* were used to root resultant trees. No suitable outgroup sequences were available for other tineid taxa. These two outgroup taxa were selected from GenBank (www.ncbi.nlm.nih.gov) based on the availability of comparable sequences for most of the fragments included in the present study.

Molecular techniques

Total genomic DNA was extracted from caterpillars using the DNeasy® Blood and Tissue kit (QIAGEN Inc.) according to the manufacturer's recommendations. Standard polymerase chain reactions (PCR) were set up (see for example Mortimer & Jansen van Vuuren, 2007) to amplify the gene fragments. Published primers were used for COI (LCO1490 and HCO2198; Folmer *et al.*, 1994), COII (C2-J-3138 and C2-N-3661; Simon *et al.*, 1994) and EF-1α (EF1-For3 and Cho10; Danforth & Ji, 1998). PEPCK primers (Friedlander *et al.*, 1996) for Lepidoptera (284dF and 511dR) obtained from Regier's (2007) protocols had low amplification success. Genus-specific forward (F) and reverse (R) primers, designated PEPCK_F (5'- CCTTAAACCCAATTCCTATG-3') and PEPCK_R (5'-CACAACGTGAATTAATAGGG-3') were designed for this gene.

Amplifications were carried out in a GeneAmp PCR 2700 system (Applied Biosystems) with a thermal profile of 30 cycles at 96˚C for 30 sec, fragment-specific annealing temperature (COI = 47°C; COII = 54°C; EF1-α = 55°C and PEPCK = 52°C) for 30 sec and 72˚C for 50 sec. The profile was preceded by an initial denaturation of 5 min at 96˚C and completed by a 10 min extension cycle at 72˚C. Amplicons were purified with the Wizard purification system (Promega) and sequenced using Big Dye chemistry (Applied Biosystems). Reactions were run on an ABI 3170 DNA automated sequencer (Applied Biosystems) after which sequence electropherograms were aligned,

edited and exported using GENEIOUS Pro™ v5.5.4 software (Biomatters Ltd, New Zealand; Drummond *et al.*, 2011).

Data analyses

Standard summary diversity indices for each of the four gene fragments (gene diversity (H_d), nucleotide diversity (π) and number of alleles (N_a)) was estimated for all four islands included here (ARLEQUIN v3.5.1.2; Excoffier & Lischer, 2010). To assess the level of genetic differentiation among the four islands, uncorrected sequence divergences were calculated in DNASP v5 (Librado & Rozas, 2009). These results were confirmed in PAUP* v4.0b10 (Swafford, 2002) by means of a manual inspection between pairwise sequences.

The phylogeny for the species was constructed using parsimony and Bayesian inference methods which were inferred from each of the genes separately as well as from the combined sequence data set. For the purpose of data interpretation, emphasis will be placed on the results from the combined data set. Parsimony analyses were conducted using standard tree building algorithms implemented in PAUP*. Searches for optimal trees were performed using a heuristic search with stepwise addition and TBR branch swapping. All the characters were weighted equally and a 50% majority rule consensus was built if more than one equally most parsimonious tree was recovered. Support for internal nodes was obtained with 1000 bootstrap replicates. The final parsimony tree was viewed in FIGTREE v1.3 (Rambaut, 2009) and INKSCAPE (Bah, 2007). Bayesian trees with posterior probabilities for nodes were constructed in MRBAYES v3.2 (Ronquist *et al.*, 2011). The appropriate evolutionary model was chosen based on a likelihood ratio test conducted in JMODELTEST (Posada, 2008). The data partitions were specified based on each of the four genes, and the optimal model, HKY+ I + G, assigned to each of the sets prior to analysing the data. A Markov Chain Monte Carlo (MCMC) sampling approach was used and five chains (four heated and one cold chain) were run simultaneously for five million generations with trees sampled every 100 generations. In all runs, the standard deviation of split frequencies fell below 0.01 indicating convergence onto a stationary posterior probability distribution of the simultaneous and independent runs conducted by MRBAYES. ESS values were above 200. The first 25% of the sampled trees were discarded as burn-in and the consensus tree constructed from the remainder of the trees. All Bayesian runs were repeated with different random starting values to confirm results. The final Bayesian trees were viewed in FIGTREE and INKSCAPE.

Tree-based criteria are often inadequate to trace a finer level of population genetic structure through space and time. Specifically, divergence values separating taxonomic units are often too low to

yield meaningful resolution. Homoplasies and among site rate variation impedes tree building algorithms and assumptions that branches are strictly bifurcating is frequently violated at the population level (Bermingham & Moritz, 1998; Goldstein *et al.*, 2000; Posada & Crandall, 2001). For these reasons, and in an attempt to obtain better resolution within species, haplotype networks for the mitochondrial data were built in HAPSTAR (Excoffier *et al.*, 1992; Teacher & Griffiths, 2011). Networks have the added advantage that they clearly indicate the number of base-pair changes between different haplotypes (Teacher & Griffiths, 2011). Although not a standard approach when dealing with higher taxonomic groupings, this approach was nonetheless included here as taxonomic affinities of specimens on the Prince Edward Islands are questionable and information about the exact branching of haplotypes may provide important information to assess the taxonomic nature of individuals on the Prince Edward Islands.

2.3 Results

All analyses reported on below were carried out for (a) all gene fragments separately, (b) combined as mitochondrial and nuclear data as well as (c) for the combined dataset including all four fragments. The results from the four gene fragments analysed separately were largely congruent as well as with that of the combined data. For ease of representation, results from the combined dataset are reported in most instances.

Genetic diversity within and among island populations

The diversity indices for the combined dataset, including number of alleles, gene- as well as nucleotide diversities are given in table 2.2. Respective values for the individual gene fragments are given in Appendix 1 (tables A2.1 – A2.4). Not unexpectedly, the majority of genetic variation detected originates from the mitochondrial DNA genes as these regions are typically more variable with a higher mutation rate.

Table 2.2: Statistics for the combined data including nucleotide diversity (mean ± SD), gene diversity (mean ± SD) and number of alleles.

Island	Number of specimens (N)	Number of alleles (N_a)	Gene diversity (H_d)	Nucleotide diversity (π)
Marion	30	19	0.965 ± 0.016	0.0035 ± 0.0019
Prince Edward	30	19	0.933 ± 0.032	0.0017 ± 0.0010
Kerguelen	20	3	0.426 ± 0.122	0.0001± 0.0001
Crozet	26	13	0.926 ± 0.027	0.0427 ± 0.0213
All islands combined	106	61	0.961 ± 0.011	0.0274 ± 0.0133

Gene diversity for specimens from Marion, Prince Edward and Crozet Islands was high (> 0.9). In contrast, gene diversity for specimens from Îles Kerguelen was notably lower (0.426) with only 3 alleles characterizing 20 specimens (see table 2.2). The lower genetic diversity for Îles Kerguelen may simply reflect a bias in sampling; all specimens were collected from a single locality. Although the genetic diversity from Îles Crozet is comparable to those from the Prince Edward Islands, it is likely to be under-represented as the specimens from Îles Crozet had notable amounts of missing data for the nuclear fragments (not the mitochondrial genes), especially for PEPCK (samples could not reliably be amplified; most probably because of mutations in the primer binding sites). The nucleotide diversity for three of the islands was comparatively low (Marion, Prince Edward and Îles Kerguelen), with Iles Crozet exhibiting a much higher value, possibly indicating divergent lineages. Sequence divergence values (uncorrected) among islands are given in table 2.3. Marion and Prince Edward were separated by the lowest divergence value with specimens from Îles Crozet being most divergent; being approximately equidistant from the Prince Edward Islands and Îles Kerguelen.

Table 2.3: Uncorrected sequence divergence values (as a percentage) between the four islands are given for each of the gene fragments separately.

a) COI

Island	Marion	Prince Edward	Kerguelen	Crozet
Marion	-			
Prince Edward	1.45	-		
Kerguelen	1.37	0.94	-	
Crozet	6.23	6.41	6.26	-

b) COII

Island	Marion	Prince Edward	Kerguelen	Crozet
Marion	-			
Prince Edward	0.35	-		
Kerguelen	1.58	1.41	-	
Crozet	7.07	7.10	7.16	-

c) EF1-α

Island	Marion	Prince Edward	Kerguelen	Crozet
Marion	-			
Prince Edward	0	-		
Kerguelen	0.99	0.99	-	
Crozet	2.61	2.60	2.69	-

Table 2.3 continued

d) PEPCK

Island	Marion	Prince Edward	Kerguelen	Crozet
Marion	-			
Prince Edward	0.01	-		
Kerguelen	0.01	0	-	
Crozet	0.68	0.67	0.67	-

Phylogenetic information

The parsimony and Bayesian inference topologies of the combined data were largely congruent. For ease of presentation and reference, only the Bayesian topology is shown (see figure 2.7, see also Appendix 1, figure A2.2), with nodal support from both parsimony and Bayesian inference indicated for the major nodes. The parsimony phylogeny is provided in the Appendices (see Appendix 1, figure A2.1). As expected, the majority of the phylogenetic signal for the species was contributed by the mitochondrial genes and the trees based on the COI and COII gene fragments (analyzed singly or combined) is congruent with the topology retrieved from the combined data. Given their more conserved nature, the two nuclear genes provide support for the separation of Îles Crozet specimens from those collected on the Prince Edward and Kerguelen Islands, but were not able to separate specimens from these latter two island groups; signalling their closer evolutionary affinity compared with the more distant *P. crozetensis*.

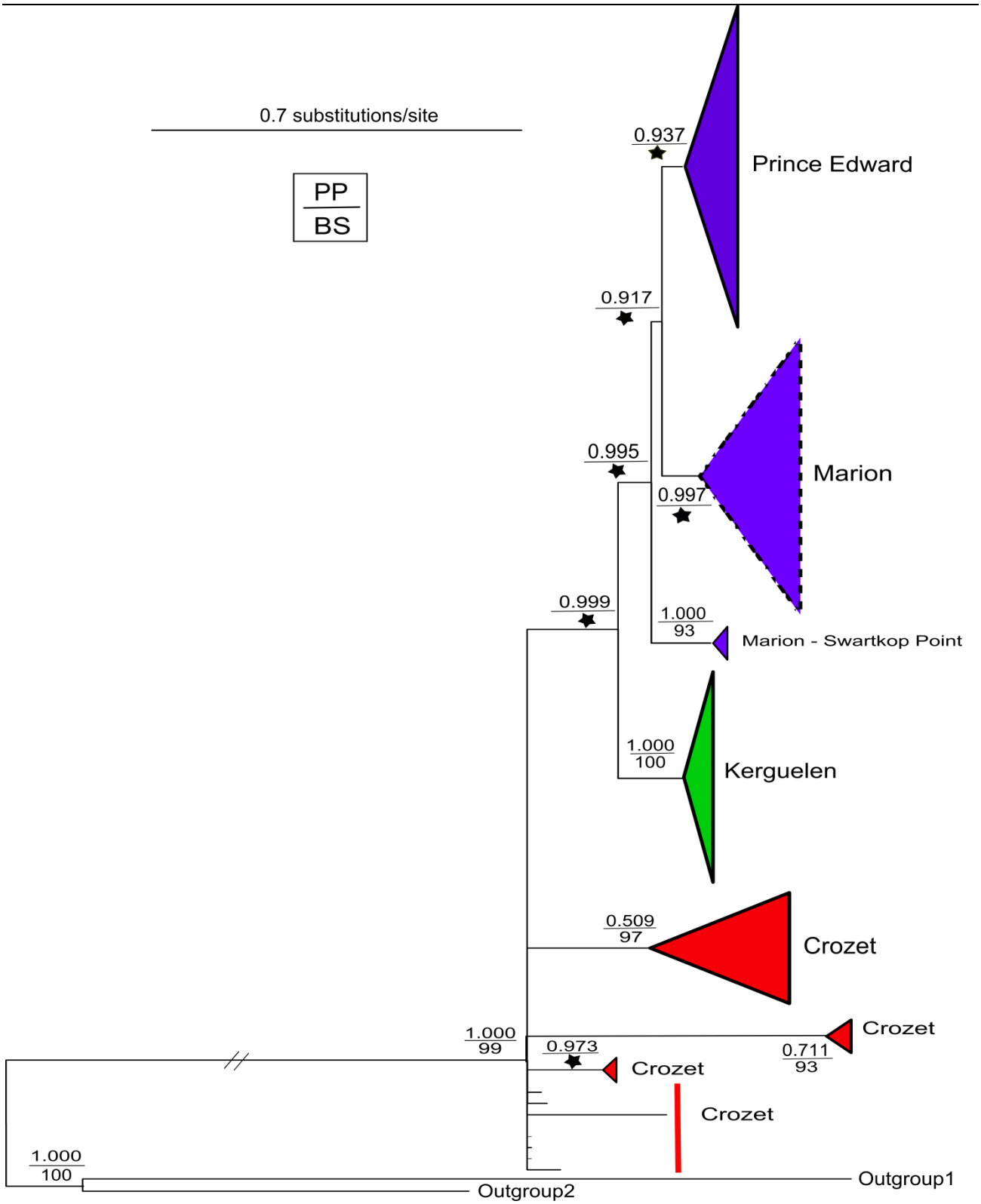

Figure 2.7: Bayesian phylogenetic tree of the *Pringleophaga* species present on four islands based on combined sequence data of four gene fragments. Posterior probabilities (PP) (above the line) and bootstrap (BS) support (below the line) for nodes are indicated. The stars (★) indicate bootstrap support value of less than 70%. Color codes: Purple = species from the Marion and Prince Edward Islands (*Pringleophaga marioni*); Green = species from Îles Kerguelen (*Pringleophaga kerguelensis*); Red = species from Îles Crozet (*Pringleophaga crozetensis*).

The presence of several lineages is seen which correspond to specimens collected from the three island groups; Crozet, Kerguelen and the Prince Edward Islands. Monophyly of *P. kerguelensis* and *P. marioni* is supported, with individuals from Îles Crozet falling into divergent lineages. Îles Crozet's individuals included here appear differentiated and may require future investigation. Without a more thorough investigation and inspection of type material, it is not possible at this stage to designate the *P. crozetensis* clade or draw meaningful conclusions on the potential number of species present on Îles Crozet. Distinct clades were retrieved from Marion and Prince Edward Island with the exception of four specimens collected from the western side of Marion Island at Swartkop Point which grouped separately.

Haplotype network

As an addition to the tree-building algorithms, a haplotype network was constructed. To avoid any biases introduced by missing data (see Joly *et al.*, 2007), and given the notable missing data for some Crozet specimens for the nuclear fragments, haplotype networks were constructed for mitochondrial fragments only. The mitochondrial haplotype network for the species is shown in figure 2.8.

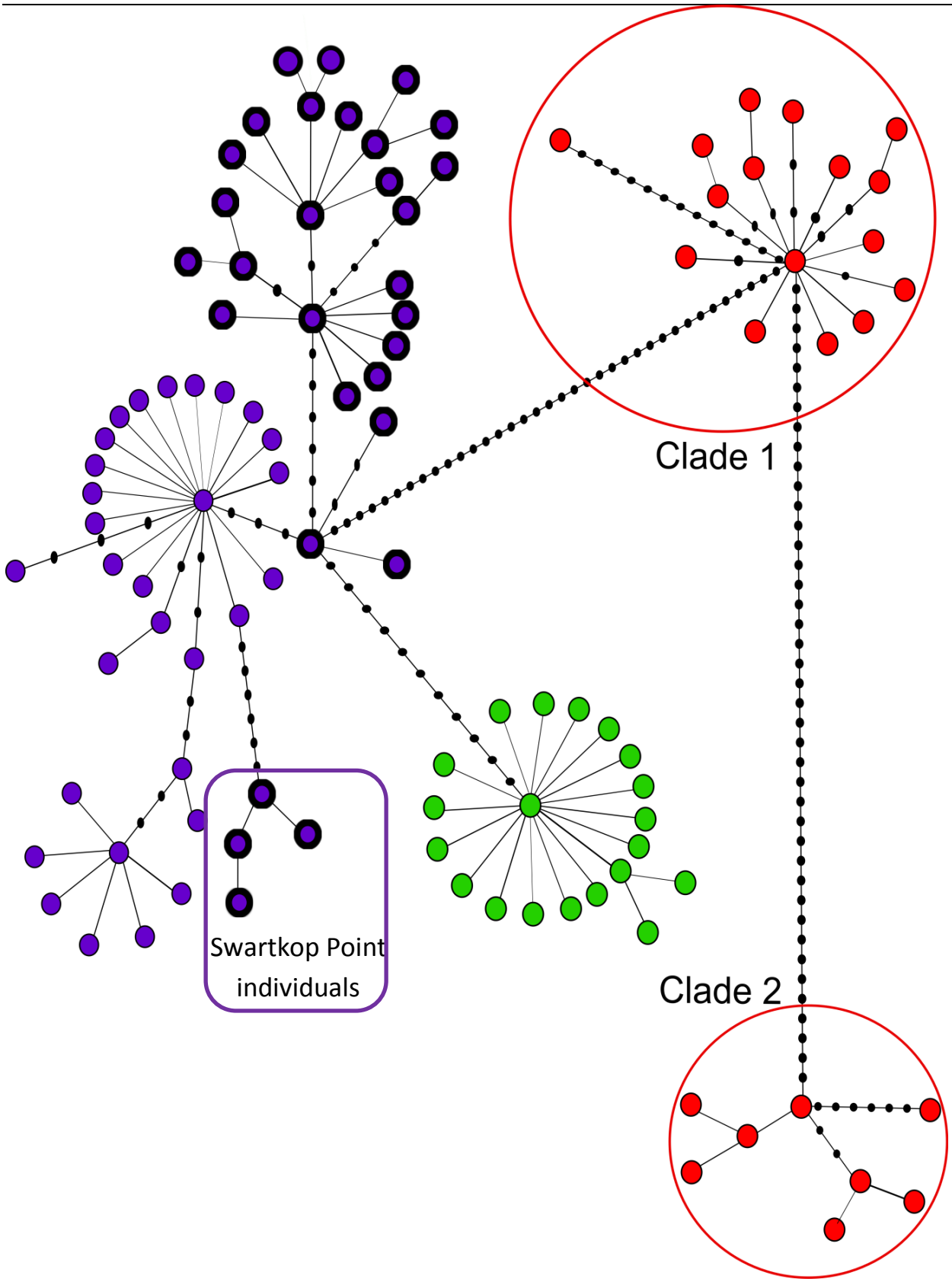

Figure 2.8: Mitochondrial haplotype network for *Pringleophaga* species collected from Marion and Prince Edward Island, Îles Kerguelen and Îles Crozet (colour-coded according to figure 2.7: purple with thin outline = Prince Edward; purple with thick outline = Marion; green = Îles Kerguelen; red = Îles Crozet). Small black-filled circles indicate missing or unsampled haplotypes.

There is overall congruence among the phylogenetic trees and haplotype network although the latter provide better resolution. Specifically, the three main lineages corresponding to the island groups are retrieved. Three distinct sub-clades are found on the Prince Edward Islands corresponding to individuals from Marion and Prince Edward Island (see figure 2.8) as well as a few individuals from Swartkop Point. The four individuals from Swartkop Point (on Marion Island) group closer to the Prince Edward individuals in the parsimony tree as well as the haplotype network, with low support for these individuals grouping outside Prince Edward and Marion in the Bayesian topology. This locality on the western side of Marion Island is proving to be more interesting than previously anticipated since gene flow amongst the Prince Edward Islands is a rare event (Grobler *et al.*, 2011b). Based on the haplotype network, individuals from Crozet group into two divergent clades; this is an unexpected finding given the assumption that only one species was collected on this island, but would explain the high nucleotide diversity observed for Crozet individuals. The distant relationship between the first group of *P. crozetensis* individuals (Clade 1) and the other *Pringleophaga* species is clearly evident from the network. Up to 36 mutational steps separates Clade 1 of *P. crozetensis* from the closest *P. marioni* individual. The second group of Îles Crozet individuals (Clade 2) is even more distant to the other *Pringleophaga* species, but also surprisingly different from Clade 1. Even more genetic differentiation is found between the two clades from Îles Crozet, than between the individuals from Clade 1 and the other two species (*P. marioni* and *P. kerguelensis*).

2.4 Discussion

Flightless sub-Antarctic moth species of the genus *Pringleophaga* is distinguished based on relatively variable and sometimes problematic morphological characters (Enderlein, 1905; Viette, 1968; Vari, 1971; Crafford, 1987). As a result, some questions surrounded the distributions of *P. marioni* and *P. kerguelensis* which were separated based on wing venation, minor male genitalia differences and size. The caterpillar stages of these two species are morphologically indistinguishable. In the present study, molecular characters are added to the morphology in an attempt to address some of the evolutionary questions about the taxonomic status and distribution of the species. The validity of the three species, based on their morphological characters, is confirmed with molecular data supporting the presence of at least three lineages in the genus. However, *Pringleophaga* from Îles Crozet are genetically more differentiated than anticipated and may be in need of a taxonomic revision. Genetic data do not support the presence of *P. kerguelensis* on the Prince Edward Islands. These results are discussed in detail below.

Pringleophaga taxonomy

Molecular data support the distinctness of three lineages within *Pringleophaga* which correspond to the different island groups and which in broad terms support the recognition of the currently described three species. Some unexpected findings include distinct lineages within island groups including on Îles Crozet as well as the Prince Edward Islands.

What was initially regarded as *Pringleophaga crozetensis* from Îles Crozet (i.e. a single species) comprised at least two divergent lineages. One possible explanation for the presence of such genetically divergent lineages may be the possible misidentification of specimens. The only other Lepidopteran species known to occur on Îles Crozet is *P. kerguelensis* and *Embryonopsis halticella* Eaton (according to Davies, 1973). Misidentification of individuals included here as *P. crozetensis* is a possibility, given that *E. halticella* specimens had previously been misidentified as *Pringleophaga* in a study on the Lepidoptera of Heard Island (Brown, 1964; Common, 1970). Brown (1964) described what he reckoned was a new *Pringleophaga* species present on Heard Island, namely *P. heardensis*, which subsequently was identified as *E. halticella*. Images of *E. halticella* are provided in the supplementary material (Appendix 1, figure A2.3).

However, the most distant clade for the Îles Crozet individuals (Clade 2) is representing a group of caterpillars (see e.g. figure 2.6 (b)) and as can be seen from the image of an *E. halticella* caterpillar (Appendix 1, figure A2.3 (b)), the coloration of this species is markedly different from *Pringleophaga* species in general (see e.g. figure 2.3 (d)). Molecular data from *E. halticella* would provide necessary insights into this issue. Unfortunately no such specimens were available for inclusion here. To further investigate this, sequence data available for another moth species that were classified in the same family as *E. halticella*, namely *Plutella xylostella* (Yponomeutidae), was compared with the sequence data for *Pringleophaga* from Îles Crozet. Comparisons among COI data from the two clades of the Crozet islands to those from *P. xylostella*, revealed an equidistant relationship between both of the clades and *P. xylostella*. In other words, the most distant clade (Clade 2) is not closer related to *P. xylostella*, as might have been expected if they were *E. halticella* specimens.

Alternatively, if the individuals included here from Îles Crozet were indeed correctly identified as *Pringleophaga*, then the genus is in need of a taxonomic revision and may in fact represent multiple species.

Pringleophaga marioni and *P. kerguelensis* are separated by relatively low sequence divergence values (0.94 – 1.37% COI sequence divergence), but in the light of these two groups being reciprocally monophyletic and given morphological (albeit subtle) differences, they should be regarded as distinct species. Low sequence divergence values amongst species are common for Lepidopterans. For example, a study on torticid moths reported COI sequence divergences of <1% and up to 2.5% for sister species (Kruse & Sperling, 2001). In lime swallowtails, values of <0.5% were estimated between closely related subspecies of the *Papilio* genus and up to 4% between more distant subspecies (Zakharov *et al.*, 2004). Renewed scrutiny of *Pringleophaga*'s morphology may reveal previously overlooked differences to separate species.

Although not without detraction (see e.g. Salomone *et al.*, 2002), it is possible to apply a standard molecular clock to obtain estimates of species divergence times. For the COI gene in arthropods, mutation rates of between 1.5 - 2.3% per million years are assumed (Brower, 1994; Salomone *et al.*, 2002; Heethoff *et al.*, 2007; McGaughran *et al.*, 2008). If applied to *Pringleophaga*, it would place the separation of *P. marioni* (from Marion Island) from *P. kerguelensis* between 600,000 to 900,000 years ago (1.37% COI sequence divergence). This date, although taken only as an indication, corresponds roughly with the estimated age of Marion Island (see McDougall *et al.*, 2001); suggesting that *Pringleophaga* colonized Marion Island shortly after its formation.

With the exception of a few individuals from Swartkop Point on Marion Island, *Pringleophaga* on the Prince Edward Islands belong to distinct clades separated by 1.45% COI sequence divergence (comparative values for individual gene fragments are given in table 2.3). If we apply a molecular clock, the divergence time between these islands are very similar to the divergence times estimated between the Kerguelen and Prince Edward Islands. This would suggest that colonization of Prince Edward Island was again soon after its emergence. This colonization is likely to have taken place from Marion Island rather than from Îles Kerguelen as no evidence was found for *P. kerguelensis* on Prince Edward Island. The previous report of *P. kerguelensis* on Prince Edward Island was most probably misled because of size variation within *P. marioni* on the two islands (Vari, 1971).

The Prince Edward Islands

An interesting phenomenon from the Prince Edward Islands is the few individuals from Swartkop Point on Marion Island's west coast that are more closely related to individuals from Prince Edward Island (as shown by the haplotype network). Grobler *et al*. (2011b) reported low levels of uni-directional inter-island gene flow (from Marion Island to Prince Edward Island) for the flightless beetle *Bothrometopus huntleyi*, however, the beetle species is more robust compared with

Pringleophaga. Several hypotheses may account for *Pringleophaga*'s genetic link between the two islands which include wind-mediated gene flow, historic events, indirect anthropogenic or seabird transport and ancestral polymorphism. These are explored in more detail below (see also Mortimer *et al*. 2012 for a development of related arguments).

Natural movement of individuals between Prince Edward and Marion Island may explain shared ancestry. One mechanism for gene flow might be through wind dispersal, although probably not easily achievable. The predominant wind direction is from the west, with north-westerlies being the most common and strongest wind followed by south-westerlies (Schulze, 1971; Rouault *et al.*, 2005; Le Roux & McGeoch, 2008). Prince Edward Island is located to the north-east of Marion Island and therefore dispersal from Prince Edward to Marion Island would have to occur against the prevailing wind. Even though the hypotheses of colonization against the dominant west wind drift is not new (proposed by Dreux & Voisin, 1987; Dreux & Voisin, 1989), Swartkop Point is located on the western side of Marion Island and therefore has no direct connection with Prince Edward Island.

Another possibility to consider is historic land bridges connecting the islands. Global sea level fluctuations are thought to have occurred during the Oligocene and Pleistocene (2.5 million – 12,000 years ago), which could have resulted in sea-levels being roughly 100 - 160 m below the present level (Hallam, 1992; Linder, 2003). It was suggested that for southern Africa the sea levels during the Oligocene may have been as far as 500 m below the current level (Siesser & Dingle, 1980; Rogers, 1987). Prince Edward and Marion Island are known to be connected by an inter-island shelf of ~200 m in depth, with some even shallower parts such as Natal Bank (see topography map of the Prince Edward Islands in Pakhomov & Froneman, 1999). These areas or parts thereof might have been exposed during these periods of low sea-levels and provide connections between the islands. However, the northern and north-eastern parts of Marion Island are more likely to have been in contact with Prince Edward Island as opposed to the western side of Marion Island (where Swartkop Point is located).

The Prince Edward Islands' history is well known for all their shipwreck incidences, which could also provide a mechanism for the movement of species between the islands (Terauds *et al*., 2010). One such event occurred in 1912 when a ship wrecked on Prince Edward Island which led to a number of crossings to Marion Island via lifeboats. The stranded men were led by T.C. Hystad, whose name is carved into soft rock at Swartkop Point.

Apart from possible anthropogenic influences, Wandering Albatrosses or other large seabirds might also have facilitated the transport of moth eggs or small caterpillars between the islands. They travel freely, swiftly and fairly regularly between islands and might carry soil or plant material on their feet/feathers (Dell, 1964; Solem, 1968; Gressitt, 1970; Brown & Oatley, 1982). Even though Swartkop Point is not one of the main nesting sites for seabirds (Underhill *et al.*, 2003), the transfer of propagules between the islands is a possibility.

The last and probably most likely explanation for the genetic link between Marion Island's Swartkop Point and Prince Edward Island might be attributed to ancestral polymorphism. The individuals collected from Swartkop Point could represent the last remnants of an ancestral population that still hasn't fully diverged from the individuals on Prince Edward Island. Prince Edward Island was most probably colonized from Marion, and as such *Pringleophaga* on these two islands share a recent common ancestor.

The Biogeography of the Southern Ocean Islands

Understanding the distribution of diversity across a region such as the Southern Ocean islands plays an important role in conservation management planning (Whittaker *et al.*, 2005). A recent study by Terauds *et al.* (2012) suggested that areas should be managed according to a structure based on their biogeographic differences at large spatial scales and also with further differentiation within specific areas. The Prince Edward Islands is a prime example of such a system, being biogeographically distinct from the other islands (Chown *et al.*, 2001) and also harbouring definitive differences at a phylogeographic level between Marion and Prince Edward Island (Grobler *et al.*, 2006; Grobler *et al.*, 2011a) as well as genetic structuring of species on Marion Island (Myburgh *et al.*, 2007; McGaughran *et al.*, 2010a; Born *et al.*, 2012; Mortimer *et al.*, 2012).

Molecular studies in Antarctica and the surrounding Southern Ocean Islands have shown that the biogeography of these landscapes are more complicated than originally thought, but they are providing valuable insight into the possible origins of the species and estimating the time at which these colonizations might have taken place (Stevens *et al.*, 2006; Chown & Convey, 2007; Wagstaff & Hennion, 2007; Convey *et al.*, 2008; De Wever *et al.*, 2009; Mortimer *et al.*, 2011; Wagstaff *et al.*, 2011). The Prince Edward Islands has insect species and/or sister species in common with the geographically closer Crozet archipelago and also with other islands in the vicinity such as the Kerguelen, Heard and McDonald islands (Jeannel, 1965; Gressitt, 1970; Morrone, 1998; Delettre *et al.*, 2003). This is true for *Pringleophaga marioni*, where this study has indicated a close relationship with the species from Îles Kerguelen. A more distant connection is found with Îles

Crozet, which could open the possibility of *P. crozetensis* being the ancestral (source) population of *Pringleophaga* for the other islands. Îles Kerguelen has previously been regarded as the source of biotas for Southern Ocean Islands since it is the oldest (~39 – 100 Myr) and largest archipelago in the group (Nougier, 1972; Crafford, 1990a). But according to a review on sub-Antarctic entomology and biogeography, Îles Crozet (0.2 - 9 Myr; LeMasurier & Thomson, 1990) proved to have a higher number of endemic species than Îles Kerguelen (Gressitt, 1970) even though Îles Crozet is the smaller of the two island groups. Insect diversity (excluding Collembola) on Îles Crozet is also substantially larger (Convey, 2007) and this archipelago can therefore not be disregarded as a probable source of species to the other islands.

CHAPTER 3

DEVELOPMENT OF A MICROSATELLITE LIBRARY FOR THE FLIGHTLESS MOTH *PRINGLEOPHAGA MARIONI* VIETTE (LEPIDOPTERA: TINEIDAE)

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Pringleophaga marioni, a species endemic to the Prince Edward Islands (Marion and Prince Edward Island), is one of three flightless tineid Lepidoptera found exclusively on islands of the sub-Antarctic (Crafford *et al.*, 1986). *Pringleophaga marioni* is characterized by several unique and unusual traits compared to other Lepidopteran species, probably as a result of limited predation and the harsh physical environment. Specifically, it apparently lacks pheromones and is flightless, with highly reduced wings and strongly developed hind legs for jumping (Crafford, 1987). Caterpillars of *P. marioni* are the major litter-dwelling detritivores on the Prince Edward Islands and play an essential role in nutrient cycling (Smith & Steenkamp, 1992b) where they annually process ~1,500 tons of dead plant material (Crafford *et al.*, 1986; Crafford, 1990a). Caterpillars also stimulate the mineralization of nutrients from plant litter in lowland plant communities (Smith, 1985; Smith & Steenkamp, 1992c; Smith & Steenkamp, 1993). On Marion Island they are under considerable threat because they form the preferred prey of invasive house mice, which are absent from the neighbouring Prince Edward Island (Crafford, 1990b; Chown & Smith, 1993). Establishing the extent of population differences in this species between the two islands is therefore essential for informing management actions, which include plans for mouse eradication on Marion Island (Davies *et al.*, 2007; Wanless *et al.*, 2010).

To do so, and to investigate various other components of population variation in *P. marioni*, species-specific microsatellite markers were developed. Caterpillars were collected at Swartkop Point on Marion Island within Wandering Albatross nests. Genomic DNA was extracted from 44 individuals (caterpillars) with the Qiagen DNeasy® Blood and Tissue Kit. An enriched microsatellite library was constructed in association with Inqaba Biotechnical Industries (Pty) Ltd (http://www.inqababiotec.co.za) by means of the FIASCO method (Zane *et al.*, 2002). The library was constructed using the protocol from Zhang *et al.* (2008) with two oligonucleotides (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') as adapters and the adapter-specific primer *Mse*I-N (5'-GATGAGTCCTGAGTAAN-3'). Genomic DNA was enriched using $(AC)_{12}$, $(TA)_{12}$ and $(CT)_{12}$ di-nucleotide repeat probes. A total of 128 positive clones were sequenced of which 38 contained microsatellite motifs of five or more repeats which also had sufficient flanking regions for primer design. Primers were designed for these clones using Primer 3 Plus (Untergasser *et al.*, 2007) and ordered from Applied Biosystems with a fluorescent dye (6-FAM, PET, VIC or NED) associated with the forward primers. Primers were tested for amplification and polymorphism. Sequences of the clones (Pm01 - Pm35) were deposited in GenBank (accession numbers HM035496 - HM035510). The Qiagen Multiplex PCR Kit was used to amplify markers in a single PCR reaction following a protocol which included Q-solution (Qiagen). Multiplex reactions (numbered 1 - 3) consisted of the following primer pairs: 1) Pm01,

Pm05, Pm23, Pm30; 2) Pm04, Pm06, Pm14, Pm16 and 3) Pm15, Pm20, Pm31, Pm35. Amplifications were performed (GeneAmp 2700 Thermocycler; Applied Biosystems) in a final volume of 10 µl containing 2 µl (±80 ng) of DNA, 5 µl of 2X QIAGEN Multiplex PCR Master Mix, 1 µl of Q-solution (5X), 1 µl of primer mix (2 µM) and 1 µl of ddH_2O. Amplifications comprised an initial denaturation at 95°C for 15 min, 30 cycles at 94°C for 30 sec, 60°C for 90 sec and 72°C for 50 sec. A final elongation step at 60°C for 30 min completed the reactions. Genotyping was performed in an ABI 3730 automatic sequencer (Applied Biosystems) using 0.2 µl of GS500LIZ size standard (Applied Biosystems). Alleles were scored using GENEMAPPER v3.7 (Applied Biosystems).

Following the initial screening process, fifteen microsatellite markers were selected for further use given their successful amplification and ease of inclusion into a PCR multiplex. These primer sequences, repeat motifs and GenBank accession numbers are given in table 3.1. Of these, 12 markers were polymorphic in our study population. The three monomorphic markers (Pm07, Pm08 and Pm26) are also listed, but excluded from subsequent analyses. Table 3.1 also includes summary statistics for the markers. The number of alleles per locus varied between 2 and 7 (average of 3.83 alleles per locus). GENEPOP v3.4 (Raymond & Rousset, 1995; Rousset, 2008) was used to perform Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) tests as well as to calculate the observed and expected heterozygosities. The population was in HWE for all the markers except for Pm06 (p-value = 0) and Pm15 (p-value = 0.0003). This deviation was attributed to the occurrence of null alleles as evidenced by an excess of homozygotes at a frequency of 0.2451 and 0.2524 respectively (MICROCHECKER; Van Oosterhout *et al.*, 2004; see also Okello *et al.*, 2005). Lepidoptera species are notorious for the occurrence of null alleles (Meglecz *et al.*, 2004; Chapuis & Estoup, 2007). Even though two of the microsatellite markers showed signs of null alleles in the study population, it might still be useful in other *Pringleophaga* populations, and the occurrence of null alleles would have to be tested on a case-by-case basis. No significant LD could be detected after Bonferroni corrections (p-value adjusted to 0.0012). The markers can therefore be considered as independent. Observed (H_O) and expected heterozygosities (H_E) were calculated according to Raymond & Rousset (1995) and Weir & Cockerham (1984). Observed and expected heterozygosities ranged from 0.047 to 0.727 and 0.046 to 0.753, respectively (see table 3.1). These markers will be useful in studies that hold implications for the management and conservation of this unique species on the Prince Edward Islands.

Table 3.1: The primer sequences, repeat motif, GenBank accession numbers and basic statistics of the microsatellite loci developed for *Pringleophaga marioni*.

Locus name	Primer sequences (5'-3')	Dye	GenBank accession number	Repeat motif	Size range	N	N_A	H_O	H_E
Pm01	F: CGTGAGGACCAGTTTCTTCC R: ACGGTTAGTGTTGGCACCAT	6-FAM	HM035496	$(TG)_4CG(TG)_6CT(TG)_5$	340-386	44	7	0.267	0.360
Pm04	F: AGTCGCCAGTGACAAGTGTG R: CCTCGCAGTGCGTCATAGT	NED	HM035497	$(CA)_{10}$	331-339	43	3	0.302	0.367
Pm05	F: AGCTGGCTTCATTGATACCG R: TCAAAGTGCCACCGCTAAGT	6-FAM	HM035498	$(CA)_6TA(CA)_8$	263-265	43	2	0.256	0.260
Pm06	F: GATGACGTGATGTGATGGCTA R: GAGATCACCAAATCCCACGA	PET	HM035499	$(TG)_9AG(TG)_{12}$	270-280	44	4	0.295	0.647
*Pm07	F: CATACGTCGAAGGCCACTTT R: AAGTTCCCAATCCACACTGG	VIC	HM035500	$(TG)_7$	n/a	n/a	n/a	n/a	n/a
*Pm08	F: TGTGTCTAGCAACTGCCAAAA R: ATGAGCCGCAAGAGTAGAGG	NED	HM035501	$(TG)_6GG(TG)_7$	n/a	n/a	n/a	n/a	n/a
Pm14	F: TTCCTGTAGCACCAACTATTATCAG R: GCGTTATACTCACACCAGCGTTA	PET	HM035502	$(TA)_6$	85-87	43	2	0.047	0.046
Pm15	F: GCCTATGGGTGCTCCTTTC R: AGATACAGAGGCACGAAGACAGT	VIC	HM035503	$(CT)_4CW(CT)_9$	74-86	44	5	0.167	0.407

Table 3.1 continued

Locus name	Primer sequences (5'-3')	Dye	GenBank accession number	Repeat motif	Size range	N	N_A	H_O	H_E
Pm16	F: GCTTGCGTGCGTGTGTAA R: GCCTCTTACTCTGTTTCCATCC	NED	HM035504	$(TG)_{12}$	60-70	44	3	0.500	0.549
Pm20	F: CGATATGTGTTTGCGTACGTG R: AGCTGGTGTAATGATGATGGTG	VIC	HM035505	$(TG)_{11}$	379-401	44	5	0.568	0.568
Pm23	F: CCCAACCTCTGCACTAGACG R: GTTGTTCCAACTTTCTGCCTTA	VIC	HM035506	$(TG)_8AG(TG)_{10}$	244-278	44	5	0.727	0.753
*Pm26	F: GTGAGGAAATCCCGCACTT R: GGGTGGCCAGAGACATACAC	PET	HM035507	$(TG)_7$	n/a	n/a	n/a	n/a	n/a
Pm30	F: TACAGTTCGTGTGCGTGTGT R: AGCCGCAAGAGTAGAGGCTA	6-FAM	HM035508	$(TG)_6CG(TG)_7$	81-87	44	3	0.295	0.320
Pm31	F: GCAAAGCATGATAGCAAATAGG R: CATTACACACGCACAAACACTT	NED	HM035509	$(TG)_{13}$	250-277	44	4	0.568	0.577
Pm35	F: CGGAAGCTTGGCAAATGTAT R: TTGAGGATATAAGCGTGTGTGC	NED	HM035510	$(AC)_5CC(AC)_4$	171-179	44	3	0.091	0.130

N=sample size, N_A= number of alleles, H_O=observed heterozygosity, H_E=expected heterozygosity. Monomorphic markers indicated with *

CHAPTER 4

PHYLOGEOGRAPHY OF *PRINGLEOPHAGA MARIONI* (LEPIDOPTERA: TINEIDAE) ON SUB-ANTARCTIC MARION ISLAND

4.1 Introduction

The geography (topography, climate, soil, vegetation) and geology (origin, history, structure, composition) of a landscape significantly impact species' genetic patterns (see e.g. Manel *et al.*, 2003; Storfer *et al.*, 2007; Manel & Segelbacher, 2009; Schoville *et al.*, 2012). Population genetic studies are therefore critical for understanding the effects of species' responses to various environmental and landscape changes including geological events and climate change (Nason *et al.*, 2002); such an understanding also allows predictions of future change. Over the years, phylogeographic studies (sensu Avise *et al.*, 1979) have highlighted processes involved in shaping species' distributions across a landscape including the identification of barriers to gene flow as well as refugia where species/populations survived unfavourable conditions (see e.g. Parker & Markwith, 2007; Beheregaray, 2008). Islands in the sub-Antarctic are particularly interesting as they typically have a history of volcanic activity and/or glaciation events (Nougier, 1972; Mercer, 1983; Hall, 1990; McDougall *et al.*, 2001; Hall, 2002; Ruddell, 2006; Hall *et al.*, 2011). These events have had a significant impact on landscape subdivision, forced persistence in small isolated refugia, and subsequently shaped the distribution of genetic variation within species (see e.g. Stevens *et al.*, 2006; Mortimer & Jansen van Vuuren, 2007; Myburgh *et al.*, 2007; Born *et al.*, 2012; Mortimer *et al.*, 2012).

The focus here is on Marion Island, a sub-Antarctic island within the South Indian Ocean Province (SIP) which has experienced several glacial cycles and volcanic events (see e.g. Hall *et al.*, 2011; Mortimer *et al.*, 2012). There have been a variety of studies on Marion Island that described phylogeographic patterns for species such as springtails (Myburgh *et al.*, 2007; McGaughran *et al.*, 2010a), mites (Mortimer & Jansen van Vuuren, 2007; Mortimer *et al.*, 2012), weevils (Grobler *et al.*, 2006; Grobler *et al.*, 2011b) and the cushion plant *Azorella selago* (Mortimer *et al.*, 2008; Born *et al.*, 2012). Consistent findings emerged from these studies including complex genetic patterns driven, in part, by geological features (such as lineaments), historic events and current climatic conditions.

Specifically relating to Marion's landscape, a recently described geological discontinuity (N26.5E° lineament, see figure 4.1) running across the island is broadly in line with the genetic structuring of *Halozetes fulvus* (Mortimer *et al.*, 2012). Although previous glacial reconstructions suggested that the island was almost entirely covered by ice (Hall, 1978; Hall, 1979; Hall, 1982), it has become increasingly clear (both from geological reconstructions as well as genetic evidence) that specific areas have remained ice-free (Nel *et al.*, 2003; Hall *et al.*, 2011; McGaughran *et al.*, 2011; Mortimer

et al., 2011). Three such areas include Long Ridge and the Feldmark plateau as well as Katedraalkrans, an elevated grey lava outcrop ~750 m a.s.l. (Chown & Froneman, 2008; Hall *et al.*, 2011). From a genetic point of view, these areas are characterized by high haplotype diversity (at least in the mite *Halozetes fulvus*; Mortimer *et al.*, 2012) which is in keeping with predictions for a refuge population (see e.g. Jansen van Vuuren & Robinson, 1997; Hewitt, 2000; Rowe *et al.*, 2004; Stevens *et al.*, 2007).

Other consistent findings related to genetic structure include markedly different evolutionary histories for populations on the eastern and western sides of Marion Island (see e.g. Mortimer & Jansen van Vuuren, 2007; Myburgh *et al.*, 2007; Born *et al.*, 2012; Mortimer *et al.*, 2012). These differences may, at least in part, be driven by different climatic conditions and microclimatic differences that exist on the two sides of the island (see e.g. Nyakatya & McGeoch, 2007; Le Roux, 2008; Le Roux & McGeoch, 2008). The southern and western parts of Marion Island are known for frequent volcanic activity, with recent eruptions documented in the west (Verwoerd *et al.*, 1981; Meiklejohn & Hedding, 2005). Furthermore, distinct western and eastern episodes of grey lava successions (Verwoerd, 1971; Hall, 1978) may have contributed to adaptive processes within species although only minor geochemical differences are evident among these lava flows (Kable *et al.*, 1971). Wind patterns differ across the island (Muñoz *et al.*, 2004; Felicísimo *et al.*, 2008; Le Roux, 2008; Le Roux & McGeoch, 2008) which has an effect on seed and pollen dispersal in the cushion plant *Azorella selago* (Born *et al.*, 2012) and which may similarly affect dispersal in other plant species and small invertebrates. Spatial variation on Marion Island also exists at a microclimatic level, where significant differences were detected in relation to altitude as well as with regard to the side (west/east) of the island (Nyakatya & McGeoch, 2007). Whether these differences are enough to affect genetic patterns is still not certain, but what has been shown is that metabolic rate variation in *Cryptopygus antarcticus travei* may be correlated with microclimate variability. Individuals from the western side of the island had a higher mean metabolic rate that those from central or eastern populations (McGaughran *et al.*, 2010a).

This study aims to test the null hypothesis that genetic variation on Marion Island is not geographically structured. As an alternative, previous hypotheses regarding genetic structuring of species on Marion Island related to the island's complex landscape, climate and history will be tested and improved on by investigating the phylogeography of an endemic flightless moth species, *Pringleophaga marioni*. All studies to date were based on mitochondrial markers alone. Although providing useful information, the need to add information based on nuclear markers was demonstrated by Born *et al.* (2012) where variable microsatellite markers allow resolution at a

much finer scale. To this end, the present study includes information from microsatellite markers in addition to mitochondrial DNA. The addition of another species to current knowledge allows more comprehensive testing and refinement of current hypotheses. Molecular data are interpreted based on three main structuring trends evident on Marion Island: a N26.5E° geological lineament; coastal and high altitude glacial refugia and east-west climatic differences.

Secondly, the null hypothesis that genetic variation on Marion Island is not geographically structured will be investigated. As an alternative hypothesis, structured genetic variation will be interpreted in the light of the variable climate, prominent geomorphological features as well as the known glacial and volcanic history of the island.

4.2 Material and Methods

Study Island

Sub-Antarctic Marion Island (46°54'S, 37°45'E) is situated some 2,300 km south-east of Cape Town, South Africa (Berry *et al.*, 1978; Chown & Language, 1994) between the sub-Tropical Convergence to the north and the Antarctic Polar Front to the south. Marion Island is an intra-plate shield volcano and is in geological terms young, approximately 500,000 years old (McDougall *et al.*, 2001). The history of the island includes at least five glaciations during the Quaternary and at least eight volcanic episodes, with the most recent eruption in 2004 indicating that the island is still volcanically active (Verwoerd *et al.*, 1981; McDougall *et al.*, 2001; Meiklejohn & Hedding, 2005).

Some of the more prominent landscape features include the Santa Rosa Valley on the south coast of Marion Island which is suggested to be the result of landslides due to volcanic activity (Chevallier, 1986). Two other large topographical landforms include Long Ridge (to the north-east) and the Feldmark Plateau (to the south). The initial possibility of these structures originating due to faulting associated with glacial activity was recently disputed (Hall, 1982; Hall, 2004; Hall *et al.*, 2011), and the mechanism behind the formation of these structures remains uncertain (see Verwoerd, 1971; Hall, 1982; Chevallier, 1986; McDougall *et al.*, 2001; Hall, 2004; Hall *et al.*, 2011). A geological discontinuity was identified by Mortimer *et al*. (2012) as a N26.5°E lineament connecting the western scarps of Long Ridge and the Feldmark Plateau. This is close to the tectonic system of the island characterized by N20°E and E-W structures which correspond with the set of known fracture zones as discussed in Chevallier (1986). Another feature of Marion Island's topography is two major grey lava successions, designated as eastern (older) and western (younger), originating from two eruptive centres (Verwoerd, 1971; Chevallier, 1986; McDougall *et al.*, 2001).

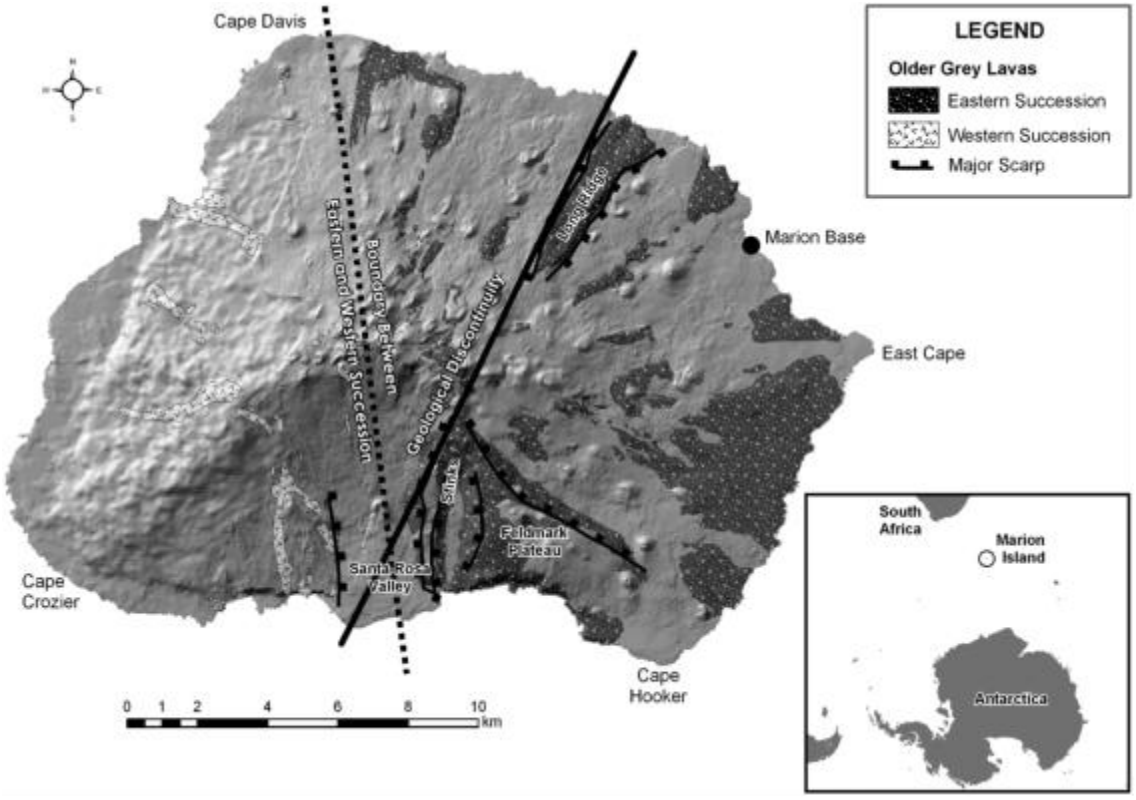

Figure 4.1: Geological map of Marion Island indicating the discontinuity created by the NE lineament (digital elevation model of Marion described in Meiklejohn & Smith 2008) as well as the boundary between eastern and western grey lava successions (Map taken from Mortimer *et al.* (2012)).

Glacier margins for Marion Island during the LGM were reconstructed because of recent evidence that faulting and deglaciation does not co-occur (Hall *et al.*, 2011, figure 4.2). Based on these reconstructions, certain areas are likely to have remained ice-free during the last glaciation period; these would have acted as refuge areas. Katedraalkrans is suggested as a refuge area for arthropod species such as *Cryptopygus antarcticus* (Myburgh *et al.*, 2007; McGaughran *et al.*, 2010a) and possibly also for *Halozetes fulvus* (Mortimer *et al.*, 2012).

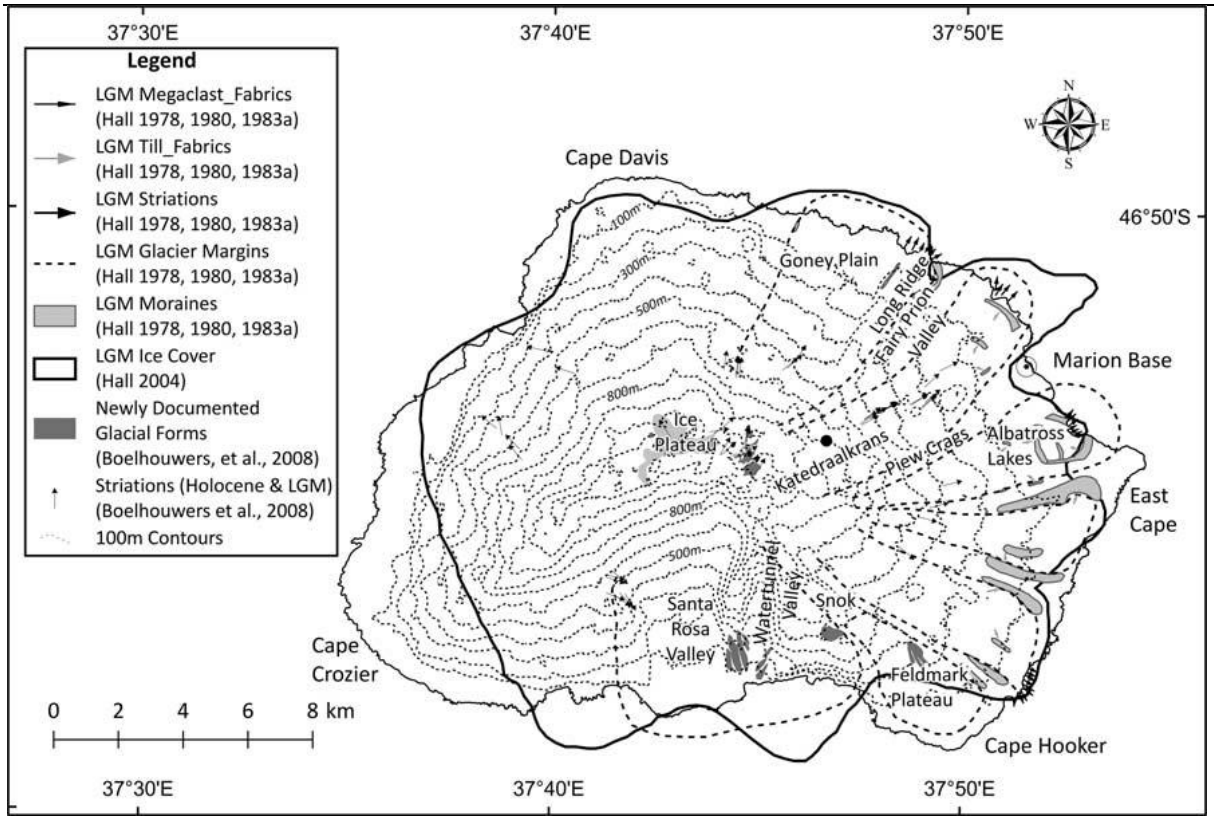

Figure 4.2: Glacial evidence and proposed extents of glaciation on Marion Island (Map taken from Hall *et al*. (2011)).

<u>*Pringleophaga marioni* as study species</u>

Pringleophaga marioni individuals were collected from several localities around the island (see table 4.1 and figure 4.3). Although methodical, sampling was designed to allow the testing of specific hypotheses including those on landforms, glacial refugia and climate. All the individuals collected were preserved in absolute ethanol.

Table 4.1: Information on the *Pringleophaga marioni* collection from Marion Island.

Number ID of locality	Locality	GPS coordinates		Year collected	Number of specimens analysed (COI / microsatellite / total)
		Latitude	Longitude		
1	Long Ridge/Sea Elephant Bay[1]	-46.8449	37.8070	2010	10 / 12 / **12**
2	Rockhopper Bay[1]	-46.8745	37.8560	2009	13 / 0 / **13**
3	Boulders Beach[1,2]	-46.8777	37.8592	2008/2009	5 / 12 / **12**
4	Trypot Beach[1,2]	-46.8844	37.8674	2008/2009	5 / 12 / **12**
5	Archway Bay[1,2]	-46.8969	37.88843	2008/2009	5 / 12 / **12**
6	Bullard Beach[1,2]	-46.9251	37.8805	2010	8 / 8 / **8**
7	Sealer's Cave[1,2]	-46.9499	37.8703	2010	2 / 8 / **8**
8	Funk Bay[1,2]	-46.9548	37.8237	2008	5 / 11 / **11**
9	Kildalkey Bay[1,2]	-46.9646	37.8493	2010	5 / 9 / **9**
10	Watertunnel[1,2]	-46.9631	37.7454	2010	10 / 11 / **11**
11	Goodhope Bay[1,2]	-46.9665	37.7060	2008	5 / 12 / **12**
12	Rook's Bay[1,2]	-46.9689	37.6631	2008/2010	5 / 19 / **19**
13	Swartkop Point[1,2]	-46.9247	37.5947	2008/2009	10 / 12 / **12**
14	Neville[2]	-46.8773	37.6332	2008	0 / 3 / **3**
15	Mixed Pickle Cove[1,2]	-46.8747	37.6355	2008	7 / 16 / **16**
16	Azorella kop[1,2]	-46.8598	37.6677	2008	3 / 3 / **3**
17	Tweeling[2]	-46.8368	37.6866	2010	0 / 8 / **8**
18	Cape Davis[1,2]	-46.8294	37.7087	2010	10 / 12 / **12**
19	Repetto's[2]	-46.8386	37.7662	2008	0 / 28 / **28**
20	Prinsloomeer[1,2]	-46.8447	37.7873	2008	5 / 9 / **9**
21	Katedraalkrans[1,2]	-46.8975	37.7795	2009	14 / 14 / **14**

[1]Samples analyzed using mitochondrial COI data; [2]Samples analyzed using microsatellites.

Bold numbers indicate the total number of individuals for which data are available.

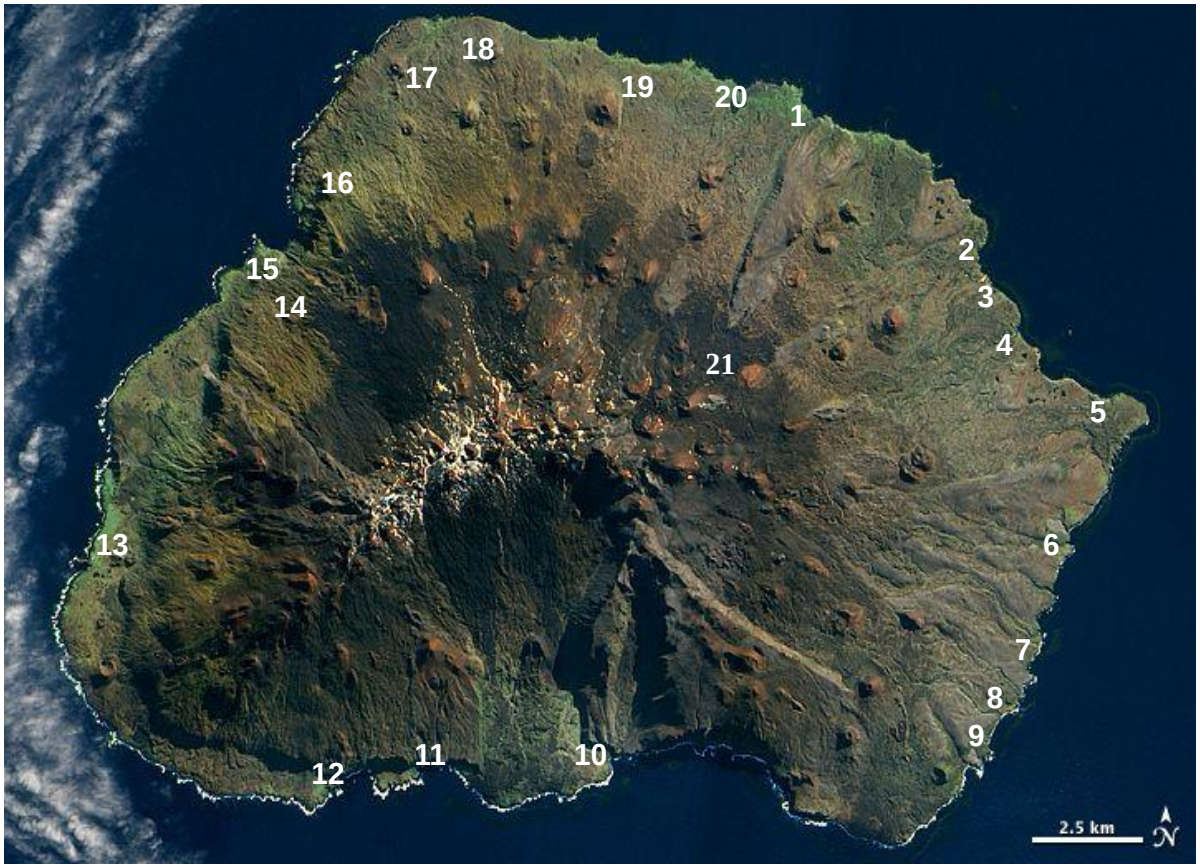

Figure 4.3: Satellite image of Marion Island (Meiklejohn & Smith, 2008) indicating the sampling localities (according to the number ID given in table 4.1) of *Pringleophaga marioni*.

Molecular markers

The distribution and spread of genetic variation across Marion Island was investigated using mitochondrial and nuclear markers. The mitochondrial protein-coding gene, cytochrome oxidase subunit I (COI), is frequently used for testing genetic variability within and between populations (including Lepidoptera species; see e.g. Segraves & Pellmyr, 2001; Simonato *et al.*, 2007; Lohman *et al.*, 2008) and is also the gene used in barcoding efforts (see Hebert *et al.*, 2003). In addition, nuclear microsatellite markers provide a powerful tool for inferring population structuring at a finer level of resolution (Jarne & Lagoda, 1996; Zane *et al.*, 2002; Born *et al.*, 2012); the usefulness of these types of markers have been demonstrated for recently diverged lineages or populations of close geographic proximity (Lukoschek *et al.*, 2008). Microsatellite markers are characterized by their locus specificity, co-dominance, neutrality and high levels of polymorphism and are therefore commonly used to infer population structure and genetic diversity (Chapman *et al.*, 2008; Bourgeois & Rinderer, 2009; Delaney *et al.*, 2009; Kence *et al.*, 2009; Soland-Reckeweg *et al.*, 2009). Twelve polymorphic species-specific microsatellite markers were developed in this study (see Groenewald *et al.*, 2011; see also Chapter 3 in this thesis).

Molecular techniques

Total genomic DNA was extracted from caterpillar tissue (air-dried to remove any trace of ethanol) using the DNeasy® Blood and Tissue kit (Qiagen) according to the manufacturer's recommendations.

Mitochondrial COI amplification and sequencing

Standard polymerase chain reactions (PCR) were set up to amplify the COI gene fragment using published primers for COI (LCO1490 & HCO2198, Folmer *et al.*, 1994). Amplifications were carried out in a GeneAmp PCR 2700 system (Applied Biosystems) with a thermal profile of 30 cycles at 96˚C for 30 sec, 47°C (annealing temperature) for 30 sec and 72˚C for 50 sec. The profile was preceded by an initial denaturation of 5 min at 96°C and completed by a 10 min extension cycle at 72˚C. Amplicons were purified with the Wizard purification system (Promega) after which the strands were sequenced using BigDye chemistry (Applied Biosystems). Reactions were run on an ABI 3130 DNA automated sequencer (Applied Biosystems).

Microsatellite genotyping

Complete details of microsatellite development and amplifications are given in Chapter 3. In short, the Qiagen Multiplex PCR Kit was used to amplify 12 microsatellite markers in a multiplex fashion following the manufacturer's protocol which included Q-solution (Qiagen Inc.). Multiplex reactions (numbered 1 - 3) consisted of the following primer pairs: 1) Pm01, Pm05, Pm23, Pm30; 2) Pm04, Pm06, Pm14, Pm16 and 3) Pm15, Pm20, Pm31, Pm35. Amplifications were performed (GeneAmp 2700 Thermocycler; Applied Biosystems) in a final volume of 10µl containing 2µl (±80 ng) of DNA, 5µl of 2X Qiagen Multiplex PCR Master Mix, 1µl of Q-solution (5X), 1µl of primer mix (2µM) and 1µl of ddH_2O. Amplifications comprised an initial denaturation at 95°C for 15 min, then 30 cycles at 94°C for 30 sec, 60°C for 90 sec, 72°C for 50 sec and a final elongation step at 60°C for 30 min completed the reactions. The PCR-products from the three multiplex reactions was combined and, together with a positive and negative control, genotyped in an ABI 3730 automatic sequencer (Applied Biosystems) using 0.2µl of GS500LIZ size standard (Applied Biosystems). Alleles were scored using GENEMAPPER v3.7 (Applied Biosystems).

Data analyses

Mitochondrial COI

Sequence data obtained for 127 individuals from 15 localities (see table 4.1) were aligned, edited and exported using GENEIOUS Pro™ v5.5.4 software (Biomatters Ltd; Drummond *et al.*, 2011). Standard genetic diversity indices including haplotype diversity (H_d, an indication of the number

and frequency of different haplotypes in a population) and nucleotide diversity (π, an indication of the levels of polymorphism in a population) were estimated in ARLEQUIN v3.5.1.2 (Excoffier & Lischer, 2010). The average level of nucleotide differentiation among populations characterising the eastern, northern and south-western sections of Marion Island was assessed by means of DNASP v5.1 (Librado & Rozas, 2009). To investigate the level of connectedness among haplotypes, a parsimony haplotype network with 95% connection limit, was constructed in TCS v1.21 (Clement *et al.*, 2000). Multiple possible connections among haplotypes were resolved by assuming a higher probability for haplotypes to connect to haplotypes on the inside of the network than to other tip haplotypes (Crandall & Templeton, 1993; Pfenninger & Posada, 2002). A visual representation of the haplotype diversity on Marion Island was done in PHYLOGEOVIZ v1.0 (Tsai, 2011), which constructs pie charts of haplotype distribution for each locality which was subsequently plotted onto a map of the island.

The spread of genetic variation across different hierarchical levels (AMOVA) was determined with ARLEQUIN. To obtain an indication of population differentiation, fixation indices (ϕ-statistics) were calculated for all populations combined as well as between populations in a pairwise manner. To correct for multiple comparisons, Bonferroni corrections were performed on all pairwise ϕ_{ST} p-values, with the significance value adjusted to 0.0059 (Rice, 1989). Population specific ϕ_{ST}-values were estimated using 5000 random permutations and pairwise ϕ_{ST} between populations using 1000 random permutations. To explore whether a spatial component is present in the overall genetic variation, SAMOVA v1.0 (Dupanloup *et al.*, 2002) was implemented. The aim of this analysis is to maximize ϕ_{CT} (the proportion of total genetic variance due to differences between groups of populations) which translates to the most likely spatial group structure for the sampled populations. Since SAMOVA results are sensitive to isolation-by-distance (IBD) when only one locus (in this case, the COI gene) is considered, a Mantel test (implemented in ARLEQUIN) was performed to determine whether there is a correlation between genetic and geographic distances between populations (Mantel, 1967). Calculations were performed at the scale of the whole island as well as for groups of populations from different sections of the island (north-east, south-east and south-west). The latter approach was implemented given that different patterns may characterize different parts of the island. Geographic distances between localities were estimated based on the most likely route of colonization for insects, which would be around the island along the lower planes as opposed to traveling over the high mountainous interior of the island with sparse and patchy vegetation cover.

Microsatellites

Twelve microsatellite loci were previously screened for deviations from Hardy-Weinberg equilibrium (HWE), linkage disequilibrium (LD) between loci and were tested for the presence of null alleles in *P. marioni* (see Chapter 3). Notwithstanding, genotypes derived from the 231 individuals taken from 16 sample sites across Marion Island (see table 4.1) were again screened for HWE (GENEPOP v3.4; Raymond & Rousset, 1995; Rousset, 2008) and for the presence of null alleles (MICROCHECKER; Brookfield, 1996; Van Oosterhout *et al.*, 2004) following Bonferonni corrections. To assess the level of the genetic diversity across loci, various basic statistics were estimated in ARLEQUIN. These include the number of alleles (N_A), allelic richness (A_R), observed heterozygosity (H_O) and expected heterozygosity (H_E). These statistics were obtained per locus for each of the populations. In addition, the average number of private alleles (N_P) over all loci was determined for each population (see table 4.4).

To quantify the informativeness of the microsatellite loci in detecting genetic structure among populations, a multivariate co-inertia analysis (MCOA; Laloë *et al.*, 2007; Berthouly *et al.*, 2008) was conducted in R v2.12 (Ihaka & Gentleman, 1996; R_Core_Development_Team, 2006) using the ADE-4 package (Chessel *et al.*, 2005). A reference topology is created from common information obtained from separate analyses conducted on allelic frequencies of the loci. The efficiency of a locus is determined by its topological value (Tv), which equates to the product of the variance (Var, variance of genetic diversity within each locus) and Cos^2 (congruence with the consensus). In other words, the Tv of a locus refers to the contribution of the marker to the reference topology.

The level of genetic structuring across Marion Island was assessed by means of two complementary methods: F_{ST}, as a measure of genetic differentiation among defined populations, and a Bayesian clustering method where no prior spatial information is provided (STRUCTURE, Pritchard *et al.*, 2000). F_{ST}-values were estimated between pairs of populations as well as for the populations combined (ARLEQUIN). To correct for multiple comparisons, Bonferroni corrections were performed on all pair-wise F_{ST} p-values, with the significance value adjusted to 0.0049 (Rice, 1989). In contrast to using predefined populations, the program STRUCTURE assigns individuals to clusters based on a set of allele frequencies at each locus. Each individual will have a percentage membership assigned to one or more cluster. Since the number of clusters (K) is unknown, the analysis were run with K-values ranging from 1 to 20 (burn-in = $1x10^6$, MCMC = $1x10^6$ permutations). To estimate the true number of clusters, calculations were done according to the method described in Evanno *et al.* (2005). The average membership of all the individuals collected

at a particular locality in each of the genetic clusters is visually represented on a map of Marion Island (PHYLOGEOVIZ).

Phylogeographic patterns of species may be misinterpreted if populations are characterized by clinal variation. To test for IBD, a Mantel test was performed (ARLEQUIN). Populations were grouped in the same manner as described for the COI data analyses namely at the scale of the whole island as well as for groups of populations from different sections of the island (north-east, south-east and south-west).

4.3 Results

Mitochondrial COI results

A 647 bp fragment (the barcoding fragment) of the COI gene was sequenced for 127 individuals taken from 15 localities. Twenty-six haplotypes were identified, with haplotype diversity for localities ranging from 0 (Goodhope Bay, Rook's Bay and Archway Bay) to 0.858 (Katedraalkrans) (see table 4.2). The overall estimate of haplotype diversity across all populations was 0.925 ± 0.011. Nucleotide diversities for the populations ranged between 0 and 0.008 (see table 4.2), with an average of 0.007 ± 0.0003 across all populations.

Table 4.2: Summary statistics for the COI data obtained from 15 localities on Marion Island.

Locality no. (as on sampling map, figure 4.3)	Sampling locality (N = sample size)	Number of Haplotypes	Haplotypes	Haplotype diversity, Hd (± SD)	Nucleotide diversity, π (± SD)
1	Long Ridge/Sea Elephant Bay (N=10)	3	Hap_3 Hap_8 Hap_23	0.511 (± 0.164)	0.003 (± 0.001)
2	Rockhopper Bay (N=13)	3	Hap_3 Hap_18 Hap_19	0.692 (± 0.075)	0.007 (± 0.002)
3	Boulders Beach (N=5)	2	Hap_4 Hap_5	0.600 (± 0.175)	0.002 (± 0.001)
4	Trypot Beach (N=5)	3	Hap_1 Hap_13 Hap_26	0.800 (± 0.164)	0.007 (± 0.002)
5	Archway Bay (N=5)	1	Hap_1	0.000 (± 0.000)	0.000 (± 0.000)
6, 7	Bullard Beach/Sealer's Cave (N=10)	4	Hap_1 Hap_6 Hap_7 Hap_22	0.800 (± 0.089)	0.006 (± 0.002)
8, 9	Kildalkey/Funk Bay (N=10)	3	Hap_7 Hap_10 Hap_11	0.622 (± 0.138)	0.003 (± 0.001)
10	Watertunnel (N=10)	2	Hap_11 Hap_13	0.356 (± 0.159)	0.001 (± 0.000)

Table 4.2 continued

Locality no. (as on sampling map, figure 4.3)	Sampling locality (n = number of individuals)	Number of Haplotypes	Haplotypes	Haplotype diversity, Hd (± SD)	Nucleotide diversity, π (± SD)
11	Goodhope Bay (N=5)	1	Hap_11	0.000 (± 0.000)	0.000 (± 0.000)
12	Rook's Bay (N=5)	1	Hap_21	0.000 (± 0.000)	0.000 (± 0.000)
13	Swartkop Point (N=10)	5	Hap_3 Hap_16 Hap_17 Hap_24 Hap_25	0.844 (± 0.080)	0.007 (± 0.002)
14, 15	Mixed Pickle Cove/Azorella kop (N=10)	5	Hap_2 Hap_3 Hap_5 Hap_16 Hap_17	0.844 (± 0.080)	0.008 (± 0.002)
17, 18	Cape Davis (N=10)	3	Hap_3 Hap_8 Hap_9	0.644 (± 0.101)	0.004 (± 0.001)
20	Prinsloomeer (N= 5)	3	Hap_3 Hap_8 Hap_20	0.800 (± 0.164)	0.006 (± 0.002)
21	Katedraalkrans (N= 14)	7	Hap_1 Hap_3 Hap_11 Hap_12 Hap_13 Hap_14 Hap_15	0.857 (± 0.065)	0.006 (± 0.001)

The largest portion of genetic variation lies within populations (~60%), with ~40% of the variation accounted for by the among population component (ϕ_{ST} = 0.396; $p < 0.001$). Pairwise ϕ_{ST}-values between sampling localities (see table 4.3) were mostly significant (in more than two-thirds of pair-wise comparisons). Populations that were not significantly different to more than two-thirds of the populations are Long Ridge / Sea Elephant Bay (locality 1), Rockhopper Bay (locality 2), Prinsloomeer (locality 20), Archway Bay (locality 5), Bullard Beach (locality 6/7), Mixed Pickle Cove (locality 15/16) and Katedraalkrans (locality 21). Interestingly, these localities are in the vicinity of proposed refugia or regions that remained ice-free during the last glacial cycle.

Table 4.3: Pairwise ϕ_{ST}-values separating localities (pairwise comparisons) with significant p-values, i.e. < 0.05, are indicated in bold (Bonferroni corrected significance are indicated with * for p-values < 0.0059, Rice 1989).

POPULATION	LR	RH	BB	TB	AB	BU	KB	WT	GB	RB	SP	MP	CD	PM	KT
Long Ridge/Sea Elephant Bay (LR)	0														
Rockhopper Bay (RH)	0.103	0													
Boulders Beach (BB)	**0.281**	0.149	0												
Trypot Beach (TB)	**0.252**	0.042	**0.363**	0											
Archway Bay (AB)	**0.317**	0.172	**0.700**	0.214	0										
Bullard Beach (BU)	**0.219***	**0.159**	**0.374***	-0.013	0.086	0									
Kildalkey/Funk Bay (KB)	**0.383***	**0.271***	**0.607***	0.138	**0.452***	0.111	0								
Watertunnel (WT)	**0.852***	**0.581***	**0.919***	**0.52941**	**0.958***	**0.630***	**0.746***	0							
Goodhope Bay (GB)	**0.816***	**0.493**	**0.914**	0.421	**1.000**	**0.535***	**0.662**	**0.710**	0						
Rook's Bay (RB)	**0.862***	**0.561***	**0.933**	**0.560**	**1.000**	**0.643***	**0.782***	**0.803***	**1.000**	0					
Swartkop Point (SP)	**0.632***	**0.427***	**0.644***	**0.308**	**0.627**	**0.466***	**0.533***	**0.405***	**0.403***	0.214	0				
Mixed Pickle Cove/Azorella kop (MP)	0.132	0.013	**0.167**	-0.019	**0.185**	0.131	**0.243**	**0.542***	**0.443**	**0.506**	**0.312**	0			
Cape Davis (CD)	0.097	**0.165**	**0.356**	0.179	**0.294**	**0.181**	**0.258**	**0.755***	**0.678***	**0.760***	**0.563***	**0.163**	0		
Prinsloomeer (PM)	0.025	0.046	**0.305**	-0.046	0.208	0.015	0.048	**0.713***	**0.587**	**0.712**	**0.444**	0.021	-0.023	0	
Katedraalkrans (KT)	**0.264**	0.106	**0.353**	-0.120	0.255	0.091	0.167	**0.368**	0.281	**0.450***	**0.323***	0.047	**0.228**	0.034	0

It is clear from the significant overall ϕ_{ST}-value as well as large portion of significant pairwise comparisons that there is genetic structuring across Marion Island. The spatial analyses of molecular variance (SAMOVA) indicated that variance among groups is maximized at three groups (ϕ_{CT} = 0.452; p < 0.001). These groups correspond to (i) the locality of Swartkop Point (locality 13), (ii) a southern group comprising Watertunnel (locality 10), Goodhope Bay (locality 11) and Rook's Bay (locality 12) and (iii) the remainder of the localities stretching from Mixed Pickle in the north-west (locality 15) clockwise to Kildalkey Bay in the south-east (locality 9). Genetic and geographic distances including all populations around the island were significantly correlated (regression coefficient (r) = 0.012, p = 0.015). The same result holds for sub-sets of populations on the north-eastern (r = 0.008, p = 0.037) and south-eastern (r = 0.031, p = 0.001) sides of the island. Populations in the south-west did not show signs of IBD (r = 0.016, p = 0.318).

The relationships among the 26 haplotypes retrieved for Marion Island is shown in figure 4.4. A high number of private haplotypes was found in localities on the western side of the island as well as for the locality of Katedraalkrans (locality 21, see table 4.2); this signature is typical for refugial sites. Most of the haplotypes are connected by a single mutational step with only a few unsampled or missing haplotypes. The most frequent haplotype (Hap 3) characterizes 19% of the individuals. This haplotype forms the central point in a star-like haplotype network pattern; typical of a population expansion. The high haplotype and comparatively low nucleotide diversities further suggest demographic change over time. As pointed out earlier, high levels of haplotype sharing was detected for the Katedraalkrans population which is characteristic of a refugial (source) population.

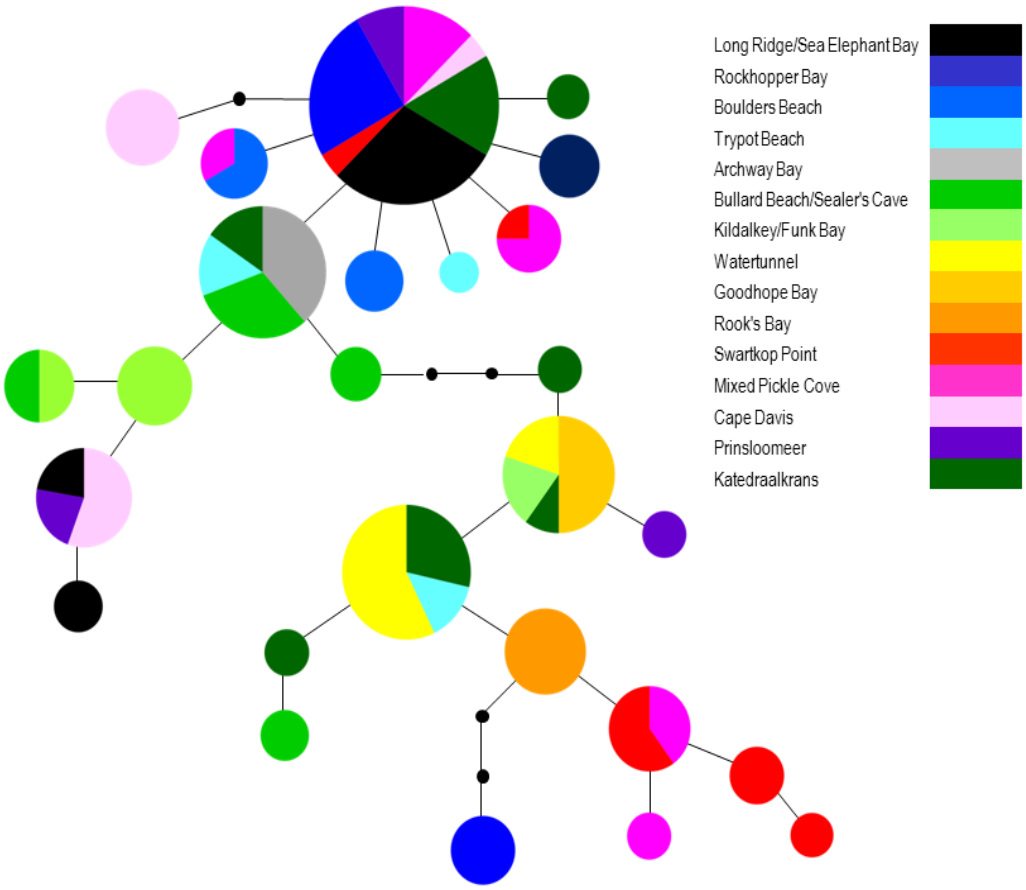

Figure 4.4: Haplotype network for COI data from Marion Island's *P. marioni* populations represented by 15 sampled localities (colour-coded). Missing or unsampled haplotypes are indicated by small black filled circles.

The distribution of haplotypes across Marion Island is indicated in figure 4.5. It is clear from this map that certain localities are genetically more diverse that others (indicated by the number of haplotypes detected). For example, localities in the south (i.e. Rook's and Goodhope Bay) are characterized by very few haplotypes whereas areas such as Katedraalkrans (locality 21) and western localities of Swartkop Point (locality 13) and Mixed Pickle Cove (locality 15/16) had a higher number of haplotypes. Haplotypes from Katedraalkrans are spread throughout most of the network and are the most represented in a number of populations.

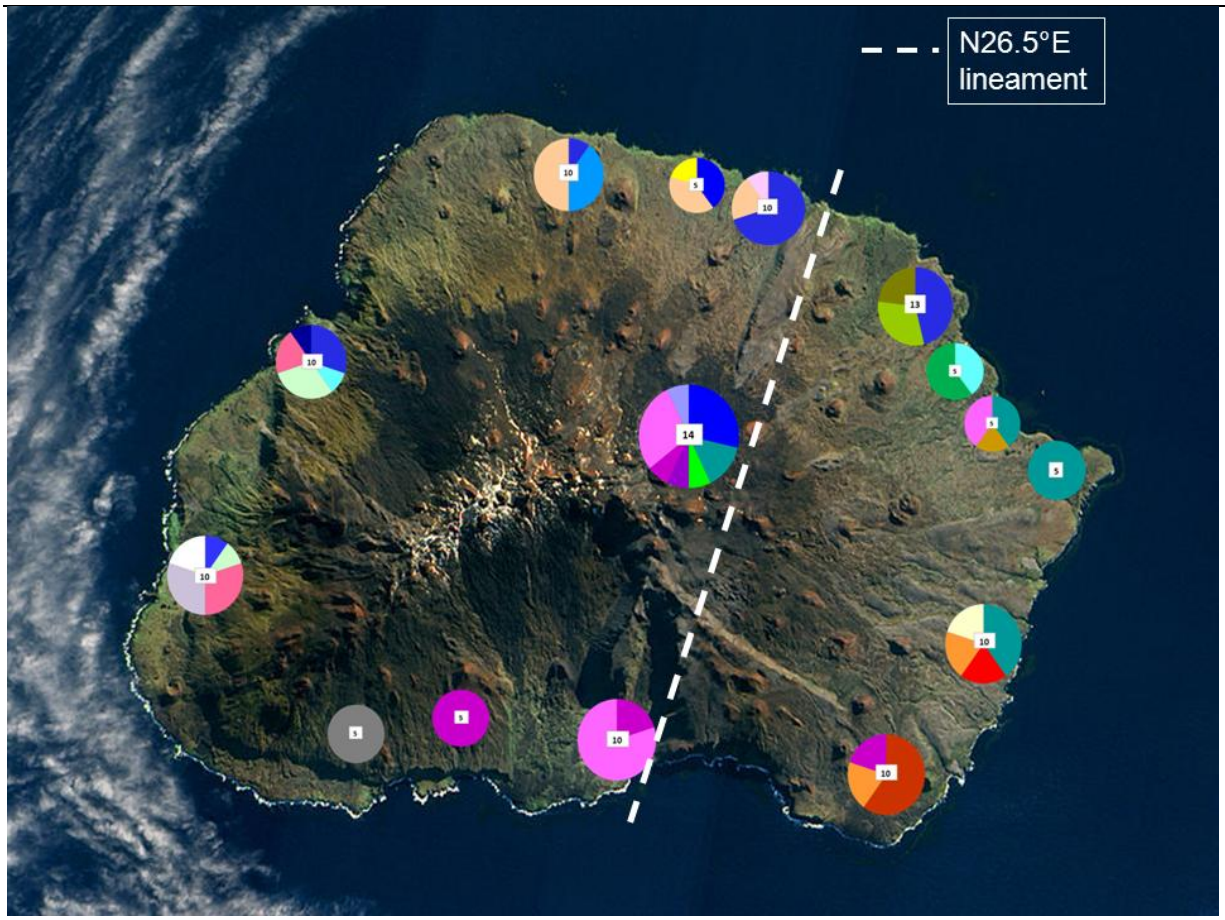

Figure 4.5: Distribution of COI haplotypes on Marion Island from 15 sampled localities. Pie charts are indicating the contribution of each haplotype to the population, with the number of individuals indicated in the centre.

Microsatellite results

Several of the microsatellite markers were susceptible to null alleles. Although unfortunate, the presence of null alleles seem almost the norm for Lepidopteran species (see e.g. Meglecz *et al.*, 2004 and Chapuis & Estoup, 2007). However, only three of the twelve markers had a high probability for null alleles with frequencies of 0.21, 0.24 and 0.16 for markers Pm05, Pm06 and Pm15 respectively (if we accept the frequency of 0.2 as indicative of null alleles; following Brookfield, 1996; Van Oosterhout *et al.*, 2004). The high null allele frequencies for these markers are due to the excess of homozygote genotypes and since some of the alleles are one repeat unit apart, stuttering may further add to scoring errors. Six other markers (Pm01, Pm16, Pm20, Pm23, Pm30 and Pm31) indicated an excess of homozygotes but at very low frequencies (ranging from 0.037 – 0.0752). The three remaining markers (Pm04, Pm14 and Pm35) did not show any evidence for homozygote excess (frequencies of 0.0163 and less).

Due to the occurrence of a deficit in heterozygote genotypes in three of the loci (Pm05, Pm06 and Pm15), the populations were not expected to be in HWE and this was confirmed by global Hardy Weinberg exact tests (GENEPOP results). All of the populations, except Boulders Beach (locality 3), Bullard Beach (locality 6) and Watertunnel (locality 10) deviated from HWE. However, failure of loci to comply with HWE expectations (random mating and no mutation, drift or migration) is not a sufficient reason for discarding loci (Selkoe & Toonen, 2006) as they may contribute important information about population processes. The distribution of null alleles was not confined to specific localities, but occurred equally across all populations (as evidenced from observed and expected heterozygosity values). Also, the efficiency of markers Pm05 and Pm15 to contribute to the overall topology was high (>30% and ~20% respectively, see figure 4.6) with Pm06 (~7%) being average. The analyses for this study was based on all markers included and results were confirmed by repeating analyses excluding these three loci susceptible to null alleles and no significant difference was seen.

Overall, the loci displayed acceptable levels of variation (see table 4.4) with the number of alleles varying between 32 and 47 per population. Average heterozygosity between the 12 markers varied between 0.242 (Bullard Beach, locality 6) and 0.424 (Swartkop Point, locality 13). Similarly, the highest allelic richness was detected for Swartkop Point (1.443). A high number of private alleles were detected in the Katedraalkrans (locality 21) and Kildalkey Bay (locality 8/9) populations.

Table 4.4: Summary statistics for 12 microsatellite genotypes from 16 localities.

POPULATION		LOCUS												Average over all loci
		Pm01	**Pm04**	**Pm05**	**Pm06**	**Pm14**	**Pm15**	**Pm16**	**Pm20**	**Pm23**	**Pm30**	**Pm31**	**Pm35**	
Long Ridge / Sea Elephant Bay	N	12	12	12	12	12	4	12	12	12	12	12	12	11.333
(N_p = 1)	N_A	5.000	2.000	2.000	4.000	1.000	1.000	4.000	6.000	6.000	3.000	4.000	1.000	3.250
	A_R	1.638	1.159	1.083	1.634	1.000	1.000	1.373	1.775	1.743	1.467	1.703	1.000	1.381
	H_O	0.583	0.000	0.083	0.083	0.000	0.000	0.417	0.583	0.583	0.583	0.417	0.000	0.278
	H_E	0.611	0.153	0.080	0.608	0.000	0.000	0.358	0.743	0.712	0.448	0.674	0.000	0.365
Boulders Beach	N	12	12	12	12	12	11	12	12	12	12	12	12	11.917
(N_p = 1)	N_A	4.000	2.000	2.000	3.000	1.000	1.000	1.000	4.000	2.000	2.000	4.000	2.000	2.333
	A_R	1.525	1.159	1.507	1.163	1.000	1.000	1.000	1.699	1.431	1.159	1.707	1.159	1.292
	H_O	0.333	0.167	0.167	0.167	0.000	0.000	0.000	0.750	0.417	0.167	0.833	0.167	0.264
	H_E	0.503	0.153	0.486	0.156	0.000	0.000	0.000	0.670	0.413	0.153	0.677	0.153	0.280
Trypot Beach	N	12	12	12	12	12	7	12	12	12	12	12	12	11.583
(N_p = 2)	N_A	4.000	3.000	2.000	2.000	1.000	1.000	4.000	3.000	6.000	2.000	4.000	1.000	2.750
	A_R	1.370	1.304	1.290	1.290	1.000	1.000	1.308	1.554	1.801	1.159	1.482	1.000	1.297
	H_O	0.417	0.333	0.000	0.000	0.000	0.000	0.333	0.500	0.583	0.167	0.500	0.000	0.236
	H_E	0.354	0.292	0.278	0.278	0.000	0.000	0.295	0.531	0.767	0.153	0.462	0.000	0.284
Archway Bay	N	12	12	12	12	12	10	12	12	12	12	12	12	11.833
(N_p = 3)	N_A	3.000	3.000	3.000	2.000	2.000	2.000	2.000	4.000	7.000	2.000	4.000	1.000	2.917
	A_R	1.236	1.236	1.301	1.344	1.083	1.337	1.228	1.511	1.797	1.228	1.533	1.000	1.320
	H_O	0.250	0.250	0.167	0.083	0.083	0.000	0.250	0.500	0.750	0.250	0.500	0.000	0.257
	H_E	0.226	0.226	0.288	0.330	0.080	0.320	0.219	0.490	0.764	0.219	0.510	0.000	0.306
Bullard Beach	N	8	8	8	8	8	4	8	8	8	8	8	8	7.667
(N_p = 1)	N_A	3.000	2.000	1.000	2.000	2.000	1.000	3.000	2.000	4.000	1.000	3.000	1.000	2.083
	A_R	1.242	1.125	1.000	1.233	1.125	1.000	1.608	1.458	1.642	1.000	1.658	1.000	1.258
	H_O	0.250	0.125	0.000	0.000	0.125	0.000	0.750	0.125	0.750	0.000	0.750	0.000	0.240
	H_E	0.227	0.117	0.000	0.219	0.117	0.000	0.570	0.430	0.602	0.000	0.617	0.000	0.242

Table 4.4 continued

POPULATION		LOCUS Pm01	Pm04	Pm05	Pm06	Pm14	Pm15	Pm16	Pm20	Pm23	Pm30	Pm31	Pm35	Average over all loci
Sealer's Cave	N	8	8	8	8	8	5	8	8	8	8	8	8	7.750
(N_p = 2)	N_A	2.000	1.000	3.000	2.000	2.000	3.000	2.000	5.000	5.000	2.000	3.000	2.000	2.667
	A_R	1.125	1.000	1.575	1.400	1.125	1.689	1.125	1.667	1.817	1.125	1.542	1.125	1.360
	H_O	0.125	0.000	0.375	0.000	0.125	0.200	0.125	0.875	0.625	0.125	0.500	0.125	0.267
	H_E	0.117	0.000	0.539	0.375	0.117	0.620	0.117	0.625	0.766	0.117	0.508	0.117	0.335
Kildalkey Bay / Funk Bay	N	20	20	20	19	19	17	18	20	19	20	20	20	19.333
(N_p = 6)	N_A	5.000	1.000	4.000	4.000	1.000	3.000	6.000	6.000	8.000	3.000	4.000	2.000	3.917
	A_R	1.429	1.000	1.433	1.734	1.000	1.314	1.517	1.663	1.802	1.099	1.568	1.185	1.395
	H_O	0.300	0.000	0.400	0.211	0.000	0.000	0.500	0.550	0.737	0.100	0.400	0.200	0.283
	H_E	0.419	0.000	0.423	0.715	0.000	0.304	0.503	0.646	0.781	0.096	0.554	0.180	0.385
Watertunnel	N	11	11	11	11	11	9	11	11	11	11	11	11	10.833
(N_p = 3)	N_A	5.000	1.000	2.000	2.000	1.000	3.000	3.000	5.000	6.000	2.000	6.000	3.000	3.250
	A_R	1.519	1.000	1.485	1.173	1.000	1.569	1.255	1.762	1.827	1.173	1.745	1.255	1.397
	H_O	0.455	0.000	0.182	0.182	0.000	0.333	0.091	0.818	0.909	0.182	0.818	0.273	0.354
	H_E	0.496	0.000	0.463	0.165	0.000	0.537	0.244	0.727	0.789	0.165	0.711	0.244	0.378
Goodhope Bay	N	12	12	12	12	12	12	11	10	12	12	12	12	11.750
(N_p = 1)	N_A	2.000	1.000	2.000	3.000	1.000	4.000	2.000	5.000	7.000	2.000	3.000	2.000	2.833
	A_R	1.159	1.000	1.159	1.594	1.000	1.649	1.368	1.758	1.826	1.083	1.540	1.228	1.364
	H_O	0.167	0.000	0.000	0.583	0.000	0.500	0.455	0.500	0.750	0.083	0.417	0.250	0.309
	H_E	0.153	0.000	0.153	0.569	0.000	0.622	0.351	0.720	0.792	0.080	0.517	0.219	0.348
Rook's Bay	N	18	18	18	18	18	16	19	17	19	19	18	19	18.083
(N_p = 3)	N_A	6.000	1.000	2.000	4.000	1.000	2.000	1.000	4.000	7.000	1.000	4.000	3.000	3.000
	A_R	1.435	1.000	1.386	1.543	1.000	1.498	1.000	1.629	1.811	1.000	1.652	1.432	1.366
	H_O	0.389	0.000	0.056	0.222	0.000	0.188	0.000	0.706	0.789	0.000	0.611	0.526	0.291
	H_E	0.423	0.000	0.375	0.528	0.000	0.482	0.000	0.611	0.789	0.000	0.634	0.421	0.355
Swartkop Point	N	12	12	12	12	12	12	12	12	12	12	12	12	12.000
(N_p = 3)	N_A	4.000	2.000	2.000	5.000	1.000	4.000	4.000	5.000	7.000	2.000	3.000	2.000	3.417
	A_R	1.612	1.083	1.228	1.670	1.000	1.605	1.533	1.656	1.819	1.391	1.554	1.159	1.443
	H_O	0.333	0.083	0.250	0.333	0.000	0.500	0.500	0.667	0.833	0.500	0.583	0.000	0.382
	H_E	0.587	0.080	0.219	0.642	0.000	0.580	0.510	0.628	0.785	0.375	0.531	0.153	0.424

Table 4.4 continued

POPULATION		LOCUS Pm01	Pm04	Pm05	Pm06	Pm14	Pm15	Pm16	Pm20	Pm23	Pm30	Pm31	Pm35	Average over all loci
Mixed Pickle Cove /	N	22	22	22	22	22	19	22	22	22	22	22	22	21.750
Azorella kop / Neville	N_A	2.000	2.000	3.000	3.000	1.000	2.000	4.000	5.000	6.000	2.000	4.000	1.000	2.917
(N_p = 1)	A_R	1.045	1.333	1.394	1.354	1.000	1.508	1.539	1.720	1.748	1.045	1.544	1.000	1.353
	H_O	0.045	0.409	0.045	0.045	0.000	0.684	0.455	0.818	0.545	0.045	0.318	0.000	0.284
	H_E	0.044	0.325	0.385	0.346	0.000	0.494	0.527	0.704	0.731	0.044	0.532	0.000	0.344
Cape Davis / Tweeling	N	20	20	20	20	19	19	20	20	20	20	20	20	19.833
(N_p = 1)	N_A	3.000	1.000	2.000	4.000	1.000	1.000	4.000	5.000	7.000	3.000	2.000	3.000	3.000
	A_R	1.440	1.000	1.185	1.383	1.000	1.000	1.387	1.788	1.756	1.445	1.450	1.488	1.360
	H_O	0.450	0.000	0.000	0.050	0.000	0.000	0.350	0.450	0.750	0.350	0.350	0.400	0.263
	H_E	0.429	0.000	0.180	0.374	0.000	0.000	0.378	0.769	0.738	0.434	0.439	0.476	0.351
Repetto's	N	28	28	28	28	28	16	28	28	28	28	28	28	27.000
(N_p = 2)	N_A	5.000	4.000	2.000	5.000	1.000	1.000	3.000	5.000	9.000	4.000	4.000	2.000	3.750
	A_R	1.320	1.232	1.382	1.613	1.000	1.000	1.350	1.736	1.856	1.571	1.745	1.070	1.406
	H_O	0.321	0.250	0.000	0.321	0.000	0.000	0.357	0.607	0.893	0.500	0.786	0.071	0.342
	H_E	0.314	0.228	0.375	0.602	0.000	0.000	0.344	0.723	0.841	0.561	0.732	0.069	0.399
Prinsloomeer	N	9	9	9	9	9	6	9	9	9	9	9	9	8.750
(N_p = 2)	N_A	3.000	3.000	2.000	3.000	1.000	1.000	2.000	5.000	5.000	2.000	5.000	3.000	2.917
	A_R	1.216	1.503	1.209	1.680	1.000	1.000	1.471	1.614	1.484	1.425	1.712	1.216	1.378
	H_O	0.222	0.333	0.000	0.222	0.000	0.000	0.444	0.667	0.556	0.111	0.556	0.222	0.278
	H_E	0.204	0.475	0.198	0.642	0.000	0.000	0.444	0.580	0.457	0.401	0.673	0.204	0.356
Katedraalkrans	N	14	14	14	14	14	1	14	14	14	14	14	14	12.917
(N_p = 5)	N_A	6.000	3.000	4.000	4.000	1.000	1.000	5.000	4.000	6.000	3.000	5.000	2.000	3.667
	A_R	1.627	1.362	1.563	1.323	1.000	1.000	1.667	1.563	1.788	1.421	1.328	1.138	1.398
	H_O	0.500	0.286	0.214	0.214	0.000	0.000	0.571	0.571	0.857	0.286	0.357	0.143	0.333
	H_E	0.605	0.349	0.543	0.311	0.000	0.000	0.643	0.543	0.760	0.406	0.316	0.133	0.384
Average over all populations	A_R	1.371	1.156	1.324	1.446	1.021	1.261	1.358	1.660	1.747	1.237	1.591	1.153	
	H_O	0.321	0.140	0.121	0.170	0.021	0.150	0.350	0.605	0.708	0.216	0.543	0.149	
	H_E	0.357	0.150	0.312	0.429	0.020	0.247	0.344	0.634	0.718	0.228	0.568	0.148	
Total over all populations	N	230	230	230	229	228	168	228	227	230	231	230	231	
	N_A	16	5	5	10	3	7	8	12	16	5	15	5	

N = sample size; N_A = number of alleles; A_R = allelic richness; H_O = observed heterozygosity; H_E = expected heterozygosity and N_P = number of private alleles (in parenthesis).

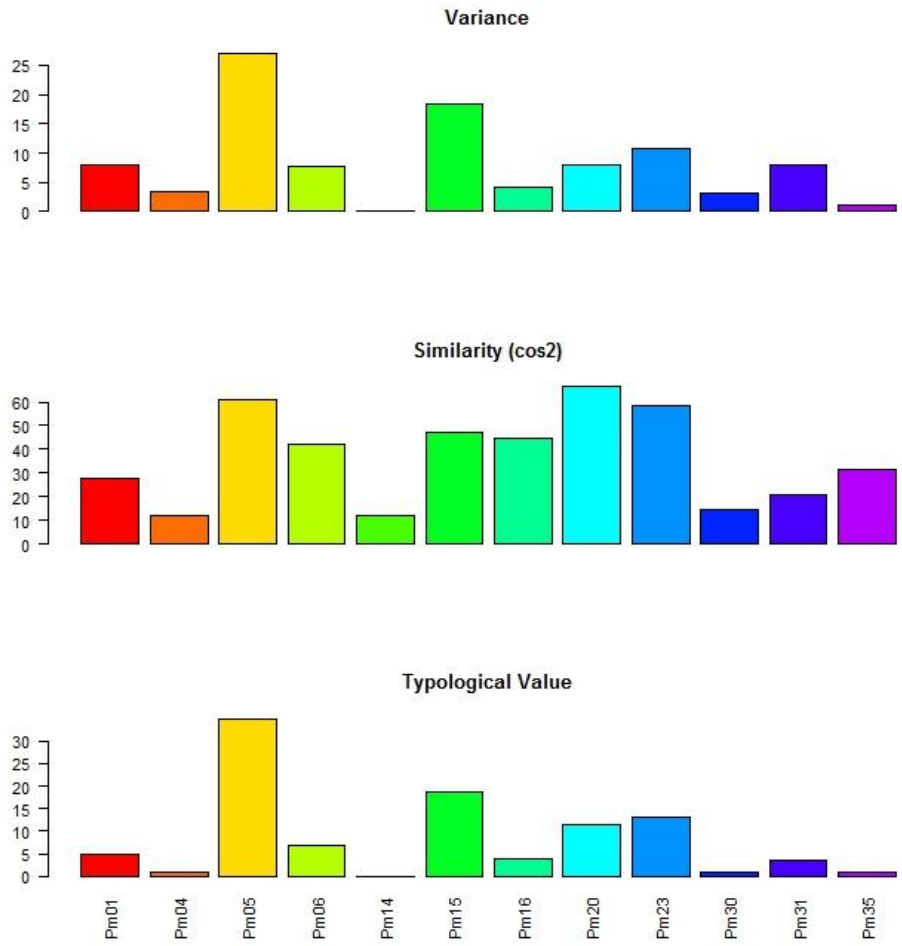

Figure 4.6: Distribution of values for components (percentages) related to efficiency among loci.

The largest percentage of the variation (averaged over the 12 loci) was found within populations (~88%), with the remainder (~12%) of the variation among populations. The overall fixation index for Marion Island (F_{ST} = 0.125; $p < 0.001$) indicated population structure / subdivision. Pairwise F_{ST}-values (table 4.5) were significant for virtually all the comparisons. Exceptions include the localities of Archway Bay (locality 5), Trypot Beach (locality 4) and Sealer's Cave (locality 7).

Table 4.5: Pairwise F_{ST}-values separating localities (pairwise comparisons) with significant p-values, i.e. < 0.05, are indicated in bold (Bonferroni corrected significance are indicated with * for p-values < 0.0049, Rice 1989).

POPULATION	LR	BB	TB	AB	BU	SC	KB	WT	GB	RB	SP	MP	CD	RP	PM	KT
Long Ridge (LR)	0															
Boulders Beach (BB)	**0.140***	0														
Trypot Beach (TB)	**0.125***	**0.050**	0													
Archway Bay (AB)	**0.133***	**0.117***	0.014	0												
Bullard Beach (BU)	**0.148***	**0.204***	**0.098***	**0.109***	0											
Sealer's Cave (SC)	**0.119***	**0.091***	0.043	0.036	**0.138***	0										
Kildalkey Bay (KB)	**0.095***	**0.149***	**0.093***	**0.108***	**0.146***	**0.056**	0									
Watertunnel (WT)	**0.104***	**0.094***	**0.092***	**0.129***	**0.190***	**0.059**	**0.068***	0								
Goodhope Bay (GB)	**0.231***	**0.199***	**0.214***	**0.224***	**0.292***	**0.077**	**0.148***	**0.149***	0							
Rook's Bay (RB)	**0.106***	**0.107***	**0.101***	**0.134***	**0.208***	**0.052**	**0.091***	**0.062**	**0.131***	0						
Swartkop Point (SP)	**0.061***	**0.115***	**0.062***	**0.091***	**0.130***	**0.064**	**0.040**	**0.074***	**0.181***	**0.064***	0					
Mixed Pickle Cove (MP)	**0.141***	**0.162***	**0.142***	**0.141***	**0.178***	**0.100**	**0.111***	**0.103***	**0.197***	**0.133***	**0.117***	0				
Cape Davis (CD)	**0.084***	**0.135***	**0.087***	**0.091***	**0.150***	**0.088***	**0.092***	**0.092***	**0.221***	**0.108***	**0.068***	**0.141***	0			
Repetto's (RP)	**0.049**	**0.143***	**0.123***	**0.113***	**0.174***	**0.101***	**0.089***	**0.118***	**0.167***	**0.106***	**0.086***	**0.127***	**0.085***	0		
Prinsloomeer (PM)	**0.082***	**0.159***	**0.125***	**0.129***	**0.150***	**0.145***	**0.133***	**0.168***	**0.246***	**0.170***	**0.126***	**0.142***	**0.118***	**0.055**	0	
Katedraalkrans (KT)	**0.185***	**0.149***	**0.118***	**0.153***	**0.217***	**0.112***	**0.130***	**0.132***	**0.164***	**0.170***	**0.102***	**0.218***	**0.152***	**0.175***	**0.227***	0

As was the case for the mitochondrial data, variation is optimally partitioned into three groups (following STRUCTURE analyses and implementing the method of Evanno *et al*. (2005)). These groups include localities from (i) the south-west and central portion of the island, (ii) the northern part and (iii) the eastern part of the island (see figure 4.7). However, very few individuals had 100% membership to any specific group, indicating some admixture (gene flow) across the island. The structure around the N26.5°E geological lineament is indicated on a map of Marion Island with pie charts representing the membership of the sampled individuals (see figure 4.8). Mantel tests revealed no significant correlation between genetic and geographic distances at a whole island level ($r = 0.002$, $p = 0.074$) as well as for populations on the south-western side ($r = 0.010$, $p = 0.09$) of Marion Island. Populations on the eastern section of the island, however, did indicate a possible IBD signature (north-east: $r = 0.004$, $p = 0.026$ and south-east: $r = 0.007$, $p = 0.004$).

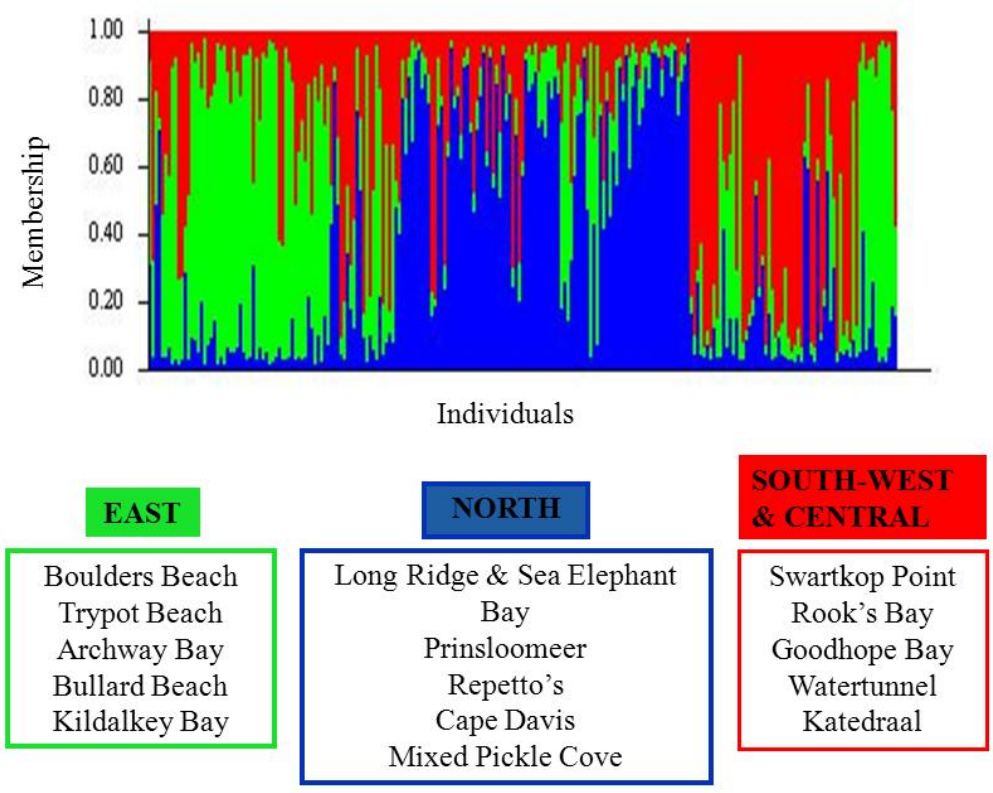

Figure 4.7: Membership of each individual from the 16 localities in the 3-group structure (STRUCTURE, Evanno *et al.*, 2005).

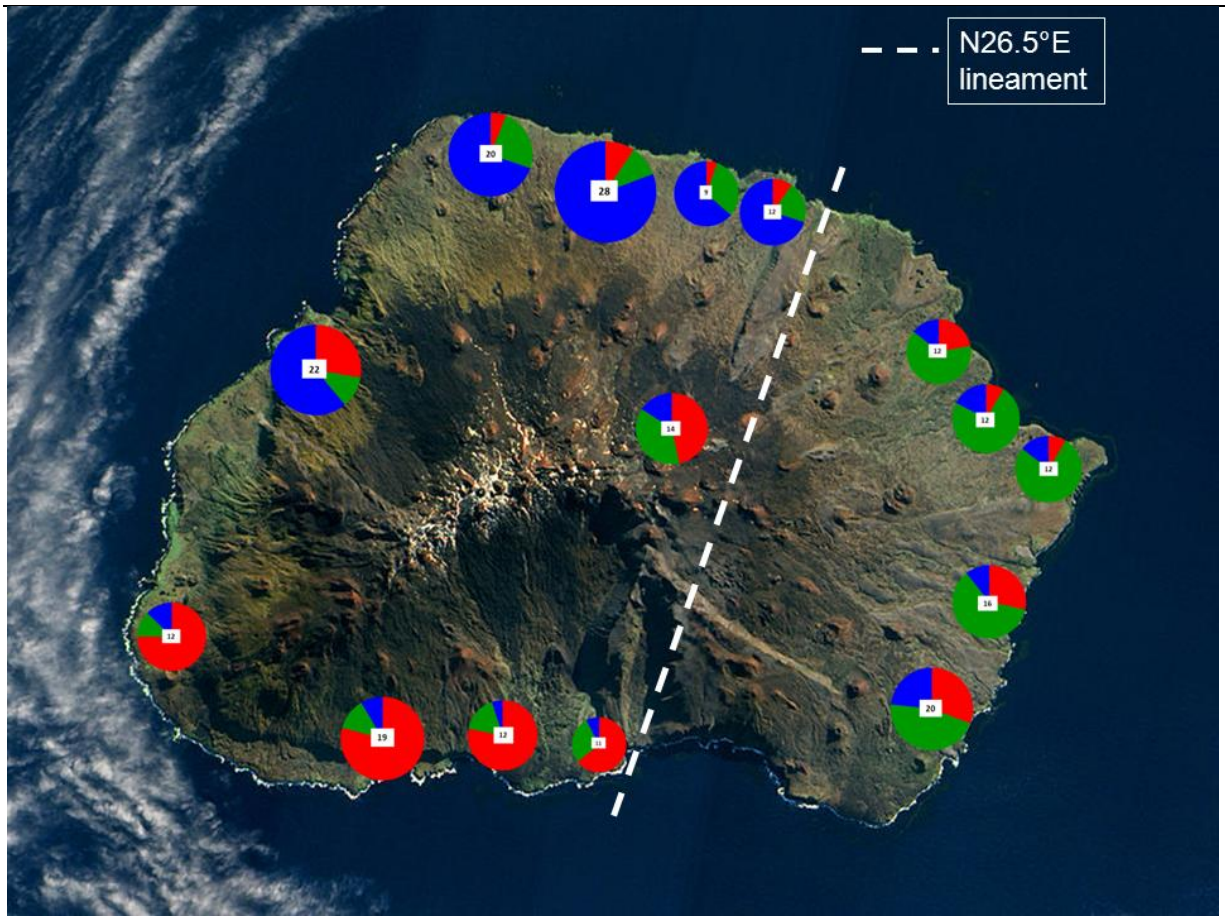

Figure 4.8: Structure of three genetic groups (red, blue and green) on Marion Island. Pie charts indicate the genetic membership of each population to the three groups.

4.4 Discussion

Patterns of genetic diversity as seen today are shaped by the relative contributions of selection (adaptation) and drift, which are affected by landscape, climate and history (Storfer *et al.*, 2010). Specifically relating to Southern Ocean islands, the interplay amongst these factors is driving complex genetic patterns (see e.g. Mortimer *et al.*, 2012). Studies on other islands systems such as the Galápagos, Hawaiian and Canary islands have demonstrated insight into both geological and phylogeographic history, emphasizing the effect that landscape or historic events has had on species' population structure (Beheregaray *et al*. 2003; Gillespie 2004; Emerson *et al*. 2006). To this, the flightless moth on Marion Island is no exception with signatures of refugia, fragmentation, and possibly local adaptation which could be a response to a combination of historical and contemporary environmental variability. The inclusion of both mitochondrial and microsatellite data allowed finer resolution of genetic patterns with several interesting findings which both confirm and extend previous hypotheses.

Molecular diversity

The relative number of haplotypes detected for *P. marioni* was consistently less than for any of the other endemic arthropods studied on Marion Island. Previous studies on endemic mites (*Eupodes minutus*, *Halozetes fulvus*), springtails (*Cryptopygus antarcticus*, *Tullbergia bisetosa*) and a weevil (*Bothrometopus huntleyi*) displayed haplotype counts of more than 15% than reported for *P. marioni* with *B. huntleyi*, in particular, being exceptionally diverse (Mortimer & Jansen van Vuuren, 2007; Myburgh *et al.*, 2007; McGaughran *et al.*, 2010a; Grobler *et al.*, 2011b; Mortimer *et al.*, 2012). Importantly, however, arthopods such as *H. fulvus* can reach population densities of up to ~700 individuals per m^2 (Barendse & Chown, 2001), whereas *P. marioni*'s biomass are estimated at ~1.19 g/m^2 in manured *P. cookii* grassland vegetation (Crafford & Scholtz, 1987). Based on an average caterpillar mass of ~ 0.10 g (data obtained from caterpillars collected for this project), this biomass estimate would correspond to a density of roughly 12 individuals per m^2. This significant difference in density may explain the comparatively lower observed genetic diversity in *P. marioni*.

When accounting for the number of individuals, haplotype and nucleotide diversity estimates for the island are in keeping with values obtained for other arthropods, with haplotype diversities being generally >0.8 and nucleotide diversities <0.007 (for mitochondrial COI comparison see e.g. Goodall-Copestake *et al*. 2012). However, haplotype diversities of individual populations were intermediate and generally lower than for other arthropods, with individuals from three localities (Archway Bay, Goodhope Bay and Rook's Bay) represented by only one haplotype for each of the populations. Low levels of genetic diversity in the southern region of the island might be attributed to extensive volcanism experienced by this region (Santa Rosa Valley being a significant black lava feature) which could have led to population subdivision/extinction and subsequent loss of genetic diversity.

Genetic variation based on microsatellite markers are in line with values reported for other Lepidopteran species such as the pine processionary moth, *Thaumetopoea wilkinsoni* Tams (Lepidoptera: Notodontidae) (Simonato *et al.*, 2007). Since this study is the first study utilizing microsatellite data for inferring population structure of terrestrial invertebrates from the sub-Antarctic, a comparison of microsatellite variation among arthropods from a similar environment was not possible.

Certain localities across Marion Island displayed high levels of genetic diversity for either, or both, mitochondrial and nuclear markers. These localities include Swartkop Point, Katedraalkrans, Long Ridge (Prinsloomeer and Repetto's in close proximity) and Kildalkey Bay. Several of these

localities, such as Swartkop Point and Kildalkey Bay, have also been singled out in previous studies for being highly diverse and significantly differentiated from other populations (see results for *E. minutus* and *C. antarcticus* populations; Mortimer & Jansen van Vuuren, 2007; Myburgh *et al.*, 2007; McGaughran *et al.*, 2010a).

Demographic changes – result of recolonization from glacial refugia?

Demographic changes are evident for the species in terms of their diversity indices, but also in the way the haplotypes link in the mitochondrial COI network. The high haplotype and low nucleotide diversities can be indicative of a population expansion, which is supported by a star-like pattern in a section of the haplotype network. The network is in fact characterized by two demographic signatures. The first is the star-like pattern where 24 individuals share the major haplotype with a few haplotypes separated from the main haplotype by only one or two mutational differences. The second part of the network reveals a more stable population pattern where there are more nucleotide differences separating haplotypes and fewer shared haplotypes. Most of the shared haplotypes in this section are in the same geographic vicinity namely localities of Trypot, Archway Bay and Bullard Beach; Cape Davis, Prinsloomeer and Long Ridge; Kildalkey, Watertunnel and Goodhope Bay as well as Swartkop Point and Mixed Pickle Cove. To date, all haplotype networks constructed for other arthropod taxa on Marion Island also suggest demographic changes due to the typical star-like pattern and high haplotype and low nucleotide diversity indices. Population expansion signatures are to be expected given the glacial history of Marion Island where species would have been confined to glacial refugia during the LGM. Molecular studies continue to report ages for taxa predating recent glacial maxima (see e.g. Nolan *et al.*, 2006; Stevens *et al.*, 2006; De Wever *et al.*, 2009; McGaughran *et al.*, 2010b; Mortimer *et al.*, 2011). Although these findings were at first controversial, the importance of Pleistocene glacial refugia has become generally accepted as it explains the persistence of populations through historical glacial cycles (Stevens *et al.*, 2007) and unites the ages of taxa based on molecular studies with glacial histories (see e.g. Myburgh *et al.*, 2007).

Glacial reconstructions based on extensive geological evidence (see figure 4.2) suggests that Marion Island was almost entirely glaciated, except for a few coastal areas and highly elevated grey-lava outcrops (Hall, 2002; Hall *et al.*, 2011; Mortimer *et al.*, 2012). Previous genetic studies have hinted at the presence of refugia on Marion Island (Myburgh *et al.*, 2007; McGaughran *et al.*, 2010a; Mortimer *et al.*, 2012). For springtails such as *C. antarcticus* and *T. Bisetosa* refugia possibly included Katedraalkrans due to its high level of intra-population variability (Myburgh *et al.*, 2007; McGaughran *et al.*, 2010a) and also due to haplotype sharing with other localities

(McGaughran *et al.*, 2010a). Divergent haplotypes were also found for *H. fulvus* in several coastal and high-altitude (not including Katedraalkrans due to low sample size) localities that are presumed to have been ice-free during the LGM (Mortimer *et al.*, 2012). These results are to be expected of a refuge population as these areas essentially act as a source for recolonization and one would therefore expect high genetic diversity with little to no differentiation from other localities (Jansen van Vuuren & Robinson, 1997; Hewitt, 2000).

Several lines of evidence from the flightless moth support the presence of refugia which correspond to proposed ice-free sections. First, although the majority of localities are significantly differentiated from others based on pairwise comparisons, a few localities show little differentiation. These include the high altitude locality of Katedraalkrans, localities in the vicinity of Long Ridge (specifically Rockhopper Bay and Prinsloomeer) as well as coastal localities in the north-western side (around Mixed Pickle Bay) and south-eastern side (around Bullard Beach and Kildalkey Bay) of Marion Island. Also, these areas are typical of population persistence since they are characterized by high haplotype diversity with high numbers of private haplotypes and private alleles. Katedraalkrans, in particular, presented the largest number of haplotypes which are shared with 12 other populations and are well represented in the haplotype network.

Population structure including the N26.5°E geological lineament

A consistent finding of genetic studies on various species occurring on Marion Island to date is population fragmentation. This is not surprising given the complex geological and climatic history of the island (Verwoerd *et al.*, 1981; McDougall *et al.*, 2001; Meiklejohn & Hedding, 2005). In addition, the effects of habitat fragmentation may be more emphasized in organisms such as arthropods with limited dispersal capabilities (Marshall & Coetzee, 2000). The flightless moth conforms to previously described patterns with significant structure shown based on both mitochondrial and nuclear data. For the mitochondrial DNA, three genetic groups are retrieved which correspond to populations on the southern part of the island (to the west of the southern section of the N26.5°E lineament), the single locality of Swartkop Point and the remainder of the localities. SAMOVA analyses obtained no significant structuring across the northern section of the lineament recently described by Mortimer *et al*. (2012). However, based on pairwise ϕ_{ST}-values Long Ridge and Sea Elephant Bay individuals (to the west of the lineament) was significantly differentiated from populations on the east of the lineament (with the exception of Rockhopper Bay).

Although somewhat congruent, analyses of the microsatellite data uncovered patterns largely shaped by the lineament with three groups which correspond to localities on the southern side of the island (to the west of the lineament, including Swartkop point), localities on the north-western side of the island (also to the west of the lineament) and a third group comprising localities to the east of the lineament (see figure 4.8).

Genetic clusters can easily be biased by the presence of IBD in the data set. Specifically, the genetic structure that results from IBD is not the result of geological or climatic factors but rather driven by the biology of the species (dispersal distance). Mantel tests on the mitochondrial data revealed significant IBD on a whole-island level; however, microsatellite data did not support IBD across the populations. Since *P. marioni* is predominantly a male-dispersive species, with females mostly sedentary, it might be expected that the COI gene (female inherited) would be more susceptible to an IBD pattern as opposed to microsatellite loci (bi-parentally inherited).

East-west population differentiation

Previous studies have reported different evolutionary patterns for the western and eastern side of the island, driven in part by different climatic and volcanic histories. Specifically, recent population divergences with high gene flow were suggested for the western side compared to a more stable pattern with IBD for the east. Also, strong to gale-force westerly winds are typical on the western side for most of the year (Le Roux, 2008; Le Roux & McGeoch, 2008) with more recent and frequent volcanic eruptions (Verwoerd *et al.*, 1981; McDougall *et al.*, 2001; Meiklejohn & Hedding, 2005). In contrast, the eastern side of the island provides a more stable environment. In the flightless moth, IBD (for both mitochondrial and nuclear data) was detected for the north-eastern and south-eastern sides of Marion Island which agrees with previous studies. Populations on the western side had no significant IBD at neither the mitochondrial nor nuclear level, with could be attributed to the more unstable environment on this side of the island.

In contrast to previous findings, the populations on the western side of Marion Island are differentiated from several localities which would suggest isolation rather than continued colonization and population extinctions. Why this is the case remains unclear. Although speculative, it may be that adaptations to local environments play an important role. Microsatellite markers, which are more sensitive to recent changes, may detect signatures of adaptation and although not specifically tested in the present study, it may be that adaptation to specific microenvironments is driving population division in the flightless moth.

CHAPTER 5

PRINGLEOPHAGA MARIONI (LEPIDOPTERA: TINEIDAE) IN TWO DIFFERENT HABITAT TYPES (NESTS VERSUS VEGETATION): GENETIC RELATEDNESS ASSESSMENTS

5.1 Introduction

It has long been recognized that birds, specifically the Wandering Albatross (*Diomedea exulans*), play a major role in marine-terrestrial transfers as well as shaping terrestrial landscapes on sub-Antarctic islands (Smith, 1976; Smith, 1979; Joly *et al.*, 1987; Ryan & Watkins, 1989; Smith & Froneman, 2008; Zmudczyńska *et al.*, 2012). Specifically, these large birds build elevated nests where they lay eggs in mid-summer and raise a chick throughout winter until they fledge anytime between December and February (Cooper & Brown, 1990; Marchant & Higgins, 1990; Ryan & Bester, 2008). In addition, Wandering Albatrosses are contributors of nutrients to the system, as well as causing changes to vegetation (notably trampling at a localized scale) where nests are constructed. These activities (nest building and nutrient input through droppings and regurgitations) have an impact on numerous arthropod species (Joly *et al.*, 1987; Sinclair & Chown, 2006; Ryan & Bester, 2008). One of the species that appears to be impacted by Wandering Albatross activities on Marion Island is the flightless moth, *Pringleophaga marioni*. On Île de la Possession (Crozet archipelago), Joly *et al*. (1987) reported a decrease in *Pringleophaga kerguelensis* (a closely related species; see Chapter 2) numbers in areas affected by Wandering Albatrosses (areas surrounding Albatross nests) although several arthropod species showed an increase in numbers (see table 3 in Joly *et al*., 1987). Sinclair and Chown (2006) indicated a marked increase in the biomass of *P. marioni* caterpillars in Wandering Albatross nests on Marion Island compared to surrounding vegetation (Crafford, 1990a; Sinclair & Chown, 2006; Tanya Haupt and Rina Groenewald, personal observation). The biomass of caterpillars is not only higher in nests compared with other plant communities (with the exception of biotic herbfield, Smith & Steenkamp, 2001), but the variance in biomass estimates is also much lower for nests than elsewhere (Sinclair & Chown, 2006) indicating consistent occupation of nests. Recently abandoned nests are also more caterpillar dense than nests abandoned the previous year, suggesting that the numbers decline as the nest gets older.

Given that no significant difference was found between the nutrient composition of nests (recently abandoned or old; Smith, 1978) and several vegetation complexes (with the exception of phosphorus which was higher in vegetation outside nests; Sinclair & Chown, 2006), higher nutrient levels are unlikely to account for the higher caterpillar biomass in nests. Rather, Sinclair and Chown report temperatures in occupied nests that are more than 5 °C higher than that measured in older abandoned nests or vegetation away from nests (as a result of birds occupying nests on a permanent basis). The temperatures recorded in nests were close to the optimum temperature for the growth and feeding of the caterpillars (see figure 2b in Sinclair & Chown, 2006) leading these authors to

argue that it may be the thermal advantage provided by occupied nests that drives higher biomass in nests.

Although a compelling argument, the exact mechanism(s) which cause the higher biomass in nests remain unclear. Several hypotheses were developed by Steven Chown and Brent Sinclair for a grant proposal and were further elaborated on by Tanya Haupt and Justine Shaw. First, female moths may actively seek out nests to lay their eggs in; similarly, caterpillars may themselves actively seek out nests. Secondly, a higher survival rate in nests compared to surrounding vegetation (given that temperatures in nests are unlikely to fall below the chill coma temperature for this species as reported by Klok & Chown (1997)) may account for the higher biomass. Thirdly, the nest building behaviour of Albatrosses (where adult birds bring vegetation to the nests which may contain larvae or eggs) might be a contributing factor in introducing caterpillars or moth eggs into the nest (Justine Shaw, personal communication).

This study contributes information to help distinguish between these hypotheses based on the genetic relatedness of caterpillars in nests compared to those in surrounding vegetation. To this end, a microsatellite library was developed (Groenewald *et al.*, 2011; see also Chapter 3) as these types of markers are routinely used in parentage studies and to calculate relatedness (see e.g. Jouventin *et al.*, 2007). The expectation is that relatedness in nests would be lower, and hence inbreeding lower, if moths actively seek out nests to oviposit their eggs in when compared with a patch of vegetation outside the nest where no such preference draws multiple moths to lay their eggs. The rationale is that the caterpillars in a nest would be the result of several moths laying their eggs compared with the caterpillars in a random patch of vegetation which would be the offspring of only one, or in the least, fewer moths laying eggs. As such, caterpillars from random sites would potentially be more related (i.e. have a higher inbreeding value) compared with those from nests. On the other hand, if Wandering Albatrosses significantly contribute to the caterpillar biomass in nests through their nest building activities, it would be informative to group the caterpillars according to their weight (a rough indication of age). The relatedness of size (age) cohorts may reveal whether they were randomly collected by the Albatrosses and placed in nests. Prior to nest sampling, the nests were occupied for about a year and abandoned for at least 4 months. Since little or no maintenance of the nests, i.e. the addition of nest/plant material, is evident throughout nest occupation, it can be hypothesized that the largest caterpillars in the nest must have been randomly added to the nest. In terms of relatedness estimates, the largest of individuals are expected to be less related than the smallest individuals in the group, due to hypothetical random selection of individuals. Based on the relative age of the nests when sampled, it would imply that the very small caterpillars could not

have been added to the nest during its construction phase and would have had to be laid in the nest (or walked into the nest) at a later stage.

5.2 Materials and Methods

Study species

Pringleophaga marioni, an endemic flightless moth of the Prince Edward Islands (Marion and Prince Edward), fulfils an important role in ecosystem functioning on the islands (Crafford *et al.*, 1986). The species is one of a few invertebrate detritivores known from sub-Antarctic islands where they significantly enhance the rates of nutrient release from a vast amount of plant litter and peat (Burger, 1978; Crafford *et al.*, 1986; Smith & Steenkamp, 1992b; Smith & Steenkamp, 1993). On Marion Island, larvae of the species can live for several years (anything from two to five years), while the adults are short-lived (three to four weeks) (Crafford, 1987). The males are the most conspicuous in the field due to their active nature. Female moths emerge from pupae with most of their eggs already ripe and do not disperse far before mating and ovipositing. They lay an average of 173 eggs in a variety of plant complexes (Crafford, 1987). *Pringleophaga marioni* is found in a large variety of habitats, most notably in the saltspray and biologically influenced communities along the coast but also in highly elevated sites of up to 800 m a.s.l. (Crafford, 1990a).

Sampling design

To assess the relatedness of *P. marioni* in Wandering Albatross nests on Marion Island, relatedness is also calculated for caterpillars collected from random vegetation. This serves as a reference point in comparison with nest values. In an attempt to standardize collection effort (specifically collection patch size), all caterpillars were collected from various plant communities within an area similar to the base of a Wandering Albatross nest. Although this does not account for the volume of a nest (taking the height component into account), it does provide a valid measure of the ground area covered by nests. These collection sites from random plant communities are from here onwards referred to as quads (quadrants of 1 m x 1 m).

Collection from nests

Four recently abandoned Wandering Albatross nests and two older nests on the eastern side of Marion Island were selected for the sampling of *Pringleophaga* caterpillars (see Appendix 2, table A5.1, for nest collection information). The nests are composed of plant material and peat from the surrounding area and are on average ± 25 cm in height (from ground level) and ± 102 cm in diameter (see figures 5.1 & 5.2 for images of the nests). The relative age of the nest was scored

based on its condition, signs of bird activity (the presence of egg shells or feathers) and plant growth on and around the nest. All the nest material was carefully separated by hand (twice) and all the caterpillars in the nests collected. After thorough inspection, the nest material was placed back in the original shape of the nests as far as was possible (following the protocol described by Sinclair and Chown).

Figure 5.1: An unoccupied sampled nest (showing signs of mouse burrow damage).

Figure 5.2: Examples of occupied Wandering Albatross nests (not sampled for this study).

Collection from vegetation quadrants within suitable plant complexes

Sampling sites along the east coast of Marion Island were selected based on the preferred habitat of *Pringleophaga* caterpillars (other than Wandering Albatross nests). This includes vascular plants such as *Rananculus biternatus*, *Cotula plumosa*, *Poa cookii*, *Poa annua* (alien), *Sagina procumbens* (alien), *Agrostis stolonifera* (alien), *Agrostis magellanica* and *Juncus scheuchzerioides* as well as mosses such as *Sanionia uncinata*. Caterpillars were especially abundant in the alien moss, *Sagina procumbens*, provided that it was not too dry nor too wet (water logged). After the areas for sampling were selected, a 1 m x 1 m quadrant was placed over the suitable vegetation (see figure

5.3) and all the caterpillars that could be found within the first 1 - 2 cm of soil and roots were collected. For some sites, up to four quads were searched within a specific area with a distance of 1 m to 11 m between the quads (see Appendix 2, table A5.2, for quad collection information).

Figure 5.3: Example of vegetation sampling in a quadrant (1 m x 1 m) in *Poa annua* (left) and *Agrostis magellanica*, *Poa cookii* and *Sagina procumbens* (right).

Laboratory protocols

All the individuals collected from nests and quads were weighed within a few hours after collection. The wet mass of the individuals (live mass) was recorded up to the fifth decimal (Appendix 2, tables A5.1 and A5.2). Following this, caterpillars were placed in absolute ethanol for storage and subsequent DNA analyses. Total genomic DNA was extracted from caterpillar tissue using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's recommendations. Individuals were genotyped for 12 polymorphic species-specific microsatellite markers (see protocol in Chapter 3). One of the markers (Pm15) was problematic and failed to amplify in 10% of the individuals. However, because of the high overall informativeness value of this marker (see Chapter 3 and Chapter 4 for details) as well as the fact that analyses (computer programmes) allow (and correct) for missing data, it was decided to retain this marker in all analyses.

Data analyses

The genotypes obtained from all nest and quad individuals was screened for the presence of null alleles in the program MICROCHECKER (The University of Hull, Brookfield, 1996; Van Oosterhout *et al.*, 2004). The relatedness and inbreeding coefficients of individuals was calculated using COANCESTRY (Wang, 2011) which implements seven different relatedness estimators and four different inbreeding coefficients from multilocus genotype data. After an initial pilot analysis, the two maximum likelihood estimates of relatedness that accounts for genotyping errors were selected for final analyses. This includes the triadic likelihood estimator (TrioML) and a dyadic

likelihood estimator (DyadML). The difference between the two methods is that the TrioML method uses a third individual as a reference when calculating the relatedness between two other individuals. This is done to limit the chances of mistakenly referring to individuals identical by state (i.e. identical alleles) as identical by descent. The relatedness of all the individuals collected from Wandering Albatross nests was compared to those found within quads of a variety of plant communities. No correction for overall inbreeding was done as the flightless moth population on the eastern side of Marion Island is considered to be sufficiently large for overall inbreeding not to be a factor; also, the flightless moth population on this side of the island is not genetically structured or geographically separated (see Chapter 4 and also Burger, 1978; see Crafford 1990 for coastal biomass estimates). Inbreeding was calculated in COANCESTRY using two approaches namely those from from Ritland (1996) and Lynch & Ritland (1999), denoted as Ritland and LynchRd respectively.

Effect of sampling size on relatedness estimates

Recently abandoned Wandering Albatross nests contain a higher abundance of caterpillars than other vegetation samples on the island. To incorporate the effect of sample size differences between quads and nests, 30 individuals from a nest with high caterpillar abundance was randomly re-sampled (repeated five times). Relatedness and inbreeding was calculated for these sub-samples of the nests and the average was compared to the average relatedness estimated for all the individuals within a nest.

Size cohorts

Since *P. marioni* is in its larval stage from anything between two to five years, a variety of instars are found within any specific sampling site (being either a nest or a quad). A mixture of caterpillars from different generations (ages) may therefore be present at any particular time and this may significantly bias relatedness estimations (given that female moths lay their eggs within a relatively short time period). This was overcome by grouping caterpillars of similar weight together as weight (size) is a rough proxy for age. Moths can lay their eggs only once and therefore the relatedness of individuals of roughly the same age may be indicative of the number of moths that laid eggs at a particular stage. Four nests (Nest 2, Nest 3, Nest 5 and Nest 6) and four quads (Quad F, Quad G, Quad K and Quad L) were selected for analyses on size cohorts. These nests / quads were selected based on the sampling sites where the maximum number of caterpillars was collected.

Hypotheses expectations

A comparison of relatedness within size cohorts between nests and random quads may provide an indication of whether moths actively seek out nests to lay eggs. If moths actively seek out nests, one would expect more moths to lay eggs in nests (as indicated by lower relatedness and lower inbreeding for cohorts in nests) compared to random quads. This same comparison may shed light on whether Wandering Albatrosses contribute to the high biomass in nests through their nest building activities. The assumption is that birds would collect vegetation from random areas (and not continuously from the same area) and as such, caterpillars in nests would represent a random collection from vegetation. It is generally accepted that Wandering Albatrosses add the majority (if not all) of nest material during the initial building of the nests, with very little material added after the nest has been constructed (other than sporadic maintenance; Aleks Terauds and Mia Cerfonteyn personal communication). As such, a comparison of caterpillar age cohorts within a nest may add valuable information. The expectation is that older caterpillars should be less related compared with younger caterpillars as these older caterpillars may have been randomly added to the nest during nest building whereas younger caterpillars would be in the nest because moths laid eggs in the nest or caterpillars crawled into the nest. The weights of all caterpillars collected from specific nests / quads were plotted in Excel to assess whether distinct sizes (indicative of cohorts) can be distinguished. In most instances, there was a gradual increase in weight (see Appendix 2, figures A5.1 and A5.2). As such, the assignment of cohorts is somewhat arbitrarily. To minimize any errors, only the smallest (weight between 0.02 g to 0.06 g) and largest (weight between 0.2 g to 0.3 g) caterpillars collected from nests was included (and considered as two distinct cohorts). Caterpillars taken from quads tended to be smaller than those from the nests. The youngest quad cohort was defined as individuals weighing between 0.001 g to 0.04 g, with the oldest cohort included individuals weighing between 0.11 g to 0.26 g.

Statistical significance

To determine whether the relatedness estimates between nests and quads are statistically significant, t-tests or Mann-Whitney U-tests (depending on whether the data are normally distributed or not) were conducted in SPSS Statistics 17.0 software (SPSS_Inc., 2008). Significant differences would be indicated by p-values of less than 0.05. In addition, statistical significance of relatedness estimates as a function of location (nest/quad) and/or size (age) was calculated in R v.2.12 (Ihaka & Gentleman, 1996; R_Core_Development_Team, 2006).

5.3 Results

Two of the microsatellite markers (Pm05 and Pm23) proved to be susceptible to null alleles with estimated frequencies of 0.2817 and 0.1876 respectively. Errors associated with the null presence alleles as well as general genotyping errors (such as PCR failure rate) were taken into account (and corrected) for each marker during the relatedness and inbreeding calculations.

Effect of sampling size on relatedness estimates

The numbers of caterpillars occupying nests were much higher than collections made from quads. Notwithstanding, relatedness and inbreeding coefficients do not show any significant correlation with number of individuals (see Appendix 2, figures A5.3 – A5.6). Furthermore, calculations based on subsets of individuals taken from nests were similar to estimates derived when all the individuals from specific nests were included. The effects of sampling size differences between nests and quads seem to be minimal. Also, calculations for nests are based on all the individuals collected (unless otherwise specified).

Inbreeding and relatedness coefficients

The relatedness and inbreeding coefficients (with their variances) obtained for all the sampled nests and quads are given in table A5.3 of Appendix 2. The averages of these coefficients are given below in table 5.1. Individuals from quads were, on average, more related (and more inbred) that those collected from nests. These differences proved to be statistically significant based on a non-parametric Mann-Whitney U-test (U = 114, p-value = 0.043). Statistics based on the several relatedness and inbreeding estimates gave congruent results and therefore only the p-values from the TrioML estimates are reported.

Table 5.1: Relatedness and inbreeding coefficients averaged for all the nests (n = 6) and quads (n = 14).

Population	**Number of individuals**	**Relatedness**		**Inbreeding**	
		TrioML	**DyadML**	**Ritland**	**LynchRd**
Nests	776	0.22603	0.27883	0.13711	0.18946
Quads	365	0.29054	0.35519	0.28572	0.24344

Size cohorts

There was a significant difference between the relatedness of size (age) cohorts in both nests and quads (t-value = -3.476, p-value = 0.006), with the larger (older) cohorts being consistently more related than the smaller (younger) cohorts (see table 5.2). The relatedness and inbreeding estimates for the size cohorts of each of the nests (n = 4) and quads (n = 4), are given in table A4 of Addendum 2. In addition, a comparison between size cohorts from nests and quads, revealed no significant difference among the relatedness of cohorts of similar size (t-value = 1.585, p-value = 0.144).

Table 5.2: Average relatedness and inbreeding coefficients of all the individuals, as well as size cohorts, from 4 nests and 4 quads collectively (all = total number of caterpillars sampled, S = number of small caterpillars, L = number of large caterpillars).

Population	Number of individuals	Relatedness		Inbreeding	
		TrioML	DyadML	Ritland	LynchRd
Nests (all)	303	0.21624	0.26825	0.14589	0.18502
Nests (S)	34	0.10950	0.14586	0.16614	0.17903
Nests (L)	26	0.18858	0.22035	0.19704	0.17305
Quads (all)	111	0.27977	0.34316	0.16504	0.28508
Quads (S)	13	0.16477	0.18904	0.18041	0.20817
Quads (L)	24	0.22586	0.29047	0.17574	0.18697

5.4 Discussion

The high biomass of *Pringleophaga marioni* in recently abandoned Wandering Albatross nests on Marion Island is a well-known phenomenon and has been documented in several studies (Sinclair & Chown, 2006; Chown & Convey, 2007; Convey *et al.*, 2007). Possible explanations to account for this phenomenon was initially a higher nutrient content in nests as a result of the presence of Wandering Albatrosses for most of the year, but this hypothesis was discarded by Sinclair & Chown (2006). Instead, these authors suggested that elevated temperatures prevent exposure to critical chill coma temperatures (i.e. -0.6 °C; Klok & Chown, 1997) which in essence optimizes *P. marioni*'s growth rate and feeding success (Sinclair & Chown, 2006). The beneficial effect for caterpillars being in nests (thermal benefit as well as reduced risk of predation from mice) compared to vegetation is similarly reflected by the notable higher overall wet mass of caterpillars from nests compared to the mass of caterpillars from random quads. The optimal thermal environment provided by occupied nests was termed thermal ecosystem engineering (Sinclair &

Chown, 2006). However, the exact mechanism(s) which drives higher biomass in nests remains unclear. Possible drivers include that (1) Wandering Albatrosses contribute to the higher biomass by introducing eggs / caterpillars to the nest during nest building, (2) caterpillars may actively seek out nests, or (3) female moths may actively seek out nests to lay their eggs in. Information on the genetic relatedness of caterpillars provide useful information which, when taken with current knowledge, may help to extend our understanding of this unique and fascinating system on Marion Island. These possible scenarios are discussed in more detail below.

Nest building

Wandering Albatrosses construct large nests from vegetation (Warham, 1997; Sinclair & Chown, 2006). It is therefore possible that caterpillars or eggs may be added to the nests during nest building. Although the nests are maintained (although not marginally) throughout the duration that the nest is occupied, the largest amount of nest material is added during the initial nest building phase (Mia Cerfonteyn and Aleks Terauds, personal communication). One would therefore expect older (larger) caterpillars to be less related compared with younger ones. The rationale is that older caterpillars may be randomly added to the nests during nest building, whereas the younger ones must have either occupied the nest by themselves (when taking the age of the nest into account) or alternatively, resulted from eggs being laid in the nest. If caterpillars are added to the nest by the bird during occupation, it would account for only a small number of individuals as nests are not regularly maintained. The genetic data do not provide any support for this hypothesis. Older caterpillars are in fact consistently more related than younger cohorts. The larger size cohorts from four nests were on average related by estimated values of 0.19 (TrioML) or 0.22 (DyadML), whereas the smallest group of individuals were less related with average values of 0.11 (TrioML) or 0.15 (DyadML). Although this hypothesis cannot be completely discarded, it would argue against a significant random contribution of eggs/caterpillars to the nests during the initial building phase, and are therefore not a very likely explanation as to the high observed caterpillar abundances.

Caterpillars crawling into nests

If we accept that Wandering Albatrosses do not significantly contribute to the high biomass of caterpillars in nests as a result of their nest building, then the large numbers of caterpillars in nests must be the result of caterpillars either occupying nests by themselves (actively seek out nests) or female moths laying eggs in nests. If caterpillars seek out nests by crawling into nests, one would expect the relatedness of these caterpillars to be lower than the surrounding vegetation as the caterpillars represent a large number of offspring from nearby vegetation. If there is no preference for a particular area, the caterpillars would be more likely to be closer related since they would

hypothetically not disperse very far from where they hatched. Preliminary genetic data do seem to support this hypothesis. Specifically, caterpillars from nests are less related than those collected from vegetation quads. Caterpillars from the six sampled nests were on average related by estimate values of 0.23 (TrioML) or 0.28 (DyadML), whereas caterpillars from quads were on average more related with estimates of 0.29 (TrioML) or 0.36 (DyadML). However, other lines of information suggest that caterpillars would not actively seek out nests. First, Joly *et al*. (1987) reported fewer caterpillars (*Pringleophaga kerguelensis*) on Île de la Possession in areas surrounding Wandering Albatrosses nests as opposed to sites not influenced by the presence of the birds, implying that the biological footprint of the Wandering Albatross (either nutrients in the surrounding vegetation or the activities of these large birds) somehow deter caterpillars. Also, the areas around nests are typically trampled with little or no vegetation, decreasing in vegetation the longer the nest is occupied. As such, it does not seem plausible that caterpillars that feed almost continuously, and which are also rather sedentary, would move through bare soil patches to reach nests.

Moths lay eggs in the nests

The last, and possibly most plausible, explanation to account for the higher biomass in nests is that moths actively seek out nests to lay their eggs in (Sinclair & Chown, 2006). There is support for this hypothesis from the genetic data. The relatedness of caterpillars in nests are lower compared with those from quads, providing evidence that the offspring in nests have more mothers than those in random vegetation. This holds, notwithstanding that female moths can lay as many as 173 eggs (Crafford, 1987), and given the caterpillar count in nests, a single moth may biologically be the mother to all or most of the caterpillars in a specific nest. If this hypothesis is accepted, it would imply that there must be cues that actively attract female moths to nests. A current Ph.D. study by T.M. Haupt will investigate possible cues, such as the smell of caterpillars or nest material, through preference experiments.

In conclusion, genetic information on relatedness of caterpillars in nests and random vegetation add useful information to help unravel this conundrum. Although not conclusive by itself, the genetic information when taken with other lines of evidence, suggest that female moths may actively seek out nests to lay their eggs. An important piece of information to add to this would be whether some cue could possibly attract moths to the nests.

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APPENDICES

APPENDIX 1: CHAPTER 2

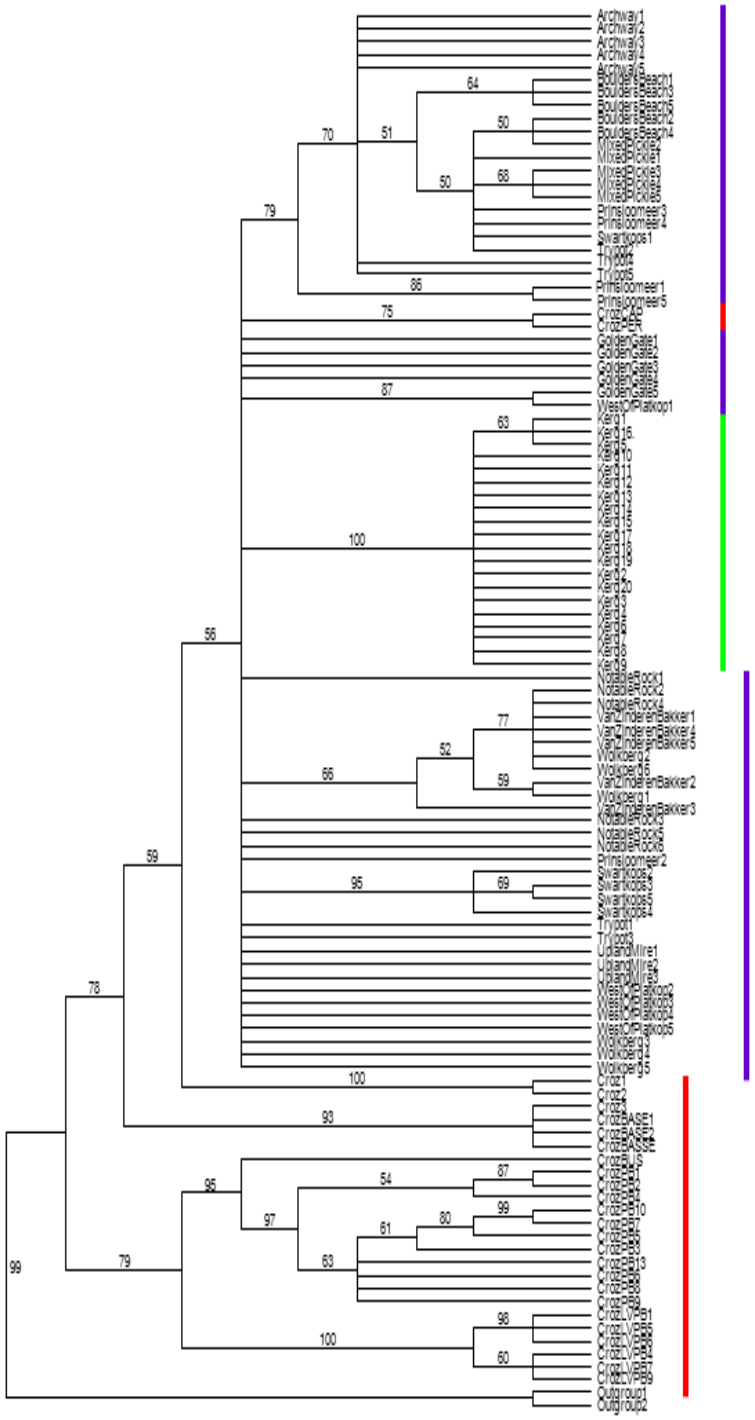

Figure A2.1: Maximum parsimony tree for the combined sequence data set (mitochondrial genes: COI and COII; nuclear genes: EF1α and PEPCK) of the *Pringleophaga* species from four islands and/or archipelago's (Marion Island, Prince Edward Island – both purple, Îles Kerguelen - green and Îles Crozet - red). Bootstrap support (%) for the various branches are indicated.

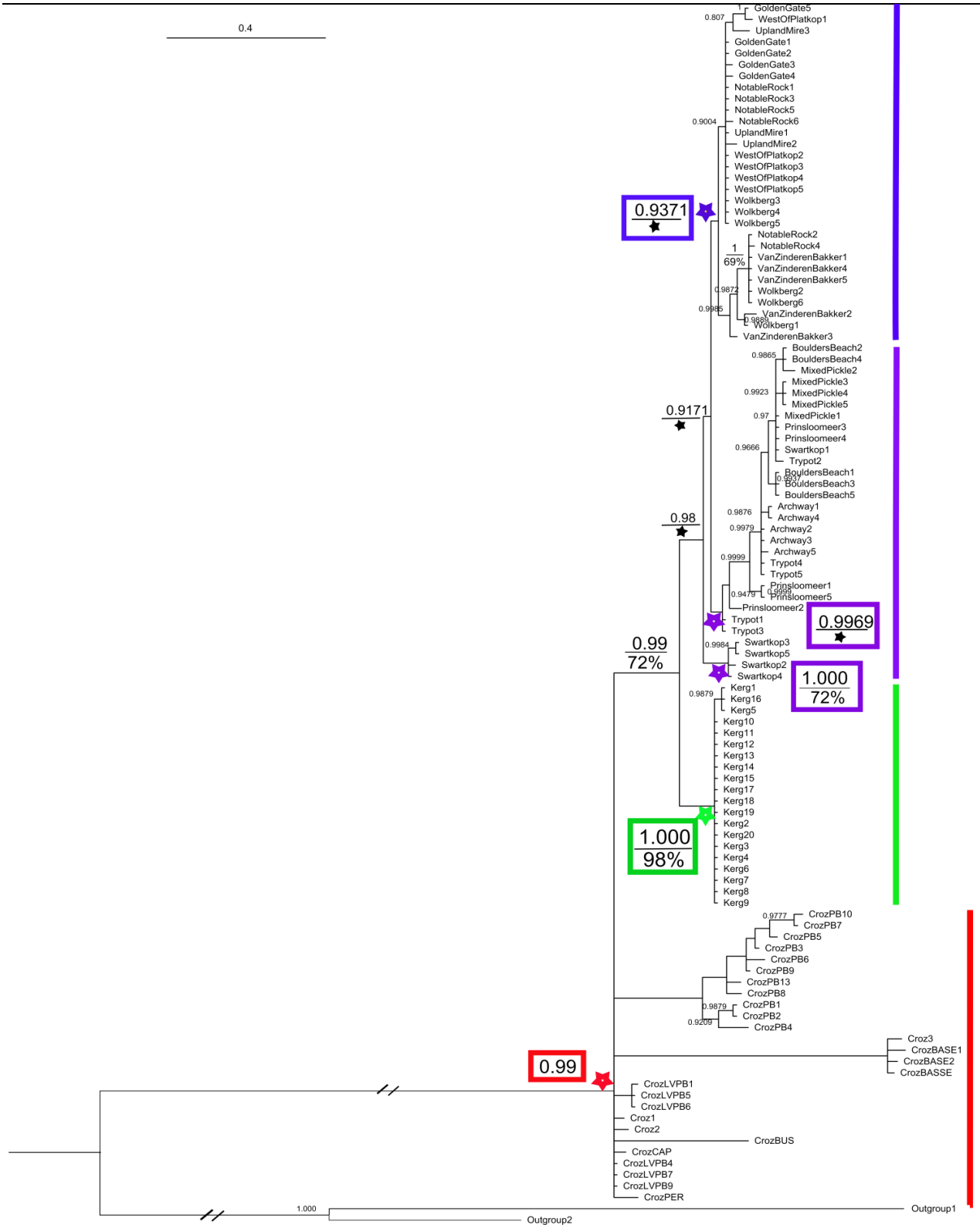

Figure A2.2: Bayesian tree for the combined sequence data set (mitochondrial genes: COI and COII; nuclear genes: EF1α and PEPCK) of the *Pringleophaga* species from four islands and/or archipelago's (Marion Island, Prince Edward Island, Îles Kerguelen and Îles Crozet). The black filled stars (★) indicate bootstap support of less than 70%. Colored stars indicate the location of the nodes that support the important clades relating to the different species and/or different islands.

Table A2.1: Statistics for COI gene fragment on each of the islands.

Islands	Number of haplotypes	Haplotype diversity	Nucleotide diversity
Marion	11	0.9080 +/- 0.0285	0.0073 +/- 0.004
Prince Edward	8	0.7057 +/- 0.0729	0.0033 +/- 0.0021
Kerguelen	2	0.2684 +/- 0.1133	0.0004 +/- 0.0006
Crozet	15	1.0000 +/- 0.0137	0.0423 +/- 0.0215
All islands combined	43	0.9404 +/- 0.0129	0.0296 +/- 0.0147

Table A2.2: Statistics for COII gene fragment on each of the islands.

Islands	Number of haplotypes	Haplotype diversity	Nucleotide diversity
Marion	7	0.7747 +/- 0.0473	0.0039 +/- 0.0025
Prince Edward	7	0.6368 +/- 0.0815	0.0023 +/- 0.0017
Kerguelen	1	0.0000 +/- 0.0000	0.0000 +/- 0.0000
Crozet	16	0.9900 +/- 0.0161	0.0499 +/- 0.0254
All islands combined	37	0.8866 +/- 0.0203	0.0323 +/- 0.0161

Table A2.3: Statistics for EF1α gene fragment on each of the islands.

Islands	Number of alleles	Gene diversity	Nucleotide diversity
Marion	7	0.5908 +/- 0.0995	0.0006 +/- 0.0009
Prince Edward	7	0.5816 +/- 0.0962	0.0007 +/- 0.0010
Kerguelen	1	0.0000 +/- 0.0000	0.0000 +/- 0.0000
Crozet	14	1.0000 +/- 0.0120	0.0190 +/- 0.0105
All islands combined	35	1.0000 +/- 0.0013	0.0130 +/-0.0073

Table A2.4: Statistics for PEPCK gene fragment on each of the islands.

Islands	Number of alleles	Gene diversity	Nucleotide diversity
Marion	3	0.1310 +/- 0.0821	0.0002 +/- 0.0004
Prince Edward	2	0.4046 +/- 0.0777	0.0000 +/- 0.0000
Kerguelen	2	0.1895 +/- 0.1081	0.0000 +/- 0.0000
Crozet	1	0.0000 +/- 0.0000	0.0000 +/- 0.0000
All islands combined	12	1.0000 +/- 0.0017	0.0012 +/- 0.0011

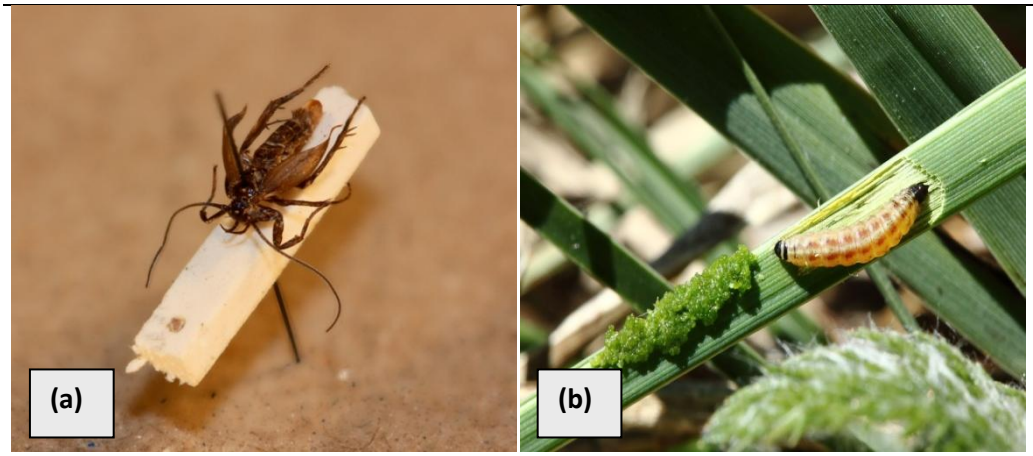

Figure A2.3: Images of *Embryonopsis halticella* Eaton: (a) Museum moth specimen obtained from Ditsong Museum, Pretoria; (b) Caterpillar feeding on *Poa cookii* (photo: Otto Whitehead).

APPENDIX 2: CHAPTER 5

Table A5.1: Collection of *P. marioni* caterpillars from Wandering Albatross nests, with their wet mass indicated in grams (ID = identification number for individual caterpillars collected from the nests).

Nest 1			Nest 2			Nest 3			Nest 4			Nest 5			Nest 6		
Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)
Boulders Beach	N1.1	0.03156	Archway	N2.1	0.23713	Boulders Beach	N3.1	0.29909	Trypot	N4.1	0.10935	Rockhopper Bay	N5.1	0.10024	Rockhopper Bay	N6.1	0.17698
	N1.2	0.10166		N2.2	0.13703		N3.2	0.17580		N4.2	0.07921		N5.2	0.06046		N6.2	0.20707
	N1.3	0.02762		N2.3	0.26661		N3.3	0.22844		N4.3	0.04002		N5.3	0.11857		N6.3	0.24958
	N1.4	0.10859		N2.4	0.15446		N3.4	0.08634		N4.4	0.06214		N5.4	0.06663		N6.4	0.23155
	N1.5	0.04955		N2.5	0.17062		N3.5	0.21905		N4.5	0.02910		N5.5	0.13894		N6.5	0.13912
	N1.6	0.09007		N2.6	0.21153		N3.6	0.14519		N4.6	0.05936		N5.6	0.13206		N6.6	0.12133
	N1.7	0.03725		N2.7	0.23411		N3.7	0.14076		N4.7	0.07143		N5.7	0.07797		N6.7	0.10174
	N1.8	0.05135		N2.8	0.14347		N3.8	0.07705		N4.8	0.03619		N5.8	0.10028		N6.8	0.09054
	N1.9	0.06475		N2.9	0.16797		N3.9	0.14101		N4.9	0.03202		N5.9	0.06456		N6.9	0.07605
	N1.10	0.04574		N2.10	0.29742		N3.10	0.07829		N4.10	0.15098		N5.10	0.09942		N6.10	0.07882
	N1.11	0.09063		N2.11	0.16396		N3.11	0.13564					N5.11	0.05731		N6.11	0.03203
	N1.12	0.03223		N2.12	0.13249		N3.12	0.09949					N5.12	0.02438		N6.12	0.09996
	N1.13	0.06179		N2.13	0.12918		N3.13	0.09490					N5.13	0.23677		N6.13	0.12462
	N1.14	0.03492		N2.14	0.12221		N3.14	0.07936					N5.14	0.10856		N6.14	0.17338
	N1.15	0.02738		N2.15	0.14343		N3.15	0.09886					N5.15	0.29297		N6.15	0.13986
	N1.16	0.06153		N2.16	0.12614		N3.16	0.25848					N5.16	0.22834		N6.16	0.12309
				N2.17	0.11136		N3.17	0.11096					N5.17	0.06246		N6.17	0.11426
				N2.18	0.12909		N3.18	0.14165					N5.18	0.08758		N6.18	0.10167
				N2.19	0.12007		N3.19	0.20106					N5.19	0.14152		N6.19	0.10489
				N2.20	0.09327		N3.20	0.12225					N5.20	0.15183		N6.20	0.06783
				N2.21	0.10952		N3.21	0.15203					N5.21	0.14269		N6.21	0.07353
				N2.22	0.12713		N3.22	0.13068					N5.22	0.12285		N6.22	0.13605
				N2.23	0.10870		N3.23	0.10192					N5.23	0.14131		N6.23	0.15344
				N2.24	0.11942		N3.24	0.10516					N5.24	0.09562		N6.24	0.05665
				N2.25	0.09285		N3.25	0.16603					N5.25	0.08956		N6.25	0.02605
				N2.26	0.13366		N3.26	0.17083					N5.26	0.04674		N6.26	0.04358
				N2.27	0.12093		N3.27	0.08346					N5.27	0.05094		N6.27	0.14771
				N2.28	0.08270		N3.28	0.20036					N5.28	0.02830		N6.28	0.12469
				N2.29	0.18019		N3.29	0.24922					N5.29	0.02886		N6.29	0.05303
				N2.30	0.06190		N3.30	0.27860					N5.30	0.04135		N6.30	0.11658
				N2.31	0.09505		N3.31	0.19598					N5.31	0.05218		N6.31	0.08683
				N2.32	0.07188		N3.32	0.25572					N5.32	0.09261		N6.32	0.17148
				N2.33	0.10679		N3.33	0.21592					N5.33	0.26194		N6.33	0.01786
				N2.34	0.06610		N3.34	0.11972					N5.34	0.11244		N6.34	0.08064

Table A5.1 continued

Nest 1			Nest 2			Nest 3			Nest 4			Nest 5			Nest 6		
Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)
				N2.35	0.07712		N3.35	0.20474					N5.35	0.24581		N6.35	0.05803
				N2.36	0.06212		N3.36	0.09871					N5.36	0.20372		N6.36	0.04103
				N2.37	0.07423		N3.37	0.15875					N5.37	0.13555		N6.37	0.03003
				N2.38	0.04796		N3.38	0.10769					N5.38	0.16881		N6.38	0.19861
				N2.39	0.05554		N3.39	0.13077					N5.39	0.14403		N6.39	0.14818
				N2.40	0.05032		N3.40	0.30278					N5.40	0.07582		N6.40	0.14171
				N2.41	0.14239		N3.41	0.10928					N5.41	0.14673		N6.41	0.17414
				N2.42	0.12584		N3.42	0.10146					N5.42	0.12674		N6.42	0.09596
				N2.43	0.07924		N3.43	0.05405					N5.43	0.12425		N6.43	0.07132
				N2.44	0.06900		N3.44	0.11452					N5.44	0.09162		N6.44	0.19111
				N2.45	0.03626		N3.45	0.03032					N5.45	0.11671		N6.45	0.12009
				N2.46	0.07699		N3.46	0.04780					N5.46	0.06428		N6.46	0.17256
				N2.47	0.04924		N3.47	0.08398					N5.47	0.05885		N6.47	0.08943
				N2.48	0.05973		N3.48	0.11472					N5.48	0.07068		N6.48	0.07243
				N2.49	0.08489		N3.49	0.07348					N5.49	0.11316		N6.49	0.15482
				N2.50	0.08927		N3.50	0.01751					N5.50	0.06333		N6.50	0.11071
				N2.51	0.12973		N3.51	0.03473					N5.51	0.06589		N6.51	0.08209
				N2.52	0.13799		N3.52	0.08059					N5.52	0.03477		N6.52	0.05533
				N2.53	0.11686		N3.53	0.05566					N5.53	0.05664		N6.53	0.16269
				N2.54	0.08434		N3.54	0.05944					N5.54	0.05216		N6.54	0.13756
				N2.55	0.10043								N5.55	0.10412		N6.55	0.08207
				N2.56	0.05611								N5.56	0.14359		N6.56	0.08182
				N2.57	0.07835								N5.57	0.10983		N6.57	0.05848
				N2.58	0.10907								N5.58	0.10996		N6.58	0.05464
				N2.59	0.11733								N5.59	0.12282		N6.59	0.10175
				N2.60	0.08957								N5.60	0.08502		N6.60	0.03806
				N2.61	0.10026								N5.61	0.13758		N6.61	0.02524
				N2.62	0.02698								N5.62	0.05314		N6.62	0.03359
				N2.63	0.28990								N5.63	0.14215		N6.63	0.04796
				N2.64	0.24364								N5.64	0.11942		N6.64	0.09703
				N2.65	0.27829								N5.65	0.09436			
				N2.66	0.17457								N5.66	0.10789			
				N2.67	0.18179								N5.67	0.09228			
				N2.68	0.14287								N5.68	0.27565			
				N2.69	0.08789								N5.69	0.13520			
				N2.70	0.10058								N5.70	0.10383			
				N2.71	0.09630								N5.71	0.21246			
				N2.72	0.06012								N5.72	0.05905			
				N2.73	0.08649								N5.73	0.12504			
				N2.74	0.13797								N5.74	0.10523			
				N2.75	0.07242								N5.75	0.13427			
				N2.X1	0.17480								N5.76	0.06245			
				N2.X2	0.05776								N5.77	0.10198			
				N2.X3	0.06592								N5.78	0.07602			
				N2.X4	0.09409								N5.79	0.07966			

Table A5.1 continued

Nest 1			Nest 2			Nest 3			Nest 4			Nest 5			Nest 6		
Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)	Locality	ID	Mass (g)
				N2.X5	0.07948								N5.80	0.09388			
													N5.81	0.10707			
													N5.82	0.09061			
													N5.83	0.05979			
													N5.84	0.09587			
													N5.85	0.09412			
													N5.86	0.13843			
													N5.87	0.11519			
													N5.88	0.02505			
													N5.89	0.08720			
													N5.90	0.11893			
													N5.91	0.08986			
													N5.92	0.03626			
													N5.93	0.01997			
													N5.94	0.02586			
													N5.95	0.04169			

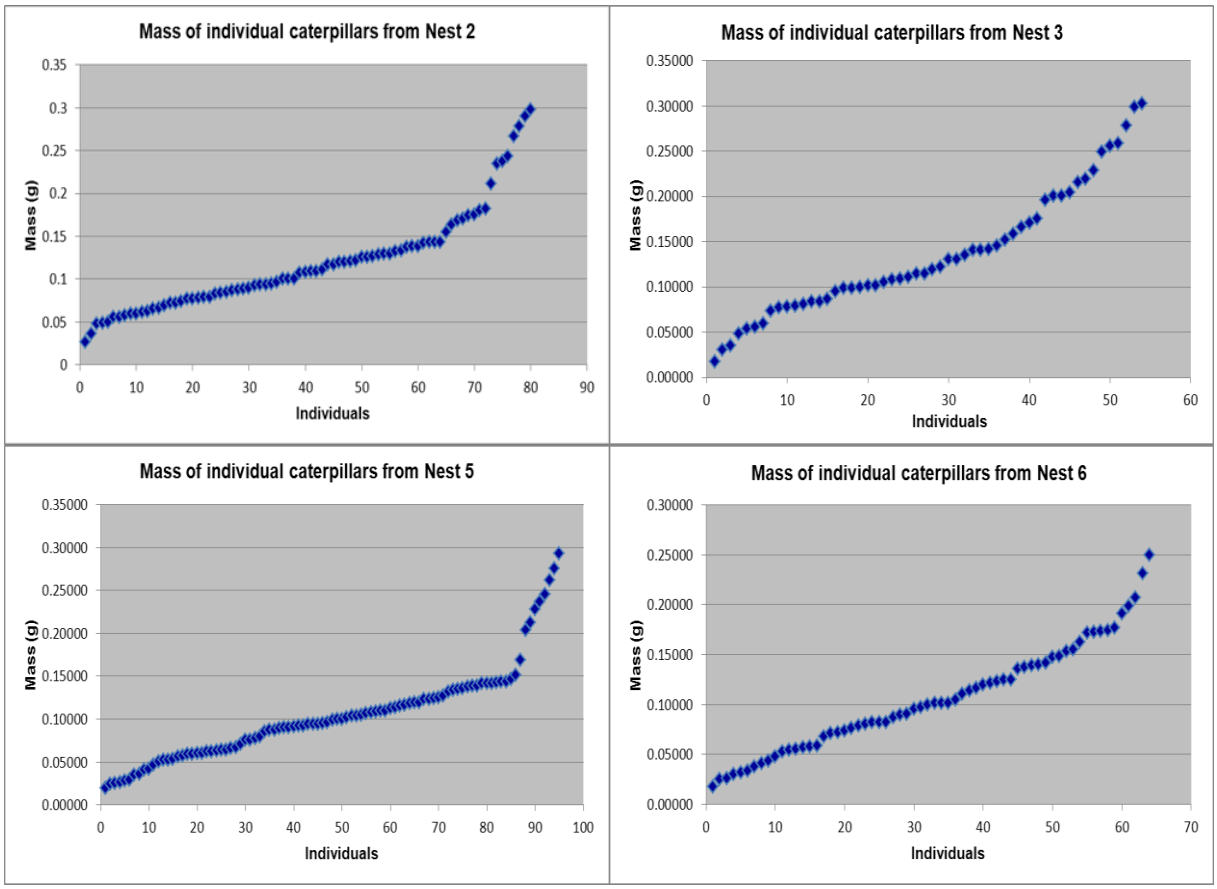

Figure A5.1: Distribution of mass (g) for caterpillars collected from nests.

Table A5.2: Collection of *P. marioni* caterpillars from vegetation quadrants with their wet mass indicated in grams (ID = identification number for individual caterpillars collected from the nests).

Site A			Site B			Site C			Site D			Site E			Site F			Site G		
Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)
Archway	A2.1	0.06578	Base	B1.1	0.14014	Base	C1.1	0.07975	Rockhopper	D1.1	0.06191	Rockhopper	E1.1	0.11683	Trypot	F1.1	0.18004	Trypot	G1.1	0.07395
	A2.2	0.12955		B1.2	0.07253		C1.2	0.06777	Bay	D1.2	0.22709	Bay	E1.2	0.05741		F1.2	0.06707		G1.2	0.13992
	A3.1	0.11569		B1.3	0.15881		C1.3	0.07913		D1.3	0.06483		E1.3	0.05636		F1.3	0.08059		G1.3	0.07405
	A3.2	0.06626		B1.4	0.03669		C1.4	0.13822		D1.4	0.04693		E1.4	0.03868		F1.4	0.18585		G1.4	0.08536
	A3.3	0.05893		B1.5	0.02488		C1.5	0.03286		D1.5	0.06491		E1.5	0.04531		F1.5	0.05948		G1.5	0.07408
	A4.1	0.03848		B1.6	0.03997		C1.6	0.06435		D1.6	0.08416		E1.6	0.06168		F1.6	0.05057		G1.6	0.05644
	A4.2	0.03636		B1.7	0.02358		C1.7	0.05745		D1.7	0.02798		E1.7	0.03439		F1.7	0.03846		G1.7	0.11427
	A4.3	0.02733		B1.8	0.02804		C1.8	0.08131		D1.8	0.04392		E1.8	0.01865		F1.8	0.05056		G1.8	0.08054
	A4.4	0.01971		B2.1	0.06604		C1.9	0.06843		D2.1	0.04677		E2.1	0.03961		F1.9	0.15663		G1.9	0.02923
	A4.5	0.07448		B2.2	0.10093		C1.10	0.09575		D2.2	0.05536		E2.2	0.08305		F1.10	0.04904		G1.10	0.05497
				B2.3	0.02348		C1.11	0.03016		D2.3	0.08427		E2.3	0.02769		F1.11	0.03429		G1.11	0.03589
				B2.4	0.09824		C2.1	0.04758		D2.4	0.12598		E3.1	0.14207		F1.12	0.03536		G1.12	0.05035
				B3.1	0.16148		C2.2	0.05571		D2.5	0.05018		E3.2	0.20239		F1.13	0.12221		G1.13	0.06593
				B3.2	0.03488		C2.3	0.07706		D2.6	0.04286		E3.3	0.13286		F1.14	0.00729		G1.14	0.01714
				B3.3	0.06452		C2.4	0.05743		D2.7	0.10540		E4.1	0.01711		F1.15	0.07047		G1.15	0.03722
				B3.4	0.12493		C3.1	0.13343		D2.8	0.05824		E4.2	0.02658		F1.16	0.04923		G1.16	0.08856
				B3.5	0.08967		C3.2	0.13367		D3.1	0.09657		E4.3	0.02925		F1.17	0.04945		G1.17	0.04959
				B3.6	0.04917		C3.3	0.08178		D3.2	0.04215		E4.4	0.01968		F1.18	0.10975		G1.18	0.02792
				B3.7	0.02528		C3.4	0.07283		D3.3	0.09456		E4.5	0.03445		F1.19	0.02286		G1.19	0.04255
				B3.8	0.03839		C3.5	0.06765		D3.4	0.11696		E4.6	0.02739		F1.20	0.08036		G1.20	0.03893
				B3.9	0.02543		C3.6	0.08505		D3.5	0.05794					F1.21	0.00962		G1.21	0.08348
				B4.1	0.21688		C3.7	0.09778		D3.6	0.13796					F1.22	0.01044		G1.22	0.01946
				B4.2	0.07160		C4.1	0.13274		D3.7	0.05976					F1.23	0.02823		G1.23	0.04244
				B4.3	0.13823		C4.2	0.14933		D3.8	0.07293					F1.24	0.08464		G1.24	0.01355
				B4.4	0.06898		C4.3	0.01662		D3.9	0.05937					F1.25	0.02279		G1.25	0.00652
				B4.5	0.06286		C4.4	0.05948		D3.10	0.07612					F1.26	0.01805		G1.26	0.03744
				B4.6	0.07208		C4.5	0.04426		D3.11	0.03801								G1.27	0.03958
				B4.7	0.03928		C4.6	0.05915		D3.12	0.05449								G1.28	0.02008
							C4.7	0.05093		D3.13	0.00554								G1.29	0.01137
							C4.8	0.0382											G1.30	0.02042
							C4.9	0.10989											G1.31	0.01416
							C4.10	0.01738												
							C4.11	0.02208												

Table A5.2 continued

Site A			Site B			Site C			Site D			Site E			Site F			Site G		
Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)
							C4.12	0.04896												
							C4.13	0.02504												
							C4.14	0.01497												
Site H			**Site I**			**Site J**			**Site K**			**Site L**			**Site M**			**Site N**		
Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)	Locality	ID	Mass(g)
Trypot	H1.1	0.08654	Trypot	I1.1	0.07985	Trypot	J1.1	0.03019	Trypot	K1.1	0.07297	Trypot	L1.1	0.11048	Rockhopper	M1.1	0.08704	King	N1.1	0.11086
	H1.2	0.07268		I1.2	0.14221		J1.2	0.06091		K1.2	0.08576		L1.2	0.01967	Bay	M1.2	0.04568	Bird	N1.2	0.12342
	H1.3	0.10133		I1.3	0.09547		J1.3	0.10549		K1.3	0.05097		L1.3	0.05969		M1.3	0.05424	Head	N1.3	0.07873
	H1.4	0.09084		I1.4	0.10624		J1.4	0.02451		K1.4	0.06231		L1.4	0.04203		M1.4	0.02967		N1.4	0.04337
	H1.5	0.10624		I1.5	0.10273		J1.5	0.07916		K1.5	0.05694		L1.5	0.06426		M2.1	0.03248		N1.5	0.11016
	H1.6	0.11065		I1.6	0.04949		J1.6	0.05822		K1.6	0.04129		L1.6	0.02578		M2.2	0.04218		N1.6	0.09875
	H1.7	0.05521		I1.7	0.06561		J2.1	0.03142		K1.7	0.05995		L1.7	0.08539		M2.3	0.07883		N1.7	0.08464
	H1.8	0.08024		I1.8	0.15826		J2.2	0.11174		K1.8	0.05716		L1.8	0.08454		M2.4	0.16057		N1.8	0.26959
	H1.9	0.02975		I2.1	0.13078		J2.3	0.05295		K1.9	0.05085		L1.9	0.04601		M2.5	0.04285		N1.9	0.07577
	H1.10	0.21292		I2.2	0.06458		J2.4	0.06646		K1.10	0.08764		L1.10	0.05117		M2.6	0.03735		N1.10	0.00893
	H1.11	0.11774		I2.3	0.08341		J2.5	0.08072		K1.11	0.04649		L1.11	0.11804		M2.7	0.05676		N1.11	0.10005
	H1.12	0.01829		I2.4	0.08776		J2.6	0.03359		K1.12	0.04109		L1.12	0.02757		M2.8	0.06525		N1.12	0.19824
	H1.13	0.13052		I2.5	0.04883		J2.7	0.10761		K1.13	0.13166		L1.13	0.03099		M2.9	0.01944		N1.13	0.09477
	H1.14	0.03273		I2.6	0.09775		J2.8	0.10277		K1.14	0.14366		L1.14	0.02773		M2.10	0.06667		N1.14	0.12544
	H2.1	0.03283		I2.7	0.21371		J3.1	0.06858		K1.15	0.07851		L1.15	0.02409		M2.11	0.10923		N2.1	0.11744
	H2.2	0.12858		I2.8	0.06717		J3.2	0.10919		K1.16	0.03589		L1.16	0.03157		M2.12	0.06782		N2.2	0.07128
	H2.3	0.03384		I2.9	0.06574		J3.3	0.0218		K1.17	0.04689		L1.17	0.06039		M2.13	0.04531		N2.3	0.06554
	H2.4	0.03658		I2.10	0.09674		J3.4	0.02978		K1.18	0.11092		L1.18	0.01447		M3.1	0.09505		N2.4	0.00716
	H2.5	0.13915		I2.11	0.07365		J3.5	0.25578		K1.19	0.06643		L1.19	0.02249		M3.2	0.04116		N2.5	0.09498
	H2.6	0.20686		I2.12	0.16628					K1.20	0.07355		L1.20	0.00836		M3.3	0.06909		N2.6	0.02751
	H2.7	0.05672		I2.13	0.10376					K1.21	0.09834		L1.21	0.01241		M3.4	0.02114		N2.7	0.07897
	H2.8	0.01661		I2.14	0.04768					K1.22	0.20852								N2.8	0.05086
	H2.9	0.18402		I2.15	0.06338					K1.23	0.09466								N2.9	0.03415
	H3.1	0.08866		I2.16	0.06010					K1.24	0.20275								N2.10	0.05604
	H3.2	0.23516		I2.17	0.13847					K1.25	0.05146								N2.11	0.05708
	H3.3	0.11992		I2.18	0.07356					K1.26	0.19467								N2.12	0.04704
	H3.4	0.10094		I2.19	0.02893					K1.27	0.09764								N2.13	0.00247
	H3.5	0.05531		I2.20	0.04488					K1.28	0.11278								N2.14	0.00116
	H3.6	0.12617								K1.29	0.21321									

A-N = sampling site; number before decimal point = quad number in the sampling site, number after decimal = number id of an individual

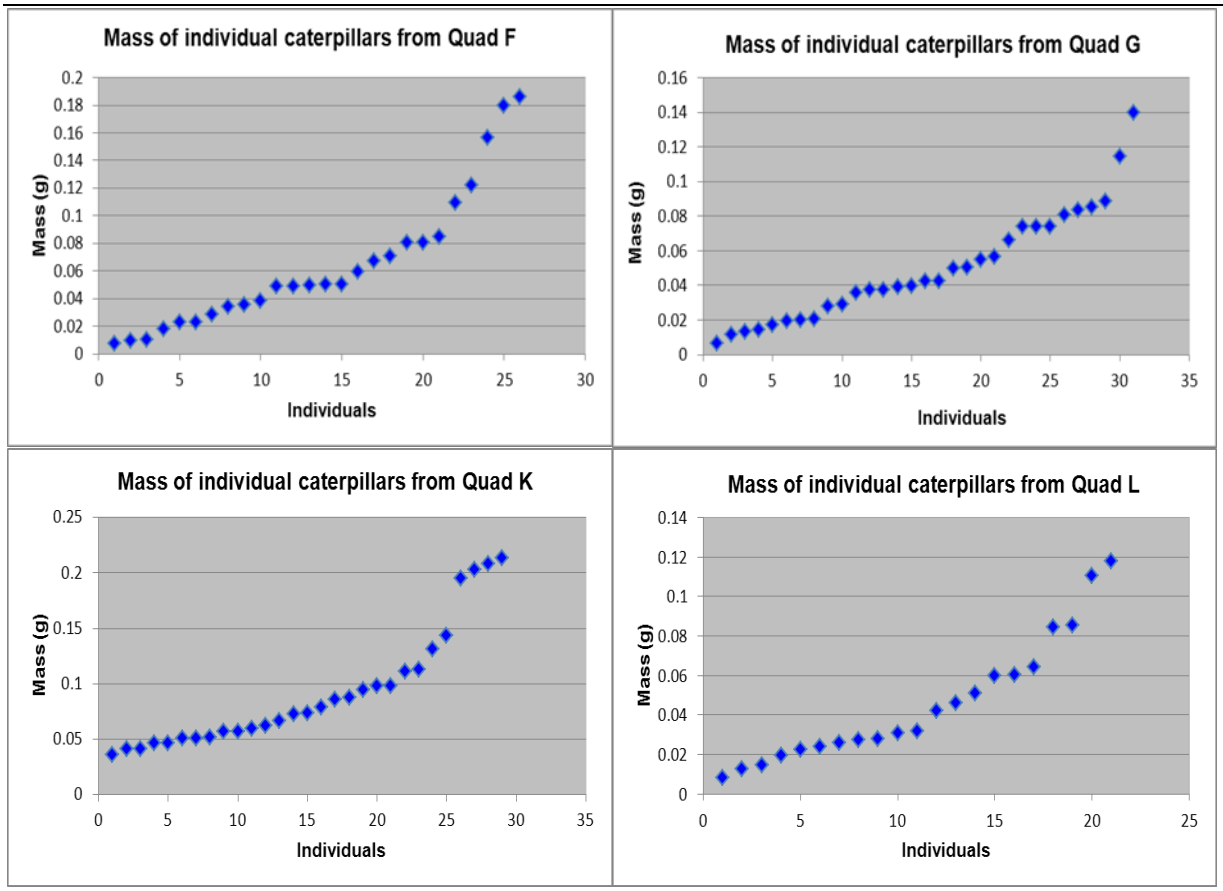

Figure A5.2: Distribution of mass (g) for caterpillars collected from quads.

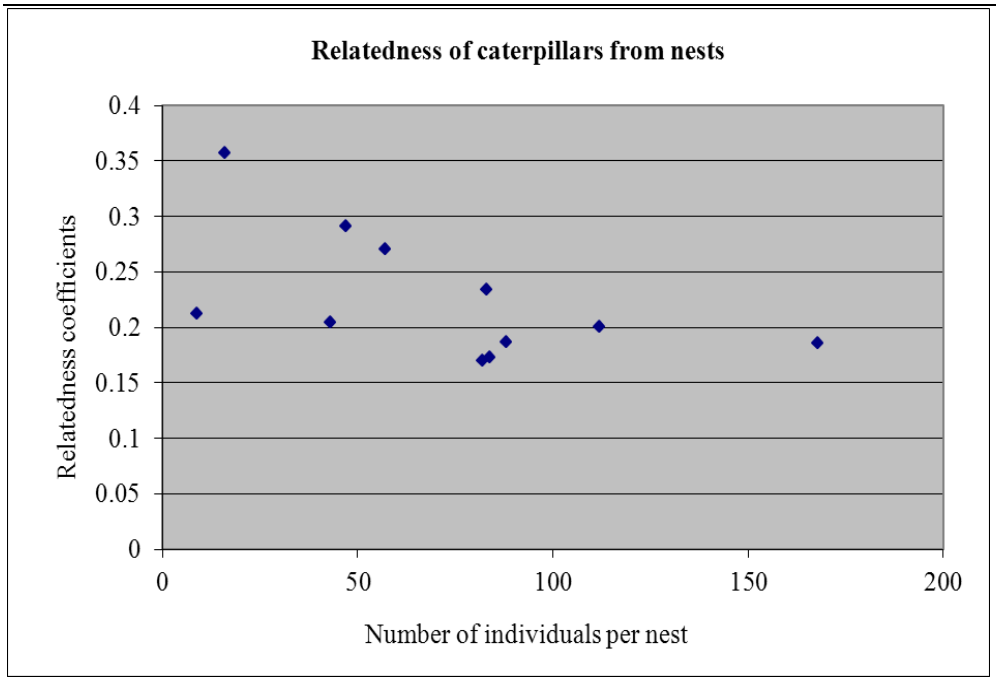

Figure A5.3: Average relatedness of caterpillars from nests.

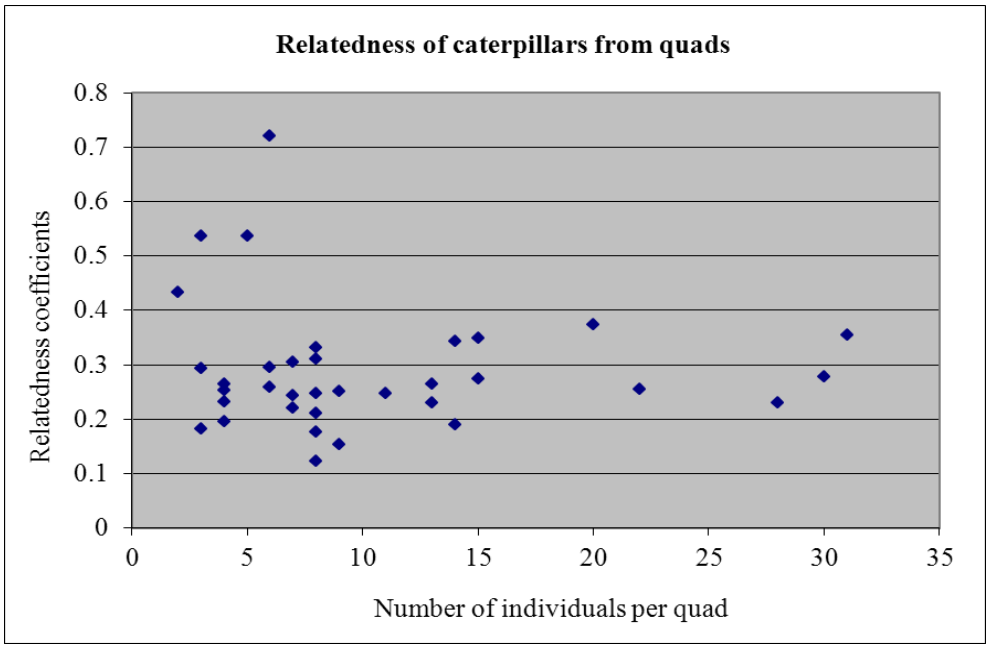

Figure A5.4: Average relatedness of caterpillars from quads.

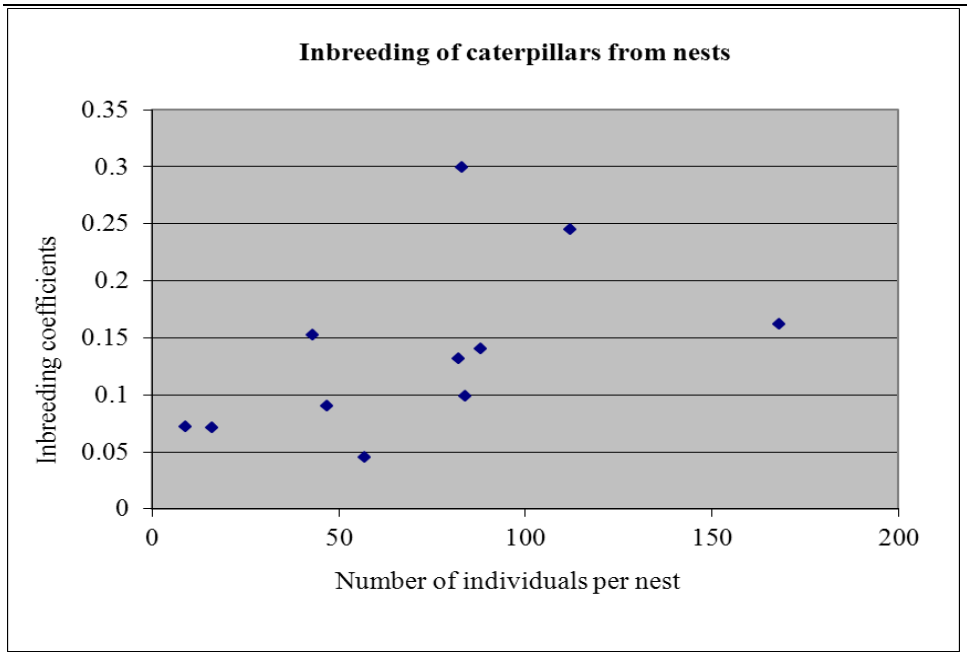

Figure A5.5: Average inbreeding of caterpillars from nests.

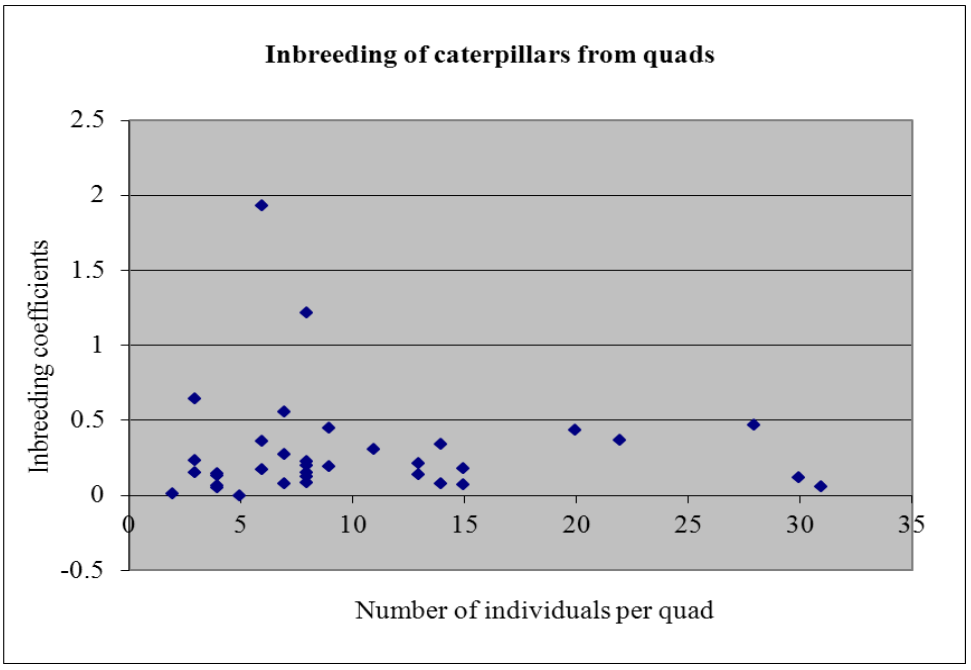

Figure A5.6: Average inbreeding of caterpillars from quads.

Table A5.3: Average relatedness and inbreeding coefficients (variances indicated in brackets) for all the nests and quads sampled for *P. marioni*.

Population (nests / random quads)	Number of caterpillars collected	**Relatedness**		**Inbreeding**	
		TrioML	DyadML	Ritland	LynchRd
Nest 1	16	0.35742 (0.04853)	0.42223 (0.0581)	0.07131 (0.00727)	0.33728 (0.08972)
Nest 2	83	0.23423 (0.0418)	0.29978 (0.05497)	0.29945 (0.22261)	0.27385 (0.09037)
Nest 3	57	0.27059 (0.04053)	0.32229 (0.05063)	0.04524 (0.00425)	0.12456 (0.05115)
Nest 4	9	0.21203 (0.03157)	0.27121 (0.0364)	0.07242 (0.01004)	0.14911 (0.05065)
Nest 5	88	0.18699 (0.03359)	0.22885 (0.04249)	0.14005 (0.16143)	0.18214 (0.05583)
Nest 6	84	0.17315 (0.03041)	0.22207 (0.04031)	0.09882 (0.02861)	0.15951 (0.05417)
Nest WNB1	47	0.29096 (0.03557)	0.34886 (0.04239)	0.09032 (0.02272)	0.16823 (0.0529)
Nest WNJ1	43	0.20431 (0.03327)	0.25871 (0.0403)	0.1523 (0.06246)	0.12876 (0.03978)
Nest WNJ2	112	0.20048 (0.03351)	0.24997 (0.04336)	0.24523 (0.48788)	0.20724 (0.07031)
Nest WNJ3	168	0.18622 (0.03001)	0.23023 (0.0384)	0.1616 (1.1196)	0.14783 (0.04185)
Nest WNJ4	82	0.1699 (0.0252)	0.21291 (0.03318)	0.13145 (0.03959)	0.20551 (0.05196)
Quad A2	2	0.5369 (0.00000)	0.6878 (0.00000)	0.006 (0.00065)	0.00435 (0.00004)
Quad A3	3	0.4341 (0.01712)	0.50573 (0.01901)	0.00707 (0.0019)	0.07413 (0.0129)
Quad A4	5	0.53651 (0.01162)	0.63901 (0.01405)	0.63778 (1.43829)	0.25076 (0.14954)
Quad B1	8	0.12214 (0.02307)	0.15947 (0.02679)	0.19131 (0.04432)	0.23736 (0.08296)
Quad B2	4	0.26395 (0.06341)	0.32127 (0.10076)	0.05638 (0.00347)	0.15888 (0.03593)
Quad B3	10	0.15308 (0.02522)	0.18934 (0.03541)	0.18951 (0.03003	0.33947 (0.08421)
Quad B4	7	0.22128 (0.02344)	0.28099 (0.03383)	0.26713 (0.13607)	0.26697 (0.07416)
Quad C1	12	0.24796 (0.03723)	0.29509 (0.04373)	0.29936 (0.50174)	0.25963 (0.0705)
Quad C2	5	0.19477 (0.03673)	0.23754 (0.03779)	0.0466 (0.01034)	0.1449 (0.07659)
Quad C3	7	0.30429 (0.06652)	0.34329 (0.07086)	0.0744 (0.02067)	0.1919 (0.12436)
Quad C4	15	0.34882 (0.0501)	0.42427 (0.06387)	0.06762 (0.00804)	0.2075 (0.09828)
Quad D1	8	0.26551 (0.04346)	0.34117 (0.05207)	0.12969 (0.06275)	0.13323 (0.02347)

Table A5.3 continued

Population (nests / random quads)	Number of caterpillars collected	**Relatedness**		**Inbreeding**	
		TrioML	DyadML	Ritland	LynchRd
Quad D2	7	0.21019 (0.04614)	0.2635 (0.06369)	0.11894 (0.01347)	0.2566 (0.11585)
Quad D3	13	0.17569 (0.0366)	0.22854 (0.04639)	1.20852 (12.49529)	0.27146 (0.13557)
Quad E1	8	0.24822 (0.03313)	0.30239 (0.04221)	0.14298 (0.01079)	0.30758 (0.05097)
Quad E2	3	0.72097 (0.00119)	0.77447 (0.00211)	1.92627 (0.00429)	0.5698 (0.01705)
Quad E3	3	0.18263 (0.01043)	0.2475 (0.01725)	0.14403 (0.00202)	0.08797 (0.00001)
Quad E4	6	0.29373 (0.02333)	0.34301 (0.02865)	0.229 (0.05579)	0.17198 (0.05958)
Quad F1	28	0.2307 (0.03159)	0.28935 (0.03919)	0.45919 (0.24154)	0.3809 (0.10083)
Quad G1	32	0.35414 (0.03935)	0.42973 (0.05269)	0.0483 (0.00234)	0.16267 (0.05193)
Quad H1	15	0.27462 (0.03294)	0.35768 (0.03952)	0.17058 (0.04554)	0.29897 (0.07231)
Quad H2	9	0.25053 (0.04894)	0.29651 (0.06396)	0.44507 (0.82105)	0.3097 (0.07262)
Quad H3	7	0.24329 (0.05592)	0.28431 (0.06738)	0.55063 (0.98346)	0.55971 (0.05257)
Quad I1	8	0.31061 (0.0348)	0.36384 (0.03649)	0.07996 (0.00864)	0.13563 (0.02671)
Quad I2	20	0.37425 (0.02868)	0.46152 (0.02995)	0.42713 (0.80461)	0.33436 (0.11883)
Quad J1	6	0.29566 (0.04292)	0.36051 (0.04932)	0.1666 (0.05769)	0.23035 (0.08928)
Quad J2	8	0.33148 (0.034)	0.41838 (0.02955)	0.21976 (0.05576)	0.3339 (0.07425)
Quad J3	6	0.25958 (0.03246)	0.39523 (0.041)	0.35518 (0.10782)	0.37725 (0.07587)
Quad K1	30	0.2781 (0.04261)	0.33929 (0.05522)	0.10958 (0.01718)	0.25389 (0.05855)
Quad L1	22	0.25612 (0.04475)	0.31428 (0.0553)	0.36325 (0.46378)	0.34285 (0.08155)
Quad M1	4	0.23043 (0.0329)	0.27933 (0.04428)	0.2075 (0.06544)	0.1836 (0.02754)
Quad M2	13	0.23207 (0.03537)	0.28482 (0.04091)	0.13632 (0.04016)	0.16536 (0.06672)
Quad M3	4	0.25412 (0.01467)	0.32688 (0.01838)	0.1263 (0.0075)	0.0881 (0.00512)
Quad N1	14	0.34291 (0.07321)	0.40402 (0.08246)	0.07109 (0.00832)	0.11204 (0.0176)
Quad N2	13	0.18949 (0.03484)	0.24158 (0.04857)	0.33328 (0.23637)	0.31667 (0.10491)

Table A5.4: Average relatedness and inbreeding coefficients of all the individuals, as well as size cohorts, from 4 nests and 4 quads respectively (all = total number of caterpillars sampled, S = number of small caterpillars, L = number of large caterpillars).

Population	Number of individuals	Relatedness		Inbreeding	
		TrioML	DyadML	Ritland	LynchRd
Nest 2 (all)	81	0.23423	0.29978	0.29945	0.27385
Nest 2 (S)	5	0.10993	0.15358	0.45210	0.38464
Nest 2 (L)	8	0.15947	0.19735	0.38483	0.27148
Nest 3 (all)	59	0.27059	0.32229	0.04524	0.12456
Nest 3 (S)	7	0.12901	0.16478	0.04037	0.05410
Nest 3 (L)	13	0.14716	0.17292	0.04393	0.07041
Nest 5 (all)	98	0.18699	0.22885	0.14005	0.18214
Nest 5 (S)	8	0.11711	0.14523	0.11578	0.19117
Nest 5 (L)	8	0.28633	0.32121	0.07793	0.16807
Nest 6 (all)	65	0.17315	0.22207	0.09882	0.15951
Nest 6 (S)	6	0.08195	0.11986	0.17990	0.06228
Nest 6 (L)	5	0.16137	0.18991	0.15788	0.20616
Quad F (all)	28	0.23070	0.28935	0.45919	0.38090
Quad F (S)	6	0.11642	0.15421	0.11988	0.22412
Quad F (L)	3	0.21280	0.24127	0.45400	0.35440
Quad G (all)	31	0.35414	0.42973	0.04830	0.16267
Quad G (S)	8	0.23854	0.27506	0.135	0.23998
Quad G (L)	2	0.27800	0.43890	0.00495	-0.0074
Quad K (all)	30	0.27810	0.33929	0.10958	0.25389
Quad K (S)	5	0.14731	0.15788	0.13220	0.08068
Quad K (L)	4	0.17027	0.21262	-0.0105	-0.00198
Quad L (all)	22	0.25612	0.31428	0.36325	0.34285
Quad L (S)	5	0.15680	0.16900	0.31586	0.20310
Quad L (L)	4	0.24235	0.26910	0.27318	0.48765