A preliminary conservation genetic study of *Pittosporum obcordatum* (Pittosporaceae), an endemic New Zealand species with a disjunct distribution

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Abstract

Pittosporum obcordatum (Pittosporaceae; heart-leaved kōhūhū) is an endemic New Zealand plant species that is classified as Threatened - Nationally Vulnerable. It has a disjunct distribution and is only known from relatively few and small populations. Using 10 Inter-Simple Sequence Repeat markers (ISSRs), we studied patterns of genetic diversity and genetic differentiation among eight out of the c. 14 populations of this species to inform its conservation management. *Pittosporum obcordatum* has low genetic diversity at the population level ($uH_e = 0.169$) compared to other long-lived and outcrossing species, but genetic diversity is relatively high in comparison

with several other threatened species. Spearman's Rank Correlation Coefficients suggest significant positive correlations between population size and genetic diversity as measured by the percentage of polymorphic loci and uH_e. *Pittosporum obcordatum* also shows relatively high levels of genetic differentiation among populations (AMOVA-derived $\Phi'_{st} = 0.508$, $P < 0.001$; all pairwise Φ_{st} values $P < 0.05$), indicating low genetic connectivity. Populations with relatively few plants are therefore prone to further reductions in genetic diversity through inbreeding and genetic drift. Of these, especially the Kaitaia, Owen Valley and Paengaroa populations are of conservation concern, because they contain private alleles, and therefore notably contribute to the genetic diversity of *P. obcordatum*.

Keywords

Genetic connectivity; Genetic diversity; habitat fragmentation; ISSR; New Zealand; population genetics; rare plant species

Introduction

A noted feature of the New Zealand flora is the current disjunct distribution of many of its uncommon plant species (de Lange et al. 2004, 2009; Shepherd & Perrie 2011). Such distribution patterns can be the result of long distance dispersal across unfavourable habitats or vicariance (McGlone 1985; Shepherd & Perrie 2011). Disjunct distributions as a result of vicariance can be of natural origin, caused by, for example, tectonic changes in land and sea patterns (McGlone 1985; Rogers 1989), or climatic changes such as glaciation (Wardle 1963; Shepherd & Perrie 2011). However, small isolated populations can also be a consequence of anthropogenic habitat destruction and modification (e.g. Armstrong & de Lange 2005). Uncommon

disjunct species are of conservation concern, because they might be particularly susceptible to losing genetic diversity through genetic drift and inbreeding (Frankel & Soulé 1981).

Pittosporum obcordatum Raoul (1844; Pittosporaceae) is a long-lived (up c. 120 years; Clarkson & Clarkson 1994) heteroblastic divaricating shrub that matures into a small tree (Figure 1). It has a dioecious mating system due to its functionally unisexual flowers (Cooper 1956; Clarkson & Clarkson 1994). *Pittosporum obcordatum* is an uncommon endemic New Zealand species with an unnatural disjunct lowland distribution (Figure 2). It is only known from about 14 populations in the North and South Islands (Clarkson & Clarkson 1994; de Lange et al. 2010), with most of these concentrated along the eastern side of the North Island and the southernmost part of the South Island (Clarkson & Clarkson 1994; de Lange et al. 2010). It has been hypothesized that *P. obcordatum* was once locally common in its formally widespread habitat, which is considered to be lowland, drought and/or frost prone alluvial forest (Clarkson & Clarkson 1994; de Lange et al. 1996; de Lange et al. 2010), but that it has become an uncommon and more disjunctly distributed species because of the destruction and fragmentation of this habitat (Given 1981; Clarkson & Clarkson 1994; de Lange et al. 2010). Many of the sites where *P. obcordatum* is currently found have fewer than 50 individuals, show little or no recruitment (Clarkson & Clarkson 1994), occur on private land, and have no legal protection. The total number of individuals of *P. obcordatum* in the wild is estimated to be about 2,500 (Clarkson & Clarkson 1994). Because this species mainly consists of small and isolated populations, and many of these show little recruitment, *P. obcordatum* has a conservation ranking of Threatened - Nationally Vulnerable (de Lange et al. 2013).

The aim of the study presented here is to contribute to the conservation management of *P. obcordatum* by providing the first insights into patterns of genetic diversity and genetic differentiation among eight out of its c. 14 populations. Inter-Simple Sequence Repeat markers (ISSRs) were selected for this purpose. ISSRs have been previously successfully used to study the genetic variation of species of conservation concern (e.g. George et al. 2009; Liu et al. 2012; Cires et al. 2013; Xing et al. 2015), including *Pittosporum* Banks ex Solander species (Carrodus 2009; Clarkson 2011; Mendes et al. 2011; Clarkson et al. 2012).

Materials and methods

Eight populations throughout the distribution of *P. obcordatum* were selected for our study (four in the North Island and four in the South Island; Figure 2). Leaf tissue samples in silica gel were obtained for six to 24 individuals per population (Table 1). Voucher specimens were lodged at the University of Canterbury herbarium (CANU; Table 1). A modified CTAB method (Doyle & Doyle 1987) was used for DNA extraction.

Using the methodology outlined below, 25 ISSR primers (Invitrogen) selected from those used by Clarkson (2011) and Mendes et al. (2011) were included in an initial screening for amplification success, reproducibility of results, and levels of polymorphism. The results of this pilot study were used to select 10 primers for subsequent analyses (Table 2). Polymerase Chain Reaction (PCR) was performed in a total volume of 15 µl. Each reaction consisted of 1 µl of unquantified template DNA, 6.13 µl of nuclease-free water, 3 µl 5× GoTaq buffer (Promega), 1.2 µl of each dNTP at 2.5mM, 1 µl of 25 pmol/µl ISSR primer, 2.4 µl of 25mM $MgCl_2$, 0.15 µl of 10 mg/ml of bovine serum albumin (BSA) and 0.12 µl of GoTaq Flexi Taq (Promega).

The cycling conditions were as follows: initial denaturation at 94° C for 4 min, followed by 40 cycles of denaturation at 94 °C for 40 sec, annealing at 50 °C for 45 sec, polymerisation at 72 °C for 90 sec, and a final extension step of 72 °C for 5 min. In addition to a negative control, two positive controls (samples that amplified in previous ISSR PCRs) were included in each PCR run to identify genotyping errors as a result of unsuccessful PCR amplification and to verify consistency of amplification across different PCRs. PCR fragments were separated by electrophoresis on 100 ml 2% agarose gels in 1× sodium borate buffer (Brody & Kern 2004) with 7 µl SYBR Safe (Invitrogen). 12 µl of PCR product was loaded into each well. 2 µl of Hyperladder 50bp (Bioline) was loaded into the first and last well of every electrophoresis gel to provide a measure of fragment length. Gels were run for five to six hours at 60 V. They were subsequently photographed using a Syngene G: BOXEF2 imager.

DNA fragments that were of an equal size were assumed to be homologous and were manually scored as either present or absent. Fragments of ambiguous presence (i.e. faint bands) were coded as missing data. Loci that frequently failed to amplify successfully were removed from further analyses. In order to calculate ISSR error rates, 20 samples were amplified twice for all ISSRs (Bonin et al. 2004).

GenAlEx v.6.501 (Peakall & Smouse 2012) was used to calculate the number of private alleles (i.e. alleles that are only found in a single population; PA) and the expected heterozygosity (H_e, and unbiased heterozygosity: uHe) for each population (Table 1). Allelic diversity and the percentage of polymorphic loci (PPL) were calculated using the rarefaction method that is implemented in AFLPdiv v.1.1 (Coart et al. 2005), with a rarefaction sampling size of six individuals (i.e. the smallest sampling size; Table 1). To test whether populations with fewer individuals have less

125 genetic diversity than populations with more individuals, the Spearman's Rank
126 Correlation Coefficients between the estimated number of individuals in each
127 population and three measures of genetic diversity (allelic diversity, PPL, uH_e) were
128 calculated in Microsoft Excel.

129 An Analysis of Molecular Variance (AMOVA; Φ_{st}, 999 permutations) in
130 GenAlEx and Bayesian model-based clustering analyses in STRUCTURE v.2.3.4
131 (Pritchard et al. 2000; Falush et al. 2003, 2007) were used to study patterns of genetic
132 differentiation between the eight *P. obcordatum* populations included in this study.
133 The STRUCTURE analyses used the admixture model and correlated allele
134 frequencies. These analyses were run with K values (estimated number of genetic
135 groups) from 1–10 and with 12 iterations for each K value. Each analysis was run for
136 700,000 generations of which the first 200,000 were discarded as burn-in. The
137 STRUCTURE output was summarized using STRUCTURE HARVESTER (Earl &
138 Von Holdt 2012) to determine the value of K that best explains the genetic structure
139 in the data. For this, both the Evanno et al. (2005) method (K with the highest value
140 of ΔK) and the method of Pritchard et al. (2000; with the highest Pr(X|K)) were used.
141 CLUMPAK 1.1 (Kopelman et al. 2015) was used for the summation and graphical
142 presentation of the STRUCTURE results.

143 Mantel tests (999 replicates) were used in GenAlEx to test for correlations
144 between geographic distance and dominant genotypic distance between individuals.
145 This was done to determine if isolation by distance can explain the patterns of genetic
146 connectivity between populations that were observed (i.e. if genetic distance is
147 positively correlated with geographic distance). The Mantel tests were executed with
148 non-transformed geographic distances as well as with a data set in which these
149 distances were log-transformed.

Results

Genetic diversity

The ISSR fragments that were scored ranged from 200 to 1200 bp. Between 11 and 20 fragments were scored for each of the 10 primers (Table 2), resulting in a data matrix with 158 fragments scored for 128 individuals from eight populations. The error rate per primer ranges from 0.4% to 10.6%, with a mean error rate of 6.4%. At the species level, the PPL is 94.9% and the uH_e is 0.274 (H_e = 0.273; Table 3). At the population level, uH_e varies between 0.142 (Kaitaia) and 0.201 (Whangarei; Table 1). Allelic diversity ranges from 1.33 (Owen Valley) to 1.45 (Whangarei) and the PPL from 35.4% (the Catlins) to 62.7% (Whangarei; Table 1). The Spearman's Rank Correlation Coefficient between measures of genetic diversity and the estimated number of individuals of each population is statistically significant at $P = 0.05$ for the PPL ($r_s = 0.76$) and uH_e ($r_s = 0.74$), but not for allelic diversity ($r_s = 0.40$).

Genetic differentiation

Private alleles were detected in four populations (Table 1). Four private alleles were found in Banks Peninsula and three in Owen Valley (South Island). Paengaroa and Kaitaia (North Island) each have one private allele. The AMOVA revealed that overall genetic differentiation among the *P. obcordatum* populations included in our analyses is significant ($\Phi'_{st} = 0.508$, $P < 0.001$). A total of 42% of the genetic variation is found among populations and 58% among individuals. All pairwise Φ_{st} values between populations are statistically significant at $P = 0.05$ after B-Y correction (Narum 2006; Table 4). The smallest Φ_{st} value is between Paengaroa and Whangarei (North Island), and the largest between Owen Valley (South Island) and

Kaitaia (North Island). The results of the STRUCTURE analysis identified four (ΔK method) or five (Pr(X|K) method) primary genetic clusters with relatively little admixture between most of them. Two of these clusters are largely restricted to the South Island and align well with Banks Peninsula and Owen Valley, with the Catlins showing some admixture between these clusters, but overall more similarity with the Banks Peninsula cluster (Figure 3). Individuals from Kaitaia, Paengaroa, Whangarei together form a central and northern North Island genetic cluster. STRUCTURE results with K=4 and K=5 differ in the clustering of individuals from Back Valley (South Island) and Tauweru (North Island). The K=4 hypothesis places individuals of these two populations in the same genetic cluster, whereas the K=5 hypothesis suggests two different clusters, with several Tauweru individuals showing admixture with Back Valley (Figure 3). A Mantel test found no significant positive correlation between genetic distance and geographic distance when all eight populations of *P. obcordatum* were included ($R^2 = 0.144$, $P = 0.27$). However, when North and South Island populations were analysed separately, isolation by distance was statistically significant within the North Island ($R^2 = 0.357$, $P = 0.03$), whereas there was no evidence for isolation by distance among South Island populations ($R^2 = 0.181$, $P = 0.18$).

Discussion

Genetic diversity

Using ISSR markers, we provide a first estimate of patterns of genetic diversity in *Pittosporum obcordatum*. At the species level, *P. obcordatum* displays high (PPL = 94.9%) or moderately high ($H_e = 0.273$, $uH_e = 0.274$) genetic diversity compared to other long-lived, outcrossing plant species of conservation concern that have been

studied using ISSR data (Table 3). In addition, at the population level, this species shows high (PPL = 49.1%) or moderately high (H_e = 0.163, uH_e = 0.169) genetic diversity relative to other threatened species (Table 3). More generally, however, *P. obcordatum* has considerably lower genetic diversity at the population level than a meta-analysis of genetic diversity in plants by Nybom (2004) indicates for long-lived perennials (H_e = 0.25) and outcrossing plants (H_e = 0.27). Although the estimates of genetic diversity reported in this meta-analysis were obtained from RAPD instead of ISSR data, those of various dominant markers (RAPD, AFLP, ISSR) are generally very similar and therefore possibly directly comparable (Nybom 2004). The highest heterozygosity (uH_e = 0.201) was observed in Whangarei, which is one of the two populations with the highest number of *P. obcordatum* individuals (Table 1). This population also had the highest allelic diversity (1.45) and PPL (62.7%). In contrast, populations with relatively few individuals display the lowest genetic diversity, in particular the Catlins, Owen Valley and Kaitaia (Table 1). Spearman's Rank Correlation Coefficients further show that this positive correlation between the number of individuals per population and genetic diversity (as measured by PPL and uH_e) is statistically significant ($P < 0.05$). The Back Valley population, however, deviates from this general pattern. Despite having the shared highest number of *P. obcordatum* individuals (c. 700), it displays relatively low genetic diversity (Table 1). This finding is surprising, because Back Valley, which is situated in Fiordland National Park, is the population that has least been affected by human land use. It largely consists of intact native vegetation, although it has been exposed to wildfires and high deer numbers (Morrison 1982; Brian Rance, Department of Conservation, pers. comm.). Potentially, Back Valley was covered with ice during the Pleistocene glaciations. It is therefore possible that the low genetic diversity at Back Valley

indicates that this area was colonized by *P. obcordatum* relatively recently and expanded from a founder population with limited genetic diversity. Alternatively, it is possible that this population experienced a genetic bottleneck during glacial times, or as a result of other historical events.

Genetic differentiation

Our analyses also provided preliminary data on genetic differentiation in *P. obcordatum*. The AMOVA results show evidence of significant differentiation between the eight *P. obcordatum* populations included in this study (Φ'_{st} = 0.508, $P <$ 0.001; all pairwise Φ_{st} values $P < 0.05$). In addition, the Φ_{st} value of this species (0.42) is considerably higher than the mean RAPD-derived estimates for long-lived perennials (0.25) and outcrossing species (0.27; Nybom 2004). Our data therefore indicate low overall genetic connectivity between populations. The distribution of private alleles (Table 1) indicates that the Banks Peninsula and Owen Valley populations (South Island) might have been genetically isolated from other *P. obcordatum* populations for a relatively long time, because they have the highest numbers of private alleles (four and three, respectively; Table 1). Owen Valley is located in north-west Nelson, which might have been a glacial refugium (McGlone 1985; Gardner et al. 2004). The relatively high number of private alleles of the Owen Valley population could indicate that this region was also a refugium for *P. obcordatum*. It is then possible that a subsequent genetic bottleneck caused the observed low genetic diversity of this population. However, it is also possible that the Owen Valley population has a more recent origin and that its private alleles are a result of genetic drift in both the Owen Valley population and its source population, or that this source population went extinct. Paengaroa and Kaitaia (North Island) are

the only other populations in which private alleles were found (one in each; Table 1). The results of the STRUCTURE analyses indicate greater genetic connectivity among the central and northern North Island populations (Kaitaia, Paengaroa, Whangarei) than with populations elsewhere in New Zealand (Figure 3). Similarly, there is evidence of past genetic connectivity between the two eastern South Island populations (Banks Peninsula and the Catlins), although admixture patterns in individuals from the Catlins also suggest some connectivity with the northern South Island Owen Valley population. Interestingly, both the K=4 and K=5 STRUCTURE results suggest genetic connectivity between Back Valley (southern South Island) and Tauweru (southern North Island; Figure 3). This finding in combination with the absence of private alleles in both populations and the discovery of somewhat lower genetic diversity in Back Valley than in Tauweru despite a larger number of individuals in the former, might indicate that the Back Valley plants descend from seeds that have relatively recently arrived from the Tauweru area. It is, however, also possible that the Back Valley population was established from seeds from a local population that was not sampled, such as the Waiau or Southland Plains population or from a population that is now extinct. More detailed molecular genetic studies in which more individuals and sites in the Back Valley and Tauweru populations are sampled are needed to test this hypothesis, as well as samples from the populations that were not sampled in our study. To better understand the observed patterns of genetic connectivity, future studies should also focus on determining the seed dispersal mechanism of *P. obcordatum*. Seed dispersal by birds and during flooding has been suggested (Sainsbury 1923; Clarkson & Clarkson 1994), but ecological research is needed to test these hypotheses.

Conclusions and conservation implications

In agreement with previous studies (Given 1981; Clarkson & Clarkson 1994; de Lange et al. 2010), the results of our preliminary conservation genetics study indicate that *P. obcordatum* is of conservation concern (de Lange et al. 2013). We provisionally conclude from our data that the genetic diversity of *P. obcordatum* at the population level is relatively low compared to other long-lived and outcrossing plant species (Nybom 2004). However, genetic diversity within the eight populations studied is relatively high in comparison with several other threatened plant species (Table 3), *P. obcordatum* populations with relatively few individuals have significantly lower genetic diversity than larger populations. Because *P. obcordatum* populations show low genetic connectivity among them, populations with relatively few plants are prone to further reductions in genetic diversity through inbreeding and genetic drift. Some of these populations are of particular conservation importance, because they have private alleles. A further loss of genetic diversity in these populations could therefore result in lower genetic diversity at the species level. For example, the Owen Valley population only has c. 23 individuals, but has three private alleles (Table 1), and is therefore of considerable conservation importance. Similarly, the Kaitaia and Paengaroa populations both have few individuals and a private allele each (Table 1). Our results therefore indicate that it is important that conservation management of these population focuses on avoiding a decline in population size, with the aim of maintaining sufficient genetic diversity at the species level to reduce the risk of extinction caused by a lack of evolutionary potential and resilience to environmental changes (Kramer & Havens 2009).

The low genetic connectivity between *P. obcordatum* populations is likely a consequence of the destruction and fragmentation of its lowland, drought and/or frost

prone alluvial forest habitat. The natural distribution pattern of this habitat in New Zealand suggests that *P. obcordatum* was probably naturally disjunct between Northland and the eastern part of the North Island, and between Nelson and eastern South Island. However, large scale land clearance and conversion is undoubtedly the primary cause of the current disjunct distribution of *P. obcordatum* and its few and often small populations. Low recruitment, competition by exotic plants and cattle browsing are among the factors that are potentially responsible for further reducing the size of *P. obcordatum* populations (Clarkson & Clarkson 1994; de Lange et al. 2010), but further research is needed to test these hypotheses.

It could be argued that the distribution of *P. obcordatum* is less disjunct than it currently appears. *Pittosporum obcordatum* is morphologically cryptic and, in the absence of reproductive parts, easily confused with a number of other small-leaved divaricating plants, such as *Myrsine divaricata* A.Cunn. and *Lophomyrtus obcordata* (Raoul) Burret. Indeed, since the detailed ecological account of this species by Clarkson & Clarkson (1994), additional populations have been found on Banks Peninsula and in Owen Valley and Southland. This indicates that it is possible that, with time, additional populations will be discovered as more people become familiar with *P. obcordatum*.

Representatives from only eight of the c. 14 known populations of *P. obcordatum* were included in our study. In the South Island, we were not able to sample at several small and isolated fragments of native vegetation north of Invercargill (Southland Plains) in which low numbers of individuals have been reported, or from the Waiau Valley, from which 11 *P. obcordatum* plants are known (Brian Rance, Department of Conservation, pers. comm.). In addition to the populations that we included in our study, *P. obcordatum* is mostly known in the

North Island from the Hawke's Bay and Gisborne regions, where most individuals are found in the vicinity of Waipukurau (c. 250 plants; Walls 1998), Wairoa (197 plants; Shaw 1990) and Gisborne (130 plants; Clarkson & Clarkson 1994). In addition, *P. obcordatum* has been reported from the Tukituki river (Clarkson & Clarkson 1994). Including samples from these populations should be a priority in future studies. Although the use of ISSR markers in this preliminary study resulted in genetic patterns that are intuitively meaningful and similar to those found in other species of conservation concern (Table 3), future studies might benefit from using co-dominant markers (e.g. microsatellites) to enable more direct estimates of heterozygosity.

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Captions

Figure 1. *Pittosporum obcordatum*. **A**, Habit of mature plant. **B**, pistillate flowers. **C**, flowers and leaves of mature plant. **D**, flowering branches of mature plant. **E**, heterophylly of juvenile plant.

Figure 2. General location of known *Pittosporum obcordatum* populations. Black circles: populations sampled for this study. White circles: unsampled populations.

Figure 3. STRUCTURE results for ISSR data of *Pittosporum obcordatum*. Each bar represents an individual plant and bar colours indicate the proportion of membership to each genetic cluster (q values). Individuals are grouped by population. **A,** K = 4. **B,** K=5.

Table 1. Sampling locations and estimates of genetic diversity for *Pittosporum obcordatum* populations. Voucher specimens are lodged at CANU. PA: number of private alleles, uH_e: unbiased expected heterozygosity, A: allelic diversity, PPL: percentage of polymorphic loci.

Table 2. Details of ISSR markers used in this study of *Pittosporum obcordatum*.

Table 3. Genetic diversity of *Pittosporum obcordatum* in comparison with examples of other plant species of conservation concern. All values calculated from ISSR data. PPL: percentage of polymorphic loci, H_e: expected heterozygosity.

Table 4. Pairwise genetic differentiation values (Φ_{st}) between *Pittosporum obcordatum* populations. All values are statistically significant at $P = 0.05$ after B-Y correction for multiple comparisons (Narum 2006).