Speciation and gene flow in Central American *Begonia* L. (Begoniaceae)

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Declaration

I hereby declare that the work contained in this thesis is my own, unless otherwise acknowledged and cited. This thesis has not in whole or in part been previously presented for any degree.

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Abstract

Begonia L. is one of the largest plant genera, comprising over 1500 species. Weak species cohesion, and the rapid evolution of reproductive barriers in allopatry, are two processes that have been postulated to explain the generation of such hyper-diversity of taxa within a single genus of plants. The aim of this thesis is to investigate whether these factors are likely to have been important contributors to the diversity of species found in Central American *Begonia*. Species cohesion was analysed in the widespread Central American species *Begonia heracleifolia* and *B. nelumbiifolia*. Interpopulation seed flow was estimated with seven plastid microsatellites. Breeding system estimates and measures of genetic differentiation at nine nuclear microsatellites were used to infer levels of interpopulation pollen flow. Controlled crosses were employed to assess the strength of reproductive barriers both between populations within species, and between species differing in ecology. The potential for gene flow between species in the wild was assessed in natural hybrid zones using molecular markers. Finally a quantitative trait locus (QTL) approach was employed to investigate the genetic basis of reproductive traits that differ between species. No plastid polymorphisms were found in *B. nelumbiifolia*, suggesting it has been through a recent population bottleneck. In contrast, *B. heracleifolia* possessed many plastid haplotypes that were strongly differentiated between populations ($G'_{ST} = 0.829$). Nuclear microsatellites showed high genetic differentiation within species, and both species were self-compatible and self-fertilize at a moderate rate (*B. heracleifolia* $F'_{ST} = 0.506$, $F_{IS} = 0.249$; *B. nelumbiifolia* $F'_{ST} = 0.439$, $F_{IS} = 0.380$). F1s between ecologically similar *B. heracleifolia* and *B. sericoneura* were partly fertile (2-5% seed set), and F1s and early generation backcrosses were found in a hybrid swarm. F1s between *B. heracleifolia* and the ecologically contrasting *B. nelumbiifolia* were pollen sterile, and 3 hybrid swarms showed no evidence of hybrids beyond the F1 generation. Seven QTL were found for reproductive traits, including: sex ratio, pollen sterility and stamen number. The population biology of *Begonia*, with limited seed and pollen dispersal, small population sizes and frequent self-fertilization predisposes them to genetic isolation, increasing the chances that reproductive barriers evolve. These characteristics may underlie the large number of endemics in *Begonia*.

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TABLE OF CONTENTS

CHAPTER 1: Introduction

There has long been an interest in plant genera and families that are species rich. The many variations in form on a common theme appeal to gardeners and plant collectors, and scientific interest is engaged by the evolutionary processes that underlie such a distinct pattern. Very large groups include the daisy family (Asteraceae), with around 23,000 species, and the orchid family (Orchidaceae), with over 22,000 species (Heywood *et al.*, 2007). Even though these large plant groups are extremely familiar, species rich groups are actually uncommon. A plot of the number of plant species per genus demonstrates the scarcity of species rich groups, as well as the commonality of species poor groups. This pattern has been called the ‘hollow curve’ (Willis, 1922; Fig. 1.1).

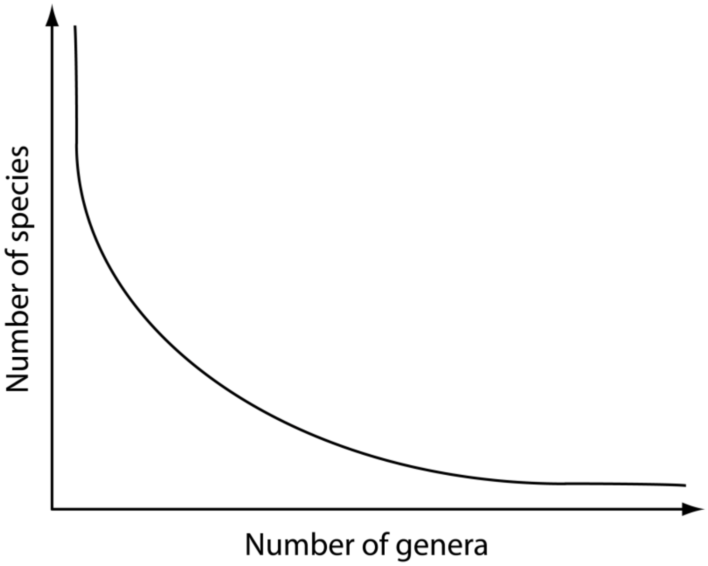


Figure 1.1. The hollow curve of genus sizes.

Expert opinions differ on the way species are delimited. Some experts divide taxa differing in only a few morphological characters (‘splitters’), while others combine broadly similar taxa under one name (‘lumpers’) (Endersby, 2009). This raises the concern that the hollow curve may be a taxonomic artefact, caused by experts delimitating species (and genera) differently. Another explanation for the hollow curve would be variation in the speciation or extinction rates between lineages.

This thesis examines speciation processes in an exceptionally specious genus. Species delimitation and generic boundaries are discussed first, as these are essential for understanding whether species rich genera are artefacts or are caused by evolution processes. The evolutionary processes that may give rise to species rich genera are then discussed, as well as the ways these can be studied. Examples of species rich genera, and the processes that have shaped them are then considered, before introducing the study system for the thesis.

1.1. Speciation and taxonomy

1.1.1. Species concepts and species delimitation

The use of species as universal biological units in evolutionary research is controversial (Coyne & Orr, 2004). Some researchers have argued that species are meaningful units that can be delimited using simple criteria (Rieseberg *et al.*, 2006), while others agree that species are real but question whether the same criteria can be applied equally to all species groups (Mishler & Donoghue, 1982). A different point of view is that species are human constructs imposed onto the continuum of biological diversity and are not meaningful units (Ehrlich & Raven, 1969; Bachmann, 1998). The ongoing disagreement over species as biological units results in a wide range of ways to delimit species boundaries.

The biological species concept (Mayr, 1942; Mayr, 1969) describes a species as interbreeding populations that are reproductively isolated from other such groups. In this case, reproductive isolation describes any barrier to gene flow between species, whether it is prior to cross-fertilization (prezygotic), or after fertilization has occurred (postzygotic). One strong criticism of the biological species concept is that it ignores the high frequency of interspecific hybridization in nature (Stace, 1975). It also ignores the high rate of interspecific gene flow that occurs during speciation (Machado & Hey, 2003; Nosil, 2008). More recent species concepts address concerns raised with the biological species concept (summarised in Mallet, 1995; Wu, 2001; Coyne &

Orr, 2004; Hausdorf, 2011), although none have been universally accepted. One example is the differential fitness species concept (Hausdorf, 2011), which defines species as 'groups of individuals that are reciprocally characterised by features that would have negative fitness effects in other groups and that cannot be regularly exchanged by groups upon contact'. This is a more accurate approximation of a species, adequately accounts for hybridization, and also covers the early stages of speciation. However, species delimitation from this concept would be far more complex than the biological species concept, as it would require the fitness of many hybrids to be studied.

Morphology alone is usually the sole criteria for species delimitation. Rieseberg *et al.* (2006) found that morphological groupings inferred from phenotypic clustering generally accord with reproductive isolation inferred from crossing barriers, supporting morphological approaches to species delimitation. Morphology is a useful proxy for species barriers as most species have been isolated for sufficient time for morphological differences to arise, and extinction of related species will further increase morphological distinction. One concern with morphological delimitation is that these traits may not be independent, due to the influence of pleiotropy or linked genes (Doyle, 1992). The study by Rieseberg *et al.* (2006) shows that non-independence of characters does not typically have a strong impact on species delimitation.

Groups where there is difficulty delimiting species using morphological characters include taxonomically complex groups, recent species radiations, and cryptic species. Taxonomically complex genera are characterised by asexual reproduction and hybridization (Ennos *et al.,* 2005) including *Euphrasia* L. (Orobanchaceae) and *Hieracium* L. (Asteraceae). While species can be found that differ in morphology, these often grade into each other making delimitation difficult. Species of recent origin will differ in few morphological characters, such as Mediterranean sexually deceptive orchids (Scopece *et al.,* 2010), and these also pose challenges for species delimitation (Shaffer & Thomson, 2007). Finally, species that are nearly morphologically indistinguishable from each other but differ in other characteristics suggesting they are separate species (cryptic species), pose major difficulties for taxonomists. One example is the liverwort genus *Herbertus* Gray (Herbertaceae), where DNA barcoding has revealed genetically distinct lineages within what was considered a single species, and morphological characters have now been found to support the genetic groups (Bell *et al.*, 2012). In these cases where choosing morphological characters to discriminate species is difficult, a more robust approach to species delimitation is to focus on multiple direct and indirect lines of evidence (e.g. crossing barriers, overlap in gene pools, differing morphologies) for reproductive barriers, which are common to all species concepts (Sites & Marshall, 2004).

While species delimitation is difficult in some complex groups, in most cases species can be recognised (Rieseberg *et al.*, 2006). However, the existence of species as evolutionary units is more important than their place in the taxonomic hierarchy. Species are a cohesive unit composed of populations that are connected by gene flow, and where new alleles arise through mutation these will be spread between populations, uniting populations within a species. This differs from higher taxonomic units such as genera, where interspecific gene flow is low compared to intraspecific gene flow (Hey & Pinho, 2012), and this tends to be restricted to secondary contact zones at range margins (Willyard *et al.*, 2009).

1.1.2. Genera and higher levels of taxonomic classification

The higher level organisation of species into genera, and genera into families, is at the core of systematics and taxonomy. However, the delimitation of such groups is controversial (Walters, 1986). Part of this controversy is because no set of criteria define hierarchical taxonomic ranks, such as genera. There are, however, two general conditions that should be met. A genus should be monophyletic, i.e. have a single common origin when the evolutionary relationships of species are assessed with molecular data (Hennig, 1966; Bremer & Wanntorp, 1978). Second, species in a genus should share a common set of morphological characters that differ from those of other genera (Stebbins, 1956). These criteria for generic limits are evidently arbitrary (Stebbins, 1956), and genera will differ in their age, size and genetic diversity. An exceptional contrast in size and age is between the monotypic genus *Ginkgo* Rich. (in the monotypic family Ginkgoaceae) that diverged from cycads ~300Ma (Pryer *et al.*, 2004), and *Astragalus* Medik. with ~2 500 species originating ~12Ma (Sanderson & Wojciechowski, 1996).

The subjectivity of genera does not invalidate them as a unit of comparison. Many genera have been relatively stabile over taxonomic history (Frodin, 2004) suggesting that at least morphologically, genera represent cohesiveness in adaptive space, and are therefore biologically relevant units. Most lineages have diversified within a single biome rather than switching between them (phylogenetic niche conservatism, Pennington *et al.*, 2009), so genera tend to be distinct in their ecology and distribution too. Therefore genera will unite species that have retained ancestral morphology and often ecological adaptations too, and where lineages have diversified extensively from their ancestral phenotype, they are more likely to be put in their own genera.

Uncertainty as to whether a group should be ranked a genus or a family can cause concerns as to whether species richness reflects speciation histories or taxonomic artefacts. However, the hierarchical level of study does not bias patterns of species richness, as similar trends of species richness emerge when the family rather than the genus is used as a unit of comparison (Clayton, 1974). For example, a similar 'hollow curve' is obtained when the number of genera per family, rather species per genus, are plotted (Clayton, 1974). Moreover, seven of the ten largest plant genera are contained within the ten largest plant families (e.g. *Senecio* L. in the Asteraceae, *Bulbophyllum* Thouars. in the Orchidaceae; www.mobot.org/MOBOT/research/APweb/; discussed below), suggesting many of the properties that have promoted speciation have been present over long periods of time. Therefore, while the genus is a much debated unit of classification, it does represent a biologically meaningful group that can be used as a unit of comparison when the reservations above are taken into account.

1.1.3. Tracing the speciation process from extant plant species

The most direct way to understand evolutionary histories of plants is by studying fossils; but individual fossils have a low information content, and do not tell us about the factors that contributed to speciation (Smith, 2009). Evolutionary biologists more typically study extant groups and use them to infer the forces behind past and current speciation events (Barraclough *et al.*, 1998). A population genetic approach is important for understanding speciation histories, as speciation is one potential outcome when gene flow is reduced between two divided populations (Wright, 1931; Slatkin, 1987; Abbott *et al.*, 2008).

The division of one continuous, randomly breeding (panmictic) population, into subdivided populations, is the first stage in the speciation process. Under these conditions, an individual is more likely to mate with another in the same population than one from another population. This means alleles are more likely to be shared within rather than between populations, and this division of genetic diversity can be summarised by the statistic F_{ST} (Wright, 1943; Slatkin, 1977; Weir & Cockerham, 1984). Factors that will increase the proportion of genetic diversity found between populations (increase the F_{ST}) are genetic drift and natural selection. These differentiation processes are opposed by interpopulation gene flow (migration), which homogenises allele frequencies between subdivided populations, decreasing the F_{ST}. When gene flow is reduced to less than one migrant per generation, population-specific mutations will accumulate between ancestrally shared gene copies (Wright, 1931), and genetic differentiation can be measured with genetic distance measures such as D (Nei, 1978).

Characterising the population substructure by calculating F_{ST}, and genetic differentiation by calculating Nei’s D, are informative ways of studying ongoing and recent evolutionary events. Understanding the strength of interpopulation gene flow can reveal the geographic scale over which speciation can occur (Fig. 1.2). Moreover, if the ongoing factors that affect genetic differentiation are assumed to be the same as those that have promoted divergence in the past, we can learn about past speciation histories from extant species (Barraclough *et al.*, 1998). For example, if related plant species have a genetic signature indicating seed dispersal limitation, one may infer that limited seed dispersal contributed to divergence in the past. In contrast, widespread species with most genetic diversity shared between populations (low F_{ST} values) may indicate gene flow between populations is common, and therefore past speciation events are more likely to be caused by strong geographic barriers than dispersal-limitation between distant populations. However the cause of geographic structuring indicated by F_{ST} values can be interpreted in different ways. For example, the species with low F_{ST} described above may have recently expanded its range, rather than being present for a long time at each site and having widespread gene flow between populations (Hey & Machado, 2003). In this case, calculating genetic diversity in each subpopulation (h_S), and analysing the number of unique genetic clusters in the sampled populations (e.g. with STRUCTURE, Pritchard *et al.*, 2000), can help discriminate between causes of structuring within natural populations.

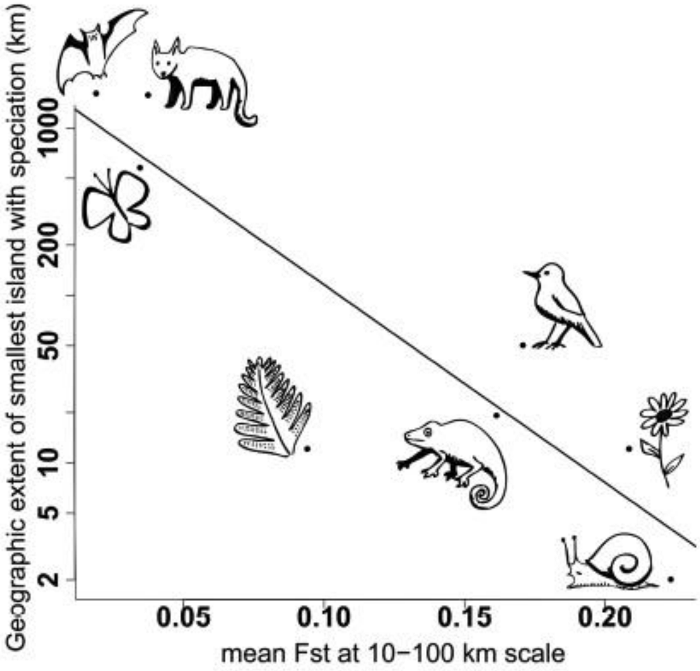


Figure 1.2. Minimum geographic scale that speciation can occur in different organisms. From a meta-analysis of island size and extent of gene flow by Kisel & Barraclough (2010).

Studies of secondary contact zones can also shed light on speciation histories. One may expect few species-specific markers (i.e. markers with fixed differences in allopatric populations) to be shared between species in areas of sympatry (Rieseberg & Ellstrand, 1993) due to reproductive barriers that prevent hybridization. However, hybridization can occur between species when speciation is generally judged to be complete (Hausdorf, 2011). Therefore, rates of introgression may be compared across the genome, which may identify genomic islands of reproductive isolation (Turner *et al.*, 2005; Feder & Nosil, 2010; Nosil & Feder, 2012) where introgression is impeded (Wu & Ting, 2004). Typically these islands include genes for species-specific adaptations (Butlin, 2010). Understanding the size of these islands, and the genes contained within them, may help us understand the evolution of reproductive isolation (Yatabe *et al.*, 2007; Strasburg *et al.*, 2012).

The process of speciation will often leave a clear genetic signature in extant plant populations. If speciation is occurring, strong patterns of population substructure and genetic differentiation would be expected. Studying introgression at species specific markers will show the strength of reproductive isolation, while genome-wide analysis with densely mapped markers will show the parts of the genome that contribute to isolation. These studies can give great insights into speciation processes, as long as suitable molecular markers are available.

1.1.4. Tools for studying the speciation process

1.1.4.1. Molecular

The increasing availability of molecular markers makes the approaches described above, such as genome scans, feasible for all plant species, not just evolutionary model systems that have large-scale genomic resources available (Ekblom & Galindo, 2011). This is largely due to next-generation sequencing (NGS) techniques, which generate a large amount of DNA sequence data at a reduced cost relative to Sanger sequencing (Metzker, 2010). The low costs make it possible to scan genomic DNA for single nucleotide polymorphisms (SNPs) in the study organism, and polymorphic sites can then be sequenced in a large numbers of individuals (Buggs *et al.*, 2010) using sequencing arrays such as Nimblegen AccuSNP (Roche, Madison, USA) or KASP (KBioScience, Hoddesdon, UK), reviewed in Seeb *et al.* (2011).

Previous to the development of next-generation sequencing, amplified fragment length polymorphisms (AFLPs, Vos *et al.*, 1995) were the principle method for large-scale genotyping. In this technique, DNA is digested with a rare-cutting restriction enzyme, the fragments amplified using the polymerase chain reaction (PCR), and the presence or absence of amplified fragments scored. The markers are dominant, which limits the type of analyses that can be done (such as calculating the inbreeding coefficient) and the information content of individual loci is low (Meudt & Clarke, 2007). However, many loci are typically generated from a single primer-combination, making them well-suited to genetic mapping and other techniques where many loci are more important than levels of polymorphism.

A third common marker type are microsatellites (also known as simple sequence repeats, SSRs, Morgante & Olivieri, 1993). These short tandem repeat sequences are amplified by PCR, and are particularly useful when only a modest number of highly polymorphic loci (5-15) are needed to answer a biological question. Microsatellites developed from enriched libraries tend to be species-specific, and take a long time to develop (Squirrell *et al.*, 2003). Using sequence libraries from next-generation sequencing of expressed genes (transcriptomes) or genomes makes the development of markers cheaper and quicker, and the primer sequences derived from transcriptome sequences are usually conserved between related species (Varshney *et al.*, 2005; Guichoux *et al.*, 2011; Lepais & Bacles, 2011). The continued popularity of microsatellite markers is mostly due to their co-dominance, revealing both alleles at a locus, which can be used to estimate the inbreeding coefficient (F_{IS}). Levels of polymorphism are also high (Zane, 2002). A major issue with microsatellites is null alleles, the non-amplification of an allele caused by mutations in the primer region, causing an individual to appear homozygous when they are heterozygous (Pemberton *et al.*, 1995). Their mutational complexity is also more difficult to model than DNA sequence data. Either each mutation is assumed to increase or decrease the microsatellite by one repeat unit in length (the stepwise mutation model, Ohta & Kimura, 1973; Valdes *et al.*, 1993, used in calculating R_{ST}, Slatkin, 1995), which is not always the case (Micheneau *et al.*, 2010), or the expected relationship between microsatellite length is ignored and only the allelic state is considered (the infinite alleles model, Ohta & Kimura, 1973; Moran, 1975, used in calculating F_{ST}, Slatkin, 1987). In either case, the high mutation rate of microsatellites can make homoplasy an issue (Estoup *et al.*, 2002).

1.1.4.2. Non-molecular

Multiple hypotheses may be proposed to explain the distribution of genetic variation in natural populations, and additional non-molecular experiments can help distinguish between these hypotheses. For example, 100 AFLP markers may show there is little introgression in a hybrid swarm, but cannot distinguish which reproductive barriers are responsible. The reproductive barriers may be investigated by using artificial cross pollinations and measures of fitness in common garden and reciprocal transplant experiments (Burke & Arnold, 2001). Experimental crosses show which genotypes are compatible independent of other reproductive barriers that interact in nature, such as pollinator preferences. The fitness of natural hybrids and those produced in experimental crosses can be assessed under common garden conditions, allowing hybrid fitness to be analysed under controlled environmental conditions, or using reciprocal transplants, which allows the fitness of hybrids to be measured directly in conditions in which the plants are known to grow (Emms & Arnold, 1997). Another example where such experiments would be useful is to identify the contribution of selection against genetic drift in patterns of genetic differentiation. Reciprocal transplants could show the fitness of genotypes in other environments, to test for local adaptation (Ågren & Schemske, 2012). Local adaptation could also be inferred by comparing morphological trait variation within and between populations with Q_{ST} analysis (F_{ST} analogue using trait variation), and comparing this to the genetic structure inferred by F_{ST} (Spitze, 1993; Waldmann & Andersson, 1999; Steane *et al.*, 2006). A significantly higher Q_{ST} than F_{ST} would be consistent with local adaptation, although this approach may be inappropriate when mutation rates at neutral markers are high, or when the adaptive role of traits have not been well characterised (Pujol *et al.*, 2008; Edelaar *et al.*, 2011).

The preceding discussion of species delimitation, generic boundaries, and how the speciation process can be studied, shows many interacting factors that make speciation biology complicated to study. While certain genetic signatures are expected when speciation is occurring, it must also be recognised that speciation is not predictable in its tempo and mode, and that 'reverse speciation' can occur (i.e. elevated gene flow merging two species, Taylor *et al.*, 2006). Studies of gene flow (described above) are a powerful way to study speciation histories, but are best set in the context of multiple different types of experiments to aid interpretation when many factors interact. This is particularly the case in large or taxonomically complex groups, where reliance on a single approach may not lead to reliable species delimitation.

1.2. Speciation histories in large plant genera

The ten largest plant genera, listed in order of ascending species number, are: *Astragalus* L. (Leguminosae), *Bulbophyllum* (Orchidaceae), *Psychotria* L. (Rubiaceae), *Euphorbia* L. (Euphorbiaceae), *Carex* L. (Cyperaceae), *Begonia* L. (Begoniaceae), *Dendrobium* Sw. (Orchidaceae), *Acacia* Mill. (Leguminosae), *Solanum* L. (Solanaceae) and *Senecio* (Asteraceae)

(Frodin, 2004). Genus-wide studies, such as phylogenies or monographs, are rare for these species rich genera because of their unmanageable sizes. This makes comparisons between them difficult, and these tend to be made on infragenic groups that can be studied in a reasonable amount of time (Bohs & Olmstead, 1997; Pelser *et al.*, 2002). It is even less likely that researchers will study related genera too, which is necessary to test if intrinsic properties of the group underlie their diversification. A rare example is a study of *Astragalus*, where elevated speciation rates were found not to be unique to the genus, but were similar to other species rich genera in the Astragalean clade (Sanderson & Wojciechowski, 1996).

Most of the ten largest plant genera are most species rich in the tropics (Davies & Barraclough, 2007; Mittelbach *et al.*, 2007). A meta-analysis of dated radiations in different plant clades by Linder (2008) showed that species diversification rates are strongly related to geography, where recent rapid radiations (i.e. increased speciation) often explain diversity in areas such as the Andes (e.g. lupins, Hughes & Eastwood, 2006), and persistence of past radiations caused by biome stability (i.e. reduced extinction) explain much of the diversity in areas such as Australia. However, the pattern of high species richness in certain areas does not tell us the factors that have promoted speciation (Weir & Schluter, 2007). The presence of *Carex*, an exclusively temperate genus, in the list of highly diverse plant genera, shows that non-tropical species can also obtain high levels of diversity. In *Carex*, diversification rates have been modest, and low rates of extinction explain its high extant species richness (Escudero *et al.*, 2012). Evidence for rapid species radiations, in addition to low rates of extinction, have also been found in temperate areas. Valente *et al.* (2010) showed the highest rates of species diversification in plants described so far were not in well known tropical radiations (e.g. *Inga* Mill., Richardson *et al.*, 2001), but in European *Dianthus* L. The relationship between extant species richness and geography is therefore complex, and requires other factors to be considered.

Sanderson & Wojciechowski (1996) proposed 4 main factors common to the largest plant genera, which are likely to have influenced their levels of species richness. These are: strong population substructure, herbaceous habit, rapid chromosome evolution and frequent transitions between ecological specializations. Each of these factors influences speciation in a different way. Many species in these genera have limited gene flow that has caused highly substructured populations (e.g. *Astragalus cremnophylax* Barneby, $F_{ST} = 0.41$, Travis *et al.*, 1996; 4 species of

Euphorbia F_{ST} = 0.237 - 0.652, Park, 2004; *Dendrobium officianale* Kimura & Migo, F_{ST} = 0.269, Li *et al.*, 2008; *Acacia raddiana* Savi, F_{ST} = 0.60, Shrestha *et al.*, 2002). Low levels of gene flow between populations leads to reduced species cohesion, and in turn may lead to allopatric divergence. This is supported by the limited distribution ranges of many species in large genera (Domínguez Lozano & Schwartz, 2005). The predominantly herbaceous growth form common in these groups means species have a short generation time relative to long-lived woody species, so mutation rates will be higher (Duminil *et al.*, 2009). Frequent chromosomal rearrangements, including whole genome duplication (polyploidy), increases the genetic diversity on which selection may act and may lead to diversification (Levin, 1983; Rieseberg *et al.*, 2006; Vamosi & Dickinson, 2006; Rieseberg & Willis, 2007). Changes in chromosome number, or large duplications and insertions, will also lead to strong reproductive barriers between incipient species (Ruhsam, in prep.; Soltis & Soltis, 2009). This is seen in *Carex*, where species have almost every even chromosome number between 2n = 12 and 2n = 124, and over 100 species have intraspecific cytological variation (Hipp *et al.*, 2009; Chung *et al.*, 2011; Escudero *et al.*, 2012). Frequent transitions between ecological specializations will cause strong prezygotic reproductive barriers, as species are unlikely to grow in sympatry. Such frequent transitions have been revealed by mapping ecology onto a species-level phylogeny of *Euphorbia*, which revealed sister-species often grow in different habitats (Frajman & Schönswetter, 2011; Horn *et al.*, 2012).

Many large genera also have unusual morphological characters, which some have considered ‘key innovations’ leading to the success of a group. For example, *Senna* Mill. (Leguminosae) species often possess extra floral nectaries, and *Senna* have diversified at a greater rate than other legume clades without extra floral nectaries (Marazzi & Sanderson, 2010). Extra floral nectaries may be a key innovation as they attract ants that protect the plants. Moreover, diversification in *Senna* occurred at the same time as diversification in ants (Marazzi & Sanderson, 2010). In many other cases however, the importance of traits that may be key innovations is hard to test, and other factors may better explain extant levels of species richness. For example, *Carex* has a sac-like structure that covers a one-seeded fruit (perigynium or utricle) that may aid dispersal (Escudero *et al.*, 2012), but frequent chromosomal rearrangements may play a bigger role in the evolutionary success of the genus. In many cases processes at or below the species level may have caused the break down of species cohesion and contributed to diversification, and key innovations may have played no significant role (Davies & Barraclough, 2007).

1.3. Evolution of diversity in *Begonia*

Most evolutionary studies in plants are confined to evolutionary model systems, which have been selected to study different evolutionary processes, such as: *Helianthus* L. for studying hybrid speciation and the evolution of reproductive isolation (Rieseberg, 1991; Rieseberg *et al.*, 1999; Rieseberg, 2000; Yatabe *et al.*, 2007; Scascitelli *et al.*, 2010; Whitney *et al.*, 2010), *Iris* L. for studying the adaptive significance of hybridization (Carney *et al.*, 1994; Arnold & Hodges, 1995; Emms & Arnold, 1997; Martin *et al.*, 2007; Arnold *et al.*, 2010), European *Senecio* for studying introgression and the origin of invasive species (Abbott *et al.*, 1992; Hiscock, 2000; Lowe & Abbott, 2004; Hegarty *et al.*, 2008; Kim *et al.*, 2008; Chapman & Abbott, 2010), *Tragopogon* L. for studying polyploidy and its adaptive significance (Soltis *et al.*, 2004; Buggs *et al.*, 2010), *Silene* L. for the evolution of sex chromosomes (Charlesworth, 1991; Charlesworth, 2002; Charlesworth *et al.*, 2005) and *Mimulus* L. for ecological and functional genetics (Schemske & Bradshaw, 1999; Fishman & Willis, 2001; Hall *et al.*, 2006; Sweigart *et al.*, 2006; Fishman & Willis, 2007; Case & Willis, 2008; Wu *et al.*, 2008; Martin & Willis, 2010).

Tropical herbaceous plants are an important species group that are not represented by current model plant species. The tropics are the most species rich region on Earth, with the Neotropics being of particular interest for its species richness (90 000 species); many of these species are in genera and families that are endemic to the region (Gentry, 1992). Between 21 and 47% of tropical plant species are found in the understory (Costa, 2004, and references therein), and developing a tropical herbaceous plant as an evolutionary model system would allow the processes contributing to species richness in these diverse tropical herbaceous genera to be investigated.

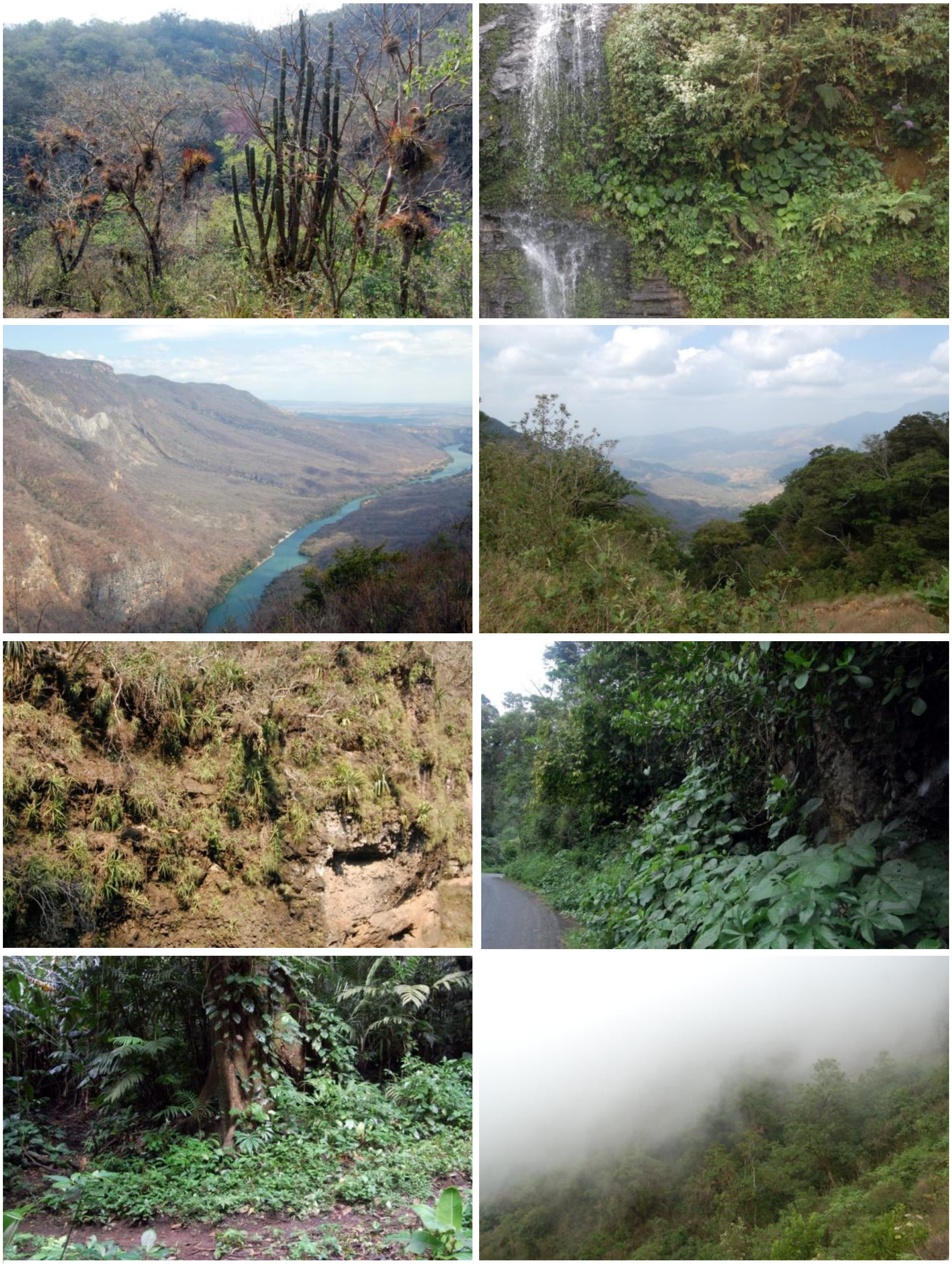
In this thesis, speciation processes are investigated in the large genus *Begonia*. We chose *Begonia*, rather than other large plant genera, for the following reasons. First, *Begonia* is amenable to genetic analysis, with most species being intercrossable, and they produce many seeds per capsule so segregation ratios can be easily tested, and mapping populations made (Neale *et al.*, 2006). Second, *Begonia* has a large range of morphologies, and there are many transitions in character states between related species, allowing the genetic basis of traits to be assessed with natural independent replicates (Forrest, 2000; Thomas *et al.*, 2011b; Harrison and Kidner, unpubl. data). Third, local monographic revisions and checklists [e.g. checklist of Southeast Asian *Begonia*, Hughes, 2008; monograph of American section *Gireoudia* (Klotzsch) A. DC, Burt-Utley, 1985] are available for some groups of *Begonia*, and many databases (e.g. The Global Biodiversity Information Facility, www.gbif.org; Tropicos, www.Tropicos.org) include occurrence records. Fourth, the distribution of the genus coincides with other large genera, such as *Peperomia* Ruiz & Pav. (Piperaceae), so evolutionary patterns in *Begonia* are likely to be of broad consequence for ecologically similar species groups. Finally, there are large research collections at the Royal Botanic Garden Edinburgh (RBGE), making it relatively well-collected and documented considering the size of the genus.

Begonia contains c.1500 species spread throughout the tropics, making it the 6th largest plant genus (Frodin, 2004). This diversity is divided between Central America (690 species), Asia (600 species) and Africa (160 species) (Goodall-Copestake *et al.*, 2010). Phylogenetic analysis of *Begonia* places African *Begonia* as early diverging, and Central American and Asian *Begonia* as more recently derived (Goodall-Copestake *et al.*, 2010). The most recent common ancestor of the genus is likely to have occurred between the end of the Cretaceous and the beginning of the Neogene (Goodall-Copestake *et al.*, 2009).

The great species richness in *Begonia* contrasts with the only other genus in the family Begoniaceae, *Hillebrandia* Oliv. (Hughes, 2002). The sole species in this genus, *H. Sandwicensis* Oliv., is restricted to the Hawaiian Islands (Clement *et al.*, 2004). *Hillebrandia* differs considerably in appearance to *Begonia*, as it has strongly differentiated petals and sepals, a semi-inferior ovary, fruits that dehisce between the styles, and a different pollen morphology (Clement *et al.*, 2004). In a broader context, the species richness in the Begoniaceae is higher than many related families in the Curcurbitales, such as the Datiscaceae (2 species) and Tetramelaceae (2 species) (Dewitte *et al.*, 2011; Schaefer & Renner, 2011), although Curcurbitaceae is also species rich (c.800, www.curcubit.org).

Begonia is morphologically recognisable by its asymmetric leaves, unisexual monoecious flowers, generally succulent petioles, twisted papillose stigmas, and dry-three winged capsules (Burt-Utley, 1985; Doorenbos *et al.*, 1998; Thomas, 2010). However, within *Begonia* there is great morphological diversity, particularly in growth form and leaf shape (simple, peltate, dissected, compound, Neale *et al.*, 2006). This morphological diversity goes hand-in-hand with the wide range of habitats *Begonia* species inhabit (Fig. 1.3). Most commonly, they grow as understory herbaceous plants, and they have become adapted to these shady conditions where less than 1% of canopy light penetrates (Chazdon & Fletcher, 1984). *Begonia* species have evolved a distinct leaf micromorphology, with both adaxial and abaxial hypodermis, and this may play a role in harvesting diffuse light (Lee *et al.*, 1990) or give structural support to the leaves (Kidner, pers. comm.). In contrast to these forest floor species, a minority of Central America *Begonia* have become adapted to very dry soils in sunny exposed positions (Burt-Utley, 1985; Fig. 1.3). The Mexican *B. peltata* Otto & A. Dietr., is one example, where its succulent leaves covered in a thick indumentum allow it to grow in very dry conditions next to cacti (A.D. Twyford, pers. obs.; Fig 1.3).

Figure 1.3. Representative habitats of species from Central American *Begonia* section *Gireoudia* (next page). Top row, left: dry canyon with cacti (*B. peltata*), right: splash zone adjacent to waterfall (*B. multistaminea* Burt-Utley); 2nd row, left: seasonally dry forest (*B. plebeja* Liebm.), right: secondary forest (*B. heracleifolia* Cham & Schltdl.); 3rd row, left: dry sun-exposed cliff face (*B. hydrocotylifolia* Otto ex Hook.), right: moist roadside (*B. nelumbiifolia* Cham & Schltdl.); Bottom row, left: wet tropical forest (*B. thiemei* C.DC. ex Donn.Sm.), right: cloud forest (*B. fusca* Liebm.).



Within *Begonia* there is evidence to support all five factors influencing diversity in large plant genera (population structure, herbaceous habit, rapid chromosome evolution, frequent ecological specializations, distinct morphological characters of the genus that may be key innovations, Sanderson & Wojciechowski, 1996). Four factors related to population biology (geographic population structure, mating system) and hybridization (evolution of reproductive traits and outcomes of secondary contact) that may promote speciation are considered in more detail below, which sets the background for the research questions that follow.

1.3.1. Dispersal limitation and population structure

Begonia have a distinct pollination biology and pattern of seed dispersal. Most *Begonia* species produce simple white or pink flowers, which are visited by generalist pollinators (Apidae, Halictidae and *Trigona* bees, Ågren & Schemske, 1991; Wyatt & Sazima, 2011). Pollinators are attracted to male flowers by a pollen reward, and visitation of female flowers is by deceit, as they produce no floral reward but are similar in appearance to the male flowers (Ågren & Schemske, 1991). It may be expected from these floral syndromes that effective pollen dispersal will be low, as insects will not travel far-a-field to collect pollen for low rewards. Interpopulation seed dispersal may also be low. *Begonia* species produce over 10 000 tiny seeds (300-600µm in length) per capsule, which dehisce from slits along the wing attachments at maturity (Thomas, 2010). In most cases, seeds are not effectively dispersed and they fall to the ground, and germinate *in situ*. This can be seen as clusters of plants in the field (Hoover, 1979).

Plants disperse their genes through pollen and seed dispersal (Ennos *et al.,* 1999). Maternally inherited plastid markers are informative of seed flow, while nuclear and plastid markers are informative for the study of pollen flow (Ennos, 1994; Mccauley, 1995). To date, studies in *Begonia* have only used nuclear markers. These studies have shown that *Begonia* populations have strong population structure, supporting the observation of dispersal limitation of pollen. Matolweni *et al.* (2000) found low interpopulation gene flow between South African populations of *B. dregei* Otto & Dietr. (F_{ST} = 0.882) and *B. homonyma* Steudl. (F_{ST} = 0.937) using allozyme markers. Hughes & Hollingsworth (2008) investigated gene flow at microsatellite loci in *B. sutherlandii* Hook. f., across fragmented forest patches in South Africa. They found significant population structure, with F_{ST} = 0.485. However, these species are all restricted to fragmented habitats and may not be indicative of widespread species that grow in more continuous habitats. Hughes & Hollingsworth (2003) investigated population structure in *B. socotrana* Hook f., using microsatellite markers. This species is endemic to the island of Socotra off the African coast, and a deviation from panmixia was detected even in the small area the species inhabits (< 15 x 10km) ($F_{ST} = 0.096$).

The likelihood of divergence in allopatry is also affected by other demographic factors. One of these is the effective population size, defined as the idealised number of individuals that would give rise to the calculated sampling variance if they breed in the manner of an idealised population (Wright, 1931; Falconer & Mackay, 1996). Typically, the effective population size is much lower than the actual population size (reviewed in Charlesworth, 2009), due to past fluctuations in population sizes. *Begonia* populations are typically small (Hoover, 1979; Hughes & Hollingsworth, 2008), and therefore particularly prone to genetic drift (Wright, 1931; Kimura, 1955; Willi & Määttanen, 2010). For example, the narrow endemic *B. mazae* Ziesenh. is known from a single population of a few hundred individuals (A.D. Twyford, pers. obs.). While we have limited knowledge of the historical survivorship of *Begonia* populations, it is likely that the high levels of genetic differentiation (e.g. *B. dregei*, Nei's D = 0.640 and *B. homonyma*, D = 0.520) could only have accumulated if populations are long-lived and stable.

1.3.2. Mating system

The mating system (degree of outcrossing) and the sex system (hermaphrodite, male or female; *sensu* Charlesworth, 2006) of a plant species affects the structuring of genetic diversity (Hamrick & Godt, 1996), and subsequently whether allopatric divergence will occur. Most *Begonia* species are monoecious (Tebbit, 2005) so their sex system is mostly uniform across the genus, however, they do vary in their mating system.

Self pollination is common in plants as it provides reproductive assurance, particularly for colonizing species where there may be no other conspecific plants with which to mate (Baker, 1955; Lloyd, 1979). Rounds of self-fertilization will decrease both components of genetic diversity, the heterozgosity and the number of alleles per locus, in comparison with their outcrossing relatives (Charlesworth, 2003; Mable & Adam, 2007). The decrease of heterozygosity in natural plant populations caused by systematic inbreeding can be calculated as the inbreeding coefficient F_{IS}. The loss of genetic diversity in populations that systematically inbreed is due to the reduction in the effective population size (Schoen & Brown, 1991; above).

Population genetic studies in *Begonia* (Matolweni *et al.*, 2000; Hughes & Hollingsworth, 2003; Hughes & Hollingsworth, 2008), as well as other experiments (Ågren & Schemske, 1991; Ågren & Schemske, 1993; Schemske *et al.*, 1996; Wyatt & Sazima, 2011), highlight the potential for self-fertilization and inbreeding in the genus. Selfed crosses produce seed in most (16/18) *Begonia* species tested to date (Ågren & Schemske, 1993; Wyatt & Sazima, 2011). The Cucurbitaceae are not thought to have self-incompatability mechanisms (Rubino & Wehner, 1986), although Brazilian species *B. integerrima* Spreng. and *B. itatinensis* Irmsch. did not produce seed when selfed in 9 and 4 self-pollinations, respectively (Wyatt & Sazima, 2011). Self-compatibility has led to inbreeding in some *Begonia* species. Consistent deviations from Hardy-Weinberg equilibrium (HWE) across loci happen in wild populations of *B. dregei* (F_{IS} = 0.273, $P < 0.001$), *B. homonyma* (F_{IS} = 0.576, $P < 0.001$; Matolweni *et al.*, 2000) and *B. sutherlandii* (F_{IS} = 0.158, $P < 0.001$; Hughes & Hollingsworth, 2008), and this may be a product of systematic inbreeding. A minor deviation from HWE was also detected in the rare *B. socotrana* (F_{IS} = 0.051, $P < 0.05$; Hughes & Hollingsworth, 2003). Ågren & Schemske (1993) demonstrated low outcrossing rates (<5%) for *B. hirsuta* Aubl. and *B. semiovata* Liebm., at a single allozyme locus. Ågren & Schemske (1993) describe a delayed selfing mechanism in these two *Begonia* species, where female flowers have a short window for outcrossing before the adjacent male flowers opens, which selfs in the absence of a pollinator. This inbreeding has a significant fitness consequence for the progeny, as selfed plants have their biomass reduced by 18-31% and seed set by 12% relative to outcrosses (Ågren & Schemske, 1993).

1.3.3. Outcomes of secondary contact

Hybridization in *Begonia* has usually been studied by horticulturists, and over 10 000 *Begonia* hybrid cultivars have been described (Tebbit, 2005). F1 hybrids have been formed between all pair-wise crosses of closely related species from section *Gireoudia*, but crosses between species from different *Begonia* sections, with different chromosome numbers, and different genome sizes, are less successful (27/156 set seed, 17.3%, Dewitte *et al.*, 2011). Despite the ease with which hybrids have been generated in cultivation, hybrids have rarely been reported in nature (Burt-Utley, 1985; Peng & Chen, 1991; Peng & Sue, 1991; Teo & Kiew, 1999; Peng & Shin-Ming, 2009; Peng *et al.*, 2010). Four natural hybrids in Taiwan have been confirmed by chromosome counts, genomic *in situ* hybridization (GISH), plastid sequencing, morphology, experimental crosses and by measuring fertility (*B.* × *buimontana*, Peng & Chen, 1991; *B.* x *breviscarpa*, Peng *et al.*, 2010; *B.* x *chungii*, Peng & Shin-Ming, 2009; *B.* x *taipeiensis* Peng & Sue, 1991). All four of these hybrids are thought to be F1s, and are sterile. Secondary contact between species where reproductive barriers are strong, such as Taiwanese *Begonia*, can be considered 'evolutionary stalemate' situations, which will not yield evolutionary significant results (Barton & Hewitt, 1985). Stalemate conditions can be caused either by strong premating isolation (e.g. non-overlapping flower times) or postmating isolation (e.g. sterile hybrids), or both. A similar situation between *Rhododendron ponticum* and *R. caucasicum* (Ericaceae) is likely caused by selection against later generation hybrids that limits hybridization to the F1 generation (Milne *et al.*, 2003).

The only known naturally occurring *Begonia* hybrid that is fertile is *B. decora* x *B. venusta*, from Peninsula Malasia. The hybrid swarm shows continuous morphological variation between that observed in the parents (Teo & Kiew, 1999), and hybrids have admixed AFLP profiles (Kiew *et al.*, 2003). In cases such as this, where reproductive barriers are weak, there are three possible evolutionary outcomes: clinal introgression, complete admixture, or reinforcement.

Widespread hybridization, and backcrossing or segregation in the hybrids, will result in the movement of genes across a species barrier (introgression). This is a common outcome of secondary contact in plants (Arnold, 1997). The direction of hybridization is influenced by many factors such as the mating system (Ruhsam *et al.*, 2010), relative abundances of the two species (Currat *et al.*, 2008; Lepais *et al.*, 2009), or their position in the local area (Lexer *et al.*, 2005). Introgression is likely to be uneven across the genome, with hybrid zones acting as selective filters only allowing some genes to pass across a species barrier (Barton & Bengtsson, 1986; Martinsen *et al.*, 2001). These genes may provide a selective advantage, such as the CYCLOIDEA-like genes RAY1 and RAY2, responsible for ray floret growth and subsequent levels of pollinator visitation, that have been introgressed from *Senecio squalidus* L. into *S.*

vulgaris L. (Kim *et al.*, 2008). Other types of genes, particularly those underlying species differences, are unlikely to be introgressed, as they are integral for the species survival under the specific ecological conditions in which they grow (Turner *et al.*, 2005; Yatabe *et al.*, 2007).

Complete admixture may occur between species if reproductive isolation is weak. This may cause the extinction of one species, particularly if it is rare. In the Canary island endemic genus *Argyranthemum* Webb., for example, the widespread *A. frutescence* Sch. Bip. invaded the rare *A. coronopifolium* Webb. (Levin *et al.*, 1996), likely leading to its extinction through introgression (Brochmann, 2002). An alternative outcome between incipient species with weak reproductive barriers is reinforcement, where selection enhances reproductive barriers between sympatic taxa completing speciation. Sympatric species must preferentially pair with conspecific rather than heterospecific or admixed individuals for reinforcement to occur (Noor, 1999). This has been seen in animals where assortative mating can be influenced by a few loci of large effect (e.g. Ortiz-Barrientos *et al.*, 2012). However, these specific circumstances are thought to be rare in plants (Marshall *et al.*, 2002), and reinforcement likely only plays a minor role in plant evolution.

1.3.4. Genetic architecture of reproductive isolating traits

The genetic architecture of a reproductive isolating trait, defined as the number of genes involved in the phenotype and their interactions, is an important factor that determines the rate at which reproductive isolation can evolve (Rieseberg *et al.*, 1999; Schemske & Bradshaw, 1999; Rieseberg *et al.*, 2003; Martin *et al.*, 2007). Some traits that cause reproductive isolation evolve roughly clock-like, i.e. the strength of reproductive isolation is proportional to genetic divergence. For example, Coyne & Orr (1989) found a significant correlation between the level of genetic divergence between closely related fruit flies (*Drosophila* spp.) and the strength of reproductive isolating barriers. A similar result was found between populations of arctic *Draba* L. (Brassicaceae) species, where geographic and genetic distances correlate with the strength of postmating isolation (hybrid sterility) (Grundt *et al.*, 2006). These cases where reproductive isolation evolves as a by-product of genetic divergence, rather than selection for particular traits, are predicted to be influenced by many loci of small effect spread throughout the genome (Coyne & Orr, 1989).

In contrast to a genetic architecture of many loci of small effect, a reproductive isolating barrier can be generated by a small number of genes of large effect. Such genes include ‘speciation genes’, which contribute to the splitting of two lineages by reducing the amount of gene flow between them (Rieseberg & Blackman, 2010). Candidate lists of speciation genes encoded by nuclear DNA in plants include those regulating floral pigments, self-incompatibility genes, and disease resistance genes (Rieseberg & Blackman, 2010). Speciation genes may be also carried by the organellar genomes. Nuclear-organelle interactions are a by-product of the co-evolution of the organelle and the nuclear genome (Rand *et al.*, 2004). Deleterious mutations that occur in the organelle can be masked by coevolving nuclear restorer loci. When an organelle is exposed to a new nuclear background through hybridization, the deleterious mutations will be unmasked and may cause sterility. *Mimulus* is a well studied plant system for nuclear-organelle interactions, where the genetic basis of mitochondrial-nuclear (Case & Willis, 2008) and cytoplasmic-nuclear interactions (Fishman & Willis, 2007) have been investigated. Other species where nuclear-organelle interactions have been studied include maize (Laughnan & Gabay-Laughnan, 1983; Dewey *et al.*, 1987) and sunflowers (Hans Köhler *et al.*, 1991; Laver *et al.*, 1991).

Different genetic architectures can give rise to similar reproductive isolating barriers. For example, hybrid sterility has an important role in isolating species during secondary contact (Barton & Hewitt, 1985; Rieseberg *et al.*, 1999). This infertility can often be explained by genome-wide divergence that prevents homologous chromosomes pairing correctly at meiosis (Tao & Hartl, 2003). Alternatively, it may be caused by genic incompatibilities at few loci, or few large karyotype differences such as deletions and inversion that differ between homologous chromosomes (Rieseberg, 2001; Brown *et al.*, 2004).

The genetic architecture of reproductive isolating traits has not been studied in *Begonia*. However, it may be predicted that reproductive barriers contributing to divergence will be caused by many mutations of small effect, as a by-product of genome-wide divergence in geographically isolated populations (Coyne & Orr, 1989). After divergence, selection on few loci of large effect may explain the frequent transitions between character states between related species. Divergent morphologies and ecologies may act as reproductive isolating barriers, as species with such different traits are unlikely to come into secondary contact, and if they did hybrids would be maladapted. Forrest (2000) mapped leaf, tepal, and ovary characters onto a phylogenetic tree of *Begonia*, and showed many characters are homoplasious. Thomas *et al.* (2011b) investigated character state evolution in Southeast Asian *Begonia*, and showed traits such as perennation organ (rhizome, tuberous, non-specialised), fruit type (fleshy, dry capsules, rain ballist capsule) and locule number often change states between sister-species. A preliminary phylogeny by Kidner (unpubl. data) shows that morphological traits, such as peltate and compound leaves, have arisen independently in American *Begonia*.

1.4. Aims of the doctoral work and structure of the thesis

This thesis addresses why there are so many species of *Begonia*. This involves a detailed investigation of the processes that contribute to speciation, using approaches targeted at different stages in the speciation process. The first part examines the evolution of reproductive barriers within species, and the second looks at the strength of reproductive isolation when species are brought together in secondary contact. Four chapters address specific research questions, and one describes the development of nuclear markers used in the chapters that follow.

What are the patterns of seed flow in widespread *Begonia* species, and how is this affected by geographic barriers? (Chapter 2)

This chapter assesses phylogeographical patterns in two widespread *Begonia* species revealed using newly designed plastid microsatellite markers. This plastid data is used to assess whether patterns of haplotype sharing are more strongly influenced by the population biology of *Begonia* (particularly seed dispersal limitation), than Pleisotcene and pre-Pleistocene geographic barriers found to affect the spatial distribution of genetic variation in other species.

Development of polymorphic nuclear microsatellite markers for population genetic analyses of Central American *Begonia*. (Chapter 3)

What ongoing population level processes may promote genetic differentiation in incipiently speciating *Begonia*? (Chapter 4)

In this chapter, two factors that contribute to speciation in *Begonia* are assessed: dispersal limitation and mating system. Patterns of pollen flow are estimated for two widespread *Begonia* species using nuclear microsatellite loci, and compared with seed flow estimates from Chapter 2. The nuclear data is used to test whether inbreeding contributes to patterns of genetic differentiation. Experimental crosses between differentiated populations test whether there are early signs of reproductive barriers evolving, and this is related to intraspecific variation in genome size.

How strong is reproductive isolation between related *Begonia* species, and which barriers contribute to isolation? (Chapter 5)

Experimental crosses are performed between ecologically similar (*B. heracleifolia* Cham & Schltdl. x *B. sericoneura* Lieabm.) and different (*B. heracleifolia* x *B. nelumbiifolia* Cham & Schltdl.) species pairs, to test the strength of post-zygotic barriers. Then the frequency of hybrids and their genotypic classes are estimated in multiple natural hybrid swarms between the two species pairs. These data are used to test the strength of reproductive isolation in a recent *Begonia* species radiation.

What is the genetic architecture of reproductive traits that have diverged since speciation? (Chapter 6)

The final research chapter uses trait segregation, and quantitative trait loci (QTL) mapping, to assess the genetic basis of important reproductive traits that differ between species.

Each of the empirical research chapters are written in the style of research articles intended for different scientific journals. This leads to some overlap in the content of the chapters, however for continuity the full list of references is given at the end of the thesis. In chapter 7, the research is summarised, and future directions for genetic studies in *Begonia* are suggested. The appendices give the full data used for genetic analyses in each chapter.

1.4.1. Study species

Five *Begonia* species are studied in the thesis, all from the large Neotropical section *Gireoudia*. This section was chosen as it has many species (c.66) that are diverse in plant form (Fig 1.4), and because a monograph has been completed for the Central American species (Burt-Utley, 1985). Species from section *Gireoudia* are thought to be uniform in chromosome number (2n = 28) unlike many other sections in *Begonia* (Legro & Doorenbos, 1969, 1971, 1973), and this removes one factor that will influence patterns of gene flow and hybridization. Preliminary phylogenetic analysis indicates that the group is paraphyletic, with the small section *Weilbachia* nested inside (Harrison and Kidner, unpubl. data).

The species examined are: *B. conchifolia* A. Dietr., *B. heracleifolia*, *B. nelumbiifolia*, *B. plebeja* and *B. sericoneura*. *Begonia heracleifolia* and *B. nelumbiifolia* are the main study species, used for comparative phylogeography (Chapter 2), population genetics (Chapter 4), and a study of hybridization (Chapter 5). These species were selected as they are widespread species, allowing estimates of gene flow over large spatial scales. They also contrast in their ecologies, allowing hypotheses relating to different ecological adaptations to be tested. The patterns of hybridization observed between *B. heracleifolia* and *B. nelumbiifolia* are supplemented with a third species, *B. sericoneura*, which readily hybridizes with *B. heracleifolia*. This allows a comparison in hybrid zone structure between different species pairs (Chapter 5). *Begonia plebeja* and *B. conchifolia* are used as parents for a genetic mapping study described in Chapter 6. They were chosen as their F1 hybrid is fairly fertile, allowing segregating populations to be generated. The species pair also differ in many morphological traits, allowing the genetics of these traits to be investigated. Each species is described in detail in the introduction to each of the chapters.

Figure 1.4. Representative species from *Begonia* section *Gireoudia* and closely related section *Wielbachia*. Labels from left to right: Top row: *B. morrisiorum* P.D.McMillan and *B. mazae* Ziesnh.; 2nd row: *B. fusca* Liebm. and *B. pseudodaedalea* P.D.McMillan & Rekha Morris; 3rd row: *B. hydrocotylifolia* Otto ex Hook. and *B. sousae* Burt-Utley; Bottom row: *B. faustinoi* Burt-Utley & Utley and *B. calderonii* Standl. White bar is approximately 10cm.

CHAPTER 2: Population history and seed dispersal in widespread Central American *Begonia* species (Begoniaceae) inferred from plastome-derived microsatellite markers

This chapter has been accepted in a special edition of the Botanical Journal of the Linnean Society entitled Neotropical evolution: assembling the big picture.

Data contributions: Nikki Harrison (University of Edinburgh) provided the plastome sequences from which the microsatellite markers were designed. Alexander Twyford designed the markers, performed all genotyping and analyses.

2.1. Chapter summary

Seven plastid microsatellite markers derived from plastome sequence data were used to study the population genetic structure in two widespread *Begonia* spp. from Central America. In *B. nelumbiifolia*, no variation was found at any locus. In contrast, significant haplotype diversity was found in *B. heracleifolia* ($h_T = 0.937$, $h_S = 0.444$, 39 haplotypes, mean of 3.3 haplotypes per population), and populations showed high absolute levels of genetic differentiation ($G'_{ST} = 0.829$, $D = 0.407$). The distribution of haplotypes showed strong phylogeographical structure ($G_{ST} = 0.526$, $R_{ST} = 0.737$, $G_{ST} < R_{ST}$, $P < 0.05$), but this pattern was poorly accounted for by commonly studied historical scenarios, such as Pleistocene refugia or Pliocene differentiation at the Isthmus of Tehuantepec. Instead, subdivision into a large number of regions, each containing local populations (e.g. when $K = 9$, $F_{CT} = 0.749$, $P < 0.05$), best explained the haplotype distribution. The lack of haplotype diversity in *B. nelumbiifolia*, a moist adapted species, suggests that it may have been severely restricted in range during dry spells in the Pleistocene, and has subsequently expanded from this recent population bottleneck. The high haplotype diversity in *B. heracleifolia* may indicate that its adaptation to drought enabled it to survive in small, but ecologically suitable, pockets of isolated habitat throughout the Pleistocene. Limited seed exchange between *B. heracleifolia* populations is likely to be responsible for its high population substructure, and provided the opportunity for divergence through genetic drift. This interpretation is consistent with previous population genetic studies in *Begonia*, and suggests a

common pattern of extremely low genetic exchange among a series of small, but long-lived, populations that may predispose the genus to rapid speciation.

2.2. Introduction

The Neotropics are the most species-rich region on Earth, harbouring over 90 000 species of angiosperms (Richardson *et al*., 2001; Antonelli & Sanmartín, 2011). The northern limit of the Neotropics passes through Mexico, one of the highest ranked countries in terms of its species richness and species endemism (Myers *et al*., 2000). The assembly of this diversity over time has been complex, with various biotic and abiotic mechanisms contributing to species diversification (reviewed in Antonelli & Sanmartín, 2011). Within this diverse array of species, there is likely to be high genetic diversity as a consequence of long-term survival in stable populations throughout the Pleistocene (Bawa, 1992; Metcalfe *et al*., 2000). However, the geographical distribution of this genetic variation is dependent on the particular history of the populations involved.

Patterns of genetic diversity in tropical Mexico may reflect pre-Pleistocene events (Ornelas, Ruiz-Sánchez & Sosa, 2010; Bryson, García-Vázquez & Riddle, 2011). In particular, the Isthmus of Tehuantepec forms a narrow strip of lowland linking the south central Mexican highlands to the uplands of Chiapas and Central America (Fig. 2.1). It has been suggested that the area may have been a historical seaway for much of the Pliocene and Pleistocene (Barrier *et al*., 1998; Morrone, 2006), and therefore a major barrier to gene flow during this period. Such a barrier may have left a genetic signature of differentiation between populations on either side of the Isthmus. This has been detected in phylogeographical studies for many mid- and high-elevation taxa (e.g. pitvipers, Castoe *et al*., 2009; harvest mice, Sullivan, Arellano & Rogers, 2000; bird-dispersed Rubiaceae, Gutiérrez-Rodríguez, Ornelas & Rodríguez-Gómez, 2011), as well as low-elevation taxa (e.g. lyresnakes, Devitt, 2006; toads, Mulcahy, Morrill & Mendelson, 2006).

A second factor potentially influencing the distribution of genetic variation is the manner in which populations were affected by climatic fluctuations during the Pleistocene. Colinvaux, De

Oliveira & Bush (2000) believed that wet tropical forests were stable during the Pleistocene and, under these circumstances, no effect of Pleistocene climatic fluctuations would be expected on the genetic structure of tropical species. Other authors have proposed that tropical lineages were affected by climatic fluctuations and only survived in areas receiving high rainfall that remained continually warm and therefore acted as refugia during cool, dry Pleistocene periods (Fig. 2.1; Haffer, 1969; Toledo, 1982; Mary & OHara, 1986; Prance, 1987). Neotropical refugia for south Mexico and Guatemala (Toledo, 1982) and elsewhere in Central America (Prance, 1987) have been proposed on the basis of concentrated areas of species diversity and species endemism. If the Neotropical Pleistocene refugia hypothesis (Haffer, 1969) is true, a signature of strong genetic differentiation among these proposed refugial areas would be expected for tropical species. To date, population genetic studies of a variety of taxa have provided no general consensus regarding the importance of Neotropical refugia during the Pleistocene (Gutiérrez-Rodríguez *et al*., 2011).

In addition to these major geological events and geographical barriers, local patterns of physiographic heterogeneity and habitat fragmentation in more recent times may also have played a role in structuring genetic diversity. Reconstructions of the range of lowland tropical wet forests in the Quaternary suggest that habitat discontinuity was accentuated during cooler drier periods (Toledo, 1982; Metcalfe *et al*., 2000; Cárdenas *et al*., 2011). ‘Islands’ of suitable habitat may have been isolated from one another by inhospitable terrain, such that migration between patches was low. Genetic drift will act strongly in small isolated populations, with genetic variation being lost within populations, and genetic differentiation between them being increased (Quinn & Harrison, 1988). The effects of habitat fragmentation are expected to affect short-lived taxa, such as herbaceous plants, more strongly than long-lived trees, as dispersal between populations is more limited, and there are a greater number of generations for mutations to accumulate, and for the action of genetic drift and selection to take place (Duminil, Hardy & Petit, 2009). If there was restricted gene flow between populations within the Quaternary, as outlined above, genetic differentiation among many different regions would be expected, the pattern showing no correspondence to pre-Pleistocene geographical barriers or putative Pleistocene refugial regions.

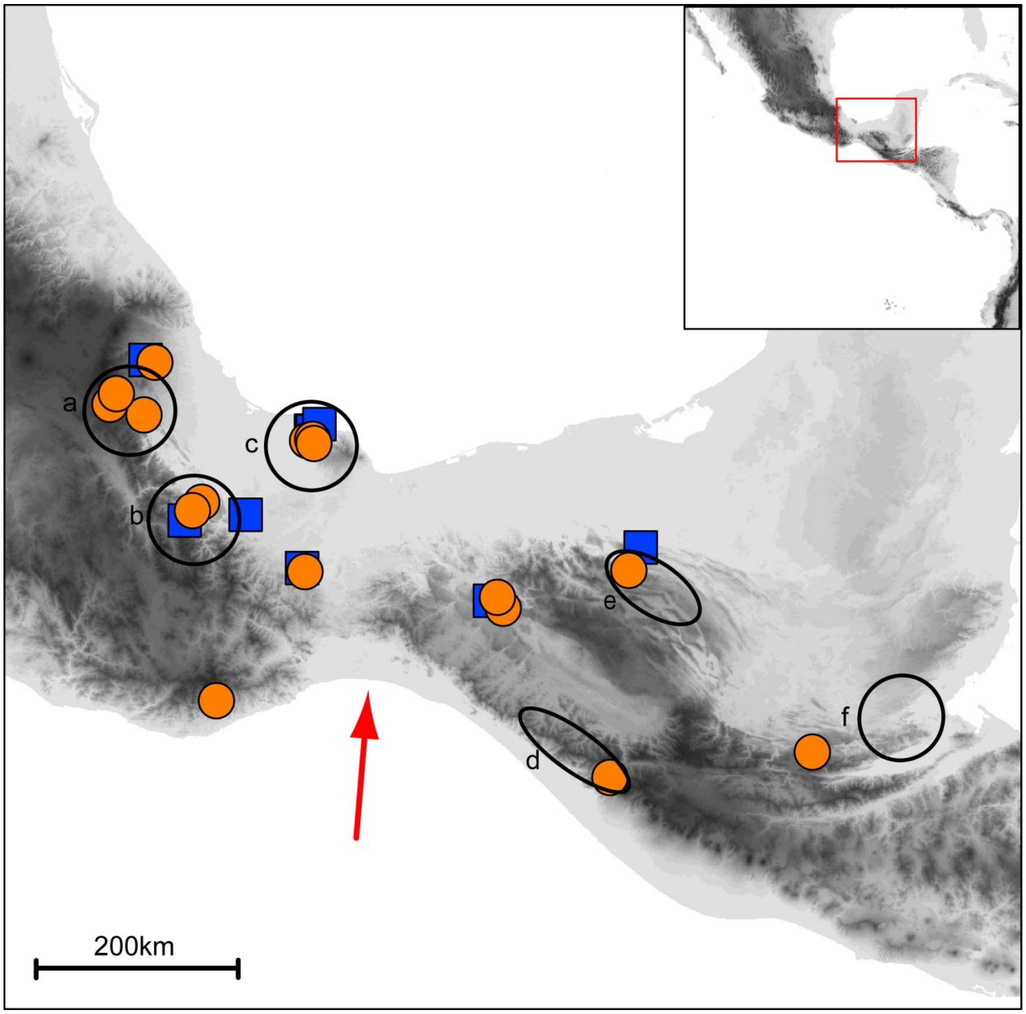


Figure 2.1. Collection sites of *Begonia heracleifolia* (orange circles) and *B. nelumbiifolia* (blue squares). Pleistocene refugia presented by Toledo (1982) are shown as black open circles, and the Isthmus of Tehuantepec is indicated by an arrow. Letters refer to the refugia listed in Table 2.1.

In order to gain an insight into the history of Neotropical plant species through genetic analysis, suitable genetic markers are required (Avise *et al*., 1987). Studies using multiple unlinked nuclear markers are needed to resolve patterns of recent gene flow and pollen dispersal, but plastid markers have some merits over nuclear markers for phylogeographical studies. These properties include the absence of recombination, low effective population size and the conservative mutation rate, allowing for primers to be designed over a wide taxonomic range

(Ennos *et al*., 1999; Provan *et al*., 1999; Provan, Powell & Hollingsworth, 2001). However, the main concern with plastid sequencing is the low rate of nucleotide substitution (Whittall *et al*., 2010), particularly when analysing relationships between groups that have undergone recent radiations (Richardson *et al*., 2001) or when resolving intraspecific relationships, where sequence divergence is predicted to be low. An alternative to sequence-based markers or single nucleotide polymorphisms (SNPs) are plastid microsatellites, which have a higher mutation rate (Provan *et al*., 1999, 2001; Jakobsson, 2007). The most direct method for locating suitable plastid microsatellite markers is to sequence the plastid genome (plastome) of individuals and select the most variable loci (Ebert & Peakall, 2009). However, until recently, this would not have been a viable option, because the generation of large quantities of sequence data is expensive. Next-generation sequencing (NGS) technologies are significantly reducing the costs of generating whole plastomes (Cronn *et al*., 2008), but have not been generated routinely for intraspecific studies to date (Whittall *et al*., 2010).

In this study, we use plastid microsatellite markers derived from plastome sequence data to investigate population genetic patterns in two widespread Central American species of *Begonia* L.: *B. heracleifolia* Cham. & Schltdl. and *B. nelumbiifolia* Cham. & Schltdl. Previous genetic studies of *Begonia* spp. have shown dispersal limitation between populations in discontinuous habitats (Matolweni, Balkwill & Mclellan, 2000; Hughes, Hollingsworth & Miller, 2003; Hughes & Hollingsworth, 2008), suggesting that *Begonia* is a good study system for understanding how geographical barriers affect patterns of gene flow and genetic differentiation. Here, we ask whether two historical scenarios, dispersal limitation across the Isthmus of Tehuantepec and survival in Pleistocene refugia, have left a detectable genetic signature in extant *Begonia* populations in Central America. If the Isthmus of Tehuantepec has influenced historical gene flow between *Begonia* populations, a clear east–west genetic break would be expected either side of the Isthmus. In contrast, if the Pleistocene glacial refugia proposed by Toledo (1982) have played a role, high genetic diversity would be expected in former glacial refugia, and haplotypes would not be shared between refugia. We also examine whether a third scenario, of dispersal limitation between long-lived isolated *Begonia* populations, fits the pattern of genetic diversity better. If this is the case, high levels of phylogeographical structure, measured using R_{ST}, and genetic differentiation, measured using statistics such as D (Jost, 2008), would be expected, which are unrelated to the patterns outlined above. In the light of our findings, we evaluate the benefits of using microsatellite markers derived from plastome sequences for studying phylogeographical patterns.

2.3. Material and methods

2.3.1. Study species

The mega-diverse genus *Begonia*, with c.1500 species, is one of the ten largest plant genera (Frodin, 2004). Approximately 690 *Begonia* spp. have been recorded in Central America (Goodall-Copestake *et al*., 2010; Dewitte *et al*., 2011), and no previous phylogeographical or population genetic studies have been conducted. *Begonia* spp. are typically herbaceous plants or understorey shrubs that grow in wet tropical lowland and montane forests, but some Central American species have a rhizomatous habit and have adapted to seasonally dry forests. They are monoecious, and their flowers are white or light pink with two petaloid sepals, which attract generalist pollinators, and pollen dispersal between populations is predicted to be low (Ågren & Schemske, 1991). The small seeds are not modified to promote dispersal by an animal vector or by wind, and are therefore expected to have limited dispersal. These attributes differ markedly from those of Neotropical trees, which have been the focus of phylogeographical studies (e.g. Cardoso *et al*., 1998; Cavers, Navarro & Lowe, 2003; Ornelas *et al*., 2010; Poelchau & Hamrick, 2012), and therefore their responses to past climatic fluctuations in relation to Pleistocene refugia are expected to differ.

The species selected, *B. heracleifolia* and *B. nelumbiifolia*, were chosen for their different ecological preferences (Hoover, 1979), being adapted to dry and moist habitats, respectively. Comparative population genetic studies of related species with different ecologies can potentially distinguish between the influence of past biogeographical barriers, which would affect the dispersal of both species, and the distribution of suitable habitat, which may affect one species more than another (Bermingham & Moritz, 1998). Their widespread distributions (*B. heracleifolia* grows from Mexico to Honduras, *B. nelumbiifolia* from Mexico to Colombia) and their tendency to grow as ruderals differ from most other *Begonia* spp., which are often narrow endemics in primary habitat (Hughes & Hollingsworth, 2008). The species are diploid ($2n = 28$;

Legro & Doorenbos, 1969) nonsister species, based on preliminary analysis using the nuclear ribosomal internal transcribed spacer (nrITS) and plastid *trnL-F* intron (C. A. Kidner, unpubl. data). *Begonia heracleifolia* has repent rhizomes and symmetrical or weakly asymmetrical lobed leaves with serrations, and varying degrees of dark blotches and coloured margins, although leaf phenotype (colour of the leaf blade and degree of leaf lobing) varies across its range (Fig. 2.2). *Begonia nelumbiifolia* has large peltate leaves and densely packed symmetrical inflorescences (Fig. 2.2), and is relatively morphologically uniform throughout its range.

Figure 2.2. Study species. Left: leaf shape variation in *Begonia heracleifolia,* photographs taken from wild collected plants grown in a common glasshouse environment. Right: *Begonia nelumbiifolia* growing on a shady rock near Motzorongo.

2.3.2. Sampling

Population samples were collected in the south Mexican states of Chiapas, Oaxaca and Veracruz (Fig. 2.1). Between three and 30 individuals per population were collected for genotyping. Individuals were selected at a minimum sampling distance of 1 m to reduce the chance of collecting clonally reproducing plants (Hughes & Hollingsworth, 2008). Flower or young leaf material was collected in silica gel stored at room temperature prior to DNA extraction (Table 2.1). A single representative of each population is lodged at the herbarium at E, except for the Guatemalan population, for which the specimen is at BIGU.

Table 2.1. Collection details for *Begonia* samples used in this study. For *B. heracleifolia*, the positions relative to the Isthmus of Tehuantepec and the nearest Pleistocene refugium (if applicable) proposed by Toledo (1982) are also indicated. Refugial area refers to those marked in Fig.2.1.

Pop. code	**Locality**	**State**	**Country**	**Latitude**	**Longitude**	**Altitude (m)**	**W/ E Isthmus**	**Refugial area**
B. heracleifolia								
h2	4km NE San Andrés Tuxtla	Veracruz	Mexico	18.47850	-95.17802	630	West	C
h3	7km NE San Andrés Tuxtla	Veracruz	Mexico	18.50341	-95.16824	883	West	C
h4	8km NE San Andrés Tuxtla	Veracruz	Mexico	18.50660	-95.16607	910	West	C
h5	9km NE San Andrés Tuxtla	Veracruz	Mexico	18.52009	-95.16176	1041	West	C
h8	Agua Azul	Chiapas	Mexico	17.22117	-92.11073	431	East	E
h9	Unión Juárez	Chiapas	Mexico	15.08881	-92.08448	1718	East	D
h10	Ocozocautla biosphere reserve	Chiapas	Mexico	16.90533	-93.45153	957	East	n/a
h12	Ocozocuatla biosphere reserve	Chiapas	Mexico	16.94645	-93.45651	625	East	n/a
h13	Near Berriozábal	Chiapas	Mexico	16.86693	-93.32781	1153	East	n/a
h14	Roadside to Fortin de las Flores	Veracruz	Mexico	18.89369	-97.01050	902	West	A
h15	5km south of Orizaba	Veracruz		18.782528	-97.08508	1300	West	A
h16	Valley 30km East of Huatusco	Veracruz	Mexico	19.20111	-96.67139	290	West	A
h21	Highway 175 between Jasaa and San Juan Bautista	Oaxaca	Mexico	17.74356	-96.32803	1246	West	B
h23	Highway 175, near Santa Maria Jacatapec	Oaxaca	Mexico	17.86422	-96.21381	60	West	B
h24	Motzorongo	Oaxaca	Mexico	18.66953	-96.78714	470	West	A
h26	San Jeronimo Zoochina	Oaxaca	Mexico	17.22117	-95.23547	109	West	n/a
h28	near Santa Maria Xanabi	Veracruz	Mexico	15.98808	-96.11061		West	n/a
h-g1	Cobán	Alta Verapaz	Guatemala				East	F

Pop. code	Locality	State	Country	Latitude	Longitude	Altitude (m)	W/ E Isthmus	Refugial area
B. nelumbiifolia								
n1	Near Los Tuxtlas biological station	Veracruz	Mexico	18.59026	-95.07876	165		
n4	8km NE San Andrés Tuxtla	Veracruz	Mexico	18.50660	-95.16607	910		
n5	9km NE San Andrés s Tuxtla	Veracruz	Mexico	18.52009	-95.16176	1041		
n7	Road to Ocosingo from Palenque	Chiapas	Mexico	17.42477	-91.99712	396		
n11	Ocozocoautla biosphere reserve	Chiapas	Mexico	16.92489	-93.45090	888		
n18	Valley 25km East of Huatusco	Veracruz	Mexico	19.20367	96.742556	635		
n21	Hwy 175 between Jasaa and San Juan Bautista	Oaxaca	Mexico	17.74356	96.328028	1246		
n25	Arroya Zacata	Oaxaca	Mexico	17.73981	95.791333	78		
n26	San Jerónimo Zoochina	Oaxaca	Mexico	17.22117	95.235472	109		

2.3.3. DNA extraction

Total genomic DNA was isolated from 25 mg of silica dried flower or leaf material using a modified protocol for the DNeasy 96-sample kit (Qiagen, Germantown, MD, USA). Dry material was disrupted for two cycles of 2 min (25 Hz), and centrifuged for 10 s (4000 rpm), prior to the recommended heated lysis, to obtain a fine powder. The heated lysis was extended from 30 min to 1 h to increase the yield of DNA. As a result of unknown inhibitors of polymerase chain reaction (PCR), all DNA was diluted 100-fold with Millipore ultrapure water prior to PCR.

2.3.4. Plastid microsatellite marker design

2.3.4.1. Plastid marker design

New plastid microsatellite markers were designed from the draft plastome of *B. nelumbiifolia* (N. Harrison & C. A. Kidner, unpubl. data). The frequency and length of microsatellite repeat motifs in the plastome were calculated using the Phobos Tandem Repeat Finder v3.3.12 plugin for Geneious v5.4 (Mayer, 2006–2010). Primers were designed around perfect (uninterrupted) microsatellite motif repeats using WebSat (Martins *et al.*, 2009). The plastome sequence was uploaded to WebSat in two FASTA files, and primers were designed to amplify PCR products between 100 and 400bp in length, with an optimal primer melting temperature (T_m) of 60°C and a primer GC content of 40%. Forty potential primer pairs located in WebSat, amplifying mononucleotide or dinucleotide repeats of at least eight repeat units in length, were annotated onto the plastome of *B. nelumbiifolia* in Geneious v5.4 (Drummond *et al.*, 2011). The primer pairs were compared with the plastome sequences of 15 other *Begonia* species (N. Harrison and C. A. Kidner, unpubl. data), and those that were not conserved between species in the same section [*Gireoudia* (Klotzsch) A.DC, 66 species; Burt- Utley, 1985] were discarded. The markers with the most variable microsatellite repeat lengths between species were further tested to determine their suitability for population genetic analysis.

2.3.4.2. Plastid marker test

Sixteen individuals from different populations of *B. heracleifolia* were used to test the amplification success and allelic diversity of the plastid markers. All primers (listed in Table 2.2) were tested individually prior to multiplex testing (below). PCRs were performed using M13-tailed fluorescent primers (Schuelke, 2000). PCRs were performed in a final volume of 10µL, containing 0.5µL of 1mM M13- tailed forward primer (Invitrogen, Grand Island, NY, USA), 1µL of reverse primer (1mM), 1µL of 1mM M13-fluorescently modified primer [6-FAM, VIC, NED, PET (Applied Biosystems, Foster City, CA, USA)], 0.25µL of bovine serum albumin (BSA, 0.4%), 1µL of 10x reaction buffer, 1µL of 2mM deoxynucleoside triphosphates (dNTPs), 0.6µL of 25 mM MgCl2, 3.6µL of double-distilled H_2O, 0.05 µL of Biotaq polymerase (Bioline, London, UK) and 1µL of dilute DNA template. PCR was performed in a Peltier Thermal Cycler and consisted of a profile of initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, followed by extension at 72 °C for 1 min. PCR products were run on a 2% agarose gel stained with SYBR Safe gel stain (Invitrogen) and visualised under UV light. One microlitre of each PCR product labelled with the four fluorescent dye colours was pooled and diluted 50x in Millipore distilled H_2O prior to fragment analysis on an ABI3730 at the GenePool (University of Edinburgh, UK) using GeneScan 500 LIZ internal size standard (Applied Biosystems, Foster City, CA, USA). Fluorescent traces were analysed automatically with manual editing using GeneMapper v4.0 (Applied Biosystems).

PCR products of microsatellite repeats are prone to PCR amplification errors caused by slipped-strand mis-pairing (Fazekas, Steeves & Newmaster, 2010). To check whether changes in the length of the target microsatellite motif correlated directly with the change in PCR product size, rather than other microsatellites in the flanking region or other indels, we sequenced a subset of individuals. Long mononucleotide repeat motifs (>15 bp) can amplify poorly. Therefore, to mitigate this problem, they were amplified using AmpliTaq Gold polymerase, as recommended by Fazekas *et al*. (2010), employing the protocol recommended by the manufacturer. Excess primers and sequencing reactions were performed using the protocol of Thomas *et al*. (2011).

Table 2.2. Plastid microsatellite primers generated for this study. Motif refers to the plastome of *Begonia nelumbiifolia; N* alleles and product size range are the number of alleles found in *B. heracleifolia* and the polymerase chain reaction (PCR) product size, respectively.

Locus	**Primer sequences**	**Multiplex (fluorophore)**	**Motif**	**N alleles**	**Product size Range (bp)**
Bnc1	F: M13-GGATTCGAGTTGGATTGGACTA R:CGAGAAAGTCTACGGTTCGAGT	1 (FAM)*	(A)$_{9}$†	3	343-347
Bnc2	F: M13-TGTGCCTTTAGTGGGCTTAGTT R:TCTGTGTTATGGTAGAAGTCGCA	5 (PET)	(T)$_{15}$	3	177-183
Bnc6	F: M13-CCCTCGATAGTTCTTTGTTTCG R:TTTGATCCCTTTATCAGCCAAC	2 (FAM)*	(AT)$_{14}$	6	398-412
Bnc7	F: M13-GGGAAGGGAGGATTCATAAAAT R:AACGGAGCACCTAACAACGTAT	3 (VIC)	(T)$_{15}$	3	189-191
Bnc9	F: M13-CGGCAGAAATAAGTGGATTCAT R:TCCTTCCGTTTCGTCTGTAGTT	5 (PET)	(T)$_{13}$	7	338-350
Bnc11	F: M13-GCTATGGTGAAATCGGTAGACA R:GCGGAAGCATATAGTCCTACAA	3 (VIC)	(C)$_{11}$	n/a‡	350-376
Bnc13	F: M13-CTTTGCCAAGGAGAAGATGC R: CGGTCAATTAGGCTTAATCTTTTT	4 (NED)	(T)$_{13}$	8	126-137
Bnc14	F: M13-GATTCAAATGGTTCTCGGAAA R: TCACTATCAGTTGATAAAGTTGAAGC	4 (NED)	(T)$_{15}$	6	181-188
Bnc16	F: M13-TAATGACCCGGGACGTAATC R: GGGTTCGAATCCCTCTCTTT	4 (NED)	(A)$_{15}$	n/a‡	235-242

*As a result of preferential amplification of one of the products, these loci were amplified individually and pooled in equimolar ratios prior to fragment analysis.

†Mutationally complex region with multiple microsatellites. Longest motif listed.

‡Not used in analyses, see text.

M13 motif, CACGACGTTGTAAAACGAC; n/a, not applicable.

2.3.4.3. *Multiplex PCR test*

The plastid microsatellite primers that amplified well individually were tested for multiplex compatibility. To reduce the likelihood of amplifying large nontarget PCR products between adjacent primer sites, the primer pairs were annotated onto the whole plastome of *B. nelumbiifolia* in Geneious v5.4, and primer combinations within 5 kb were excluded. Additional primer–primer interactions that interfere in the amplification of the template DNA were tested for using AutoDimer (Vallone & Butler, 2004). Potentially compatible multiplex partners were then tested empirically by sequentially mixing equimolar concentrations of each primer pair and testing PCR on one individual of each species. Once successful combinations were observed (amplification of both products with similar band intensities on an agarose gel, minimal primer dimers), this procedure was repeated until the optimal multiplexing combinations were derived from the available markers. Multiplex PCR assays were then conducted on population-level samples of *B. heracleifolia* and *B. nelumbiifolia*.

2.3.5. Haplotype distributions and relationship among haplotypes

A plastid haplotype can be defined as the unique combination of alleles at multiple loci from the plastid. Plastid haplotypes were defined in a Microsoft Excel (Microsoft Corporation, Washington DC, USA) spreadsheet using the Chloroplast PCR-RFLP Excel macro (French, 2003). To assess the relationships between haplotypes, we calculated haplotype connection lengths in Arlequin v3.0 (Excoffier, Laval & Schneider, 2005), and visualised the minimum-spanning tree in HapStar v0.5 (Teacher & Griffiths, 2011). We also constructed a median-joining network in Network (http://www.fluxus-engineering.com) using default parameters. We visualised the spatial distribution of plastid haplotypes by plotting haplotypes by hand onto a topographical map of Central America (downloaded from http://www.worldclim.org).

2.3.6. Population genetic diversity and genetic differentiation

A reduced dataset including only populations for which all loci amplified in at least three individuals (15 of 18 populations) was used for population genetic analyses. The total number of haplotypes and the mean number of haplotypes per population were calculated, and the intrapopulation haplotype diversity was assessed using the gene diversity index (H_e) calculated in Arlequin v3.0.

Absolute measures of differentiation (G'_{ST} and Jost's D) were calculated in SMOGD (Crawford, 2010), and their significance was calculated with 1000 bootstrap replicates. G'_{ST} is a standardised G_{ST} based on the maximum level of differentiation possible for a given set of gene diversities (Hedrick, 2005). D (Jost, 2008) is a test statistic based on the effective number of alleles, and is therefore a good measure of allelic differentiation among populations (Meirmans & Hedrick, 2011). We tested for a pattern of isolation by distance, which can indicate dispersal limitation between populations. Pairwise F_{ST} values between populations were calculated in Arlequin and their significance was calculated by 1000 permutations. Pairwise geographical distances between populations were calculated with the Geographic Distance Matrix Generator v1.2.3 (http:// biodiversity informatics.amnh.org/open_source/gdmg) (Ersts). We regressed pairwise estimates of F_{ST} /(1 - F_{ST}) against the natural logarithm of geographical distance (Rousset, 1997) and tested the significance of the relationship using a Mantel test with 10 000 permutations in Isolation by Distance (IBD) Web Service (Jensen, Bohonak & Kelley, 2005).

The relative roles of different historical scenarios on the patterns of haplotype distributions were assessed using analysis of molecular variance (AMOVA), following the barriers tested by Gutiérrez-Rodríguez *et al*. (2011). First, we evaluated the proportion of F_{ST} accounted for by the Isthmus of Tehuantepec. Second, we tested the proportion of F_{ST} accounted for by the glacial refugia presented by Toledo (1982), by placing populations in broad bins surrounding these refugia. AMOVAs were performed in Arlequin with 20 000 permutations per test, and the groups used for each AMOVA are shown in Table 2.1.

We also tested whether other groups of populations form distinct genetic clusters, without defining potential groups a priori, using spatial analysis of molecular variance (SAMOVA) implemented in SAMOVA v1.0 (Dupanloup, Schneider & Excoffier, 2002). This approach groups adjacent populations that are genetically similar to each other, whilst maximizing between-group genetic variance, without making assumptions of ploidy, linkage or Hardy–

Weinberg equilibrium. We tested all K values between K = 2 and K = 15 using 100 initial conditions per test and the sum of the squared size difference setting recommended for microsatellite data. To test whether related haplotypes are more likely to be found in geographical proximity to each other (phylogeographical structure), we compared patterns of unordered haplotype frequencies (G_{ST}) with the squared difference of the number of repeats (R_{ST}) in the program cpSSR (Pons & Petit, 1996).

2.4. Results

2.4.1. Plastid microsatellite marker design

The draft *B. nelumbiifolia* plastome contained 232 perfect mononucleotide repeats exceeding eight repeat units in length, 114 of which were longer than 10 repeat units. Repeats longer than eight repeat units in length included: 101 poly(A), 126 poly(T), four poly(C) and one poly(G) repeat. The longest repeat was a mono(A) repeat with 25 repeat units, but this result may be biased as NGS frequently underestimates the length of mononucleotide repeats (Chan, 2009), and long repeat units were often followed by a chain of unknown nucleotides (Ns). The *B. nelumbiifolia* plastome also contained seven dinucleotide repeats with at least six repeat units, six of which were poly(AT) and one poly(TG). Thirty-one of the 40 (77.5%) primer pairs that were compared across the plastome alignment were conserved between species of section *Gireoudia*.

All nine of the plastid microsatellite primers that were conserved across section *Gireoudia* applied in the first round of testing successfully amplified a fragment in both species. A multiplex PCR assay was found to incorporate all nine loci in five PCR reactions without affecting the band profile, producing large primer dimers or greatly reducing the yield of one of the products (Table 2.2). Locus *Bnc16* did not amplify well in some samples, so that these primers were not used for further genotyping. Overall, the full set of eight loci was successfully amplified in 263 individuals from 18 populations for *B. heracleifolia* and 187 individuals from 13 population of *B. nelumbiifolia* (Table 2.1). The full matrix of plastid microsatellite data is in Appendix 1.

Begonia nelumbiifolia was monomorphic for all genotyped loci, and therefore no further population genetic analyses could be conducted on this species. In contrast, *B. heracleifolia* was polymorphic for all eight loci. One locus, *Bnc11*, had a 15-bp deletion relative to the second shortest allele at the locus. As some measures of population differentiation (e.g. measures of R_{ST}) assume a stepwise mutation model, this locus was excluded from further analyses. The alleles at the remaining seven loci generally differed between the two species, with few overlapping allelic variants, and a 12-bp deletion at locus *Bnc6* consistently distinguished the two species. For *B. heracleifolia*, the mean number of alleles per locus was 5.2; the full lists of the numbers of alleles per locus and gene diversities are given in Tables 2.2 and 2.3, respectively.

2.4.2. Haplotype distributions and the relationship among haplotypes

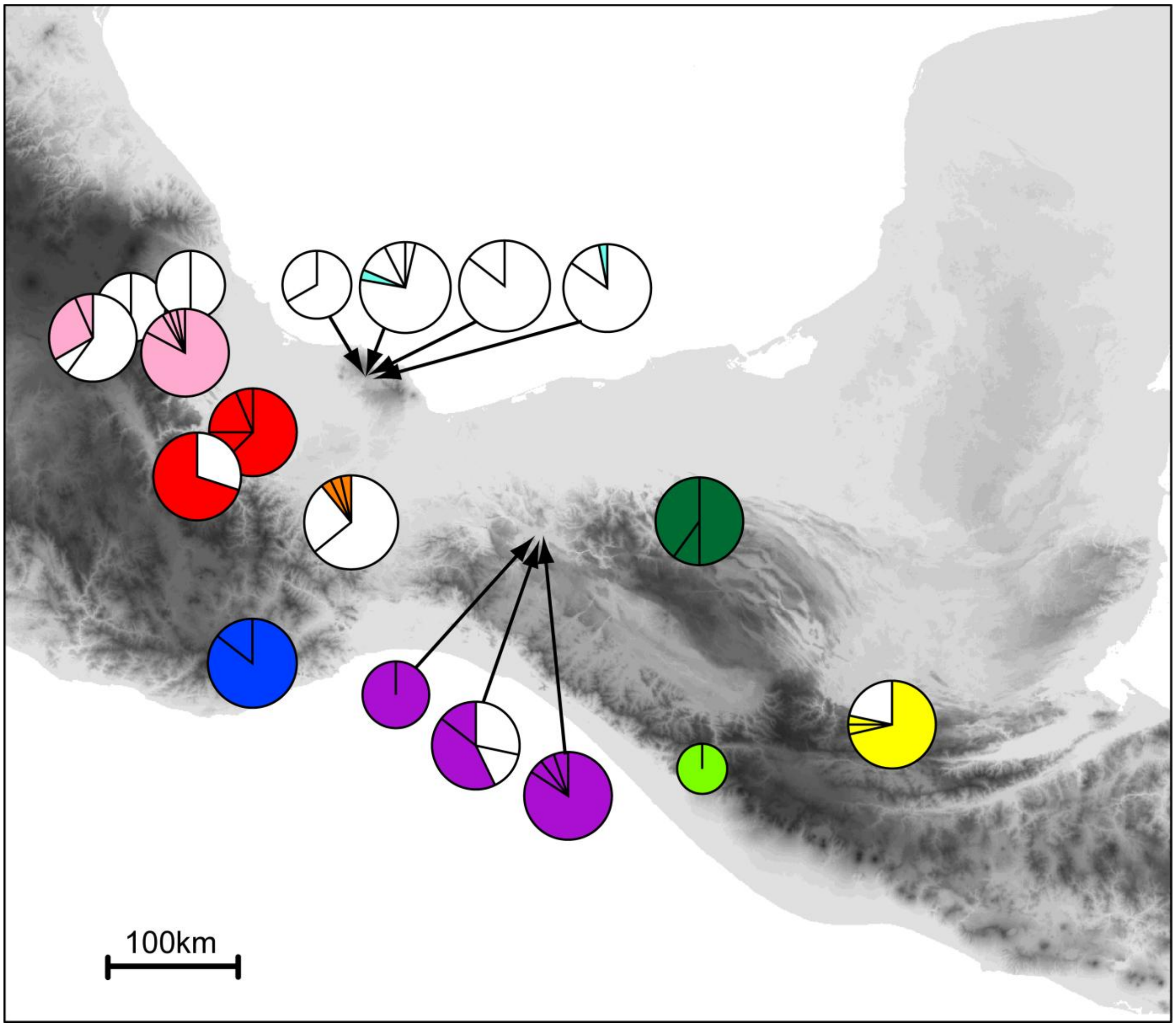
Thirty-nine haplotypes from the allelic combinations of seven plastid microsatellite loci were found in *B. heracleifolia* (Table 2.3). Only six of the 39 haplotypes (*pt1*, *pt2*, *pt4*, *pt6*, *pt21*, *pt23*) were shared between populations, with the remaining 33 (85%) being private haplotypes (Fig. 2.3). The most widespread haplotypes were *pt1* and *pt2*, which were found in five and seven populations, respectively, and *pt5* and *pt6* were shared between distant populations within the Mexican Gulf region (*sensu* Escalante *et al*., 2007). The only haplotype shared across geographical regions was *pt21*, which was common to one of the most westerly populations (h15) and the most easterly population from Guatemala (hg), separated by ~800 km. In the haplotype tree, *pt21* fell in a haplogroup with four other haplotypes from the Mexican Gulf. The minimum spanning tree (Fig. 2.4), which is a single parsimony tree reconstructing the relationship between haplotypes, showed no large-scale phylogeographical structure or distinct haplogroups. However, local geographical structure was indicated by haplotypes being clustered with others from the same area. The internal haplotypes included *pt1* and *pt2*, which were some of the few widespread haplotypes. The median-joining network also showed consistent results, with the absence of broad-scale phylogeographical structure, but local clustering (results not shown).

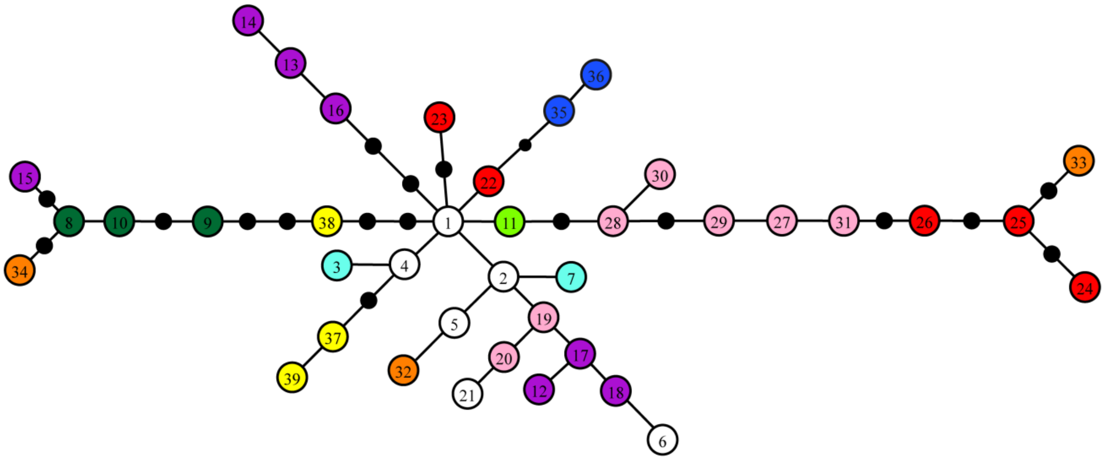
Table 2.3. Plastid haplotype frequencies and gene diversities per population. Population codes refer to Table 2.1. *N* is the number of individuals genotyped. The frequency of each plastid type (*pt*) is indicated in parentheses.

Population Code	N	Haplotypes	Gene Diversity
h2	7	*pt1*(6), *pt2*(1)	0.041
h3	27	*pt1*(1), *pt2*(20), *pt3*(1), *pt4*(3), *pt*5(2)	0.107
h4	3	*pt2*(2), *pt6*(1)	0.286
h5	33	*pt2*(28), *pt6*(4), *pt7*(1)	0.102
h8	10	*pt8*(5), *pt9*(1), *pt10*(4)	0.137
h9	1	*pt11*(1)	
h10	2	*pt12*(2)	
h12	19	*pt13*(16), *pt14*(1), *pt15*(1), *pt16*(1)	0.087
h13	7	*pt17*(3), *pt18*(1), *pt17*(3), *pt18*(1)	0.218
h14	3	*pt1*(3)	0.000
h15	15	*pt1*(9), *pt19*(1), *pt20*(4), *pt21*(1)	0.210
h16	2	*pt1*(1), *pt2*(1)	
h21	20	*pt4*(6), *pt22*(14)	0.063
h23	16	*pt23*(10), *pt24*(2), *pt25*(3), *pt26*(1)	0.364
h24	35	*pt27*(29), *pt28*(3), *pt29*(1), *pt30*(1), *pt31*(1)	0.097
h26	28	*pt2*(18), *pt5*(7), *pt32*(1), *pt33*(1), *pt34*(1)	0.184
h28	7	*pt35*(6), *pt36*(1)	0.041
h-g1	28	*pt21*(6), *pt37*(20), *pt38*(1), *pt39*(1)	0.137

Figure 2.3. Spatial distribution of plastid haplotypes in *Begonia heracleifolia* (next page, top). Pie charts are placed at the site of each collection, and the chart size is proportional to the number of individuals genotyped. The size of each segment corresponds to the number of individuals with a given haplotype. White segments represent widespread haplotypes, and coloured segments are haplotypes limited to that region. Key to colours: pink, Veracruz; red, north Oaxaca; orange, north-east Oaxaca; light blue, Los Tuxtlas; dark blue, south Oaxaca; purple, west Chiapas; dark green, north Chiapas; light green, south Chiapas; yellow, Guatemala.

Figure 2.4. Minimum spanning tree of haplotype relationships (next page, bottom). Numbers refer to the plastid types (listed in Table 2.3). Colours refer to collection sites, and are the same as in Fig. 2.3 (see legend for Fig. 2.3 for explanation). Hypothetical (unsampled) haplotypes are represented by filled black circles.


100km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2.4.3. Population genetic diversity and genetic differentiation

Begonia heracleifolia showed significant structuring of genetic diversity (G_{ST} = 0.526, h_T = 0.937, h_S = 0.444). Pairwise comparisons of population structure (F_{ST}) were also high, with 89% of pairwise F_{ST} values significant at $P < 0.05$ (data not shown). The mean value of Hedrick's (2005) standardised estimator of genetic differentiation G'_{ST} was 0.829. The value for Jost's estimator of genetic differentiation D was 0.407. The Mantel test between genetic distance and the natural logarithm of geographical distance recognised significant isolation by distance (Z = 153.7, r = 0.439, $P < 0.005$); however, this relationship explained a limited amount of the genetic structure (r^2 = 19%). To test whether plastid mutations had accumulated in each population, we calculated the level of significance between G_{ST} and R_{ST} by a permutation test. An R_{ST} value of 0.737 (0.0872 SE) was significantly greater than the G_{ST} value of 0.526 (0.0577SE, $P < 0.05$), indicating phylogeographical structuring of related haplotypes.

2.4.4. Historical barriers to gene flow

Most spatial structuring of genetic diversity was not explained by an east–west break at the Isthmus of Tehuantepec (F_{CT} = 0.08915, P = 0.15) in the hierarchical AMOVA (Table 2.4). A higher and significant level of plastid variation was partitioned between geographical clusters that corresponded to glacial refugia (F_{CT} = 0.2951, $P < 0.05$). Patterns of genetic structure influenced by geographical barriers that were not tested in the ANOVAs were assessed with SAMOVA. The highest F_{CT} value in the SAMOVA corresponded roughly to the number of broad geographical areas sampled (k = 9, F_{CT} = 0.749), or as the level of subdivision approached the actual number of populations collected (e.g. K = 13, F_{CT} = 0.746). At K = 9, a single widespread geographical cluster contained localities in the Mexican Gulf (populations h3, h4, h5, h14, h15, h26).

Table 2.4. Analysis of molecular variance (AMOVA) of *Begonia heracleifolia* by populations (a), collection groups (inferred Pleistocene refugia) (b) and west–east division at the Isthmus of Tehuantepec (c). DF – degrees of freedom.

	DF	Sum of squares	Variance components	Percentage of variation	Fixation indices
(a) Among populations	14	301.739	1.256	72.77	
Within populations	243	114.563	0.471	27.23	$F_{ST} = 0.728$
(b) Collection groups (inferred Pleistocene refugia)					
Among groups	7	222.918	0.524	29.51	$F_{CT} = 0.296$
Among populations within groups	7	78.821	0.781	43.96	$F_{SC} = 0.624$
Within populations	243	114.563	0.471	26.53	$F_{ST} = 0.735$
Total	257	416.302	1.777		
(c) W-E Isthmus Tehuantepec					
Among groups	1	41.641	0.163	8.91	$F_{CT} = 0.089$
Among populations within groups	13	260.098	1.193	65.28	$F_{SC} = 0.717$
Within populations	243	114.563	0.471	25.80	$F_{ST} = 0.742$
Total	257	504.612	2.172		

2.5. Discussion

2.5.1. Between-species comparisons of genetic diversity

Of the two species examined here, *B. heracleifolia* showed significant spatial structuring of genetic diversity, whereas *B. nelumbiifolia* showed no polymorphisms at any locus examined. There are a number of possible explanations for the reduced plastid variation within *B. nelumbiifolia* relative to *B. heracleifolia*, one of which is that it could be a genotyping artefact. We used seven plastid microsatellite loci and, by chance, the loci selected may show no variation; other loci in the plastome may show variation. However, this seems unlikely, as we used loci with the longest repeat motifs from *B. nelumbiifolia*; therefore, we do not anticipate any ascertainment bias. Further evidence for a lack of variation within *B. nelumbiifolia* comes from the absence of any SNP variation in the flanking regions of the microsatellite markers and in seven other plastid microsatellite loci used for genotyping in the marker development phase (data not shown). Therefore, it seems more likely that our results indeed represent reduced plastid diversity in *B. nelumbiifolia*.

The most likely explanation for the absence of plastid variation in *B. nelumbiifolia* is a recent population bottleneck or a selective sweep in a single refugial population prior to range expansion. Allelic diversity is reduced when a population experiences a reduction in its effective population size, and the allelic diversity after a population bottleneck is a function of the population size when it is reduced to its minimum and the rate of population growth (Nei, Maruyama & Chakraborty, 1975; Cornuet & Luikart, 1996). The recent timing of range expansion after a bottleneck in the Pleistocene could explain the lack of any post-expansion accumulation of mutations in *B. nelumbiifolia*. This is consistent with the preference of the species for moist habitats, which are likely to have been geographically more restricted in dry periods during the Pleistocene (Toledo, 1982). The fixation of a single plastid haplotype in response to recent bottleneck events has been found in other plant species over similarly large geographical areas (e.g. Mediterranean *Pinus pinea* L., Vendramin *et al*., 2008; Australian *Atherosperma moschatum* Labill., Worth *et al*., 2011).

The absence of plastid variation in *B. nelumbiifolia* contrasts with that in *B. heracleifolia*, where significant genetic diversity was found (average of 3.3 haplotypes per population; Table 2.3). This level of haplotype diversity is surprising, as a single haplotype may be expected to be fixed by genetic drift in the small populations typical of *B. heracleifolia* (Hoover, 1979; A. D. Twyford, pers. observ.). The most likely explanation for the high genetic diversity found in *B. heracleifolia* populations is that this drought-adapted species may have tolerated Pleistocene drought conditions, and populations may have survived *in situ* and not have been confined to refugia. Over a long period of time, there would have been the opportunity to differentiate by drift and selection for local adaptations with limited dispersal of the accumulated new mutations across the range of the species. This is consistent with the wide diversity of leaf morphologies seen in *B. heracleifolia* (Fig. 2.2A), which may be the product of both drift and local adaptation.

Monographic work on the section by Burt- Utley (1985) suggested that morphological variation in *B. heracleifolia* is continuous and does not warrant further division into subspecific taxa, but this question may now be readdressed considering the population level molecular evidence presented here and evidence from future molecular phylogenetic studies.

2.5.2. The relative role of historical barriers to gene flow

We evaluated whether two geographical barriers that are known to have reduced dispersal in other groups influenced the spatial structuring of genetic diversity in *B. heracleifolia*: differentiation across the Isthmus of Tehuantepec and differentiation between putative Pleistocene glacial refugia. Bryson *et al*. (2011) compiled a list of 30 phylogenetic and phylogeographical studies of highland and lowland animal species that had genealogical splits between populations within species, or between sister species, that corresponded to the Isthmus of Tehuantepec. Seventeen of these species had exclusively old (Neogene) splits and eight species had more recent (Pleistocene) splits. Gutiérrez- Rodríguez *et al*. (2011) found that an east–west intraspecific split at the Isthmus of Tehuantepec explained more genetic variation ($F_{CT} = 0.737$) than putative Pleistocene glacial refugia ($F_{CT} = 0.556$) for the understorey plant *Palicourea padifolia* (Willd. ex Roemer & Schultes) C.M.Taylor & Lorence (Rubiaceae). The Pleistocene divergence date (309 000 years ago; confidence interval, 136 000–667 000 years ago) between clades in their study suggests that the Isthmus of Tehuantepec has presented a barrier to plant dispersal in recent times, not just pre- Pleistocene. However, we found little evidence for a genetic break in *B. heracleifolia* corresponding to the Isthmus, indicated by the low F_{CT} value in the AMOVA ($F_{CT} = 0.08915$, P = 0.15, Table 2.4).

As an alternative scenario, we tested whether the spatial arrangement of genetic diversity could be accounted for by the glacial refugia proposed by Toledo (1982). We found no strong support for past refugia structuring genetic diversity in *Begonia*. Most genetic variation was found within groups that represent refugia ($F_{SC} = 0.62362$), rather than between them ($F_{CT} = 0.29510$). Moreover, much more diversity was explained when the populations were divided at a finer scale, or in the alternative groups supported by the SAMOVAs, as discussed below. These results give little support for the Pleistocene refugia proposed by Toledo (1982), but this may in part reflect the difficulty in delimiting tropical refugia, especially using methods based on species diversity and endemism. These results show that responses to Pleistocene climatic fluctuations may depend on species-specific adaptations, and therefore the pinpointing of refugia for complex tropical species assemblages will be difficult (Poelchau & Hamrick, 2012b). Some progress in locating common geographical barriers to gene flow that may represent Neotropical refugia has been made by comparing patterns of haplotype diversity in widespread Central American tropical tree species (Poelchau & Hamrick, 2012a), where barriers shared between species with different ecologies were distinguished from haplotype patterns idiosyncratic to individual species. Therefore, more phylogeographical studies of species with different life-history traits are required to understand where potential refugia are likely to be located, although the current data suggest that less clear-cut patterns may be expected than those revealed for European and North American refugia, where congruent geographical patterns of genetic diversity have shed convincing light on their likely locations (Hewitt, 2000; Petit *et al*., 2003).

2.5.3. Dispersal limitation in *Begonia* explains current patterns of genetic structure

The spatial structuring of plastid variation at a local scale provides further support for the persistence of *B. heracleifolia* populations *in situ* during dry periods in the Pleistocene, rather than preferential survival in the refugia suggested for moisture-loving tropical plants by Toledo (1982). Of the 39 plastid haplotypes found, 33 (85%) were private haplotypes restricted to a single population (Table 2.3 and Fig. 2.3). Although the frequency of plastid haplotypes is strongly influenced by the different sample sizes for each population, additional support for local population genetic structure comes from the high overall G_{ST} value and the many significant pairwise F_{ST} values. The result of the SAMOVAs also supports the subdivision of genetic variation by local geographical groups.

Significant spatial partitioning of genetic diversity and high levels of genetic differentiation (mean value of Jost's D across loci, D = 0.422, G'_{ST} = 0.829) in *B. heracleifolia* are best explained by dispersal limitation between long-lived isolated populations. *Begonia* spp. have tiny seeds that are not known to be dispersed by any animal vectors and are poorly wind dispersed, and therefore effective seed dispersal between populations is expected to be low. This is confirmed by clumps of *Begonia* seedlings growing directly below adult plants in the field (A. D. Twyford, pers. observ.). Hoover (1979) also observed the clustering of individuals in three Mexican *Begonia* spp. (including *B. heracleifolia*). Seed dispersal limitation may explain the isolation of populations for extended periods throughout the Pleistocene, during which time genetic novelties could have arisen that are not shared between populations. The pattern of local geographical structure also shows that human-mediated dispersal, facilitated by road building and translocation for horticultural purposes, has not obscured the natural patterns of geographical variation, as might be expected for a species that tends to grow as a weed in human disturbed environments.

High levels of genetic differentiation between populations have been found in other population genetic surveys of *Begonia* spp. using nuclear markers. Matolweni *et al*. (2000) found strong spatial structuring of genetic diversity in a survey of allozymes in the endemic South African *B. dregei* Otto & A.Dietr. and *B. homonyma* Steud. Hughes & Hollingsworth (2008) found a high F'_{ST} value between forest patches of the endemic *B. sutherlandii* Hook.f and a strong signal of isolation by distance among populations. They linked this micro-evolutionary pattern of local population structure to the macro-evolutionary process of allopatric species divergence that may have given rise to the large number of species present in the genus (1500 species; Frodin, 2004). These population-level surveys are supported by molecular phylogenetic studies of South-East Asian *Begonia* spp. using plastid DNA sequences, in which long-distance dispersal events appear to have been rare, with *in situ* species radiations after dispersal into each of the eastern Malesian islands (Thomas *et al*., 2011). To allow comparisons of gene flow with these previous studies, and to assess the joint role of interpopulation pollen and seed dispersal, future studies should apply nuclear markers to these widespread species. This will also allow the responses of the two species to Pleistocene refugia to be investigated in more detail.

2.5.4. Plastid microsatellites as tools to study intraspecific relationships

The use of plastid markers remains a popular approach for population genetic and phylogeographical studies of Neotropical plants (e.g. Cardoso *et al*., 1998, Fontaine *et al*., 2004).

This popularity may partly be attributed to the limited availability of variable nuclear markers; however, there are a number of properties of the plastome that make it a desirable source of data for population genetic and phylogeographical analysis of widespread groups. First, it is typically maternally inherited in angiosperms (Corriveau & Coleman, 1988) and will reflect only seed dispersal patterns, unlike biparentally inherited nuclear markers that are spread in pollen and in seed (Ennos, 1994). Seeds typically disperse less than pollen, which makes seed-specific markers more likely to track geographical barriers. Second, only seed dispersal (not pollen) can result in establishment in a new habitat, so that plastid markers can reveal species distribution changes, without the blurring of ancestral patterns of gene flow caused by subsequent pollen dispersal (Ennos *et al*., 1999; Petit *et al*., 2003). Third, plastid markers have a smaller effective population size than nuclear markers (Wright *et al*., 2008), so that genetic drift is more potent. Moreover, fewer alleles can be maintained at mutation–drift equilibrium, so that fewer individuals need to be genotyped to sample the range of allelic diversity in a population. Finally, plastid genotyping has been widely used in plant phylogeography, and there are typically many other studies in which the distributions of plastid haplotypes have been assessed over a similar geographical area, allowing for comparisons between taxa. Using NGS to generate whole plastomes, and the identification of hypervariable microsatellite markers from these data, is a novel approach to the design of variable plastid markers. This route was used successfully to find variable markers for *B. heracleifolia*, even when the plastid sequence divergence between populations was anticipated to be low.

Despite the benefits of this approach, there are a number of pitfalls associated with sole reliance on hypervariable plastid markers for population genetic and phylogeographical studies, as highlighted by the results of this study. Population genetic studies typically assume that alleles shared between individuals are the product of gene flow between the populations, and that homoplasy, where the same allele is caused by convergent mutations, violates this assumption. Microsatellite markers are particularly prone to homoplasy as the number of repeat units will not always expand, but can also contract (Provan *et al*., 2001). Moreover, mutations do not always occur in a stepwise manner (Ceplitis, Su & Lascoux, 2005), and rapid deletion of microsatellite motifs can occur [as demonstrated by Micheneau *et al*. (2010) in *Cephalanthera* Rich. (Orchidaceae)]. This may explain the disjunct fragment length seen in this study at locus *Bnc11*.

The combined influence of the complex mutation model and homoplasy makes coalescent methods for the estimation of divergence age estimates from plastid microsatellite data challenging, as it is difficult to correctly assign suitable priors. This is particularly problematic for phylogeographical studies, as a knowledge of divergence ages can help to discriminate between different hypotheses. Therefore, sequence data should be used in preference to plastid microsatellites when divergence age estimates are required.

2.6. Conclusion

This study contributes to our growing knowledge of the evolutionary processes occurring at the population level in the genus *Begonia*. The contrasting levels of plastid diversity for the two species tested here suggests the importance of species-specific ecological preferences in terms of their response to climate change in relation to glacial refugia. Dry-adapted tropical lineages, such as *B. heracleifolia*, may have survived *in situ* during dry periods in the Pleistocene, whereas moisture-loving species, such as *B. nelumbiifolia*, may have been more vulnerable and persisted only in refugia. Generalizations about refugia may therefore only hold for species with similar ecological preferences, rather than at broad taxonomic scales. This study also shows that patterns of strong population genetic differentiation and dispersal limitation may be common across the genus. Taxa with low interpopulation gene flow, such as *Begonia*, may thus be especially useful for tracking local geographical variation, as homogenizing gene flow from neighbouring populations is limited. As more data are assembled, *Begonia* is emerging as a powerful study system for testing biogeographical questions.

CHAPTER 3: Development and characterisation of microsatellite markers for Central American *Begonia* section *Gireoudia* (Begoniaceae)

Data contributions: Catherine Kidner (University of Edinburgh) provided the transcriptome and genome sequences from which the markers were designed. Alexander Twyford designed the markers, performed all genotyping and analyses.

3.1. Chapter summary

Premise of the study: Transcriptome sequence data was used to design microsatellite primers for two widespread Central American *Begonia* species, *B. heracleifolia* and *B. nelumbiifolia*, to investigate population structure and hybridization.

Methods and results: The transcriptome from vegetative meristem tissue from the related *B. plebeja* was mined for microsatellite loci, and thirty-one primer pairs amplified in the target species. Fourteen primer pairs were combined in two multiplex PCR reactions, which amplified an average of 4 alleles per locus.

Conclusions: The markers developed will be a valuable genetic resource for medium-throughput genotyping of Central American *Begonia* species. Their amplification in two divergent species within the section is promising for their transferability to related species.

3.2. Introduction

Begonia is a diverse tropical genus with over 1500 species. Evolutionary research has focused on the early diverging African species (e.g. Hughes and Hollingsworth, 2008), and the more derived Asian species (e.g. Thomas *et al.*, 2011), with the American species largely overlooked. The most recent common ancestor of Central American *Begonia* is likely to be relatively recent (Miocene, Dewitte *et al.*, 2011), and subsequent speciation has resulted in high species richness (total c.690 species, Copestake *et al.*, 2010). Population studies of Central American *Begonia* spp. will shed light on the evolution of species richness in a morphologically diverse group of

Neotropical herbs; but to date studies have been limited by the availability of suitable nuclear markers to complement plastid microsatellite markers (Chapter 2).

In this study, we describe the development a multiplex PCR assay of nuclear microsatellite markers for two Central American *Begonia* species: *B. heracleifolia* Cham & Schltdl. and *B. nelumbiifolia* Cham & Schltdl.. These species were chosen as they are two of the most widespread *Begonia* species in a genus of mostly rare endemics (Hughes and Hollingsworth, 2008). The species are known to hybridize (Burt-Utley, 1985) facilitating studies of species boundaries. The markers are developed from the transcriptome of related *B. plebeja* Liebm., and these genic markers are anticipated to amplify over a broad phylogenetic scope (Lepais and Bacles, 2011). These markers will be used to study patterns of gene flow within and between Central American *Begonia* species.

3.3. Methods and results

Microsatellite markers were designed from the transcriptome sequence of vegetive meristem tissue from *B. plebeja*, a related species from *Begonia* section *Gireoudia* (European Nucleotide Archive Sequence Read Archive accession number: ERP001195; Brennan *et al.*, *submitted*). The QDD bioinformatic pipeline (Meglécz *et al.*, 2010), which integrates microsatellite detection, a redundancy check to avoid amplifying multiple PCR products, and designs primers, was used according to Lepais and Bacles (2011). A FASTA file of the *B. plebeja* transcriptome sequence assemblies were analysed in QDD v1.3 using default parameters: selecting only primers that amplify a PCR product between 90 and 320bp in length, with a repeat motif of 2 - 6bp repeats, and a minimum length of 4 repeat units. To make microsatellite amplification in other species more likely, primers were excluded if they did not have a perfect BLAST match to the transcriptome of *B. conchifolia* A.Dietr. (section *Gireoudia*).

Table 3.1. Characterisation of nuclear microsatellites for Central American *Begonia* species. The multiplex to which the primer was assigned, primer melting temperature when amplified individually (T_m), the motif in *B. plebeja*, the number of alleles (N_a) found in *B. heracleifolia* (her) and *B. nelumbiifolia* (nel), and the observed range of PCR product sizes excluding the M13 motif, are listed.

Locus name	Primer sequences	Multiplex	Fluorscent dye	T_m (°C)	Motif	N_a her	N_a nel	Allele sizes (bp)
Multiplexed loci								
BI4329	F: M13-CAACCAACAATGGCAGCTT R:CATGGAGATAATGGAGCTGG	1	FAM	59	$(GGA)_6$	4	2	89-104
BI3043	F: M13-CGACATCCAACCAAACCTG R:TTGATAGATGGAAGGGTCGC	1	FAM	60	$(TC)_5$	1	2	173-179
BC432	F: M13-AAACTCCGATGGATTCAGCA R:TGAAATAAACACACAAACAAAGACA	1	FAM	60	$(TG)_5$	1	1	261-263
BC344	F: M13-GAGGGAGGGTCCCTTGTTAG R:CCGTCTTACGTTGCATCATC	1	VIC	60	$(GCA)_5$	1	1	105-108
BI6278	F: M13-TGTAGTTGTTGTAGTAGCAGAACTTTG R:CAGATGGGTCGGAGATTTTG	1	VIC	59	$(TCC)_7$	1	3	238-253
BI5347	F: M13-TCAGTCCATTTTCTTAATCAGACC R:CTCTATCATTTCCAAGCGATTTC	1	VIC	59	$(CTT)_6$	2	1	171-183
BC552	F: M13-TGTCTGAGATGGAAACTGCG R:TAGTCGAAGGGATCCGAATG	1	NED	60	$(GT)_5$	2	2	271-273
BI3348	F: M13-ACTTGTTTCTCGTTGGGAGC	1	PET	60	$(CT)_6$	3	3	279-283

	R:CTGCAGCCCAGTGGATTTAC							
BI06534	F: M13-CGTTGCTCTGCTCTAACCCT R:AGATACAGCCAACCGGATTC	1	PET	59	(TC)$_{6}$	6	2	97-107
BI7112	F: M13-ATCCAATGTCAACCTCTCGG R:GTGCATTAGAGTCCCGTGGT	2	FAM	60	(TCC)$_{6}$	2	2	109-115
BI3820	F: M13-AGGACCAGTTTTGACGGCTA R:GAAGCTTTTGCTCTTCTGTTGA	2	FAM	59	(CTT)$_{7}$	5	2	158-176
BI134	F: M13-ATCAGCTCACTCCCTATCCTCT R:TGCAATCTCCTTCGGTTCTT	2	VIC	60	(CT)$_{6}$	4	2	306-314
BI362	F: M13-CTTCACCTCGCCTGAACAAC R:GAGGCGAAATATTATGCGGA	2	NED	60	(ATG)$_{6}$	4	4	147-159
BC332	F: M13-GAACCAGAAGTCAAGGGTTCA R:AAACATGATTTTCCTCATCCAA	2	PET	59	(TCA)	4	2	188-200
Additional loci tested								
BI4004	F: M13-TCAGGAAATATTCGATTGGGA R:GCATTCCTCTGTGTACAATGC			59	(AT)$_{5}$	2	3	155-169
BC672	F: M13-CCTTGATCGAGAAAGAACCG R: AAAGCCAGCTCCTTCCTGTA			60	(CTT)$_{8}$	3	1	152-158
BI4477	F: M13-GGATCTCCTCTGCTTTGCTG R:GGCGAGACCAGAAGAAAAGTT			60	(CT)$_{9}$	4	2	111-119
BI06604	F: M13-ATTTTTCCACAGAAGAGCCC R:GGCAGAACCCGCAGTATATC			59	(AT)$_{8}$	6	1	111-127
BI6294	F: M13-TGCTGGTCTGAATCTTTAATCA R:TGGGGTCTTGGTACTCTTTCC			59	(AT)$_{10}$	1^{M}	1^{M}	148
BI6701	F: M13-AGAATCCCCACTCACTGCAC R:GAGATGATGAGGGTTCAGGC			60	(GA)$_{6}$	1^{M}	1^{M}	195

BI05710	F: M13-GAAAGTTTTGGAGGAAGCCC R:TGGAAGAGATCAGAAGGTACA	60	(GAA)$_{7}$	3	1	178-184
BI4848	F: M13-CGACGCCTCTCAAAGAAGAA R:GAGCTTTGAATTTCGCTACG	59	(AG)$_{6}$	4	2	71-74
BC402	F: M13-TTACTCGAGCTAGAAGCCGC R:AGGGCTTGGAGAGCTAGAGG	60	(AT)$_{5}$	1^{M}	1^{M}	92
BC932	F: M13-GTAGTCCATCAGTCCGCCAT R:GAGTGATGAAGGCGAAGAGG	60	(GA)$_{5}$	2	1	660-662†
BI3069	F: M13-AACCACAGTAATCATCCGGC R:TGTCCGGTAACTGTGGTGAA	60	(CA)$_{5}$	1	1	184-192
BI3377	F: M13-AACACAATCATCAGCCGGAC R:GAAGGAGATGATTATGACGAA	60	(AGG)$_{5}$	MP	MP	
BI5174	F: M13-GTCGCAGGGTTTGTCTAGGA R:GGAAATCAGAGTGCTGGCTC	60	(CTT)$_{5}$	1	1	118-121
BC42	F: M13-GCTATGCAGGTTCTGGTGGT R:ACTGGTTGTCACTACTGCCG	59	(TGG)$_{6}$	3	2	147-173
BI6984	F: M13-GAAGGGGTTTCTTGGTCTCA R:TTGTCAATTCTCACCAGACACA	59	(TC)$_{6}$	3	2	148-164
BI7247	F: M13-CTCTTATTCCGCGTCAAAGC R:AGCGGAGAAGTCGAAAACAG	60	(AG)$_{6}$	1^{M}	1^{M}	135
BC312	F: M13-ATTTCCTTCTGCGAACGATG R:ATCGGAACTCTGAGCCTGAA	60	(GA)$_{5}$	2	1	178-180

^{M} Monomorphic in all individuals tested

† Large product size assumed to be caused by an intron

^{MP} Multiple PCR products amplified

M13 sequence is: CACGACGTTGTAAAACGAC

Thirty-one primer pairs detected in QDD were tested for amplification in 7 individuals of both *B. nelumbiifolia* and *B. heracleifolia* from different populations. A subset of polymorphic markers that amplified reliably in both species was then tested for multiplex compatibility, by mixing equimolar ratios of each primer. The PCR multiplexes were then tested on a population of each species (20 individuals) to estimate the genetic diversity of the markers. The primers sequences were BLAST searched against the transcriptome sequence of the divergent Asian species *B. venusta* King (section *Platycentrum*), to test for likely cross-amplification of primers in other *Begonia* species.

Approximately 15mg of silica dried leaf material was extracted using DNeasy 96-sample kit (Qiagen, Germantown, MD, USA). To overcome an unknown PCR inhibitor that co-elutes with DNA extractions in *Begonia*, extractions were diluted 100-fold with Millipore dH_2O to a final DNA concentration of ~0.1-1.5µg/ml. PCR reactions were performed using the M13-tailed primer method (Schuelke, 2000) in a final reaction volume of 10µl, containing: 0.5µl of 1mM M13-tailed forward primer (Invitrogen, Grand Island, USA), 1µl reverse primer (1mM), 1µl of 1mM M13 fluorescently modified primer (6-FAM,VIC, NED, PET), 0.25µl bovine serum albumin (BSA, 0.4%), 1µl of 10x reaction buffer, 1µl of 2mM dNTPs, 0.6µl of 25mM $MgCl_2$, 0.05µl Biotaq polymerase (Bioline, UK), 1 µl dilute DNA template, and made up to the final volume using dH_2O. PCR cycles consisted of an initial denaturation of 1 minute as 95°C, followed by 40 cycles of denaturation for 1 minute at 95°C, annealing for 1 minute at 57°C followed by extension of 1 minute at 72°C. Five microlitres of each PCR product labelled with the four fluorescent dye colours was pooled and diluted 2x in Millipore dH_2O, and the GeneScan 500 LIZ internal size standard (Applied Biosystems, Foster City, Ca) was added prior to fragment analysis on the ABI3730 (GenePool, University of Edinburgh, UK). Fluorescent traces were analysed automatically with manual editing using GeneMapper v4.0 (Applied Biosystems).

A total of 136 primer pairs were located in the *B. plebeja* transcriptome using the QDD bioinformatic pipeline. All thirty one of the subset of primers tested for amplification yielded a PCR product (Table 3.1). Of these loci, 4 loci were monomorphic (BI6701, BC402, BI6294 and BI7247), and one amplified multiple PCR products (BI3377). Two PCR multiplex reactions were designed to amplify a total of 14 polymorphic loci (Table 3.1). All loci were polymorphic in at least one of the populations tested, and showed moderate genetic diversity with number of alleles per species from 1 to 5 and expected within population heterozgosity between 0 and 0.75 (Table 3.2). Twenty-one of the the 62 primers (34%) had perfect BLAST matches in the transcriptome of the divergent *B. venusta*, including both the forward and reverse primers for loci BI3348, BC932, BC552.

Table 3.2. Genetic diversity in population samples of *B. heracleifolia* and *B. nelumbiifolia*. The number of alleles per locus (N_a), expected heterozgosity (H_E) and observed heterozgosity (H_O), and the total alleles observed in the two species (N_T) are listed.

Locus	*B. heracleifolia*			*B. nelumbiifolia*			$\mathbf{N_T}$
	$\mathbf{N_a}$	$\mathbf{H_o}$	$\mathbf{H_E}$	$\mathbf{N_a}$	$\mathbf{H_o}$	$\mathbf{H_E}$	
BEI4329	3	0.400	0.524	3	0.500	0.537	5
BEI03043	4	0.000	0.444	3	0.500	0.630	4
BEC432	2	0.100	0.097	2	0.000	0.097	3
BEC344	1	-	-	2	0.000	0.097	2
BEI6278	1	-	-	3	0.353	0.668	4
BEI5347	3	0.300	0.449	1	-	-	4
BEC552	1	-	-	3	0.050	0.229	3
BEI3348	4	0.579	0.604	4	0.500	0.665	5
BEI06534	5	0.500	0.750	4	0.105	0.201	7
BEI7112	2	0.400	0.467	3	0.278	0.522	4
BEI3820	5	0.600	0.623	2	0.000	0.108	6
BEC134	4	0.611	0.732	3	0.050	0.145	5
BEI04004	2	0.059	0.059	3	0.188	0.623	4
BIC362	2	0.050	0.050	2	0.000	0.097	2
BEC332	4	0.250	0.483	3	0.154	0.495	5
Mean	3.333	0.321	0.440	2.857	0.191	0.365	4
SD	1.155	0.228	0.246	0.663	0.199	0.243	1.327

3.4. Conclusions

We have described the development of nuclear microsatellite primers that amplify in two divergent Central American *Begonia* species. Many of the primers have exact BLAST matches in the transcriptome of the South-East Asian species *B. venusta* and may therefore be transferable widely across the genus. The transferability of markers is important for the study of natural hybrids, and the development of a multiplexed assay of 14 loci should enable accurate assignment to hybrid classes (e.g. F1, backcross). Future studies will use these loci to estimate the genetic structure of populations, and the frequency of hybrids and extent of introgression in hybrid swarms.

CHAPTER 4: Genetic differentiation and species cohesion in two widespread Central American *Begonia* species

Data contributions: Andrew Matthews (Queen Mary University London) provided the genome size (C-value) estimates. Alexander Twyford performed all genotyping and experimental crosses, and all the analyses.

4.1. Chapter summary

Begonia is one of the ten largest plant genera, with over 1500 species. This high species richness may in part be explained by weak species cohesion, which has allowed speciation by divergence in allopatry. In this study, we investigate species cohesion in two widespread *Begonia* species, *B. heracleifolia* and *B. nelumbiifolia*, to test whether the population processes that may have promoted past speciation events, such as dispersal limitation and inbreeding, are ongoing in extant species. Populations from Mexico and Guatemala were genotyped at nine nuclear microsatellite loci. Crosses between divergent populations were used to test whether reproductive barriers have accumulated, and compared with genome size estimates from representative populations. Strong population substructure was found for *B. heracleifolia* ($F_{ST} = 0.364$, $F'_{ST} = 0.506$, $P < 0.05$) and *B. nelumbiifolia* ($F_{ST} = 0.277$, $F'_{ST} = 0.439$, $P < 0.05$), and Bayesian admixture analysis supports the division of most populations into discrete genetic clusters. Comparisons with plastid microsatellite data (Chapter 2) show that pollen flow is particularly limiting between populations, and this dispersal limitation has lead to significant genetic differentiation (*B. heracleifolia* Jost's $D = 0.274$, $P < 0.05$; *B. nelumbiifolia* $D = 0.294$, $P < 0.05$). Moderate levels of selfing (*B. heracleifolia* $s = 0.40$, *B. nelumbiifolia* $s = 0.55$) may further increase genetic differentiation between populations. Intraspecific genome size estimates for *B. heracleifolia* ($1C = 0.80$pg) and *B. nelumbiifolia* ($1C = 0.54$pg) were consistent across the species range, except one Oaxacan *B. heracleifolia* population that possessed a genome ~10% larger ($1C = 0.88$pg) than the species mean. Crosses with this divergent population had a 20% reduction in pollen viability compared to close outcrosses. The population genetic data suggest *Begonia* populations are long lived and stable, but only weakly connected by gene flow. This weak species cohesion has led to reproductive barriers accumulating between the most isolated

populations, supporting the hypothesis that allopatric divergence in situ is the precursor of speciation in *Begonia*.

4.2. Introduction

Species cohesion is maintained by gene flow (Slatkin, 1987; Morjan & Rieseberg, 2004). If gene flow between populations is disrupted, populations become genetically isolated from one another, and reproductive barriers subsequently evolve leading to speciation (Coyne & Orr, 2004; Martin & Willis, 2010). Studying this trajectory, from a panmictic population, to a genetically differentiated species with strong reproductive barriers, is one way to get a handle on the processes of speciation (Coyne & Orr, 1989; Hall *et al.*, 2006). Many factors will influence this trajectory, some promoting genetic differentiation between populations, others maintaining species cohesion in spite of reduced gene flow (Wright, 1943).

One major factor influencing cohesion and differentiation processes in plants is the rate of interpopulation gene flow (migration) mediated by pollen and seed dispersal (Ennos, 1994; Linhart & Grant, 1996). Pollen is the main source for gene exchange between populations, as it is normally dispersed over greater distances than seeds (Ennos, 1994; Heuertz *et al.*, 2003; Bacles *et al.*, 2006; Dick *et al.*, 2008, but exceptions include many orchid species and *Anthoxanthum odoratum* L., Freeland *et al.*, 2011). For this reason, pollen flow plays an important role in the cohesion of species (Heywood, 1991). Seed dispersal between populations has a smaller role in gene flow, but is crucial in colonization and subsequent expansion of a species range (Petit *et al.*, 2003). It is therefore important to estimate both pollen and seed flow to best understand population dynamics, and the way that species cohesion is maintained.

Mating systems affect patterns of gene exchange between populations, and therefore contribute to geographic structuring of genetic diversity that is the precursor of reproductive isolation and speciation (Hamrick & Godt, 1996; Lasso *et al.*, 2011). Obligate outbreeding species, such as dioecious or self-incompatible species, usually maintain high levels of genetic diversity in a given population (Charlesworth, 2003). In contrast, populations of species that self-fertilize or inbreed may have reduced allelic diversity (Hamrick & Godt, 1996), and populations with low genetic diversity are particularly prone to divergence through genetic drift (Vellend & Geber, 2005).

Dispersal limitation and the mating system will contribute to neutral genetic divergence between populations, in combination with other demographic factors such as the effective population size (Charlesworth, 2009). But speciation will only be an outcome if reproductive barriers accumulate between populations (Rieseberg & Blackman, 2010). These barriers may be caused by a small number of genes causing differential adaptation (‘speciation genes’ in the genic species concept, see Wu, 2001) or incompatibilities between many fixed alleles at neutral or adaptive loci between lineages [Dobzhansky-Muller (DM) incompatibilities, Dobzhansky, 1937; Muller, 1942]. Studies of species cohesion must therefore also estimate the strength of reproductive barriers between populations to test for incipient speciation.

High species richness in tropical plant genera can in part be explained by geographically structured populations where inbreeding in common, which promotes allopatric speciation (Baker-Federov hypothesis, Lasso *et al.*, 2011). The link between population patterns of genetic differentiation, and species-level patterns of lineage splitting and speciation, has been made for *Begonia* (Hughes & Hollingsworth, 2008). Population genetic analyses have been conducted for African *Begonia* species which are rare or restricted to particular habitats, and these studies have shown high levels of genetic differentiation at nuclear microsatellite (*B. sutherlandii*, F_{ST} = 0.485, F'_{ST} = 0.896; Hughes & Hollingsworth, 2008) and allozyme loci (*B. dregei*, F_{ST} = 0.882; *B. homonyma*, F_{ST} = 0.937; Matolweni *et al.*, 2000). At the species level, phylogenetic work shows geographically constrained monophyly of species radiations (Forrest & Hollingsworth, 2003; Thomas *et al.*, 2011a). Micro-evolutionary processes of high genetic differentiation, even at small spatial scales, seem to be correlated with macro-evolutionary patterns of rapid speciation (Hughes & Hollingsworth, 2008). This pattern may in part explain why *Begonia* is one of the largest angiosperm genera, with over 1500 species.

Here, we test whether there is a strong genetic signature of differentiation between populations of two widespread *Begonia* species that grow in continuous habitats in Central America. This would be consistent with weak mechanisms of species cohesion that would allow new *Begonia* species to form through divergence in allopatry. *Begonia heracleifolia* Cham. & Schltdl. and *B. nelumbiifolia* Cham. & Schltdl. are genotyped at nine nuclear microsatellite markers to estimate genetic differentiation. The data from nuclear markers are compared to plastid data (Chapter 2), to infer the ratio of interpopulation pollen and seed dispersal. The co-dominant data is also used to estimate the level of inbreeding, which may further contribute to genetic differentiation. We then test whether genetic incompatibilities have accumulated between differentiated populations, by looking at the fertility of crosses between populations, and relate the crossing data to an intraspecific survey of genome sizes. The joint genetic, crossing, and genome size data is used to test whether allopatric divergence in situ within *Begonia* species is the precursor of allopatric speciation in this large genus.

4.3. Material and methods

4.3.1. Study species

Begonia nelumbiifolia and *B. heracleifolia* were chosen as they are two of the most widespread Central American *Begonia* species, found throughout Mexico and into Central America (*B. heracleifolia* to Honduras; *B. nelumbiifolia* to Columbia, Burt-Utley, 1985), in a genus of mostly narrowly distributed endemics (Hughes & Hollingsworth, 2008). These species are easily distinguished from other related *Begonia* species by their leaf and flower morphology (Burt-Utley, 1985). The species pair are an ideal comparison for studying incipient speciation, as *B. heracleifolia* is highly variable in leaf shape and leaf colour throughout its range, whereas *B. nelumbiifolia* is more uniform. They also differ in their ecologies, with *B. nelumbiifolia* growing in moist shaded areas and *B. heracleifolia* in dry sun-exposed areas. These species typically occur in small isolated populations, although they can be locally abundant and form dense stands (Hoover, 1979). Both species can be propagated by splitting rhizomes, allowing them to be easily transported, and grown in cultivation to be used in experimental crosses.

4.3.2. Sampling and genotyping

To test patterns of genetic diversity and differentiation, an average of 25 individuals were genotyped from 13 populations of *B. heracleifolia* and 7 populations of *B. nelumbiifolia* (Table 4.1). Samples of *B. nelumbiifolia* were made in the Mexican Gulf region, while samples of *B. heracleifolia* were made over a broader sampling distance from South Mexico to Guatemala (Fig. 4.1). Population samples of less than 15 individuals were included if this was all the plants at a given sampling locality. A representative specimen from each population was placed in the herbarium in Edinburgh (E) except the Guatemalan population which is in the University of Guatemala herbarium (BIGU). DNA extraction from silica dried material was performed using a modified protocol for the DNeasy 96-sample kit (Qiagen, Germantown, MD, USA), described in Chapter 2. A preliminary test of nuclear microsatellite amplification was made with the 14 nuclear microsatellite loci listed in Chapter 3. The 9 loci which amplified uniformly across species and populations were then used for the full genotyping of the population samples, with the same PCR protocol and amplification program. Input files for genetic analysis were formatted with the Microsatellite Toolkit (Park, 2001) and the file conversion program Create (Coombs *et al.*, 2008). To test whether data from microsatellite loci are independent of each other, linkage disequilibrium between markers was tested in FSTAT 1.2 (Goudet, 1995).

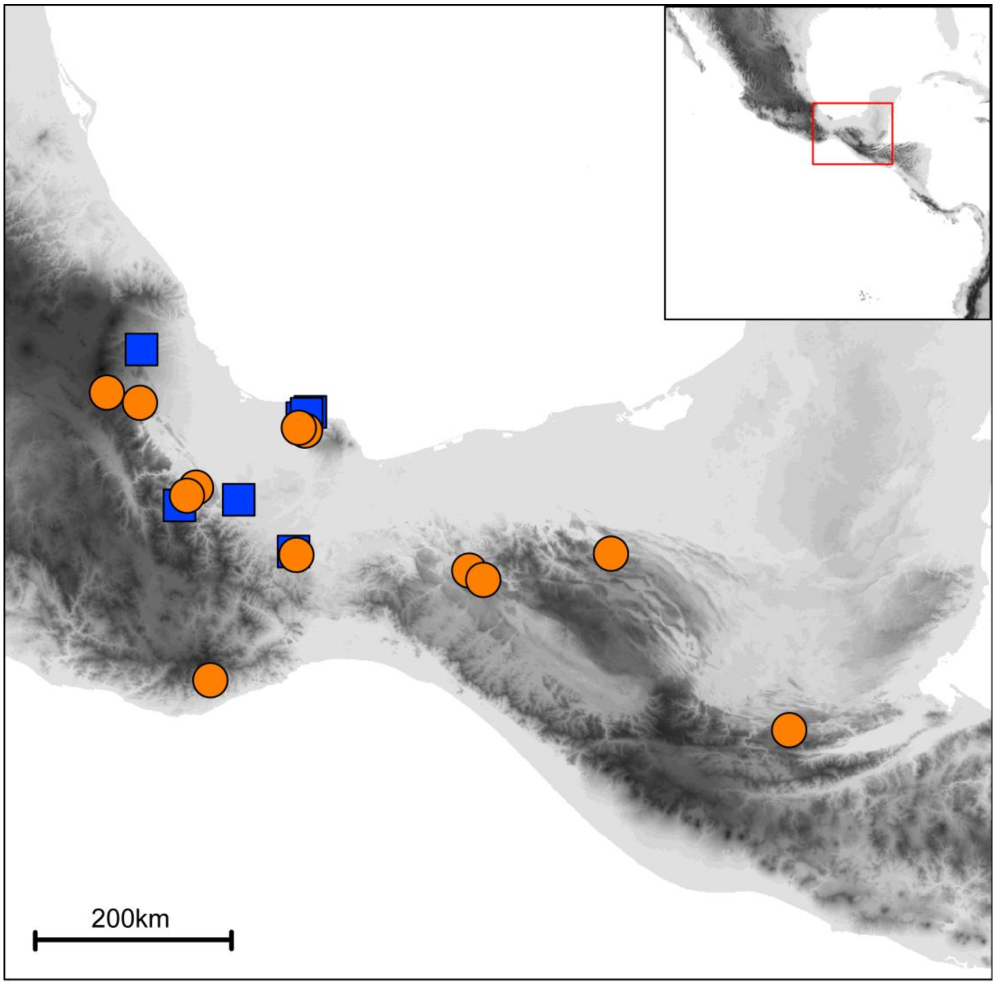


Figure 4.1. Collection sites of *B. heracleifolia* (orange circles) and *B. nelumbiifolia* (blue squares) from South Mexico and Guatemala.

Begonia species can reproduce asexually, and clumps of clonal individuals may arise by rhizomatous growth or from vegetative material being broken off and rooting (A.D. Twyford, pers. obs.). The probability that individuals in a population shared identical genotypes at all 9 loci through random mating was calculated using the approach of Parks & Werth (1993) implemented in GenClone 2.0 (Arnaud-Haond & Belkhir, 2007). Genotypes not considered a product of sexual mating (P_{sex}) at $P < 0.01$ when non-random mating was allowed (positive F_{IS} value), were removed from analyses. Individuals from populations where putative hybrids were found were only included if they were confidently considered pure species (i.e. assigned to a ‘pure’ parental class with at least 95% probability, discussed in Chapter 5). This approach was validated as removing populations where hybrids occurred did not affect values of F-statistics (results not shown).

4.3.3. Measures of genetic diversity

Diversity statistics were calculated per locus and per population in FSTAT v1.2 (Goudet, 1995). The statistics calculated were: number of alleles (A, calculated per locus only), allelic richness corrected for sample sizes (A_e, rarefaction method, Mousadik & Petit, 1996), and gene diversity (h_S, Nei, 1987). The number of private alleles per population were scored by eye.

4.3.4. Measures of inbreeding

Inferences of mating systems in natural populations, including self-fertilization and inbreeding, can be estimated from co-dominant genetic data. Weir and Cockerham’s (1984) F_{IS} is a single-locus estimator of heterozygosity deficiency, and the F_{IS} values were calculated and the average across-loci value bootstrapped in FSTAT v1.2. For each species the inferred selfing rate was calculated from the inbreeding coefficient, using the formula of Allard *et al.* (1969):

$$S = 2F_{IS}/(1+F_{IS})$$

Rates of self-fertilization were also estimated with a multi-locus inbreeding estimator in the program RMES, which considers heterozgote deficiency across all loci simultaneously, making it less sensitive to technical errors at a few loci (David *et al.*, 2007; Jarne & David, 2008).

Self-compatibility was confirmed using greenhouse-grown plants. One cultivated accession of *B. nelumbiifolia* and five accessions of *B. heracleifolia* from different populations were self fertilised, and the presence of fertilised seeds (which appear ‘filled’ rather than ‘shrivelled’) checked under a 10x dissecting microscope. Seeds were germinated in 9cm pots of finely sieved bark, which were kept in a propagator at 25°C, and germination was recorded after 6 weeks.

4.3.5. Measures of population structure and genetic differentiation

F-statistics were calculated between sampling sites for each species, as well as between the distinct genetic clusters identified in the BAPS analysis (see below). To allow like-for-like comparisons between the two species over a similar geographic area, F-statistics were also calculated for *B. heracleifolia* excluding populations from south Oaxaca (h28) and Guatemala (h-g1). Weir & Cockerham's (1984) estimator of F_{ST}, which is a measure of the genetic structure in the data, was calculated in FSTAT v1.2. A standardised measure of population structure that takes into account sample sizes and allelic diversity (F'_{ST}) was calculated by using RecodeData v0.1 (Meirmans, 2006) and FSTAT. Absolute differentiation was measured with D (Jost, 2008) using SMOGD (Crawford, 2010), and confidence intervals calculated by bootstrapping with 1 000 replicates. An F_{ST} analogue which incorporates allele size length (R_{ST}) was calculated with SPAGeDI v1.3 (Hardy & Vekemans, 2002), to test for phylogeographical structure in the data. The between species F_{ST} was also calculated, which shows the similarities in allele frequencies between the species.

The ratio of interpopulation pollen to seed dispersal can be estimated using formula 5a of Ennos (1994), which relates the F_{ST} for biparentally inherited nuclear markers ($F_{ST(b)}$) and maternally inherited plastid markers ($F_{ST(m)}$), as well as the level of inbreeding (F_{IS}). Maternal inheritance of plastids in *Begonia* have been confirmed by cytological observations (Corriveau & Coleman, 1988) and sequencing plastid DNA in experimental crosses (Peng & Chiang, 2000). No variation was found at 7 plastid microsatellite markers in *B. nelumbiifolia* (Chapter 2), so the pollen to seed ratio could not be calculated for this species. For *B. heracleifolia*, $F_{ST(m)}$ was calculated from the plastid microsatellite data in Chapter 2 using the same populations sampled for the nuclear microsatellites (this chapter), and compared to the $F_{ST(b)}$ and the F_{IS}.

To test whether dispersal between populations fits a simple model of dispersal limitation between more distant populations (the stepping stone model of dispersal, Kimura & Weiss, 1964), the relationship between genetic similarity and geographic distance was tested using isolation by distance analysis. Pairwise comparisons of F_{ST} /(1- F_{ST}) for each population were plotted against the natural logarithm of geographic distance as suggested by Rousset (1997), and implemented in the Isolation by Distance Web Service v3.21 (Jensen *et al.*, 2005).

To visualise the spatial structuring of populations, 3 complementary Bayesian clustering methods were used: STRUCTURE (Pritchard *et al.*, 2000), BAPS (Corander *et al.*, 2008) and InStruct (Gao *et al.*, 2007). A comparison was then made between the genetic clusters assigned in the three different programs, to evaluate whether the results are robust to the different assumptions made each of the programs (Kettle *et al.*, 2007). STRUCTURE assigns individuals to one (or more) of the user defined number of genetic clusters (K value), minimizing Hardy-Weinberg disequilibrium and linkage disequilibrium within each cluster (Pritchard *et al.*, 2000), and maximizing disequilibrium between clusters. Preliminary STRUCTURE results were consistent for short runs (100 00 burn-in iterations, run 100 000 iterations) and long runs (burn-in 300 000 iterations, run 1 000 000 iterations), and summary statistics were stationary for both run lengths suggesting the data had converged (Pritchard *et al.*, 2000), so the shorter run conditions were used. The admixture model was used, with sampling locality as a weak prior (Hubisz *et al.*, 2009). K values between 1 and 13 were evaluated for *B. heracleifolia*, and 1 and 7 for *B. nelumbiifolia*, with 10 independent replicates per K value. The *ad hoc* statistic ΔK was calculated across runs, and the greatest value inferred to be the optimal K value (Evanno *et al.*, 2005). For analysis of a given K value, a consensus file correcting for equally optimal solutions (multi-modality) and label switching was produced in CLUMPP (Jakobsson & Rosenberg, 2007), and the results displayed in DISTRUCT (Rosenberg, 2004).

The same data file was used as the input for BAPS 5.4 (Corander *et al.*, 2008), which uses an alternative assignment algorithm to STRUCTURE. The ‘clustering of groups of individuals’ setting was used, and the same number of genetic clusters (K) was evaluated as described above. Five replicates were made for each K value, and the results file was then used as the input for admixture analysis using the ‘mixture clustering option’. The minimum size of each population was set to 3 individuals, and runs were made of 10 000 iterations, and 5 000 reference individuals were used. The optimum value of K is automatically calculated in BAPS using a greedy stochastic optimization algorithm (Corander *et al.*, 2008). The BAPS admixture bar plots produced by the program were used to display the results, with only significant admixture shown ($P < 0.05$).

As a moderate departure from Hardy-Weinberg equilibrium was detected for both species (see results), the data was also analysed in InStruct (Gao *et al.*, 2007). This program is intended for analysing genetic structure from selfing or inbreeding species, where populations are not at Hardy-Weinberg equilibrium. The same run settings and K values were evaluated as for STRUCTURE, and the convergence of independent runs was confirmed by low Gelman-Rubin scores across runs (Gao, InStruct manual). The optimum number of K was assessed by the lowest value of the deviance information criterion (DIC). Runs were combined in CLUMPP and visualised in DISTRUCT (as above).

To visualise the relationship between populations, a neighbour-joining tree was constructed for each species. Allele frequencies from FSTAT were used as the input for POPTREE2 (Takezaki *et al.*, 2010), where a neighbour-joining tree based on Nei's (1983) measure of genetic distance was constructed. The tree was edited in FigTree v1.2.2 (available from http://tree.bio.ed.ac.uk/software/figtree/).

4.3.6. Measures of reproductive isolation

To test whether genetic incompatibilities have accumulated between differentiated populations of *B. heracleifolia*, pollen fertility was scored in artificial interpopulation crosses. It was not possible to grow crosses between every pair of populations due to the large number of populations and the limited greenhouse space, so instead three groups of crosses were made, and pollen sterility compared between the three groups. First, pollen sterility was recorded in selfed individuals, to establish a benchmark for pollen sterility. Second, pollen sterility was recorded in a small subset of crosses between populations across the Mexican gulf (the main area where population samples were collected), selecting the pollen donor at random from those in flower. Finally, pollen sterility was recorded in crosses between the population in Oaxaca (h28) and other populations, selecting the pollen donor at random. The Oaxacan population was chosen as it is the most divergent population with living material available. Seeds were germinated on finely sieved bark, and after 6 weeks seedlings were transferred to 9cm pots in sterilised potting mix (16 bark: 3 peat: 1 perlite plus finely sieved osmocote) and grown at 28°C to flowering. Pollen sterility was measured by acetocarmine staining, which is a reliable method for viability assessment in *Begonia* (Matthews, 2007; Dewitte *et al.*, 2011), and corresponds well to artificial pollen germination and fluorescent staining (Twyford and Kidner, unpubl. data). Dehiscing pollen from one flower per plant was stained with 1M acetocarmine, visualised under a Leica Microscope, and the proportion of well-stained pollen recorded out of 200 pollen grains.

4.3.7. Measures of genome size

Fully expanded leaf material from four accessions of *B. nelumbiifolia* and five of *B. heracleifolia* were selected for nuclear DNA content (C-value) estimates by flow cytometry. These accessions represent different populations from South Mexico (Table 4.3). The procedure followed Brennan *et al.* (submitted), summarised here briefly. Approximately 1cm^2 of material was chopped with an internal size standard (*Solanum lycopersicum* 'Stupiké polní rané' 2C = 1.96 pg, or *Petroselinum crispum* 'Champion Moss Curled' 2C = 4.45 pg) in 2 mL of General purpose isolation buffer (GPB; Loureiro *et al.*, 2007) with 3% PVP-40 following the procedure described by Doležel *et al.* (2007). The nuclear suspension was then filtered through a 30 µm nylon mesh to remove debris, stained with propidium iodide (Sigma-Aldrich) at a final concentration 60 µg·mL^{-1} and supplemented with 100 mg·mL^{-1} ribonuclease A (RNase A; Sigma-Aldrich). Samples were kept on ice for 15 min, and then 5 000 particles were recorded using a Partec Cyflow SL3 flow cytometer (Partec GmbH, Canterbury, UK) fitted with a 100-mW green solid state laser (Cobolt Samba, Solna, Sweden). Two to four replicates per accesion were processed and the resulting fluorescence histograms were analysed with FlowMax software (Partec GmbH). The mean and standard error for each individual, and per species, were calculated.

4.4. Results

4.4.1. Genetic diversity

A total of 306 individuals from *B. heracleifolia* and 177 from *B. nelumbiifolia* were genotyped (Apendix 2 & 3, respectively). Five individuals of *B. heracleifolia* were identical at 9 polymorphic loci, and are likely to be clones (P < 0.01), so were removed from the data set.

Table 4.1. Collection sites and estimates of genetic diversity per population. N_g, number of individual genotyped; N_{adj}, adjusted number of individuals after suspected clones removed, A_e, allelic richness rarefied to 5 individuals; h_S, gene diversity; F_{IS}, inbreeding coefficient.

Pop.	Locality	State	Latitude	Longitude	N_g	N_{adj}	A_e	h_S	F_{IS}
B. heracleifolia									
h2	4km NE San Andrés Tuxtla	Veracruz	18.4785	-95.178	19	19	1.870	0.257	0.01
h3	7km NE San Andrés Tuxtla	Veracruz	18.50341	-95.1682	34	33	1.970	0.263	0.224
h5	9km NE San Andrés Tuxtla	Veracruz	18.52009	-95.1618	26	26	1.685	0.209	0.111
h8	Agua Azul	Chiapas	17.22117	-92.1107	14	14	1.642	0.222	0.514
h12	Ocozocuatla biosphere reserve	Chiapas	16.94645	-93.4565	28	27	1.860	0.286	0.294
h13	Near Berriozábal	Chiapas	16.86693	-93.3278	6	6	1.941	0.292	0.684
h15	5km south of Orizaba	Veracruz	18.78253	-97.0851	27	27	2.004	0.300	0.083
h21	Highway 175 between Jasaa and San Juan Bautista	Oaxaca	17.74356	-96.328	31	31	2.250	0.390	0.338
h23	Near Santa Maria Jacatapec	Oaxaca	17.86422	-96.2138	25	25	2.686	0.491	0.316
h24	Motzorongo	Oaxaca	18.66953	-96.7871	37	37	1.939	0.280	0.274
h26	San Jeronimo Zoochina	Oaxaca	17.22117	-95.2355	28	28	1.927	0.269	0.171
h28	Santa Maria Xanabi	Oaxaca	15.98808	-96.1106	12	12	1.436	0.154	0.491
h-g1	Cobán	Alta Verapaz (Guatemala)	15.47552	-90.3787	26	23	1.709	0.169	0.22
Average across populations					24.1	23.7	1.918	0.276	0.287
B. nelumbiifolia									
n1	Near Los Tuxtlas biological station	Veracruz	18.59026	-95.0788	20		2.221	0.450	0.327
n3	7km NE San Andrés Tuxtla	Veracruz	18.50341	-95.1682	12		2.257	0.473	0.444
n5	9km NE San Andrés s Tuxtla	Veracruz	18.52009	-95.1618	30		2.397	0.492	0.328
n18	valley 25km East of Huastusco	Veracruz	19.20367	-96.7426	16		2.025	0.368	0.535
n21	Highway 175 between Jasaa and San Juan Bautista	Oaxaca	17.74356	-96.328	41		2.506	0.497	0.407
n25	Arroya Zacata	Oaxaca	17.73981	-95.7913	27		1.766	0.276	0.471
n26	San Jerónimo Zoochina	Oaxaca	17.22117	-95.2355	31		2.198	0.367	0.306
Average across populations					25.3		2.196	0.417	0.403

Descriptive statistics are summarised per population in Table 4.1 and per locus in Table 4.2. All loci were polymorphic, except *B5347* which was monomorphic in *B. nelumbiifolia*. Overall, the levels of genetic diversity were low for both *B. heracleifolia* (mean values across populations: A = 6.6; A_e 1.972, $h_S = 0.276$) and *B. nelumbiifolia* (A = 4.7; $A_e = 2.197$, $h_S = 0.417$). The most genetically diverse population of *B. heracleifolia* was h23 ($A_e = 2.686$; $h_S = 0.491$), and the least was h28 (A_e=1.436; $h_S = 0.154$). The most diverse population of *B. nelumbiifolia* was population n21 ($A_e = 2.51$; $h_S = 0.497$), and the least was n25 (A_e=1.766; $h_S = 0.276$). Seventeen of the 54 alleles (29.8%) detected in *B. heracleifolia* were private alleles. The most private alleles were found in populations h15 and h-g1, with 3 each, and the least were in populations h8, h23 and h28, which had no private alleles. Nine of the 44 alleles (20.4%) found in *B. nelumbiifolia* were private alleles. The most private alleles (4) were found in populations n18 and n21, and one in n26. No private alleles were found in the other populations of *B. nelumbiifolia*.

Table 4.2. Species-level estimates of genetic diversity and genetic differentiation per locus. A, total number of alleles per locus; A_e, allelic richness rarefied to 5 individuals; h_T gene diversity. *, $P < 0.05$; **$P < 0.01$.

Species	**Locus**	**A**	**A_e**	**h_T**	**F_{ST}**	**F_{IS}**
B. heracleifolia	B4329	4	2.745	0.302769	0.460**	0.118
	B3043	6	2.422	0.181231	0.462*	0.689**
	B6278	2	1.285	0.036385	0.676	0.700
	B5347	7	2.922	0.316231	0.342**	0.324**
	B3348	9	3.895	0.497615	0.312**	0.223**
	B7112	6	1.656	0.116308	0.140	0.320**
	B3820	10	4.115	0.484462	0.350**	0.046
	B134	8	3.045	0.395923	0.269*	0.244*
	B332	5	2.179	0.150308	0.492**	0.340*
	Across loci	6.3	2.696	0.275692	0.364**	0.249**
B.nelumbiifolia	B4329	5	3.838	0.535714	0.266*	0.060
	B3043	8	3.553	0.456857	0.188**	0.373*
	B6278	5	2.863	0.471857	0.144*	0.421**
	B5347	1	1	0	n/a	n/a
	B3348	4	2.747	0.487571	0.125**	0.316**
	B7112	4	2.128	0.335429	0.383*	0.372**
	B3820	4	2.929	0.301571	0.554**	0.512*
	B134	6	2.594	0.322714	0.142*	0 .234**
	B332	5	3.012	0.427714	0.315*	0.868**
	Across loci	4.7	2.7065	0.371048	0.277**	0.454**

4.4.2. Inbreeding

The average F_{IS} value across loci was 0.249 (SE 0.062) and 0.454 (SE 0.095) for *B. heracleifolia* and *B. nelumbiifolia*, respectively. F_{IS} values varied from 0.01 in population h2 to 0.684 in population h13 (*B. heracleifolia*), and from 0.306 in population n26 to 0.535 in population n18 (*B. nelumbiifolia*), see Table 4.2. The values for the inferred selfing rate (s) calculated from the inbreeding coefficient F_{IS}, were 0.399 for *B. heracleifolia*, and 0.624 for *B. nelumbiifolia*.

The multiple-locus maximum likelihood (ML) estimate of selfing (s) averaged across loci for *B. heracleifolia* was 0.154 (non-significant), and only population h23 had a selfing rate significantly different from 0 [ML s = 0.302, 95% confidence interval (CI) 0.039 - 0.474]. For *B. nelumbiifolia*, the ML estimate of s averaged across loci was 0.097 (non-significant). Only one population had a selfing rate significantly different from 0 (population n18; ML s = 0.554; 95% CI: 0.057 - 0.759).

Seed set was high (> 90%) in the self-pollination experiment with *B. heracleifolia* (n = 5) and *B. nelumbiifolia* (n = 1).

4.4.3. Population structure and genetic differentiation

Significant population substructure was found by F_{ST} analysis (average across loci, *B. heracleifolia*, $F_{ST} = 0.364$; SE 0.028, $P < 0.05$; *B. nelumbiifolia*, $F_{ST} = 0.277$; SE 0.055, $P < 0.05$), as well as when the F_{ST} was standardised for the maximum possible value for the loci sampled (*B. heracleifolia* $F'_{ST} = 0.506$, *B. nelumbiifolia* $F'_{ST} = 0.439$). Moderate levels of differentiation were found with Jost's estimator of absolute differentiation D, with average values per locus for *B. heracleifolia* (D = 0.274, $P < 0.05$), and for *B. nelumbiifolia* (D = 0.294, $P < 0.05$). Recalculating measures of population substructure and genetic differentiation for the genetic clusters identified in the BAPS analysis for *B. heracleifolia* had little effect (results not shown). R_{ST} values, which use allele size lengths and a stepwise mutation model, were significantly different from 0 (*B. heracleifolia* $R_{ST} = 0.212$; *B. heracleifolia* $R_{ST} = 0.257$), but were not significantly larger than the G_{ST} values (not shown), suggesting the absence of phylogeographical structure. Isolation-by-distance accounted for a modest amount of the genetic variance (*B. heracleifolia*, $r^2 = 0.250$, $P = 0.001$; *B. nelumbiifolia*, $r^2 = 0.289$, $P = 0.016$). The F_{ST} value between species, which is a measure of genetic similarity between them, was 0.466 ($P < 0.01$). When the Oaxacan and Guatemalan populations of *B. heracleifolia* were removed from the analyses, leaving the 11 populations from the Mexican Gulf across a similar range as *B. nelumbiifolia*, values of genetic differentiation and substructure were reduced ($F_{ST} = 0.306$, SE 0.04; $F'_{ST} = 0.437$; $D = 0.219$, $P < 0.05$), and more of the variation could be explained by isolation-by-distance ($r^2 = 0.383$, $P = 0.001$ Mantel Test $r = 0.619$).

Using the F_{ST} values from the plastid microsatellites ($F_{ST(m)} = 0.728$) and the nuclear microsatellites ($F_{ST(b)}$ 0.364), and also considering inbreeding ($F_{IS}= 0.249$), the ratio of pollen to seed dispersal for *B. heracleifolia* was 3.8. Transmission of genes through pollen is therefore c.4 times more effective than through the seed.

The optimal number of genetic clusters for *B. heracleifolia*, assessed by comparing the mean DIC value across runs for InStruct, and the maximum ΔK value for STRUCTURE, was 10. The most likely number of genetic clusters in the BAPS analysis was K=11 [log likelihood (LL) of K = -3428.614], closely followed by K = 10 (LL = -3429.484). For *B. nelumbiifolia* the optimal value for K was 7 for all three approaches.

The genetic clustering results for the three Bayesian methods gave similar major groups, but differed in the amount of admixture between them. For *B. heracleifolia*, the genetic clusters identified in BAPS were the same as the sampled populations, with two exceptions (Fig. 4.2a). Populations h3 and h5, which are separated by less than 2km, shared a common gene pool. BAPS assigned population h13, which contained only 6 individuals, as admixed between populations h3, h5, and h8 (270km away). The STRUCTURE analyses (Appendix Figure 4) differed from the BAPS analysis by having greater admixture between less clearly defined genetic clusters, and placing the closely grouped (< 5km apart) populations h2, h3 and h5 together. Population h8 was poorly defined, with a complex pattern of admixture. The InStruct analysis showed even fewer distinct genetic clusters (Appendix Figure 4), with 6 of the 13 populations being recognised as distinct clusters (h12, h21, h23, h24, h28, h-g1).

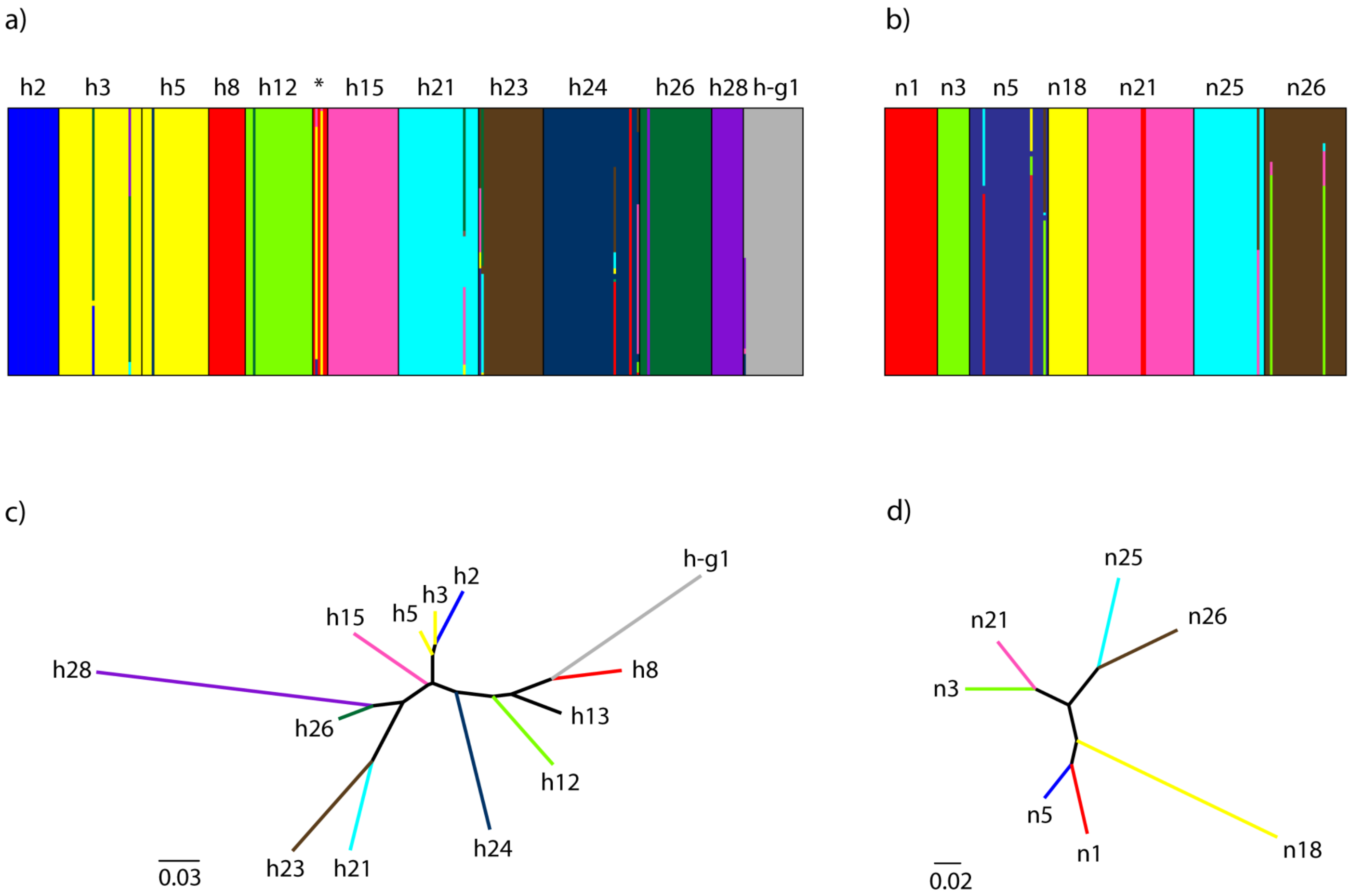


Figure 4.2. Bayesian assignment to genetic clusters and the relationships between populations. Bayesian admixture results in BAPS for *B. heracleifolia* (a) and *B. nelumbiifolia* (b). Each individual is represented by a vertical bar, and different colours represent the different genetic cluster. Asterisk indicates population h13. Neighbour-joining trees of Nei's (1983) measure of population divergence for *B. heracleifolia* (c) and *B. nelumbiifolia* (d). Branches are coloured to correspond with the genetic clusters from the BAPS analyses.

Bayesian clustering in BAPS revealed 7 main clusters corresponding to the 7 populations genotyped for *B. nelumbiifolia* (Fig. 4.2b). Levels of admixture were low, with 9 individuals (5%) having admixed ancestry. As with *B. heracleifolia*, the STRUCTURE results showed less resolution (Appendix Figure 4) than BAPS, with only 3 populations being assigned to distinct genetic cluster (populations h18, h25, h26). The InStruct analysis (Appendix Figure 4) detected even fewer distinct genetic clusters than STRUCTURE.

The average value for Nei’s 1983 pair-wise population distance measures, which were used to build the Neighbour-joining trees, were 0.231 (0.011SE) for *B. heracleifolia*, and 0.201 (0.013SE) for *B. nelumbiifolia*. The tree for *B. heracleifolia* included two long branches, connecting populations h28 (mean pair-wise D_{Nei} = 0.360) and h-g1 (mean pair-wise D_{Nei} = 0.313; Fig. 4.2c). The tree for *B. nelumbiifolia* was roughly star-shaped (Fig. 4.2d).

4.4.4. Reproductive isolation

The mean pollen stainability of selfed plants was 97.5% (SE 0.0126, n = 18), and only one plant had a value below 85%. Outcrossed plants were similarly pollen-fertile, with a mean pollen stainability of 98.7% (SE = 0.0197, n = 10). Outcrosses involving individuals from Oaxaca had a mean viability of 78.3% (SE = 0.0170, n = 3).

4.4.5. Genome size

The average 1C genome size estimated for *B. heracleifolia* and *B. nelumbiifolia* were 0.80pg and 0.54pg, respectively (Table 4.3). Genome size etimates were consistent across individuals within species, except a *B. heracleifolia* individual from population h28 that had an estimated genome size of 0.88pg, which is 10% higher than the parental mean.

Table 4.3. Collection sites and estimates of genome sizes. Standard errors of genome sizes given in brackets.

Pop. code	Locality	State	Latitude	Longitude	1C genome size in pg
B. heracleifolia					
h2	4km NE San Andrés Tuxtla	Veracruz	18.47850	-95.17800	0.79 (0.00)
h13	Near Berriozábal	Chiapas	16.86693	-93.32780	0.78 (0.00)
h16	30km east of Huatusco	Veracruz	19.2011	-96.67139	0.78 (0.00)
h21	Highway 175 between Jasaa and San Juan Bautista	Oaxaca	17.74356	-96.32800	0.78 (0.00)
h28	Santa Maria Xanabi	Oaxaca	15.98808	-96.11060	0.88 (0.00)
B. nelumbiifolia					
n7	Road to Ocosingo from Palenque	Chiapas	17.42477	-91.99712	0.55 (0.00)
n11	Ocozocuatla biosphere reserve	Chiapas	16.92489	-93.45090	0.54 (0.00)
n19	3km north of Josaa	Oaxaca	17.64247	-96.337472	0.53 (0.00)
n21	Motzorongo	Oaxaca	18.66953	-96.787139	0.54 (0.00)

4.5. Discussion

4.5.1. Strong population substructure and genetic differentiation in widespread *Begonia* species

The two widespread *Begonia* species showed strong population substructure (*B. heracleifolia* $F_{ST} = 0.364$, $P < 0.05$; *B. nelumbiifolia*, $F_{ST} = 0.277$, $P < 0.05$), and Bayesian structure analysis supports the division of most populations into discrete genetic clusters (Fig. 4.2). Moreover, levels of genetic diversity in each population were low (*B. heracleifolia* mean $h_S = 0.276$; *B. nelumbiifolia* mean $h_S = 0.417$), and populations showed significant genetic differentiation between them (*B. heracleifolia* Jost's $D = 0.274$, $P < 0.05$; *B. nelumbiifolia* $D = 0.294$, $P < 0.05$). These data suggest *Begonia* populations are isolated, with little homogenizing gene flow between them. Despite the small population sizes of the two species (c.10-300 individuals, A.D. Twyford pers obs.; Hoover, 1979), populations may be long-lived and stable allowing them to become differentiated by drift or local adaptation. These results are consistent with the plastid analysis presented in Chapter 2, where a pattern of strong genetic differentiation was found. In a broader context, long lived and isolated *Begonia* populations have been inferred from population genetic analysis of three other *Begonia* species (*B. sutherlandii*, Hughes & Hollingsworth, 2008; *B. dregei*, *B. homonyma*, Matolweni *et al.*, 2000), suggesting that a common set of population-level mechanisms (strong genetic drift in isolated populations) play a role in the evolution of intra-specific genetic diversity in *Begonia*.

Pollen-mediated gene flow was found to be low between populations of *B. heracleifolia*. A pollen to seed ratio (r) of 3.8 is at the lower end of the range seen in plants (e.g. r = 1 - 196, Ennos, 1994; Squirrell *et al.*, 2001). Similarly low pollen to seed ratios (r = 4.0) have been found for other herbaceous plants such as *Dysosma versipellis* (Hance) M.Cheng ex T.S. Ying (Berberidaceae) (Guan *et al.*, 2010), or other plant species that grow below the forest canopy, such as the epiphytic bromeliad *Vriesea gigantea* Lem. (Bromeliaceae) (r = 3.3, Palma-Silva *et al.*, 2009). This low level of pollen dispersal is likely related to the pollination biology of *Begonia*. Female *Begonia* flowers do not produce any reward for pollinating insects; instead they are intersexual mimics of the male flowers, which reward pollinators with pollen but not nectar (Ågren & Schemske, 1991; Schemske *et al.*, 1996). This low-reward strategy may only be effective for attracting naive insects, as pollinators may develop an unfavourable search image of flowers with little reward and avoid them in the future (Goulson, 2000 and references therein). By delivering little reward for pollinators, there is little incentive for pollinators to travel far-a-field to another *Begonia* plant, making the chances of interpopulation pollen dispersal low. It is however an effective strategy to reduce the high costs of producing floral rewards, and by attracting generalist pollinators seed set may be relatively high (Wyatt & Sazima, 2011) when the isolated nature of most *Begonia* populations is considered.

4.5.2. Inbreeding promotes genetic differentiation

Both *Begonia* species were found to be fully self-compatible, and a moderate amount of self-fertilization occurs in natural populations (40% *B. heracleifolia*, 62% *B. nelumbiifolia*). The occurrence of self-fertilization was supported by the consistent F_{IS} values across loci, although the average across-loci result for the multi-allelic estimator was non-significant. This is likely to be due to the limited diversity at each of the EST-derived microsatellite loci, and more loci, or loci with higher levels of polymorphism, would be required to obtain a significant result. This will also be influenced by the interpopulation variation in selfing rates (Table 4.1). A positive F_{IS} value could also be due to technical artefacts, such as null alleles (Pemberton *et al.*, 1995). The presence of null alleles seems unlikely because there were few individuals that could not be amplified for a given locus (null homozygotes), and where these did occur they did not fit any consistent pattern (e.g. being common in a particular population). Moreover, the primers were designed from transcriptome sequence data and have been shown to be conserved over a broad phylogenetic scope (Chapter 3), suggesting interpopulation polymorphisms in the primer regions are unlikely.

Most *Begonia* species are self compatible (Ågren & Schemske, 1993; Dewitte *et al.*, 2011; Wyatt & Sazima, 2011; Twyford and Kidner, unpubl. data), with only two species studied to date not setting seed in a small number of experimental crosses (Brazilian *B. integerrima* Spreng. and *B. itatinensis* Irmsch. Ex Brade; Wyatt & Sazima, 2011). Levels of inbreeding in *Begonia* species varies, from close to panmictic (*B. sutherlandii*, mean F_{IS} = 0.158, 7 microsatellies) to fully selfing (*B. hirsuta* single locus outcrossing rate 0.03 ± 0.01, 1 isozyme locus, Ågren & Schemske, 1993). The mechanisms underlying inbreeding in *Begonia* are not currently clear; it may either be autopollination or self-fertilization mediated by insect pollinators (geitonogamy). Most *Begonia* species are monoecious and functionally protandrous, producing male flowers on an inflorescence before females (Forrest & Hollingsworth, 2003). However, as many inflorescences are borne over a flowering season, there are plenty of opportunities for self-pollination. This would particularly be the case for species such as *B. nelumbiifolia*, which produces many densely-packed inflorescences that can easily become intertwined. Self-pollination in such a way would assure mating success if pollinators are rare, which may be expected in the isolated populations *Begonia* species typically grow.

4.5.3. Seed dispersal limitation and self-fertilization promote allopatric divergence

The F_{ST} values between populations for *B. heracleifolia* and *B. nelumbiifolia*, as well as those for other *Begonia* species (Matolweni *et al.*, 2000; Hughes & Hollingsworth, 2008), indicate a higher level of population structure than most other plant species (Gitzendanner & Soltis, 2000; Petit *et al.*, 2005; Hey & Pinho, 2012). Plant species with higher F_{ST} values are typically distributed across a much more heterogenous environment with large geographic barriers impeding gene flow, or are highly selfing. One example of this is the bromeliad *Pitcairnia geyskesii* L.B.Sm., where there is strong population structure (F_{ST} = 0.533) between the large emergent rocky outcrops (inselbergs) where the species grows in French Guiana (Boisselier-Dubayle *et al.*, 2010). Selfing species with high F_{ST} values include *Arabidopsis thaliana* L. (F_{IS} = 0.969, F_{ST} = 0.61, Bomblies *et al.*, 2010); *Bromus tectorum* L. (F_{IS} = 1, F_{ST} = 0.53; Ramakrishnan *et al.*, 2006) and *Medicago truncatula* Gaertn. (F_{IS} = 0.978, F_{ST} = 0.3 - 0.75, Siol *et al.*, 2008). While the highest levels of genetic divergence were found between populations separated by a geographic barrier, such as the Southern Oaxacan population h28 (mean pair-wise F_{ST} = 0.463, range 0.342 - 0.638) which is isolated from other populations by the Sierra Madre Occidental, relatively high pairwise F_{ST} values were still found between populations in close proximity across semi-continuous habitats. The absence of mechanisms that promote dispersal of pollen and seed in *Begonia* therefore appear to prevent gene flow even over short distances.

To link divergence in allopatry with the potential for speciation, we estimated pollen fertility between crosses from the isolated Oaxacan population and other populations from the Mexican Gulf. We then used flow cytometry to shed light on whether genome size changes have also occurred. We showed a ~20% reduction in pollen stainability in wide outcrosses, and a ~10% increase in C-value of the Oaxacan population relative to the species mean (Table 4.3). This marked intraspecific C-value variation, and reduced fertility of intraspecific crosses, support populations being isolated from homogenizing gene flow allowing them to diverge on a potential route towards speciation.

4.6. Conclusion

The mean F_{ST} values for these species, and the other *Begonia* species studied to date, approach or exceed the threshold value of $F_{ST} = 0.35$ suggested by Hey & Pinho (2012) for delimiting species. In addition to this strong geographic structure caused by dispersal limitation, inbreeding may further reduce the level of gene flow between populations and promote divergence. The joint role that genetic drift and inbreeding play on levels of genetic differentiation in speciose tropical plants (the Baker-Fedorov hypothesis, Lasso *et al.*, 2011) is now beginning to be appreciated. Moreover, we have shown reduced fertility in crosses between divergent populations within a species, supporting the hypothesis that differentiation leads to the accumulation of genetic incompatibilities that may be involved in reproductive isolation, in a trajectory towards allopatric speciation.

CHAPTER 5: Reproductive isolating barriers revealed by a comparative hybrid swarm analysis in a recent *Begonia* species radiation

Data contributions: Alexander Twyford collected all the genotypic and phenotypic measures and performed the analyses.

5.1. Chapter summary

Begonia is one of the most species-rich angiosperm genera (1500 species), with related species showing striking variation in their morphology and ecology. One hypothesis for this species richness is that reproductive barriers evolve rapidly between incipient species because homogenising gene flow between them is low. Introgression may be reduced further by selection acting on hybrids between ecologically divergent species. Here, we test the strength of reproductive isolation between ecologically similar *Begonia heracleifolia* and *B. sericoneura*, and between ecologically divergent *B. heracleifolia* and *B. nelumbiifolia*. Reproductive isolating barriers (hybrid vigour, sterility, phenological differences) were first tested under experimental conditions and the outcomes of natural hybridization then observed by genotyping replicate hybrid swarms with twelve nuclear and seven plastid microsatellite markers. Overall, reproductive barriers were relatively strong, with hybrid sterility, frequent self-fertilization, and differences in flowering time contributing to isolation. Most barriers were more pronounced between ecologically divergent *B. heracleifolia* and *B. nelumbiifolia*. In contact zones between *B. heracleifolia* and *B. sericoneura* F1s were common but introgression limited; in the *B. heracleifolia* x *B. nelumbiifolia* contact zones F1s were less common and introgression absent. Reproductive barriers appear to evolve rapidly between *Begonia* species, which may be further enhanced by ecological selection against hybrids. These factors, in conjunction with the population biology of *Begonia*, may have promoted speciation in this species-rich genus.

5.2 Introduction

Tropical forests harbour half the world's plant species (Corlett & Primack, 2010), with much of this species richness contributed by a few species-rich genera (or families) (Gentry, 1992;

Frodin, 2004). The cradle model of tropical evolution proposes a recent origin for this species richness (Stebbins, 1974), and this model has gained support from dated phylogenetic analyses of some large plant genera (e.g *Inga*, Richardson *et al.*, 2001; *Guatteria*, Erkens *et al.*, 2007). While much emphasis has been placed on the timing and rates of speciation in the tropics, it is currently unclear how so many new species can arise from a common ancestor over such a short period of time, when reproductive barriers are weak and homogenising gene flow may be strong (Chan & Levin, 2005). A greater understanding of the evolution of reproductive isolation in large genera may shed light on why species diversity has accumulated in biodiversity hotspots such as the tropics.

Ecological selection may play a prominent role in large genera where species richness is recent in origin, because ecological divergence evolves early during speciation and often promotes it (Butlin, 2010). The role ecological selection plays in maintaining species barriers can be tested by comparing reproductive isolation between species that share similar ecologies, and those that differ. One may predict that ecological divergence between hybridizing species will affect pre-pollination barriers more than post-pollination barriers, because ecologically divergent species are less likely to be found in sympatry, but post-pollination barriers will be influenced more by differences in genome structure than by different ecologies (endogenous selection, Jiggins & Mallet, 2000). Alternatively, post-pollination barriers may also be affected by ecological divergence, as hybrids will combine divergent alleles at adaptive loci that may be incompatible (exogenous selection, Jiggins & Mallet, 2000). To compare intrinsic barriers and the role of selection, the strength of each reproductive barrier can be tested under experimental conditions, and the expected frequency of hybrids compared with the number of hybrids in sympatric populations where all reproductive barriers interact.

A particularly suitable system for this combined experimental and field based approach is *Begonia*, one of the most species-rich plant genera (>1500 species), where species richness has accumulated during recent radiations both in the Americas and in Asia (Miocene, Goodall-Copestake *et al.*, 2009; Dewitte *et al.*, 2011). Analyses of widespread Central American *B. heracleifolia* Cham. & Schltdl. and *B. nelumbiifolia* Cham. & Schltdl., have shown gene flow between populations is low (Chapters 2 & 4), and this may have led to a break down in species cohesion with reproductive barriers evolving between divergent populations of *B. heracleifolia*

(F1 partial hybrid sterility, Chapter 4). This raises the hypothesis that allopatric speciation occurs *in situ* (autochthonously) in *Begonia*, with reproductive barriers evolving quickly due to the fixation of alleles in small founding populations, and such rapidly evolving barriers may promote speciation. However few studies have assessed the reproductive barriers that promote divergence or maintain species barriers during secondary contact between *Begonia* species.

This study uses estimates of reproductive barriers under experimental conditions, and the frequency of hybrids in nature, to shed light on the evolution of reproductive isolation between two pairs of Central American *Begonia* species. We predict that reproductive isolation between species will be relatively strong, and in conjunction with the low gene flow typical between populations (Chapter 2; Chapter 4; Matolweni *et al.*, 2000; Hughes & Hollingsworth, 2008), this will facilitate rapid speciation. The second aim is to assess the contribution ecological selection plays in maintaining species barriers, through a comparison of species pairs that differ in their ecologies. We predict a prominent role for selection in a contact zone between species that differ markedly in their ecologies, i.e. that it will contain fewer hybrids and that these will be limited to early generation hybrids. *Begonia heracleifolia* and *B. sericoneura* Liebm. represent a pair of species that grow in similarly dry or seasonally dry habitats, while *B. heracleifolia* and *B. nelumbiifolia* are two species that grow in very different habitats (dry and permanently moist, respectively). For these two pairs of species we assess the possible reproductive barriers that act in sympatry (flowering phenology, chromosomal number differences, hybrid vigour, hybrid male and female fertility) to determine whether these are greater in the more ecologically divergent species pair. We then analyse the structure of hybrid zones between these pairs of species using nuclear and plastid microsatellites to determine whether differences in experimentally assessed reproductive barriers translate into differences in interspecific gene exchange in the wild.

5.3. Material and method

5.3.1. Study species

Two species pairs that frequently hybridize in the wild were chosen for this study: *B. heracleifolia* x *B. sericoneura*, and *B. heracleifolia* x *B. nelumbiifolia* (Burt-Utley, 1985; Morris, 2008). *Begonia sericoneura* has morphological similarities to *B. heracleifolia*, but can be identified by its persistent stipules and lack of coloured leaf markings (Fig. 5.1). Both species are similar in their ecologies, most often found in dry or seasonally dry habitats near roadsides, as well as in open areas in tropical forests (Burt-Utley, 1985). One hybrid swarm between the two species was analysed (HS4, Table 5.1).

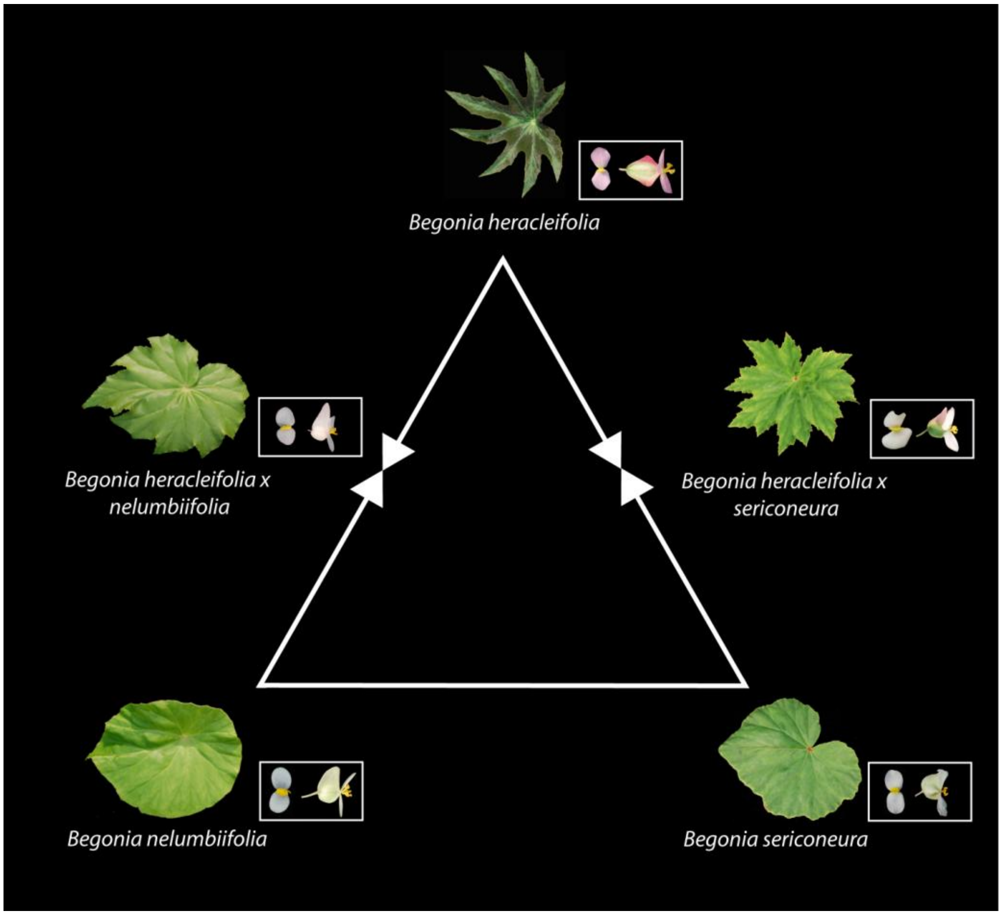


Figure 5.1. *Begonia* hybrid triangle. Parental leaf shape and staminate and pistillate flowers are shown in each corner, with F1 hybrids between them. Natural hybrids between *B. nelumbiifolia* and *B. sericoneura* have not been recorded.

Begonia nelumbiifolia is very distinct from *B. heracleifolia*, as it has large unmarked peltate leaves and inflorescences with densely packed small flowers (Burt-Utley, 1985). *Begonia nelumbiifolia* grows in more moist shaded areas (Hoover, 1979; Burt-Utley, 1985). Both species are ruderals and co-occur in sympatry at disturbed roadsides, but within sites they are usually separated into different microhabitats (Hoover, 1979). At least 4 hybrid swarms have been recorded and here we analyse three of them (Table 5.1).

Table 5.1 Collection sites of *Begonia* hybrid swarms used for this study.

Hybrid swarm number	**Species**	**Locality details**	**Latitude**	**Longitude**	**Habitat description**
HS1	*B. heracleifolia* x *B. nelumbiifolia*	9km SE of San Andres Tuxtlas	18.520090	-95.161760	Forest clearing 20m from roadside. Overgrown grasses and some small shrubs.
HS2	*B. heracleifolia* x *B. nelumbiifolia*	Highway 175 between Jasaa and San Juan Bautista	17.743560	-96.328028	Roadside cliff. Plants growing at base of cliff, little other vegetation.
HS3	*B. heracleifolia* x *B. nelumbiifolia*	San Jeronimo Zoochina	17.221170	-95.235472	Roadside bank in woodland, covered in secondary vegetation.
HS4	*B. heracleifolia* x *B. sericoneura*	Motzorongo	18.669530	-96.787139	Roadside cliff with mixed secondary vegetation of herbs and shrubs

Silica-dried material was collected for genetic analysis from South Mexico (see below), and 1 - 3 rhizomes per species were collected from each hybrid swarm and from other populations (Chapter 4) for use in experimental crosses (Table 5.2). Rhizomes were potted in compost mix (Chapter 4), and grown in a common greenhouse environment (28°C) at the RBGE until flowering size.

5.3.2. Experimental estimates of reproductive barriers

5.3.2.1. Phenology

The flowering times of species were compared, to see if a limited overlap in flowering will prevent cross-pollination. Most *Begonia* species are monoecious (Tebbit, 2005), and female flowers can be identified from males by the presence of an ovary. Nine accessions of *B. heracleifolia*, 6 *B. sericoneura* and 4 *B. nelumbiifolia* were used, collected from populations across South Mexico (Table 5.2). The numbers of open male and female flowers were recorded at weekly intervals from the start of February to the end of April 2012; flowers were counted only if the tepals were still attached to the flower. The number of male and female flowers were averaged across individuals for each species, and plotted against the time interval.

5.3.2.2. Chromosome numbers

Differences in chromosome numbers can render hybrids sterile, which will largely prevent backcrossing and introgression. Previous chromosome counts from multiple accessions of *B. sericoneura* are 2n = 28 (Matthews, 2007). Chromosome counts for *B. heracleifolia* and *B. nelumbiifolia* have only been made from a single cultivated accession, where the count is also 2n = 28 (Legro & Doorenbos, 1969). As these species are widespread and genetically differentiated (Chapters 2 and 4), and because polyploidy is common in the genus (Dewitte *et al.*, 2009; Dewitte *et al.*, 2011), chromosome counts for 3 accession of *B. heracleifolia* and *B. nelumbiifolia* (Table 5.2) were made to determine whether differences in chromosome numbers could contribute to reproductive isolation between them. Roots from 6 week old cuttings were pre-treated in Gammexane (Sigma-Aldrich, Croatia) for 4 hours prior to fixation in 3:1 ethanol to glacial acetic acid (v/v). Roots were softened in enzyme solution (4% pectinase and 4% cellulase) for 20 minutes at 37°C, and squashed in a drop of acetic acid in glycerol. Slides were frozen in liquid nitrogen, and the coverslip removed with a razor blade. The samples were stained with 1µM DAPI in Mcilvaine buffer for 10 minutes in the dark, washed in 1x PBS buffer, mounted in Vectashield (Vector Laboratories, Burlingame, USA) and covered with a glass cover slip. Samples were visualised using the DAPI filter in a Zeis Axioskop microscope.

Table 5.2. Locality details for material used in experimental crosses (X), estimates of flowering time (FT), and chromosome counts (CC). All Material wild collected in Mexico except where indicated.

Pop. code	Locality	Latitude	Longitude	Experiment
B. heracleifolia				
h2	4km NE San Andrés Tuxtla, Veracruz	18.47850	-95.17802	CC, X
h8	Agua Azul, Chiapas	17.22117	-92.11073	FT, X
h13	Near Berriozábal, Chiapas	16.86693	-93.32781	CC, FT, X
h14	Roadside to Fortin de las Flores, Veracruz	18.89369	-97.01050	X
h15	5km south of Orizaba, Veracruz	18.78253	-97.08508	FT, X
h16	Valley 30km East of Huatusco, Veracruz	19.20111	-96.67139	X
h21	Between Jasaa and San Juan Bautista, Oaxaca	17.74356	-96.32803	CC, FT, X
h24	Motzorongo, Oaxaca	18.66953	-96.78714	FT, X
h28	Near Santa Maria Xanabi, Veracruz	15.98808	-96.11061	FT, X
B. nelumbiifolia				
n3	7km NE San Andrés Tuxtla, Veracruz	18.50341	-95.16824	FT
n7	Road to Ocosingo from Palenque, Chiapas	17.42477	-91.99712	CC, FT
n11	Ocozocoautla biosphere reserve, Chiapas	16.92489	-93.45090	CC, FT
n19	3km north of Josaa, Oaxaca	17.64247	-96.33747	CC, X
n24	Motzorongo, Oaxaca	18.66953	-96.78714	FT
n/a	Cultivated accession, unknown provenance			X
B. sericoneura				
s24	Motzorongo, Oaxaca	18.66953	-96.787139	FT, X

5.3.2.3. Crossing barriers and fitness of hybrids

Low hybrid fitness will reduce the likelihood of introgression, and four measures of fitness were compared between experimental F1s and their parents: (1) seed set (2) seed germination (3) early vigour (4) pollen viability.

Hybrid seed set. Five *B. heracleifolia* plants were selected at random as maternal parents and used in three different cross types: selfs, outcrosses, and F1 crosses with *B. sericoneura*. The pollen donor for outcrosses and F1 crosses were chosen at random from plants in flower. Crosses were made by rubbing the dehiscing stamens across the stigma, and each cross repeated three times. Other male flowers in the vicinity were removed to prevent accidental cross-pollination. Seed capsules were harvested when the capsule had turned brown, and stored in glassine packets at 4°C. *Begonia* species produce thousands of seed per capsule, so seed set was estimated using the approach of Ågren & Schemske (1993), summarised here briefly. Filled seeds and shrivelled seeds can distinguished under a 10x dissecting microscope; filled seeds may be viable or have aborted late in development, while shrivelled seed never germinate (Ågren & Schemske, 1993). The average mass of an aborted seed (m_a) or a filled seed (m_f) was estimated by weighing 1000 aborted of filled seeds, pooled from each accession. For each pollinated capsule, the capsule mass (M_t), and the proportion filled seeds (p) from a sample of 100 seeds was measured. The number of filled seeds per capsule (N) was estimated as pN using the following formula (Ågren & Schemske, 1993):

$$N = M_t/[pm_f + (1-p)m_a]$$

The impact of cross type (self, outcross or F1) and maternal parent (both fixed effects) on seed set was estimated using general linear model analysis of variance (GLM-ANOVA) in Minitab Statistical Software (www.minitab.com). Seed set was also tested in 5 reciprocal F1 crosses (*B. sericoneura* x *B. heracleifolia*). The significance of the differences between means in reciprocal crosses was calculated using Student's *t*-test in Minitab.

Seed set was also analysed in F1 crosses between *B. heracleifolia* and *B. nelumbiifolia*. Two accessions of *B. nelumbiifolia* flowered in 2011, and these were used as paternal parents to 5 different maternal parents. The reciprocal cross *B. nelumbiifolia* x *B. heracleifolia* was also attempted. The significance of the difference between the mean of the reciprocal crosses, and each cross to selfs, were calculated with Student's *t*-test, as above.

Hybrid seed germination. Seed germination was compared between the three crosstypes (selfs, outcrosses, F1 with *B. sericoneura*), to test whether filled seeds are viable or if they have aborted late in development. Twenty-five to forty filled seeds per cross generated from the seed set experiment (above) were germinated on finely-sieved bark in a propagator at 28°C, watered weekly with 1:10 dilute tomato fertilizer, and the proportion germination scored after 8 weeks. The effect of cross type and maternal parent were used in a GLM-ANOVA as above. Seed germination was also attempted in the reciprocal F1 cross to *B. sericoneura*. Four different maternal parents were selected and 11 crosses made, and the significance of the differences between means was calculated using Student's *t*-test.

F1 *B. heracleifolia* x *B. nelumbiifolia* crosses were also germinated and scored, as above. The significance of the difference in the mean F1 seed and that of selfs in this experiment, and the other crossing barriers tested below (hybrid vigour, sterility, and later-generation barriers), was tested using Student's *t*-test.

Hybrid vigour. The vigour of F1s (*B. heracleifolia* x *B. nelumbiifolia*, *B. heracleifolia* x *B. sericonuera*) was compared to selfs, using the seedlings from the germination experiment (above). Four eight week old self and F1 hybrids were chosen at random and potted into 9cm pots. After a total 16 weeks, 3 plants per cross type were harvested at soil level, and the number of leaves (>3mm) counted, and the mass of the plants dried at 90°C for 4 days (dry mass herein) recorded. The final plant from each cross type was grown to maturity for pollen viability measures (below).

Hybrid sterility. Pollen from a single flower from each of the F1 crosses (from above) was stained with 1M acetocarmine, observed under a Leica microscope, and the number of well-stained pollen recorded from 200 pollen grains. Female fertility was also scored using the wild-collected F1 hybrids that flowered in the greenhouse (2 *B. heracleifolia* x *B. sericoneura* and 3

B. heracleifolia x *B. nelumbiifolia*). These were used as pollen recipient to both the parental species, and the seed set recorded (as above).

Later-generation barriers. Hybrid break down may occur after the F1 generation (Stace, 1989) so the fitness of F1 backcrosses (F1BCs) was recorded. The vigour and pollen stainability of F1BC plants generated in the hybrid sterility experiment (above) were made in the same way as before.

5.3.3. Extent of gene exchange in hybrid swarms

5.3.3.1. B. heracleifolia x B. sericoneura hybrid swarm

A total of 100 individuals were analysed at nuclear loci in the hybrid swarm. These were catagorised on the basis of morphology as: 42 *B. heracleifolia*, 28 *B. sericoneura*, and 30 putative hybrids. Sixty-three of these genotypes were generated for this study (all *B. sericoneura*, putative hybrids, and 5 *B. heracleifolia*), and added to the 37 *B. heracleifolia* genotypes generated in Chapter 4.

A total of 93 individuals were analysed at plastid loci: 35 *B. heracleifolia*, 28 *B. sericoneura*, and 30 putative hybrids. Fifty-eight of these genotypes were scored solely for this study (all *B. sericoneura* and the putative hybrids) and added to the 35 *B. heracleifolia* genotypes scored in Chapter 2.

5.3.3.2. B. heracleifolia x B. nelumbiifolia hybrid swarms

Morphometric analysis of leaf shape was conducted on hybrid swarm HS1, to test whether hybrid categories from molecular data are consistent with morphology. A large leaf was harvested from each of 57 plants (25 *B. heracleifolia*, 29 *B. nelumbiifolia*, 3 putative hybrids) and photographed against a black background with a scale, prior to analysing leaf blade dimensions and leaf shape in LAMINA (Bylesjö *et al.*, 2008). Box plots were made for each measure of leaf shape, comparing individuals assigned to pure parental classes and hybrid classes in the NewHybrids analysis (below).

The number of individuals analysed at nuclear loci was as follows: HS1 total 61 plants, 29 *B. heracleifolia*, 29 *B. nelumbiifolia*, 3 putative hybrids; HS2 total 71 plants, 30 *B. heracleifolia*, 40 *B. nelumbiifolia*, 1 putative hybrid; HS3 total 61 plants, 29 *B. heracleifolia*, 30 *B. nelumbiifolia* and 2 putative hybrids. All hybrids that were found in HS2 and HS3 were genotyped, but only 3 hybrids were collected in HS1 because access to the site was limited. Fifteen of the genotypes were newly generated for this study (6 putative hybrids, 3 *B. heracleifolia* HS1, 1 *B. nelumbiifolia* HS1) and added to the 179 generated in Chapter 4.

A total of 111 plants were analysed for plastid loci from two hybrid swarms: HS1, 33 *B. heracleifolia*, 29 *B. nelumbiifolia*, 3 putative hybrids; HS2, 20 *B. heracleifolia*, 25 *B. nelumbiifolia*, 1 putative hybrid), as well as the two hybrids from HS2. Three of the genotypes were newly generated for this study (6 putative hybrids) and added to the 105 generated in Chapter 2.

5.3.3.3. Nuclear genotyping

Plants were genotyped with the 14 nuclear microsatellite loci described in Chapter 3, and scored in the same manner.

5.3.3.4. Hybrid swarm analyses for nuclear markers

Three complementary analytical approaches were applied to the nuclear microsatellite data to assess the genetic composition of hybrid swarms. The program NewHybrids 1.1 Beta (Anderson & Thompson, 2002) assigns individuals to one of six genealogical classes of hybrids after 2 generations of crossing: parent A-type, backcross A-type, F1-type, F2-type, backcross B-type, parent B-type. Milne & Abbott (2008) extended the number of hybrid classes to the 45 unique combinations after 4 generations of crosses, and grouped these into the 6 categories described above; individuals not equivocally assigned to a single category are placed in compound categories (e.g ‘backcross to parent A or parent A’) using a MS Excel spreadsheet (made available by R. Milne, University of Edinburgh, pers. comm.). For the *B. heracleifolia* x *B. nelumbiifolia* hybrid swarms 12 individuals of each species were used as a reference in the analysis and these were taken from across the species ranges. The analysis for HS4 was run without reference populations, as no suitable populations were found in the field. This has little effect on the accuracy of hybrid assignment (Vähä & Primmer, 2006). Analyses of *B. heracleifolia* x *B. nelumbiifolia* hybrid swarms were repeated without reference populations for comparison. For each hybrid swarm, 2 and 4 generations of crosses were evaluated, using 100 000 sweeps after a burn-in period of 10 000 sweeps. In NewHybrids, individuals are assigned to one or more genealogical classes with a posterior probability score (q-value), and to reduce miss-assignment a stringency of $q > 0.9$ was used as recommended by Vähä & Primmer, 2006).

Second, the program FLOCK (Duchesne & Turgeon, 2009) was used, which allocates individuals to one of the user defined numbers of genetic clusters (K) with a log-likelihood score (LLOD score). FLOCK is a non-Bayesian approach which operates well even when admixture is high, and when reference populations are not available (Duchesne & Turgeon, 2009). Each hybrid swarm was analysed without reference populations using default parameters and with K = 2. A plot of LLOD scores was evaluated to see if there were distinct clusters of parents and putative hybrid classes, or whether there is a continuum of LLOD scores suggesting complete admixture.

Third, Bayesian clustering in BAPS (Corander *et al.*, 2008) was used to measure the contribution of each parental genome to the hybrids. BAPS reports this as a q-value. To avoid confusion between NewHybrids and BAPS q-values, these are referred to as q_{NHZ} and q_{BAPS}, respectively, herein. Preliminary runs with ‘clustering of individuals’ followed by ‘admixture based on mixture clustering’, where no *a priori* information is given about pure individuals, performed poorly (results not shown). Therefore, pure individuals were first detected in NewHybrids ($q_{\mathrm{NHZ}} > 0.9$), and then defined in the input file for ‘admixture based on pre-defined populations’, as recommended in the BAPS manual. Default options were selected, except that 10 000 iterations and 5 000 reference individuals were used.

5.3.3.5. Simulated hybrid swarms

The discriminatory power of the markers and the error rate of assigning individuals to the correct hybrid class were tested using simulated hybrid swarms. Hybrids were simulated from the allele frequencies of pure individuals in hybrid swarms ($q_{\mathrm{NHZ}} > 0.9$). This approach allows simulated and empirical datasets to be compared directly, but assumes the parents have not been introgressed by other species (Burgarella *et al.*, 2009). This assumption is supported by their distinct morphologies and their strong differentiation at nuclear and plastid loci (see results). However, if introgression had occurred, this would simply reduce the power of detecting hybrids (Burgarella *et al.*, 2009).

Pure parents from two hybrid swarms (HS1, 29 *B. heracleifolia* and 28 *B. nelumbiifolia*; HS4, 39 *B. heracleifolia* and 26 *B. sericoneura*) were used as parents for the two simulated hybrid swarms. Random mating between the parents was simulated by drawing alleles at random from the observed parental allele frequencies using HybridLab v1.0 (Nielsen *et al.*, 2006). A total of 90 hybrids (30 F1s and 30 BC to each parent) were simulated for each hybrid swarm, and these were analysed in NewHybrids (as before). The likelihood of assignment to a genetic cluster (q_{NHZ}) was averaged across each hybrid class and standard errors calculated, and the percentage of individuals assigned to the wrong category recorded.

5.3.3.6. Inbreeding

The level of inbreeding can affect the genetic composition of hybrid swarms, as pollen is most likely to be carried from the outcrossing to the selfing species (Ruhsam *et al.*, 2010). The inbreeding coefficient and inferred selfing rates for *B. heracleifolia* and *B. nelumbiifolia* were calculated in Chapter 4. For comparison, the F_{IS} value for *B. sericoneura* was calculated using individuals that were assigned as parental *B. sericoneura* in NewHybrids ($q_{\mathrm{NHZ}} > 0.9$). The same subset of 9 loci were used as Chapter 4, and the F_{IS} value calculated in FSTAT v1.2 (Goudet, 1995). The inferred selfing rate was calculated with the formula of Allard *et al.* (1969, see Chapter 4).

5.3.3.7. Plastid analyses

Maternal plastid inheritance has been found in cytological observations (Corriveau & Coleman, 1988) and plastid sequencing of experimental crosses (Peng & Chiang, 2000) in other *Begonia* species. Therefore plastid genotyping was used to detect the direction of hybridization. Plants were genotyped with 7 plastid microsatellites using the same amplification protocol and method of scoring as Chapter 2. Plastid haplotypes were defined as the unique combination of alleles at all plastid loci. The frequency of plastid haplotypes were compared between the putative hybrids and the parents for each hybrid swarm.

5.4. Results

5.4.1. Barriers to hybridization between *B. heracleifolia* and *B. sericoneura*

5.4.1.1. Experimental assessment of reproductive barriers

Phenology. A similar number of flowers were produced at peak flower time for *B. heracleifolia* (40.1, n = 9) and *B. sericoneura* (39.5, n = 6). *Begonia sericoneura* started flowering earlier than *B. heracleifolia*, although peak flowering overlapped (Fig. 5.2).

5.4.1.2. Crossing barriers and fitness of hybrids

Hybrid seed set. The number of seeds set when wild-collected *B. heracleifolia* was a pollen recipient to *B. sericoneura* (1080 ± 152; n = 15) was not significantly different to outcrosses (1055 ± 78; n = 15) or selfs (1265 ± 156; n = 15). The seed set between reciprocal F1 crosses were also not significantly different (*B. sericoneura* x *B. heracleifolia*, 1473 ± 418, n = 5). However, the effect of crosstype (self, outcross, F1 to *B. sericoneura*) was marginally significant on seed set in the ANOVA (P = 0.045, Table 5.3), with the maternal parent and the interaction effect being more significant (P < 0.001; Table 5.3). The interaction plot showed no common pattern between cross type and seed set; some F1 hybrids produced more and others fewer seeds than selfs.

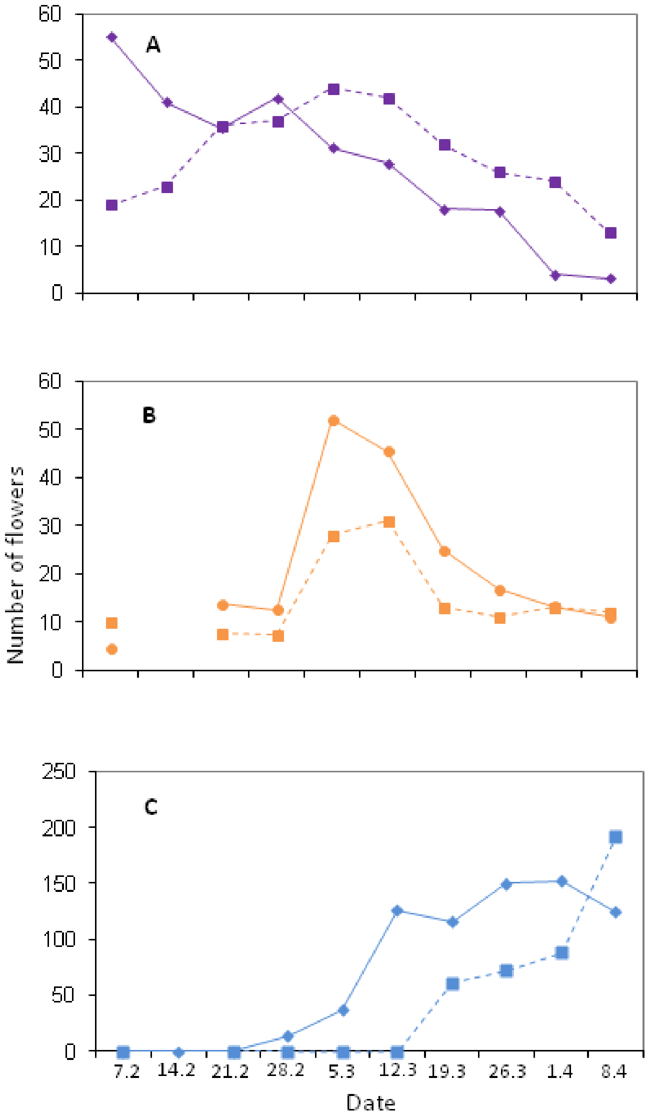


Figure 5.2. Variation in flower time for the three *Begonia* species. a) *B. sericoneura*, b) *B. heracleifolia*, c) *B. nelumbiifolia*. Number of flowers shown separately for males (solid line) and females (dashed line).

Hybrid seed germination. The germination of *B. heracleifolia* x *B. sericoneura* F1 plump seeds (70.0% ± 6.7; n=15) was not significantly different from selfs (64.1% ± 8.7; n=15) or outcrosses (79.5% ± 7.5; n=15). Only the effect of maternal parent was significant on seed germination in the ANOVA (Table 5.3).

Table 5.3. Effect of maternal parent and cross type (self, outcross, or F1) on seed set and germination analyzed with GLM-ANOVA. DF, degrees of freedom; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

Fitness measure	Source	DF	Mean square	F
Seed set	Maternal parent	4	1645540	28.58***
	Cross type	2	198144	3.44*
	Maternal parent x cross type	8	377129	6.55***
	Error	30	57585	
Germination	Maternal parent	4	3785.3	8.25***
	Cross type	2	908.3	1.98
	Maternal parent x cross type	8	10209.5	2.24
	Error	30	458.7	

Hybrid vigour. The vigour of *B. heracleifolia* x *B. sericoneura* F1 plants (number of leaves = 5.8 ± 0.2; dry mass = 0.33g ± 0.075; n = 9) was not significantly different to selfed plants (number of leaves = 6.1 ± 0.3; dry mass = 0.42g ± 0.08; n = 18).

Hybrid sterility. The 2 wild-collected F1 *B. heracleifolia* x *B. sericoneura* hybrids had low pollen fertility, one plant dropped male flowers, and the other had little stainable pollen (4.8%); none of the experimental F1s flowered in their first year (n = 7). The number of filled seed was not significantly different when the F1 was used as pollen recipients to *B. heracleifolia* (40 ± 10 seeds, n = 8) or *B. sericoneura* (12 ± 3 seeds, n = 6).

Later-generation barriers. Germination of seeds in the F1BC to *B. sericoneura* was significantly higher (33.3% ± 5, n = 9) than F1BC to *B. heracleifolia* (11.5 ± 3, n=6). The F1BC seedlings with *B. sericoneura* as a paternal parent (leaves = 6.7 ± 0.5; dry mass = 0.41 ± 0.11g; n = 6) were not significantly more vigorous than when *B. heracleifolia* was used (leaves = 4.8 ± 1.1 leaves; dry mass = 0.18 g ± 0.15, n = 2).

5.4.1.3. Analysis of hybridization in nature

The 14 nuclear microsatellite loci amplified successfully in both species and their putative hybrids (Appendices 5 – 8). Loci BI5347 and BI3348 were excluded due to uneven amplification or unexpected repeat motif lengths. Analyses using 10 loci common to both species pairs gave similar results to the full dataset of 12 loci (results not shown). All loci were polymorphic, except BE332 which was monomorphic in HS4.

Breeding system. Four loci were polymorphic in pure *B. sericoneura* individuals. The F_{IS} value across loci was 0.415, which gives an inferred selfing rate of 0.59 (Table 5.4).

Table 5.4. Inbreeding coefficients and inferred selfing rates for the three study species.

Species	F_{IS}	Inferred selfing rate	Reference
B. heracleifolia	0.249	0.399	Chapter 4
B. nelumbiifolia	0.454	0.624	Chapter 4
B. sericoneura	0.415	0.590	This study

***Hybrid swarm structure*.** The NewHybrids analysis using 6 hybrid catagories assigned 27 individuals as pure *B. sericoneura* (mean q_{NHZ} = 0.999), 41 pure *B. heracleifolia* (mean q_{NHZ} = 0.999), 25 F1s (mean q_{NHZ} = 0.980) and 2 BCs to *B. sericoneura* (q_{NHZ} = 0.935; Fig. 5.3). Five individuals were not placed in a single category using a stringency of $q_{NHZ} > 0.9$. When stringency was lowered, these were classified as: 3 F1s (q_{NHZ} = 0.75), 1 BC to *B. sericoneura* (q_{NHZ} = 0.76) and 1 pure *B. sericoneura* (q_{NHZ} = 0.55). There was no evidence for any backcrosses to *B. heracleifolia* or F2s. When 4 generations of crosses were considered (Milne & Abbott, 2008), 39 individuals were assigned as *B. heracleifolia*, 29 *B. sericoneura*, 30 F1 or F2s, and 1 BC to *B. heracleifolia* or pure *B. heracleifolia*.

The FLOCK analysis generally supported the NewHybrids results. Three main clusters corresponded to *B. heracleifolia* (mean LLOD 11.28 ± 0.36SE, n = 41), F1s (mean LLOD -11.90 ± 0.44SE, n = 28) and *B. sericoneura* (mean LLOD -26.73 ± 0.22SE, n = 28). Two individuals placed between the F1s and *B. sericoneura* are likely to be BC (mean LLOD -21.59 ± 0.52SE). The BAPS analysis classified 28 individuals as pure *B. sericoneura* (P = 1.0), 41 as pure *B. heracleifolia* (P = 1.0) and 31 individuals as admixed (average q_{BAPS} -value across admixed individuals = 0.40 ± 0.01). Five of these admixed individuals (average q_{BAPS} = 0.279, range 0.19 - 0.33) are more consistent with being backcrosses than F1s.

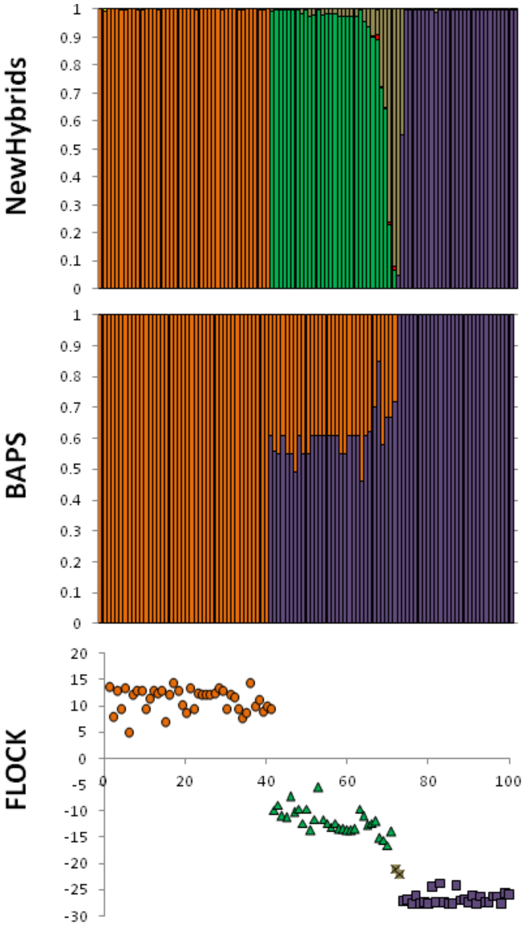


Figure 5.3. Genetic composition and classification of hybrids in hybrid swarm 4 (*B. heracleifolia* x *B. sericoneura*). NewHybrids analysis with 6 hybrid catagories (top row): orange, *B. heracleifolia*; purple, *B. sericoneura*, green, F1 hybrid; red, F2; yellow, BC *B. heracleifolia*; dark blue, BC *B. sericoneura*. BAPS (centre row): orange, *B. heracleifolia*, purple, *B. sericoneura*. FLOCK (bottom row) coloured by most likely class in NewHybrids: orange circles, *B. heracleifolia*; green triangles, F1 hybrids; grey crossed squares, putative backcrosses to *B. sericoneura*, purple squares, *B. sericoneura*.

5.4.1.4. Accuracy of assignment in simulated hybrid swarms

The classes that individuals in simulated hybrid swarms were assigned to were compared to their actual genotypic class, to test the accuracy of assignment with the markers. When 6 hybrid classes were considered, 143 plants (91.7%) were correctly assigned, and the other 13 (8.3%) were not assigned to a single category. Assignment was also high when 45 hybrid classes were considered: 140 plants (89.7%) were correctly assigned, and the others were assigned to compound categories (9 plants, 5.8%), remained unassigned (4, 2.6%), or were assigned to the wrong category (3, 1.9%).

5.4.1.5. Plastid genotyping and direction of hybridization

The plastid microsatellites revealed 3 haplotypes in *B. heracleifolia* and 1 in *B. sericoneura* in HS4. No haplotypes were shared between species. The 30 putative hybrids all had the plastid haplotype of *B. sericoneura*.

5.4.2. Barriers to hybridization between *B. heracleifolia* x *B. nelumbiifolia*

5.4.2.1. Experimental assessment of reproductive barriers

Phenology. *Begonia nelumbiifolia* produced many more flowers at peak flowering (>150, n = 4) than *B. heracleifolia* (40.1, n = 9; Fig. 5.2). Flowering times for *B. heracleifolia* and *B. nelumbiifolia* overlapped, but peak flowering for *B. nelumbiifolia* was at least 5 weeks later.

Chromosome numbers. A diploid chromosome number of 2n=28 was found in both *B. heracleifolia* and *B. nelumbiifolia* from each of 3 accessions (Fig. 5.4).

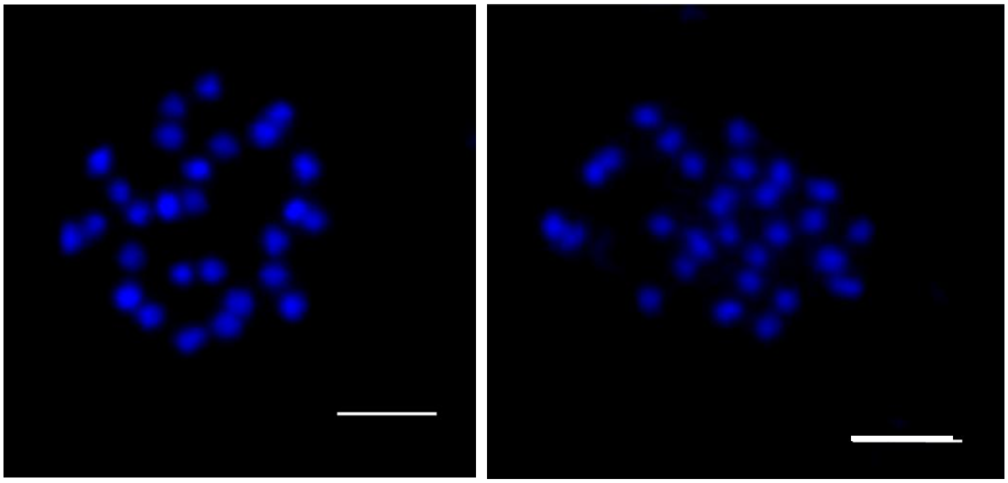


Figure 5.4. Chromosome spreads of *B. heracleifolia* (left) and *B. nelumbiifolia* (right). Scale bar is 10µM.

5.4.2.2. Crossing barriers and fitness of hybrids

Hybrid seed set. The number of seed set in *B. heracleifolia* x *B. nelumbiifolia* F1 crosses (503 ± 86, n = 11), was significantly reduced relative to selfed *B. heracleifolia* (1265 ± 156; n = 15, $P < 0.05$). The reciprocal cross (*B. nelumbiifolia* x *B. heracleifolia*) also set seed (394 ± 230, n = 3), with the mean not significantly different from the self.

Hybrid germination. Germination success of *B. heracleifolia* x *B. nelumbiifolia* F1 hybrids was 45.2% ± 9% (n = 11), which was not significantly different from selfed *B. heracleifolia* (64.1% ± 9%, n = 15).

Hybrid vigour. The vigour of *B. heracleifolia* x *B. nelumbiifolia* F1s (number of leaves = 5.3 ± 0.6; dry mass = 0.37g ± 0.09; n = 4) was not significantly different from selfs (number of leaves = 6.1 ± 0.3; dry mass = 0.42g ± 0.08; n = 18).

Hybrid sterility. The three wild-collected F1s were largely infertile, producing no viable pollen nor setting any viable seed when selfed. Experimental F1s were also male sterile (4/6 dropped male flowers, 2/6 had 0% stainable pollen). However, wild collected F1s did set a low number of seeds as a pollen recipient to either of the parents, which did not differ significantly when *B. heracleifolia* (43.5 ± 11.2, n = 9) or *B. nelumbiifolia* (57.4 ± 15.2, n = 14) were the pollen donor.

Later-generation barriers. The germination of F1BC seed to *B. heracleifolia* (41.1% ± 6.5, n = 8) was not significantly different from the BC to *B. nelumbiifolia* (47.6% ± 6.1, n=10). No difference was found in the vigour between the backcross plants to *B. heracleifolia* (number of leaves = 6.2 ± 0.8, dry mass = 0.22 ± 0.05g, n = 5) and *B. nelumbiifolia* (number of leaves = 6.5 ± 0.2, dry mass = 0.33 ± 0.11g, n = 4).

5.4.2.3. Analysis of hybridization in nature

Morphometrics. *Begonia heracleifolia* and *B. nelumbiifolia* were clearly distinguished by morphometric analyses of leaf shape, with the 3 hybrids placed as intermediate (e.g. Fig. 5.5 for leaf circularity index, the percentage of pixels shared between the leaf and a superimposed circle with same width/height as the leaf).

Hybrid swarm analyses. The 14 nuclear loci amplified successfully in both species and their hybrids, two loci were excluded (BI6534 and BC332) due to uneven amplification or unexpected repeat motif lengths.

All three analytical methods (BAPS, NewHybrids and FLOCK) identified few hybrids in each hybrid swarm (total of 6: 3 in HS1, 2 in HS2, and 1 in HS3) that are likely to be F1 hybrids, with no other hybrid classes present (Fig. 5.6). NewHybrids analyses strongly supported the 6 hybrid individuals as F1s (mean q_{NHZ} with reference populations = 0.986; without = 0.953). The genotypic classes accorded exactly to their morphology (Fig. 5.5). The mean contribution of each parents' genome to the 6 hybrids (estimated in BAPS) was 0.48:0.52 (*B. heracleifolia*:*B. nelumbiifolia*, not significantly different from 50:50), and in each case FLOCK identified clusters of *B. heracleifolia* (mean LLOD = -27.06 ± 0.52SE, n = 88) and *B. nelumbiifolia* (mean LLOD = 27.98 ± 0.48SE, n = 99), with putative hybrids intermediate (mean LLOD 3.91 ± 0.73SE, n = 6).

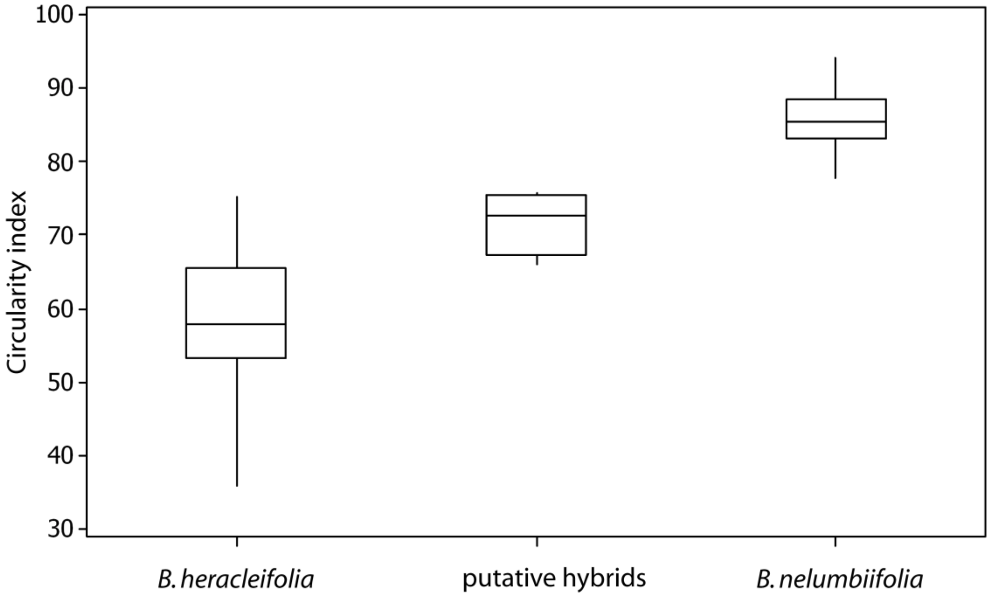
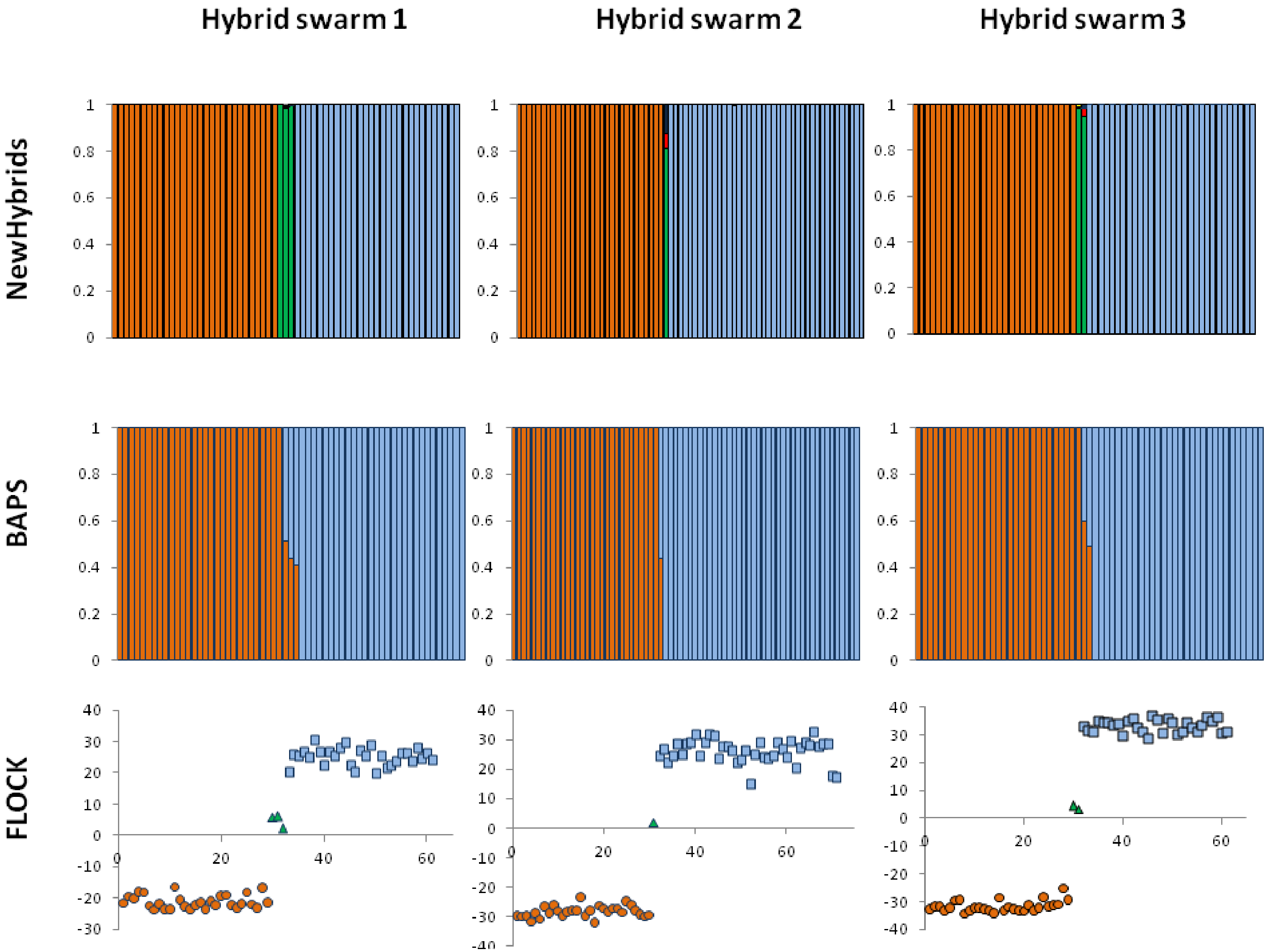


Figure 5.5. Morphometric analysis of leaf shape in hybrid swarm 1 (*B. heracleifolia* x *B. nelumbiifolia*).

When 4 generations of crosses were considered in NewHybrids (Milne & Abbott, 2008): 84 individuals were considered to be *B. heracleifolia*, 98 *B. nelumbiifolia*, 3 F1 or F2s, 4 BC to *B. heracleifolia* or parental *B. heracleifolia*, 1 BC *B. nelumbiifolia* or parental *B. nelumbiifolia*, and 3 unclassified (any hybrid class). The classification of some hybrids as potential backcrosses was not supported by any of the other analyses.

Figure 5.6. Genetic composition and classification of hybrids in three *B. heracleifolia* x *B. nelumbiifolia* hybrid swarms (next page). NewHybrids analysis (top row): orange, *B. heracleifolia*; blue, *B. nelumbiifolia*; green, F1 hybrid; red, F2; yellow, BC *B. heracleifolia*; dark blue, BC *B. nelumbiifolia*. BAPS (centre row): orange, *B. heracleifolia*; blue, *B. nelumbiifolia*. FLOCK (bottom row) coloured by most likely class in NewHybrids: orange circles, *B. heracleifolia*; blue squares, *B. nelumbiifolia*; green triangles, F1 hybrids.


Hybrid swarm 1
Hybrid swarm 2
Hybrid swarm 3
NewHybrids
BAPS
FLOCK
0
0.2
0.4
0.6
0.8
1
-40
-30
-20
-10
0
10
20
30
40
20
40
60

5.4.2.4. Accuracy of assignment in simulated hybrid swarms

Most individuals were correctly assigned when 2 generations of crossing were considered without reference populations, although some individuals were unassigned (Table 5.5). Increasing the number of hybrid classes to the 45 unique categories after 4 generations of crosses (Milne and Abbott, 2008) marginally increased the assignment error, and many individuals were assigned to pooled categories (Table 5.5).

Table 5.5. NewHybrids assignment for the *B. heracleifolia* x *B. nelumbiifolia* hybrid swarm with different settings. † MCMC analysis consistently ended prematurely.

Number of hybrid categories used	Reference populations	Assigned to correct category	Assigned to compound category	Unassigned	Incorrect category
6	Yes	141 (95.3%)	n/a	6 (4.1%)	1 (0.75)
6	No	143 (96.6%)	n/a	5 (3.4%)	0
45	Yes	104 (70.3%)	34 (23.0%)	6 (4.1%)	4 (2.7%)
45†	No				

5.4.2.5. Plastid genotyping and direction of hybridization

In the three *B. heracleifolia* x *B. nelumbiifolia* hybrid swarms, a single plastid type was found in *B. nelumbiifolia*, and a total of 9 in *B. heracleifolia*. No haplotypes were shared between species. All 6 putative hybrids had the plastid type of *B. nelumbiifolia*.

5.5. Discussion

5.5.1. Evolution of reproductive isolation in *Begonia*

The hypothesis that relatively strong reproductive barriers accumulate between related *Begonia* species is supported by analyses of two pairs of hybridizing *Begonia* species. In contact zones between both species pairs heterospecific mating will be uncommon due to frequent self-fertilization (inferred selfing rates 40 – 62%) and differences in flowering times (Fig. 5.6), and introgression is unlikely due to low fertility of F1 hybrids (0 – 5% stainable pollen; discussed below). These barriers translated to structured hybrid swarms in the field, composed of parents and mostly early generation hybrids. Other studies of *Begonia* hybrids are mostly consistent with the hypothesis of rapidly evolving reproductive barriers. Wide-outcrosses between *B. heracleifolia* populations have a modest reduction in fertility (Chapter 4). Hybridizing *Begonia* species in Taiwan have strong crossing barriers, with sterile F1s and no later generation hybrids, with these reproductive barriers likely to be explained by differences in chromosome number (e.g. *B.* x *buimontana*, Peng & Chen, 1991, *B.* x *breviscarpa*, Peng *et al.*, 2010; *B.* x *chungii*, Peng & Shin-Ming, 2009; *B.* x *taipeiensis*, Peng & Sue, 1991). Whether hybrids between *B. decora* and *B. venusta*, closely related species from section Platycentrum (Thomas *et al.*, 2011b), fit this pattern is unclear. Hybridization leads to a wide range of hybrid morphologies (Teo & Kiew, 1999) and admixed AFLP profiles (Kiew *et al.*, 2003), but the classes of hybrids have not been estimated from the genetic data.

The strength of reproductive isolation between *Begonia* species contrasts with many other plant hybrid zones between diploid species, where widespread introgression is common (e.g. *Aquilegia*, Hodges & Arnold, 1994; *Helianthus*, Scascitelli *et al.*, 2010; Louisianan *Iris*, Arnold *et al.*, 2010; *Populus*, Lexer *et al.*, 2005; *Silene*, Minder *et al.*, 2007). The scarcity of studies that report limited introgression likely represents publication bias, however structured hybrid swarms, like *Begonia*, give a unique insight into the evolution of reproductive isolation. Similar hybrid swarm structure, where F1s are present but backcrosses rare or absent, have been found between *Rhododendron* species and between some *Quercus* species. In two pairs of hybridizing *Rhododendron* species, F1 hybrids are fertile and the absence of BCs may be caused by extrinsic habitat selection (Milne *et al.*, 2003; Milne & Abbott, 2008). Hybrids between *Quercus kelloggii* and *Q. wislizenii* var. *frutescens* are also thought to be fertile, and the absence of BCs has been attributed to competition with the parents (Nason *et al.*, 1992).

We tested whether limited introgression in contact zones is caused by ecological selection against hybrids, by comparing reproductive barriers under experimental conditions with the frequency of hybrids in the field between an ecologically similar (both dry-adapted) and more divergent (dry-adapted and moisture-loving) species pair. The frequency and extent of hybridization between ecologically similar *B. heracleifolia* and *B. sericoneura* was much greater than between ecologically divergent *B. heracleifolia* and *B. nelumbiifolia*. This was the case even though hybrids between both species pairs were relatively vigorous under experimental conditions, although this must be interpreted with caution due to the small sample sizes and the lack of field testing. Strong support for assortative mating and a bimodal distribution of hybrid indices (i.e. hybrid swarm dominated by parental individuals), has been considered evidence for the role of ecology rather than genomic incompatabilities in hybridization (Jiggins & Mallet, 2000). However, we could not estimate the role of selection against hybrids independently of intrinsic reproductive barriers, as intrinsic reproductive barriers were also weaker between *B. heracleifolia* x *B. sericoneura* than *B. heracleifolia* x *B. nelumbiifolia*. Specifically, *B. heracleifolia* and *B. sericoneura* overlap in their flowering time for longer than *B. heracleifolia* and *B. nelumbiifolia* (Fig. 5.2), produce more seed in F1 crosses, and some F1 plants produce viable pollen. The stronger reproductive barriers and reduced frequency of hybrids between the ecologically more divergent species may be explained by negative interactions between divergent adaptive loci (Presgraves *et al.*, 2003), or through hybrids having a maladaptive mix of traits ‘blended’ between the parents (Burke & Arnold, 2001), both of which would affect the more ecologically divergent species pair to a greater extent. Alternatively, adaptive loci to particular environments, or prezygotic barriers (flowering time), may have a pleiotropic effect on intrinsic reproductive barriers (e.g. F1 hybrid sterility). One example would be if an adaptive trait is located within an inversion polymorphism that reduces homologous chromosome pairing in meiosis, causing sterility (Hoffmann & Rieseberg, 2008).

A non-mutually exclusive explanation would be that strong reproductive isolation in crosses with *B. nelumbiifolia* reflects its unique demographic history, rather than being related to ecological selection. This species frequently self-fertilizes, and may have also been through a population bottleneck (Chapter 2). Both these factors will fix alleles through genetic drift, which may then be incompatible with other *Begonia* genomes when brought together through hybridization. Deleterious alleles fixed through drift have been proposed to explain the low fertility of interspecific crosses between the highly selfing *Glycine falcata* and other *Glycine* species, which does not follow the trend between genetic distance and reproductive isolation found with other *Glycine* crosses (Moyle *et al.*, 2004). It may also be that nuclear-cytoplasmic incompatibilities in interspecific crosses are caused by the fixation of a single plastid haplotype in south Mexican populations of *B. nelumbiifolia*. Maternal effects have also been proposed to explain why crosses between another pair of *Begonia* species, *B. formosana* Hayata (Masamune) and *B. aptera* Blume, can only be crossed in one direction (Peng & Chiang, 2000). Future studies to distinguish whether ecological selection or demographic history are the main drivers of strong reproductive isolation in *Begonia* may look to other hybridizing species, or compare patterns of sequence divergence at neutral and putatively adaptively loci underlying ecologically relevant traits.

5.5.2. Experimental limitations of hybrid swarm comparisons

An important confounding factor that may influence the results of our study is the phylogenetic relatedness of the species pairs (Funk *et al.*, 2006). Reproductive isolation will usually increase with genetic divergence (Gleason & Ritchie, 1998), as increasingly divergent species are likely to differ for traits that cause prezygotic isolation (e.g. flower colour), and have accumulated genetic incompatibilities and chromosomal differences that cause post-zygotic barriers. This has been shown in fungi (Le Gac *et al.*, 2007) and animals, including: salamanders (Tilley *et al.*, 1990), fruit flies (Gleason & Ritchie, 1998), frogs (Sasa *et al.*, 1998), toads (Malone & Fontenot, 2008), fish (Mendelson, 2003), butterflies and moths (Presgraves, 2002) and birds (Price & Bouvier, 2002). In plants, postmating reproductive isolation correlates with genetic distance in *Silene* (Caryophyllales), *Glycine* (Fabaceae), food-deceptive Mediterranean orchids, but not *Streptanthus* (Brassicaceae) or sexually-deceptive orchids, likely due to insufficient time since divergence for reproductive barriers to evolve (Moyle *et al.*, 2004; Scopece *et al.*, 2007). Our current understanding of evolutionary relationships in Central American *Begonia* is poor (Dewitte *et al.*, 2011), but preliminary phylogenetic analysis with nuclear and plastid sequence data place both species pairs as non-sister species that are similar in their genetic distances (patristic distance: *B. heracleifolia* x *B. sericoneura*, 0.0025; *B. heracleifolia* x *B. nelumbiifolia*, 0.0016; Harrison, University of Edinburgh, pers comm.). Therefore phylogenetic distance does not seem to explain the observed differences in hybrid swarm structures. The second concern with a comparative hybrid swarm approach is that reproductive barriers may not have driven divergence, but instead accumulated after speciation (Scopece *et al.*, 2010). It can however be argued that all reproductive isolating barriers are important to the isolation of species upon secondary contact, even if they have not directly promoted divergence. In *Begonia*, the interspecific barriers described in this study have also been found between divergent populations (e.g. pollen sterility, chapter 4; flower time differences, A.D. Twyford, unpubl. data), therefore it seems likely that these barriers evolved during divergence, and may have promoted it.

5.5.3. Reproductive barriers in *Begonia*

The main barriers detected in this study were differences in flowering time, hybrid sterility, and inbreeding, and the pattern of mating observed in hybrid swarms fitted predictions from the species population biology and the barriers detected under experimental conditions. For example, all natural hybrids had the plastid type of the parent that is more highly selfing (*B. nelumbiifolia* and *B. sericoneura*) supporting the more highly outcrossing *B. heracleifolia* as the paternal parent as would be expected due to its pollen being more competitive (Ruhsam *et al.*, 2010). Many other reproductive isolating barriers likely play a role in maintaining species barriers in *Begonia*. This study focused on crossing barriers in sympatry, and strong prezygotic barriers will prevent contact in the first place (Barton & Hewitt, 1985; Widmer *et al.*, 2009). The narrow ecological niches and limited distribution ranges of *Begonia* species, such as species in *Begonia* section *Gireoudia* that are adapted to such habitats as dark moist tropical forest (e.g. *B. thiemei* C.DC. ex Donn.Sm. and *B. sousae* Burt-Utley) and sun baked cliffs (*B. peltata* Otto & A. Dietr. and *B. hydrocotylifolia* Otto ex Hook.), will play a critical role in whether species co-occur. Other post-pollination barriers that were not tested in this study include pollen-stigma interactions, which have been described in *Iris* (Carney *et al.*, 1994) and *Senecio* (Allen *et al.*, 2011).

Future studies should characterize the genetic basis of the reproductive isolating barriers that we detected. Many loci of small effect would be predicted to influence these barriers if they have evolved incrementally over time, such as those evolving during allopatric speciation (Coyne & Orr, 1989). However, this does not preclude species differences evolving through mutations at loci of large effect after initial divergence. It will be of particular interest to characterize F1 hybrid sterility, as it is common between many pairs of *Begonia* species (Dewitte *et al.*, 2011; Twyford and Kidner, unpubl. data). Strong hybrid sterility in F1 crosses can be caused by: genome-wide divergence (e.g. between *Drosophila* species, Tao & Hartl, 2003), a few sterility loci of large effect (e.g. *Mimulus*, Fishman & Willis, 2007), or chromosome rearrangements such as inversions or deletions (e.g. *Helianthus*, Lai *et al.*, 2005). This study, and others in *Begonia* (Chapter 4), support hybrid sterility evolving more rapidly than hybrid inviability (Malone & Fontenot, 2008). Genetic characterization of hybrid sterility can be achieved through the analysis of populations that segregate for fertility (Chapter 6). This approach would also be useful for other reproductive traits that differ between species, such as flowering time.

5.5.4. Evolutionary outcomes of hybridization in *Begonia*

Predicting the long-term evolutionary outcomes of hybridization from young hybrid swarms is difficult. The *Begonia* hybrids swarms were all by roadsides where the surrounding vegetation was well established, suggesting there has been plenty of time for species with a short generation time (1 year; A.D. Twyford pers. obs.) to hybridize. Strong fertility barriers and rare F1 formation in groups such as *Iris* and *Helianthus* has not prohibited introgression and hybrid speciation over long time periods (Arnold, 1993; Cruzan & Arnold, 1993; Ungerer *et al.*, 1998; Yatabe *et al.*, 2007). In irises however, backcrossing may be promoted by preferential pollinator visitation (Emms & Arnold, 2000) and hybrid superiority (Cruzan & Arnold, 1993), which have not been found in *Begonia*. Moreover, dispersal distances of *Begonia* seed are low (Chapter 2), and dispersal away from parental progenitors is important for the establishment of homoploid hybrid species (Gross & Rieseberg, 2005). Whether past hybridization events have occurred in

Begonia could be addressed by comparative phylogenetic analysis with unlinked markers, or through the analysis of introgressed genes in areas of sympatry using densely mapped markers (Twyford & Ennos, 2012).

In conclusion, relatively strong reproductive isolating barriers were found between two pairs of *Begonia* species, which in conjunction with limited gene flow from neighbouring populations (Chapter 2, Chapter 4, Matolweni *et al.*, 2000; Hughes & Hollingsworth, 2008), makes homogenizing gene flow unlikely to disrupt the speciation process. The wide range of habitats to which species are adapted further contributes to isolation, through reducing the number of species that co-occur in sympatry, as well as the putative role disruptive selection at adaptive loci plays on hybrid fitness. Taken together, the population biology and evolution of reproductive isolation may allow rapid divergence in the early stages of speciation, and may partly explain the high species richness in *Begonia*. Whether similar evolutionary processes have played a role in other large genera has yet to be tested.

CHAPTER 6: Quantitative trait locus (QTL) analysis of sex ratios and inflorescence architectures in backcross *Begonia* populations

Data contributions: Keith Gardner (University of Edinburgh) made the measures of flower number, flower time, and inflorescence branch patterns. Chris White (University of Edinburgh) measured stamen number. Alexander Twyford collected measures of pollen and seed viability, and performed all analyses.

6.1. Chapter summary

Inflorescence architecture describes the branching pattern and position of flowers on an inflorescence as determined by the developmental patterns of the inflorescence meristem. These decisions concern the production of branches via axillary meristems, and in monoecious plants, the sex of floral meristems. Variation will affect the sex ratio, the investment in flowers and support structures, and the presentation to pollinators, as well as flower number. Here, we use quantitative trait locus (QTL) analyses to studying the genetics of sex ratios and branching patterns, and other reproductive traits, in backcross populations between Central American *B. plebeja* and *B. conchifolia*. Five QTL of moderate effect (13.4 - 18.7% variance explained, VE) are found for variation in sex ratio and related traits, and these map to 2 different linkage groups. A QTL for number of male flowers co-localizes with a QTL for meristem decisions for either axillary branches or female flowers, showing the close link between inflorescence architecture and sex ratio. One QTL is detected in the reciprocal cross suggesting some conserved mechanisms underlie these traits. QTL for other reproductive traits include one of major effect for stamen number (30% VE), and one for pollen sterility (12.3% VE). We propose that biased sex ratios in *Begonia* are caused by selection on loci responsible for inflorescence branching. The consequences of such changes for inbreeding and pollination are also discussed.

6.2. Introduction

Sex allocation is the relative investment in male and female sexual functions. Most plant species are hermaphrodites; in these species sex allocation refers to the investment in male and female organs within a flower (Charlesworth, 2006). Sex systems that partition male and female functions between unisexual flowers (dicliny), such as monoecy, are less common, but may confer an advantage as sex ratios can be changed in response to resource availability, or to the optimum value given the mean gender of the population (Charlesworth & Morgan, 1991; Ashman & Diefenderfer, 2001; Dorken & Barrett, 2003). Most often plants will minimize investment in expensive female flowers, and develop a male-biased sex ratio (Delph & Lloyd, 1991). A female-biased sex ratio may be advantageous in less resource limiting environments, or when other individuals have male-biased sex ratios (Ashman & Diefenderfer, 2001; Stehlik *et al.*, 2008).

Flowers are arranged on inflorescences, and these can vary in the branching pattern and position of flowers in a range of forms: panicles, racemes or cymes (Schoen & Dubuc, 1990; Ashman & Hitchens, 2000; Prusinkiewicz *et al.*, 2007; Ortiz *et al.*, 2009). Inflorescence architecture, the branching pattern of inflorescences and position of flowers, is dictated by the frequency and pattern in which axillary meristems and floral meristems are produced by the inflorescence meristem and the pattern of internode lengths (Prusinkiewicz *et al.*, 2007). Understanding inflorescence architecture is particularly important in the study of sex ratios in monoecious plants, as flowers of each sex are often produced only at certain points on an inflorescence (Dorken & Barrett, 2003).

Begonia is a large genus (>1500 species), where almost all species are monoecious and functionally protandrous (Tebbit, 2005). *Begonia* have conserved inflorescence architectures, always maturing from the bottom up (acropetal), and usually produce male flowers at each branch point and female flowers at the terminals of each branch (Matzke, 1938). *Begonia* species are pollinated by deceit, as female flowers are non-rewarding intersexual mimics of males, which deliver a pollen reward (Ågren & Schemske, 1991; Castillo *et al.*, 2002). Sex ratios may be under sexual selection as female-biased inflorescences are less attractive to pollinators, while increasingly male-biased inflorescences will set less seed (Castillo *et al.*, 2002). Sex ratios within individual *Begonia* plants can change in response to pollen availability (Lopéz & Domínguez, 2003), showing that sex ratio is a flexible trait of importance in natural populations.

Here we investigate the genetic control of sex ratio determination in *Begonia* species. Sex ratios and inflorescence branch patterns are scored in backcross (BC) mapping populations between Central American *B. plebeja* Lieabm. and *B. conchifolia* A. Dietr. (Fig. 6.1; Brennan *et al.*, submitted), which differ in their sex ratios and branching patterns. This allows the number of chromosome segments and their effects to be studied by quantitative trait locus (QTL) mapping, without a priori assumptions of gene homology from model species. We also QTL map other reproductive traits (days to first flower, stamen number, pollen stainability and seed set), to compare the number of QTL and their location between reproductive traits. Understanding the genetic control of sex ratios in *Begonia* species, in particular through changes in inflorescence architecture, will enable us to better understand how monoecious species change their sex ratio in response to selection.

Figure 6.1. Study species. *Begonia plebeja* (left) and *B. conchifolia* (right). White bar is approximately 10cm.

6.3. Material and method

6.3.1. Study species and the generation of backcross populations

Begonia plebeja and *B. conchifolia* were chosen as they differ in reproductive strategies (Fig. 6.2). *Begonia plebeja* is widespread in dry forests in Mexico, and produces few large inflorescences, typically 3-12cm in diameter, with approximately 40 flowers (Burt-Utley, 1985;

A.D. Twyford, pers. obs.). These flowers are c.2cm in length (stigma-capsule length), and typically have between 11 - 21 stamens (Burt-Utley, 1985; Table 6.2). *Begonia conchifolia* is restricted to wet tropical forests in Costa Rica, and produces many small inflorescences (2 - 10.5cm diameter), which have around 20 flowers. These flowers are 3 - 7mm in length, and have fewer stamens (6 - 13) (Table 6.2; Burt-Utley, 1985). Morphological (Burt-Utley, 1985) and preliminary molecular phylogenetic analyses (N. Harrison, University of Edinburgh, pers. comm.) using nuclear and plastid sequence data support the species as closely-related, and they produce an F1 that is partly fertile (Brennan *et al.*, submitted).

QTL analyses compare fixed genetic differences between parents of a cross, which may differ from average species values due to intraspecific polymorphism. In this case the parents of the backcross differed in inflorescence branch patterns and symmetry in flower production (Fig 6.2), which are important traits as they may be mechanisms responsible for modifying the sex ratio. The *B. plebeja* parent was roughly symmetrical in branching, and most branches terminated at the same tier (Fig. 6.2a). The *B. conchifolia* parent was asymmetric in branching, and some branches terminated early in a female flower (Fig. 6.2b). The number of female flowers on each half of the inflorescence (i.e. all branching points that arise from one of the two first branches; grey square in Fig. 6.2) was also asymmetric, with *B. plebeja* producing more female flowers on one side of the inflorescence than the other (Fig. 6.2).

The parents of the cross are non-inbred cultivated accessions of unknown provenance. F1 hybrids were generated by pollinating a plant of *B. plebeja* with pollen from a *B. conchifolia* individual, using the pollination procedure described in Chapter 4. F1 plants were raised to flowering on sterilised compost (16 bark: 3 peat: 1 perlite plus finely sieved osmocote) in a glasshouse at 28°C, and used to generate backcross populations. One F1, labeled CKB137_8, was used as pollen recipient to the *B. plebeja* parent, to generate the *B. plebeja* backcross (hereafter PBC) population, the focus of this study. An additional population, generated using a different F1 plant (CKB137_6), was produced using *B. conchifolia* as the pollen donor (*B. conchifolia* backcross, CBC hereafter). This comparative mapping approach allows QTL to be compared in the different genetic backgrounds.

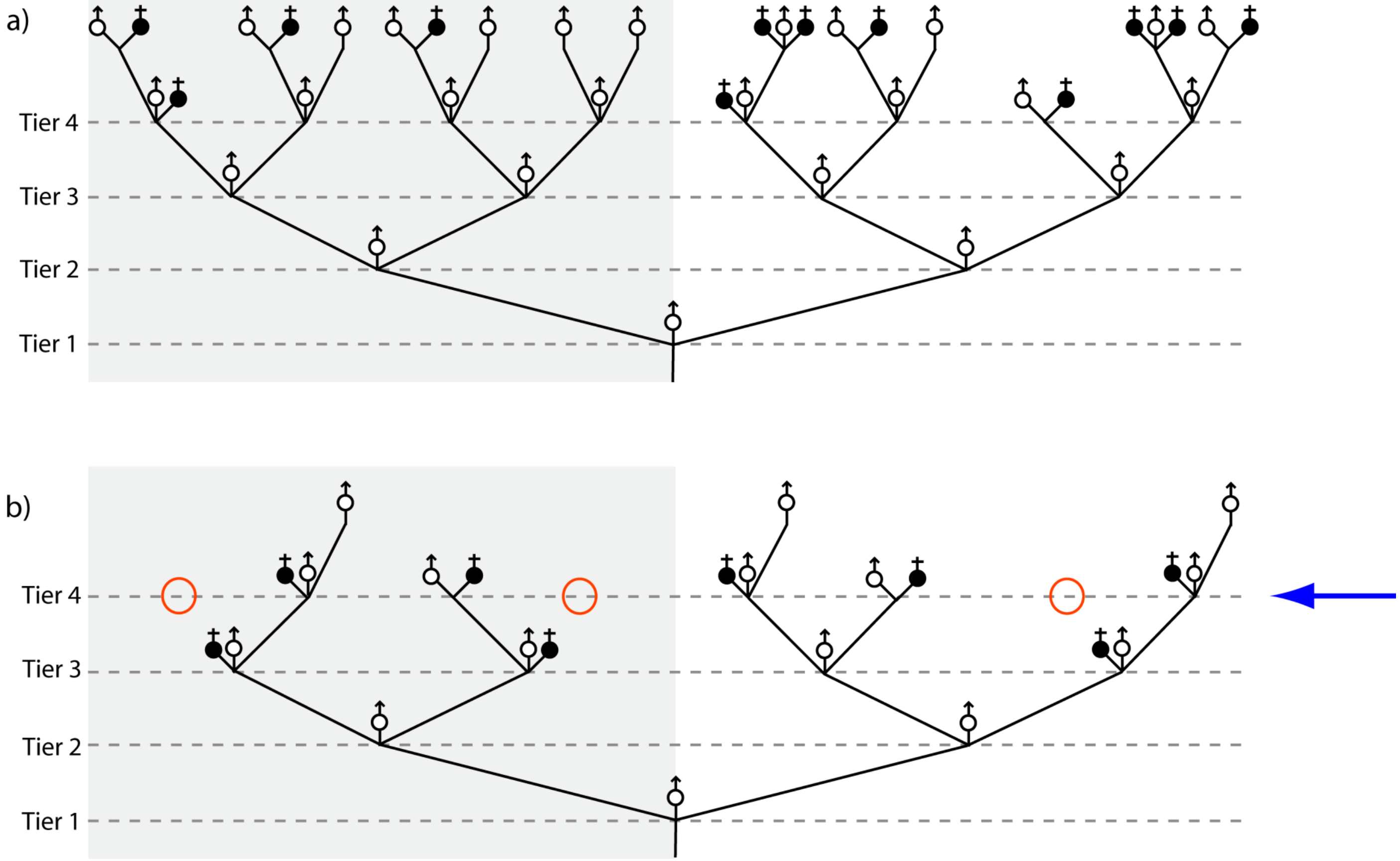


Figure 6.2. Representative inflorescence architecture for *Begonia plebeja* (top) and *Begonia conchifolia* (bottom). Male and female flowers are shown on inflorescence branches (solid black lines). Tiers of branches are shown by dashed grey lines. The blue arrow shows an unfilled tier. Red circles show where predicted branches are missing (branch asymmetry).
The large light grey square represents one half of the inflorescence.

Reproductive and inflorescence architecture traits were scored in the two segregating backcross populations (PBC 225 individuals, CBC 117 individuals) and at least 8 clonal replicates of the parents. The mapping populations and parents were grown in the same glasshouse conditions (described above) throughout the phenotyping. *Begonia* can change their sex ratios in response to pollen loads (Lopéz & Domínguez, 2003), and the closed glasshouse conditions mostly prevented pollinating insects visiting, so sex ratios can be observed under pollen-starved conditions.

6.3.2. Phenotyping

Fourteen reproductive traits were scored in the backcross populations, listed in Table 6.2. Traits were scored on the first inflorescence except where noted below, and measured as follows. A diagram was drawn of the inflorescence branching structure and the position of male and female flowers, and this was updated twice weekly through the flowering season. The number of male and female flowers was calculated from the inflorescence diagram once flowering had finished. The sex ratio was calculated as the number of male flowers over the total number of flowers across all inflorescences on a plant. The number of tiers of branches (tiers herein) was counted from the inflorescence diagram (Fig. 6.2), as a measure of inflorescence size. Inflorescence asymmetry was calculated by scoring the proportion of flowers of each sex on one half of the inflorescence (i.e. all branching points arising from one of the two first branches; shaded grey on Fig. 6.2) out of the total produced on the inflorescence. This was calculated separately for male (asymmetry males) and female flowers (asymmetry females), and standardised by recording the frequency on the side of the inflorescence with less of the given sex, giving values between 0 and 0.5.

Additional traits that were scored were stamen number and flower time, and two measures of fertility (pollen stainability and seed set). The stamen number was counted on the first flower on the first inflorescence. The tepals were removed and the stamens counted at 10x magnification. The date the first male and female flower opened was recorded, and used to calculate the days to first flower for each sex. To measure pollen sterility, 200 pollen grains from a subset of 125 PBC plants were stained with acetocarmine, and the percentage well-stained recorded (as in Chapter 3). Well stained pollen grains are likely to be viable (Matthews, 2007). Seed set was measured using 48 PBC plants as pollen recipients to *B. plebeja*, allowing seed set to be assessed independent of pollen viability (Sweigart *et al.*, 2006). Crosses were performed as in Chapter 4. The number of seeds per capsule was estimated with the formula of Ågren & Schemske (1993; see Chapter 4).

The developmental decisions that give rise to different branch patterns were scored in three different ways in the PBC. Two measures compared the number of male and female flowers from each tier to the model of Cozza (2008), which assumes a male flower and two branches at each internal node, and a female flowers at the branch terminals (Table 6.1). The first measure was the number of tiers with fewer male flowers than predicted by the model (unfilled tiers, herein; blue arrow Fig. 6.2). As male flowers occur at branching points, this represents the number of tiers where branches have terminated early. The second measure was the sum of the fraction of missing nodes in each tier (branch asymmetry herein; red circle Fig. 6.1); unlike the measure of unfilled tiers, this incorporates the magnitude of asymmetry at each tier. For these two measures, the final tier was ignored as it is always unfilled. Finally, the developmental decisions that give rise to branch patterns were compared on a tier-by-tier basis. The proportion of female flowers or axillary branches that arise from each node was averaged across the tier; male flowers were ignored because there is always one per internal node (Fig. 6.2).

Table 6.1. Predicted branching patterns in a symmetrical *Begonia* inflorescence. Adapted from Cozza (2008)

Number of levels of branching (tiers)	Number of male flowers	Number of female flowers	Total flowers	Sex ratio
1	1	2	3	0.33
2	3	4	7	0.43
3	7	8	15	0.47
4	15	16	31	0.48
5	31	32	63	0.49
6	63	64	127	0.50

6.3.3. Segregation patterns and quantitative analyses

Quantitative analyses were performed separately for the 14 traits from the 2 populations. The distribution of each trait was assessed by drawing histograms. Thirteen traits were then tested for transgressive segregation with the approach of Kim & Rieseberg (1999). Transgressive segregation for flower time could not be assessed as seeds were not available from the parent plants for comparison. Non-normally distributed traits were first transformed to fit the assumption of normality, count data was square root transformed and proportions arcsine transformed. Traits were considered transgressive if the number of progeny that exceed the higher parental value by 2, 3, or 4 standard deviations, was greater than the number expected by chance given the population size. Trait values for the parents were the average scores from at least 8 clonal replicate plants.

Many traits are not expected to be independent of each other, such as sex ratio and number of male and female flowers, and this was tested by correlation analysis between a subset of reproductive traits. Fifteen correlation analyses were performed, listed in Table 6.3. Pearson's correlation analyses were performed in Minitab Statistical Software (www.minitab.com), and P-values were adjusted with Bonferonni correction to account for multiple testing. The correlations between traits were compared to the location that QTL mapped to (below), to test whether related traits actually represent a single trait.

Measures of vegetative vigour from a parallel QTL study (Mobina Shaukat Ali, University of Edinburgh, pers. comm.) were compared with reproductive output (number of inflorescences, inflorescence tiers, number of flowers), to test whether reproductive output is constrained by the size of the plant (Schoen & Dubuc, 1990). For each PBC plant, the largest leaf was harvested 6 months after germination, photographed against a black background, and the surface area calculated using LAMINA (Bylesjö *et al.*, 2008). The mass of this leaf was then measured after drying at 60°C for 5 days (dry mass). The number of leaf scars along each rhizome was added to number of leaves, representing the number of leaves produced in the life of the plant (number of leaves herein). The number of leaves was multiplied by the leaf surface area to estimate a fourth proxy of vigour, total leaf surface area. Correlation analyses were performed between the four proxies of vigour and the three proxies of reproductive output using Minitab, with P-values adjusted using Bonferonni correction.

6.3.4. QTL analyses

QTL analyses were performed using the genetic map of Brennan *et al.* (submitted). The map is constructed using co-dominant SNP markers in candidate developmental genes, and dominant AFLPs. The combined dataset for the PBC was 162 markers (80 AFLPs and 82 SNPs) for 225 individuals, and for the CBC 155 markers (74 AFLPs and 81 SNPs) for 117 individuals. The markers were spread across the 14 distinct linkage groups, and the maps were mostly syntenous in their marker order. The total length of the *B. plebeja* map was 1099-1110cM, and the *B. conchifolia* map 1034-1043cM (depending on the approach used; Brennan *et al.*, submitted).

QTL were detected with composite interval mapping, which accounts for co-factors (other QTL that affect a given trait). All QTL analyses was performed in QGene v4.0 (Joehanes & Nelson, 2008). Step-wise cofactors were selected, and the scan interval set to 10cM. The significance of each trait ($P < 0.05$, < 0.01) was tested using 1000 permutations. Traits that were not normally distributed were transformed prior to QTL analysis (as above).

The data for average proportion of axillary branches or female flowers per node, for each tier, were skewed and could not be transformed to a normal distribution. These traits were first analysed using composite interval mapping, as above, which is usually robust to traits that deviate from normality (Knott, 2005). These QTL results were confirmed with non-parametric analyses; traits were coded as ordinal data and used in single-marker analyses in QGene.

The likelihood of finding significant QTL may be reduced when genotype data are missing. Therefore, a second dataset was prepared, where missing BC genotypes were replaced with the most likely genotype inferred from the surrounding markers. Genotypes were arranged in map order in a MS Excel spreadsheet, and different colours used for homozygote and heterozygote genotypes. Missing genotypes within a colour block were filled in assuming no recombination is more likely than two recombination events. Missing data was not imputed at the ends of linkage groups, or where the surrounding markers show a genotype transition. All analyses where marginally significant QTL (i.e. $P = 0.05 - 0.10$) were detected, were then repeated using the imputed dataset.

6.3.5. Candidate gene detection

The genetic basis of QTL localizing between AFLP markers were not investigated further. Those located within ~5cM (~2.4Mb; Brennan *et al.*, submitted) of a SNP marker were investigated for potential candidate genes. First, the transcriptome isotig from which the SNP marker was designed was BLAST searched against the draft *B. conchifolia* genome (Kidner, unpubl. data), to retrieve the surrounding sequence. The open reading frames (ORFs) within the genome contig were then located with the EMBOSS tool getorf (Rice *et al.*, 2000). Finally, ORFs were BLAST searched against the annotated protein sequence database for *Arabidopsis* (TAIR; Swarbreck *et al.*, 2008), to find the putative function of the surrounding genes. For each likely match ($e < 10^{-40}$) the gene name, gene ontology (GO) annotation, expression profile, and mutant phenotypes were recorded.

6.4. Results

6.4.1. Trait segregation

The two species differed for most reproductive traits that were measured (Table 6.2). *B. plebeja* produced fewer inflorescences than *B. conchifolia*, and these inflorescences had more tiers of branching, and double the number of male and female flowers. *Begonia plebeja* had inflorescences with roughly symmetrical branching, while *B. conchifolia* had 23% fewer internal branches than predicted by the model of Cozza (2008), and half the tiers did not have the full complement of branches. These measures of inflorescence branching had large variances in the parents (Table 6.2). The distribution of male flowers was roughly equal, but both species had an unequal distribution of females across the inflorescences, and this was more pronounced in *B. plebeja*. *Begonia conchifolia* flowers had two-thirds fewer stamens than *B. plebeja*.

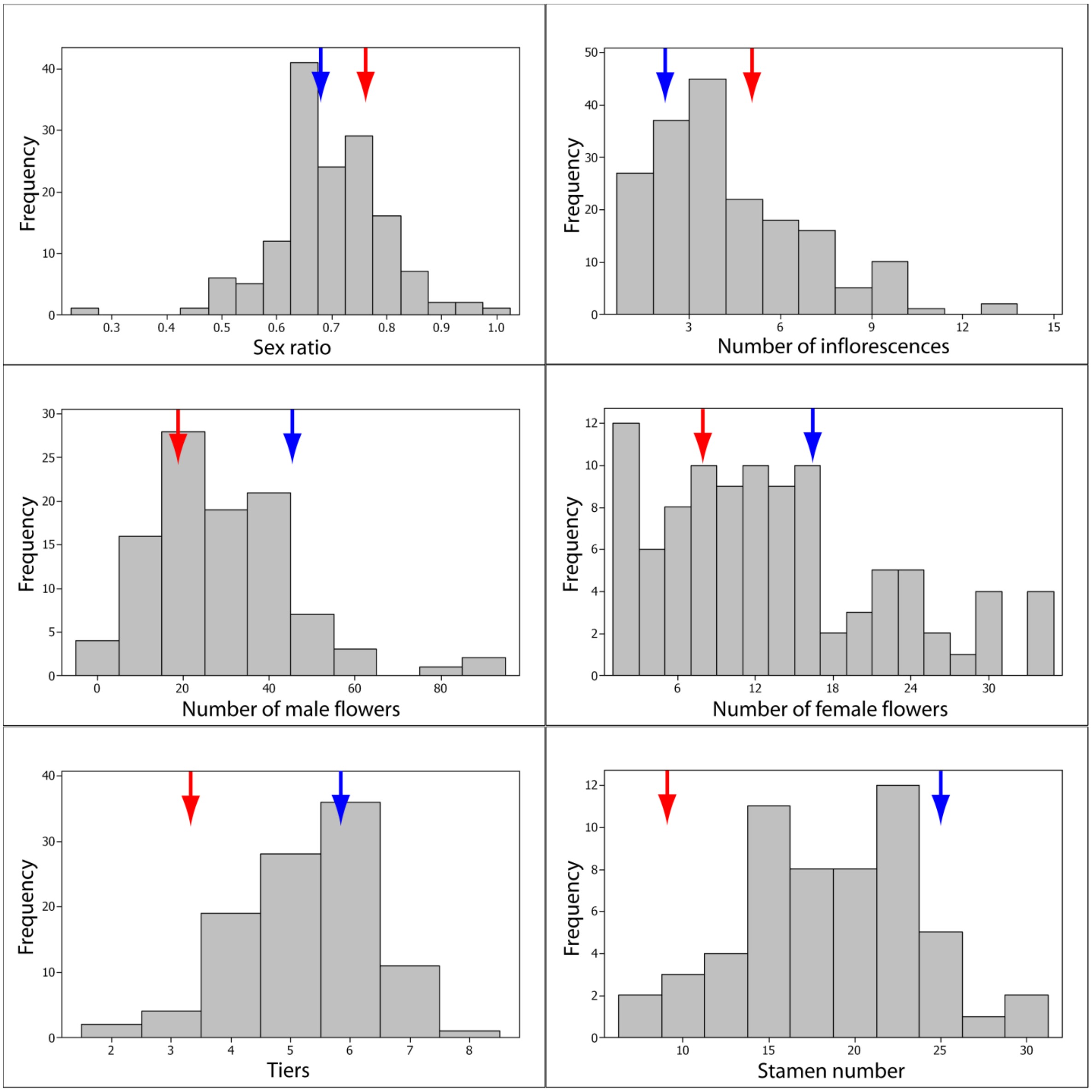


Figure 6.3. Trait segregation in the PBC population. Blue arrow: *B. plebeja* average parental value; red arrow, *B. conchifolia* average parental value.

All trait differences segregated in the backcross populations (Fig 6.3). Some PBC individuals exceeded parental values for ten traits (number of inflorescences, male flowers, female flowers, stamen number, number of tiers, branch asymmetry, unfilled tiers, asymmetry males, asymmetry females, sex ratio). Transgressive segregation was found for seven traits, including: sex ratio (P

< 0.001), four measures of asymmetry (unfilled tiers, P < 0.01; branch asymmetry, P < 0.05; asymmetry males, P < 0.05; asymmetry females, P < 0.05) and two other traits (number of inflorescences, P < 0.001; number of tiers, P < 0.05). Transgressive segregation was only found for a single trait in the CBC, number of male flowers (P < 0.05). Transgressive segregation could not be tested for number of inflorescences as the data could not be transformed to fit the assumption of normality.

Table 6.2. Average trait values for the parents of the mapping populations, with standard errors in brackets (n = 8).

Trait	***B. plebeja***	***B. conchifolia***
Number of inflorescences	2.1 (0.3)	5.2 (0.8)
Male flowers 1st inflorescence	38 (6.4)	19.1 (4.4)
Female flowers 1st inflorescence	16.7(3.7)	7.9 (3.2)
Total flowers 1st inflorescence	54.7 (9.6)	27.0 (7.5)
Number of tiers 1st inflorescence	5.9 (0.4)	3.3 (0.6)
Sex ratio	0.68 (0.03)	0.76 (0.03)
Unfilled tiers	0.13 (0.13)	0.5 (0.34)
Branch asymmetry	0.05 (0.05)	0.23 (0.17)
Asymmetry males	0.46 (0.08)	0.45 (0.03)
Asymmetry females	0.24 (0.09)	0.33 (0.02)
Days to first male flower	n/a	n/a
Days to first female flower	n/a	n/a
Pollen viability	> 90%	> 90%
Seed set	n/a	n/a
Stamen number (flower from 1st tier 1st inflorescence)	25.0 (0.9)	7.9 (0.7)

Only one significant correlation was found in the 12 pairwise comparisons between four proxies of vegetative vigour (area largest leaf, leaf dry mass, number of leaves, total leaf surface area) and three proxies of reproductive output (number of inflorescences, number of tiers and total flowers on the first inflorescence) after correcting for multiple testing. This was between the number of leaves and the number of inflorescences ($r^2 = 0.296$, $P < 0.05$).

Significant correlations were found between the number of flowers of each sex and the number of tiers on an inflorescence, with a greater proportion of variance explained for male than female flowers (Table 6.3). Similarly, the likelihood of producing axillary branches (as opposed to female flowers) on tiers 4 and 5 explained more variance in the male than female flowers. The number of tiers and number of inflorescences were weakly correlated, but no correlation was found between the number of flower on an inflorescence and the number of inflorescences, or the sex ratio and number of tiers. Stamen number was not correlated with number of male flowers.

Table 6.3. Pearson's correlation between reproductive traits. * P < 0.05; ** P < 0.01

Traits		Correlation
Number of male flowers	Number of female flowers	0.728**
Number of male flowers	Total flowers	0.970***
Number of female flowers	Total flowers	0.874**
Number of male flowers	Number of tiers	0.836**
Number of female flowers	Number of tiers	0.758**
Total flowers	Number of tiers	0.861**
Sex ratio	Number of tiers	-0.248
Sex ratio	Total flowers	-0.272
Number of male flowers	Axillary branches tier 4	0.775**
Number of female flowers	Axillary branches tier 4	0.668**
Number of male flowers	Axillary branches tier 5	0.823**
Number of female flowers	Axillary branches tier 5	0.650**
Tiers	Number of inflorescences	0.261*
Total flowers	Number of inflorescences	0.128
Stamen number	Number of male flowers	0.056

6.4.2. QTL mapping

Five significant QTL were detected for reproductive traits in the PBC, and these were for different measures of inflorescence branching or sex ratios (Table 6.4; Fig. 6.4). Two marginally significant QTL (P = 0.05 - 0.10) were significant when missing genotype data were imputed (pollen sterility, stamen number). QTL contributed 12.2 - 30% of the phenotypic variation explained (PVE), with only a single trait, stamen number, controlled by a locus of large effect (i.e. PVE >25%, Kim & Rieseberg, 1999, and references therein). The 7 QTL were distributed across 4 linkage groups, with 4 QTL found on linkage group 11. Two of the QTL, for number of flowers and number of female flowers, overlapped in their one-LOD support interval. In addition, a marginally significant QTL ($P < 0.1$) was found for female flower time in the PBC [linkage group 2, LOD = 3.428, PVE = 19.2%, position 36cM (33.0 - 37.0, 1 LOD support range)]. The full QTL data are given in Appendices 9 and 10.

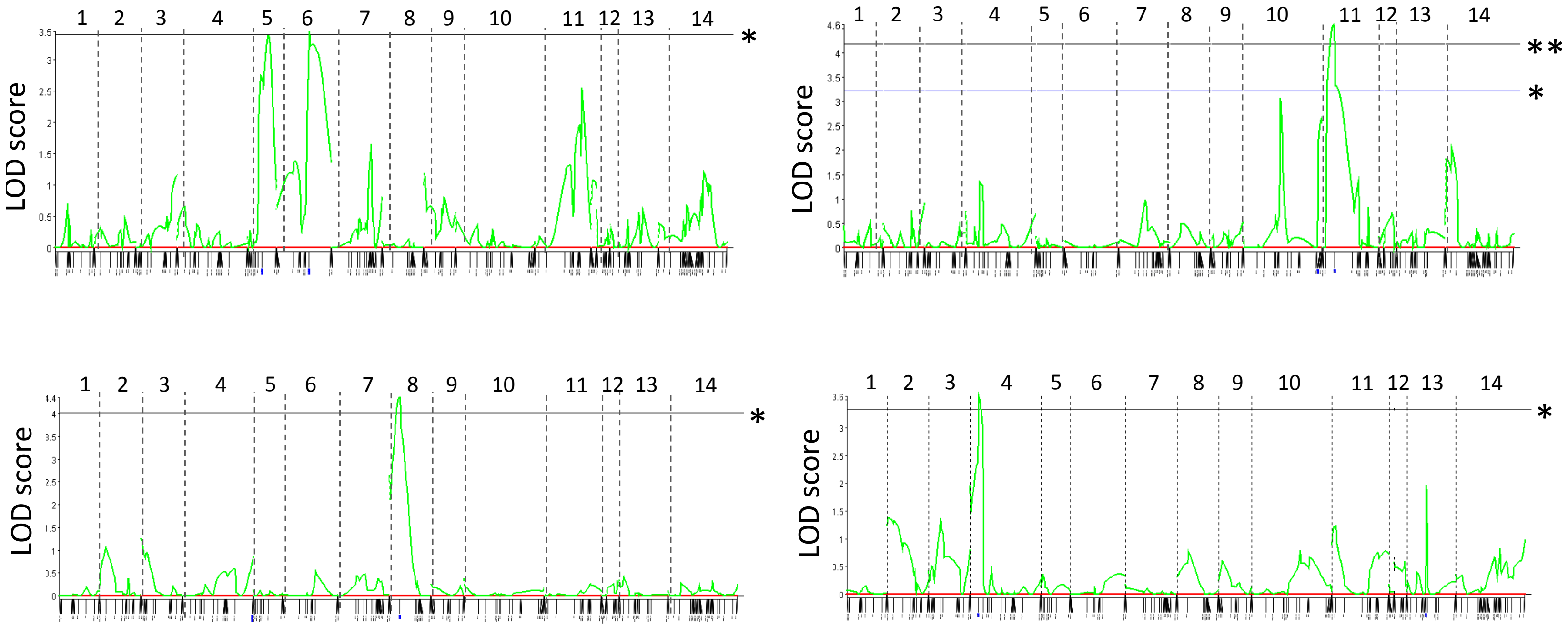


Figure 6.4. Representative QTL plots for traits in the PBC: number of male flowers (top left), sex ratio (top right), stamen number (bottom left, shown for imputed data), pollen stainability (bottom right). The LOD score (green line) is plotted against each marker (bottom axis) on a given linkage group (top axis). The imputed threshold is indicated by a solid horizontal lines, * P < 0.05; ** P < 0.01.

Two QTL were found in the CBC, both for sex ratio, and these explained 19-22% of the phenotypic variation. One QTL for sex ratio in the CBC mapped to the same linkage group, and overlapped in 1-LOD interval, as the male flower QTL in the PBC.

Additional QTL were found for meristem decisions at each tier of branching (Table 6.5). Significant QTL were found with single-marker regression for tiers 4, 5 and 6, with the QTL for axillary branches and female flowers on linkage group 6 overlapping in their 1-LOD interval. Only one of these QTL, for female flowers on tier 4 (linkage group 6), was also found with composite interval mapping. This QTL overlapped in 1-LOD interval with the PBC QTL for number of male flowers, and was on the same linkage group as a sex ratio QTL in the CBC (Table 6.4).

6.4.3. Candidate genes

Eleven QTL localised within 5cM of a SNP marker, with 9 of the 11 SNPs being different. Five SNPs had ORF matches to genes in the *Arabidopsis* database, however none of these matched candidate genes directly related to inflorescence or flower development, or transcription factors.

Table 6.4. Putative QTL for reproductive traits in two backcross *Begonia* populations. Imputed values are reported in brackets after the original dataset. PVE: percentage variance explained. Population: PBC, *B. plebeja* backcross; CBC, *B. conchifolia* backcross. The direction of additive effects are shown in square brackets after the value; C, *B. conchifolia* alleles overrepresented; P, *B. plebeja* alleles overrepresented. ^{ms}, marginally significant 0.10 > P > 0.05; * P < 0.05; ** P < 0.01.

Population	**Trait**	**Linkage group**	**LOD score**	**PVE (%)**	**Additive effect**	**Position cM (1 LOD interval)**	**Nearest SNP**
PBC	Pollen sterility	4	3.061^{ms} (3.569*)	10.6 (12.3)	10.039 [C] (13.484, C)	11.0 (10.0-16.0)	BLP1
PBC	Number of male flowers	6	3.434*	14.5	13.004 [P]	41.0 (38.0-58.0	n/a
PBC	Number of female flowers	11	3.396*	14.8	6.491 [P]	67.6 (62.6-71.6)	BMYBTF5
PBC	Total number of flowers	11	4.007*	16.7	18.494 [P]	71.6 (69.7-76.6)	BMYBTF5
PBC	Asymmetry males	11	4.133*	18.7	0.11 [C]	59.6 (58.6-62.6)	BSBP1
PBC	Sex ratio	11	4.575**	13.4	0.077 [C]	27.6 (18.6-29.6)	BP2
PBC	Stamen number	8	3.311^{ms} (4.4*)	23.8 (30.0)	5.264 [C] (5.594, C)	27.7 (19.7-39.7)	BDFL2
CBC	Sex ratio	6	3.557*	19.9	4.704 [C]	58.0 (57.0-60.0)	BEIN1, BMYBTF6
CBC	Sex ratio	12	3.987*	22.0	0.08 [C]	21.0 (13.0-34.0)	BHP1

Table 6.5. QTL mapping of meristem decisions and inflorescence branching in the PBC. Phenotypic scores are the average probability of producing female flowers or axillary meristems across meristems at the given tier of branching. Analysis: SMR, single-marker regression; CIM, composite interval mapping.

Analysis	**Trait**	**Linkage group**	**LOD score**	**PVE (%)**	**Additive effect**	**Position cM (1 LOD interval)**	**Nearest SNP**
CIM	Tier 4 female flowers	6	3.86*	15.9	0.209 [C]	51.0 (37.0-64.0)	n/a
SMR	Tier 4 female flowers	6	4.146**	16.9	7.246 [C]	41.5 (37.6-68.6)	n/a
SMR	Tier 4 axillary branches	6	3.661**	15.1	7.232 [P]	41.5 (37.6-68.6)	n/a
SMR	Tier 5 axillary branches	5	2.581*	11.5	5.045 [P]	13.2 (0-36.4)	BTF33
SMR	Tier 6 axillary branches	5	2.16*	9.2	0.859 [P]	0 (0-19.2)	BTF33

6.5. Discussion

6.5.1. QTL for reproductive traits in *Begonia* mapping populations

The two study species shared the same inflorescence architectures (i.e. branch pattern and positional differences in flower sex), but differed in their sex ratios, with *B. conchifolia* having a more male-biased sex ratio than *B. plebeja*. Differences in sex ratios could not be explained by the number of tiers on an inflorescence (Table 6.1, 6.3), nor by developmental constraint of investment in reproductive structures as more vigorous plants did not produce bigger inflorescences (or have more female-biased sex ratios). Correlation analyses in the PBC showed the number of tiers strongly dictates the number of male flowers per inflorescence ($r^2 = 0.970$, $P < 0.01$), but the number of female flowers is less closely linked to architecture ($r^2 = 0.874$, $P < 0.01$), as found in *Begonia urophylla* Hook. (Cozza, 2008). This raises the prospect that genes underlying inflorescence architecture affect both male and female flower number, with additional genes further modifying the number of female flowers.

The differences in sex ratios between parents (Fig. 6.2) allowed genetic analysis through QTL mapping. Five QTL were found for traits related to sex ratios in the PBC (number of males, number of females, total flowers, asymmetry in males, sex ratio; Table 6.4). However, the strong correlation between many of these traits in the PBC (Table 6.3), and the co-localization of 4 QTL to linkage group 11, suggests these traits are not independent. This is most obvious for number of female and total number of flowers, where QTL overlapped in their 1-LOD interval and had similar additive effects. Whether the other QTL represent different genes is hard to tell with the sparse marker coverage on linkage group 11. Correlations between traits in natural populations would help distinguish the independence of traits.

To investigate the way these QTL influence the phenotype, we investigated meristem decisions (female flower or axillary branches) at each tier. The most striking result was that a QTL for number of male flowers mapped to the same position on linkage group 6, as a QTL for meristem decisions on tier 4 in the PBC. This meristem decision QTL also co-localised with a sex ratio QTL in the CBC. This supports *Begonia* inflorescences differing in their sex ratios by terminating some inflorescence branches in a female flower earlier than others. This gains further support from the strong correlation between meristem decisions at tier four and the number of both male and female flowers (Table 6.3). An alternative mechanism that sex ratios could differ, independent of branching, is by selectively aborting male or female flowers. Cozza (2008) suggested that *Begonia urophylla* selectively aborts female flowers, as shown by the weaker correlation between inflorescence architecture and female flowers, than with male flowers. Moreover, two small female buds are visible at each branch terminal, not all of which develop. It also seems likely selective abortion of female flowers occurs in the PBC, as the correlation between sex ratio and number of tiers of branching or total flowers was not significant after Bonferonni correction, and the correlation only explained a modest amount of variance in the sex ratio.

Some QTL for sex ratio may be shared between species, as shown by sex ratio QTL in the CBC mapping to syntenous markers to a QTL for number of male flowers in the PBC. Whether the second QTL for sex ratio in the CBC (linkage group 12) represents a novel mutation, as it had no similar QTL in the PBC, is currently unclear, as a different F1 parent was used to generate the two backcross populations. Moreover, the CBC had a smaller population size and so the ability to detect QTL is reduced.

Comparisons of QTL for inflorescence traits with QTL for vegetative traits will be necessary to more fully understand the interacting mechanisms that give rise to differences in sex ratios. The non-independence of vegetative and inflorescence traits is suggested by the moderate amount of variance explained in the number of inflorescences by the number of leaves on a plant ($r^2 = 0.296$, $P < 0.05$). It will also be important to understand phenotypic plasticity in reproductive traits under different environments with different levels of resources available, or with different rates of pollinator visitation, as these factors have been found to affect sex ratios in *Begonia* (Lopéz & Domínguez, 2003).

In addition to QTL relating to sex ratios, a QTL of large effect was found for stamen number (30% VE, imputed data), which differs greatly between the two mapping parents (Table 6.2). This shows the different reproductive strategies of the species, with *B. plebeja* making few large inflorescences with expensive flowers (large tepals, many stamens), and *B. conchifolia* more inflorescences with less expensive flowers (smaller tepals, fewer stamens). The genetics of stamen number are not well understood in model systems, in part due to the conserved stamen number of 6 found in all Brassicaceae species, preventing mapping of interspecific differences between *Arabidopsis* species (Edwards & Weinig, 2011). However, candidate genes for differences in stamen number would include those that control meristem activity such as *AGAMOUS* (Yanofsky *et al.*, 1990) and *CLAVATA* (Clark *et al.*, 1993). Having a single locus of large effect controlling stamen number has important evolutionary implications, as an allele at this locus could be quickly fixed by strong selection (Louthan & Kay, 2011), or may be lost by genetic drift in small populations. Future fine mapping and comparisons of sequence variation at this locus in other *Begonia* species may elucidate more about this important reproductive trait.

A single QTL of modest effect (12.3% VE, imputed data) was found for pollen sterility. The position of the sterility QTL does not correspond to markers that segregate in a non-Mendelian manner (transmission ratio distortion), nor map close to recombination breakpoints (Brennan *et al.*, submitted). Phenotyping more individuals, or having more markers on the map, will help resolve the genetic basis of pollen sterility in the *Begonia* BC population. This warrants further work as pollen sterility is common in F1 crosses in *Begonia* (Matthews, 2007; Dewitte *et al.*, 2011), and plays an important role in maintaining species identities in secondary contact (Chapter 5). Different genetic architectures have been found for pollen sterility in other organisms. Two pollen sterility QTL of large effect were found in a BC_1 *Helianthus* mapping population (*H. annuus* x *H. debilis* ssp. *cucumerifolius*; 38.2-38.8% VE; Kim & Rieseberg, 1999), whereas pollen sterility was polygenic in a higher resolution BC_2 map between different species (*H. annuus* x *H. petiolaris*; 11 QTL, 5-26% VE; Lai *et al.*, 2005).

6.5.2. Candidate genes for inflorescence development in *Begonia*

Begonia species have a distinct inflorescence architecture (Fig. 6.2; Cozza, 2008), and this will be determined by the position of floral and inflorescence meristems (Prusinkiewicz *et al.*, 2007). In *Begonia* the developing inflorescence meristem rapidly undergoes a transition to flower meristem, terminating the inflorescence in a male flower. After this, two new axillary inflorescence meristems develop, which give rise to subsequent branches and flowers. This sympodial pattern of inflorescence architecture bears some resemblance to tomato (*Solanum* *lycopersicum* L.), except that the axillary meristems in tomatoes first produce leaves (i.e. they start as vegetative meristems before becoming inflorescence meristems) (Park *et al.*, 2012), whereas in *Begonia* they produce inflorescences. A gene that may play a role in inducing axillary inflorescence meristems is *TERMINAL FLOWER 1* (TFL1; Alvarez *et al.*, 1992; Liljegrena *et al.*, 1999; Conti & Bradley, 2007), as *TFL1* is involved in shoot meristem activity, and *tfl1* mutants produce flowers instead of shoots on the inflorescence (Bradley *et al.*, 1996). *FLOWERING LOCUS F* (*FT*) has the opposite function (Hanano & Goto, 2011), and could be responsible for terminating inflorescences in a flower.

This study investigated the ways that monoecious plants can change their sex ratios; however, the molecular genetic mechanisms for determining unisexual flowers are still unclear. Understanding sex determination in *Begonia* inflorescences will help shed light on how monoecy has evolved and is maintained. Studies of sex determination in cucumbers, which are also monoecious, and are in the same order as *Begonia* (Curcubitales), have shed some light on this question, and some mechanisms may be conserved. One hypothesis proposes that sex determination is hormonally regulated. Ethylene, stimulated by auxin, arrests anther development in female flowers, and the positional sex effects in *Begonia* inflorescence may be due to lower expression of ethylene receptors (e.g. CsETR1) at more distal nodes as is the case in cucumber (Wang *et al.*, 2010). Alternatively, differential expression of *AGAMOUS* (AG, Yanofsky *et al.*, 1990) in different flower meristems may cause sex differences. Two homologs of *AGAMOUS* (*CUM1* and *CUM10*) have been cloned in cucumber (*Cucumis sativus*), and these genes regulate floral organ identity, with mutants converting sepals into carpel-like structures and petals into stamens (*CUM1*), and petals into anther-like structure (*CUM10*) (Kater *et al.*, 1998). Studying hormonal effects on sex ratios, as well as candidate genes such as *AG*, will be important for understanding the evolution of monoecy. However, hundreds of genes are differentially expressed between unisexual cucumber flowers and gynodioecious mutants, including hormone signaling genes and transcription factors, suggesting the process of sex determination is complex (Guo *et al.*, 2010; Wu *et al.*, 2010).

6.5.3. Adaptive significance of inflorescence architectures

The distinct inflorescence architecture (bisexual, protandry, acropetaly) described here is common to most *Begonia* species. The large Asian section Petermannia (> 270 species) is an exception, as species are protogynous, producing a two flowered female inflorescence distal to a many-flowered male inflorescence (D. C. Thomas, University of Hong Kong, pers. comm.). There are also rare reports of dioecy (Tebbit, 2005) or plants with unisexual inflorescences (Forrest, 2000). It does however appear that most *Begonia* species share an inflorescence architecture with sex ratios related to the arrangement of flowers and the pattern of inflorescence branching. What are the reasons that inflorescence architectures are conserved with only minor modification across *Begonia*? Many other families have a conserved inflorescence type, and these include: the compound umbel of the Apiaceae (Umbelliferae), cymose capitulum of the Asteraceae (Compositae), ultimate spike of the Poaceae, spadix of the Araceae, and the helicoid cyme of the Boraginaceae (Tucker, 1999). Inflorescence types are even shared between related families (Endress & Doyle, 2009). One hypothesis is that a single inflorescence type needs only minor modifications to take a large range of forms. In this case, terminating inflorescence branches early can change the sex ratio in response to a variety of selection pressures, without changing the whole inflorescence form.

Begonia species tend have the joint syndromes of acropetaly, protandry, and bee pollination (Wyatt & Sazima, 2011). This combination of characteristics are predicted to be maladaptive (Darwin, 1877) as bees typically visit inflorescences from the bottom up (Harder & Barrett, 2000; Jordan & Harder, 2006), and this would cause frequent self-pollination and reduce pollen movement between plants (pollen discounting, Harder & Barrett, 1995). This assumes that the foraging paths of solitary bees follow those bee species that have been studied to date (Jordan & Harder, 2006), and it may be that temporal separation of sexes reduces self-pollination in these fully compatible species (Ågren & Schemske, 1993; Wyatt & Sazima, 2011). However, this inflorescence architecture may be advantageous in other ways. Most notably, producing cheap male flowers early in the flowering season may provide ample opportunities for cross-pollination, with the number of expensive female flowers determined later depending on the available resources.

The inflorescence architecture may also bring fitness benefits when the phase that flowers open across the inflorescence, which will alters the floral display as seen by a pollinator at a given time (the display architecture), is considered (Jordan & Harder, 2006). Producing rewarding male flowers first may be necessary to attract pollinators to visit the deceptive female flowers that follow (Ågren & Schemske, 1991; Wyatt & Sazima, 2011; Castillo *et al.*, 2012). Differences in display architecture that may be related to pollinator visitation can be seen between the study species, with *B. plebeja* producing a large display of female flowers on the terminal tier, whereas *B. conchifolia* has a longer period of time where a similar number of male and female flowers are open together (Fig. 6.2). It may therefore be that species differences in sex ratios could be a by-product of pollinator selection for display architecture. *Begonia conchifolia* inflorescences with female flowers only at the terminal tier may be selected against as the female-biased display would not be as attractive to pollinators, and instead inflorescences with females dispersed over multiple tiers next to male flowers may be favoured. In contrast, the larger terminal tier on a *B. plebeja* inflorescence has more positions available for female flowers to occur with male flowers. The smaller display of female flowers at a given time on *B. conchifolia* may also promote selfing (Harder & Barrett, 1995), which is supported by the more homozygous transcriptome of *B. conchifolia* relative to *B. plebeja* (*Brennan et al.*, submitted). Finally, inflorescence architectures will affect other factors not considered in this study, such as the transport of resources (Schoen & Dubuc, 1990), and the structural support of fruit (Stebbins, 1973), therefore this architecture may have evolved in response to other selection pressures.

CHAPTER 7: General conclusions

7.1. Evolution of diversity in *Begonia*

Studying the evolutionary mechanisms that promote diversification in speciose genera is difficult because of their unmanageable sizes, and because many different processes interact to shape patterns of species richness. For these reasons, evolutionary histories may be investigated in a subset of species where patterns are more broadly applicable to the group of interest. This thesis used genetic markers from two genomes (nuclear and plastid), in different contexts (phylogeography, population genetic, hybridization), to study population processes in two widespread *Begonia* species. The results have shed light on the processes that promote speciation in *Begonia*, and the main findings are outlined below.

First, *B. heracleifolia* had strong population genetic structure at plastid loci (Chapter 2). This shows that without mechanisms to promote seed dispersal, or any known seed dispersal vector, inferred seed dispersal distances are low and populations are weakly connected by seed-mediated gene flow. While seed dispersal is usually low relative to pollen dispersal (Ennos, 1994), and plays a limited role in maintaining species cohesion, it is essential for colonizing new habitats. Species with poor dispersal are less likely to invade other congeneric species, which can cause species collapse through introgression, especially in the early stages of speciation when reproductive barriers are weak.

Second, low pollen-mediated gene flow in these extremely widespread species leads to a break down in species cohesion (Chapter 4). *Begonia heracleifolia* has a distribution range of ~3200km, from West Mexico to Panama, and *B. nelumbiifolia* ~2000km, from Central Mexico to Honduras; high gene flow may be expected to maintain species cohesion over such distances. However, interpopulation pollen flow inferred from nuclear markers was low, with populations showing strong geographic structure. This result is supported by populations of *B. heracleifolia* being divergent in their morphology (leaf shape), as well as accumulating genetic incompatibilities causing interpopulation crosses to have a reduced fertility (Chapter 4). Genetic divergence may be ongoing in these species, with speciation a possible outcome. This is supported by narrow endemic species being present at the edge of the range of *B. heracleifolia*, with these species distinguished from *B. heracleifolia* by few morphological characters (e.g. *B. philodendroides* Zeisenh.). While a break down in species cohesion is consistent with the data, it may actually be that weak species cohesion is maintained by strong selection for advantageous alleles at loci for adaptive traits (Rieseberg & Burke, 2001; Morjan & Rieseberg, 2004). In this way genome wide divergence can occur (as observed with high mean F_{ST} at 9 nuclear microsatellites), and cohesion is maintained by low gene flow and strong selection at specific loci. This seems most possible for *B. nelumbiifolia*, given its wide distribution and morphological uniformity throughout its range. To definitively understand mechanisms of species cohesion, it will be necessary to understand levels of divergence across the genome, including at loci that may contribute to adaptive differences.

Third, both species were found to be self-compatible and have moderate levels of inbreeding (Chapter 4), similar to other *Begonia* species (Ågren & Schemske, 1993; Matolweni *et al.*, 2000). Inbreeding is common in herbaceous plants, which on average have a four-fold higher average F_{IS} value than woody plants (Duminil *et al.*, 2009). There is clear potential for inbreeding to promote the genetic isolation of populations by reducing interpopulation gene flow, and population structure measured with F_{ST} is correlated with breeding system estimates in plants (Duminil *et al.*, 2009). Inbreeding may therefore promote speciation in tropical herbs (Lasso *et al.*, 2011). Some *Begonia* species may have evolved to promote inbreeding by densely packing self-compatible flowers on inflorescences, while others discourage it by more strongly separating male and female flowers both spatially and temporally. Levels of inbreeding in *Begonia* are comparable with other understory tropical plants such as bromeliads, and like bromeliads outcrossing rates and morphologies to promote inbreeding appears to differ between species (Palma-Silva *et al.*, 2009).

Fourth, reproductive isolating barriers were relatively strong in sites of secondary contact between two pairs of species (Chapter 5). Species barriers were maintained by a number of pre- and post-zygotic reproductive isolating barriers, including differences in flowering time and F1 hybrid sterility. Such differences have also been found between divergent populations within *B. heracleifolia*, suggesting some of these barriers may have evolved during divergence. The complexity of reproductive isolation between diploid species in conjunction with strong population structure points to allopatric speciation as an important mode of speciation in the group. The genetic mechanisms that underlie traits that differ between species were then tested by QTL analysis, focusing on reproductive traits (Chapter 6). This analysis, as well as a parallel study of ecophysiological and micromorphological traits in the same BC populations (Mobina Shaukat Ali, unpubl. data) show that some QTL of moderate to large effect influence important species differences. Rapid evolution for such traits with a simple genetic architecture may reduce the likelihood of introgression between species.

Taken in concert, poor pollen and seed flow between populations, frequent self-fertilization, and reproductive barriers that evolve quickly, may pre-dispose the genus to rapid speciation. These processes are unlikely to be unique to *Begonia*, and the findings in this thesis are consistent with the predictions made for other large plant genera (Sanderson & Wojciechowski, 1996). In particular, support is given for species in large genera having a geographic population structure with restricted gene flow (Sanderson & Wojciechowski, 1996). An explanation for a second factor, that large genera are typically composed of many ecologically specialised species, can also be proposed from the data presented here. Rapid shifts in ecology may occur when selection acts on a trait with a simple genetic architecture, and low gene flow between incipient species will allow divergence for these adaptive traits. Moreover, ecological isolation may influence the outcomes of secondary contact, with selection against hybrids or low intrinsic fitness of hybrids caused by divergence of adaptive loci causing conflicts.

7.1.1. Comparison with *Senecio*

Common characteristics that promote speciation in phylogenetically divergent lineages can be identified by comparing *Begonia* with other species-rich genera. These common characteristics will influence speciation histories, even if genera are found in different geographic areas and have diversified over different time scales. Little is known about the population biology and evolutionary genetics of most species-rich plant genera, with *Senecio* being a notable exception. Many broad parallels can be drawn between *Senecio* and *Begonia*, such as their wide variety of vegetative and floral forms (*Senecio* species are very variable in: leaf shape, indumentums type, inflorescence type, flower colour, Barkley, 1978, cited by Pelser *et al.*, 2007) and the habitats species are adapted to, the frequent occurrence of hybridization and polyploidy (Lowe & Abbott, 2000; Kirk *et al.*, 2004; Lowe & Abbott, 2004; James & Abbott, 2005; Chapman & Abbott, 2010), and the tendency for some species to grow as invasive ruderals over large distribution ranges (e.g. *S. vulgaris* L.). Other life-history traits are quite dissimilar. For example self-incompatibility is common in *Senecio* but not know from any *Begonia* species. Here, the four factors proposed to promote diversification in *Begonia* (above) are compared to *Senecio*, to see whether similar characteristics promote diversification in both groups.

The first factor to consider is whether seed dispersal limitation affects patterns of genetic diversity in *Senecio*. *Senecio* fruits (achenes) have a pappus to promote wind dispersal, and trichromes to aid animal attachment and subsequent dispersal (Schmitt, 1980; Mcevoy & Cox, 1987), so high seed dispersal between populations would be expected. No correlation was found between geographic distance and genetic divergence at plastid loci in the Mediterranean *Senecio gallicus* Chaix, suggesting long-distance seed dispersal and Pleistocene range expansion have obscured geographic structuring of genetic diversity, as would be expected if seed dispersal was limiting (Comes & Abbott, 1998). Studies of Israeli *Senecio glaucus* L. and *S. vernalis* Walst. and Kit. show plastid haplotypes are widespread and this is likely caused by long-distance seed dispersal (Comes & Abbott, 1999). Overall, with seeds showing adaptations to promote dispersal, and plastid haplotypes often being widespread, seed dispersal appears to be an important mode of gene dispersal in *Senecio*. This contrasts with *Begonia* where seed dispersal is limiting (Chapter 2). However, species in *Senecio* section *Senecio* shed their pappus at maturity, so may not spread their seeds as far as other *Senecio* sections. Significant geographic structure of plastid haplotypes have been found in Moroccan *Senecio leucanthemifolius* Poiret var. *Casablancae* Alexander (Coleman & Abbott, 2003), supporting seed dispersal limitation in section *Senecio*.

The second factor proposed to promote speciation in *Begonia* is limited pollen dispersal. Most *Senecio* species are generalists in the pollinators they attract, like *Begonia*, with pollinators including: solitary bees, syrphid flies, and other dipterids (Schmitt, 1980; Comes & Abbott, 1998). Most of these pollinators are likely to travel short-distances between plants (Schmitt, 1980), promoting the geographic structuring of genetic diversity. This is supported by high F_{ST} values for some *Senecio* species (Swiss *Senecio vulgaris*, $F_{ST} = 0.39$ with RAPD's, Müller-Schärer & Fischer, 2001; $F_{ST} = 0.49$ with AFLP's, Steinger *et al.*, 2002; *S. leucanthemifolius*, F_{ST}

= 0.30 with RAPD's, Coleman & Abbott, 2003). Interpopulation gene flow does not prevent drift or selection occurring in populations of *Senecio gallicus* and *S. glaucus* (Comes & Abbott, 1998; Comes & Abbott, 1999), and this has in turn lead to morphological differentiation between populations, as seen in *S. leucanthemifolius* (Coleman & Abbott, 2003). Therefore, pollen dispersal between populations does appear to be low. Exceptions include those North American *Senecio* species that are pollinated by butterflies, which often travel between geographically isolated populations (Schmitt, 1980).

The third factor proposed to promote genetic differentiation between *Begonia* populations is frequent selfing. *Senecio* are more varied in their mating systems than *Begonia*, with some *Senecio* species being fully self-compatible and others self-incompatible. Ferrer & Good-Avila (2007) estimated that ~63% of Asteraceae species are self-incompatible, including many *Senecio* species. Self-incompatability in the Asteraceae is sporophytic, and pseudo-self incompatibility in *Senecio squalidus* and potentially other *Senecio* species is caused by unlinked modifiers of the self-incompatibility locus (Hiscock, 2000). Some colonizing *Senecio* species have a mutation at the *RAY* locus (Kim *et al.*, 2008) stopping them producing showy ray florets that attract pollinators, subsequently increasing their selfing rates (Marshall & Abbott, 1982). The impact of selfing on the genetic structure of *Senecio* populations is not clear, as most population genetic studies have used dominant markers and therefore have not calculated the inbreeding coefficient or inferred selfing rate. *Senecio vulgaris* is a commonly selfing species, and selfing may have contributed to the highly structured populations (described above). Other population genetic studies have been conducted on the largely outcrossing section *Senecio*, although Comes & Abbott (1998) reported population F_{IS} values up to 0.21 at allozyme loci in *Senecio gallicus*, and this may be explained by non-random mating which may hence influence the genetic structure of populations (discussed above).

The final factor proposed to promote speciation in *Begonia* is strong reproductive isolating barriers. Hybridization in *Senecio* has been studied in a number of species; here two pairs of hybrids are considered where experimental crossing barriers and observations of hybrids have been made under natural conditions. *Senecio eboracensis* Abbott & Lowe is a recent allotetraploid with strong reproductive barriers to its parental progenitors *S. squalidus* and *S. vulgaris* (Lowe & Abbott, 2004). Artificial F1 hybrids between *S. squalidus* and *S. vulgaris* are relatively fertile, with pollen stainability and seed viability when open-pollinated greater than 60% of the parental means. Hybrids are extremely rare in the wild, likely due to a combination of pre- and post-zygotic barriers (Lowe & Abbott, 2004). A second experimental system for hybridization in *Senecio* is between closely related self incompatible *S. jacobaea* and *S. aquaticus* Hill.. F1 crosses between *S. jacobaea* and *S. aquaticus* produce significantly more seeds, and the offspring are more vegetatively vigorous, than intraspecific *S. jacobaea* crosses. The ease of producing F1 hybrids is similar to F1 *Begonia heracleifolia* x *B. sericoneura* crosses. The F1 *Senecio* hybrids also perform well in both parental habitats (Kirk *et al.*, 2005a). Seed production is then reduced in F2s and BCs (Kirk *et al.*, 2005b). Hybrid swarms have admixed AFLP profiles, and likely include later-generation backcrosses (Kirk *et al.*, 2004). Much like *Begonia*, the outcomes of hybridization in *Senecio* are dependent on the species being crossed. Many of the same barriers contribute to reproductive isolation between sympatric *Begonia* and *Senecio* species, such as the high frequency of self-fertilization and differences in flowering time (Chapter 5). There is also evidence that reproductive barriers are evolving within *Senecio* species, like seen in *Begonia heracleifolia* populations (Chapter 4). Seasonal cohorts within *S. vulgaris* flower at three different times of year, and may co-occur in sympatry, although only a small amount of genetic diversity is partitioned between cohorts (Haldimann *et al.*, 2003).

Hybridization has also been shown to have evolutionary significant consequences in *Senecio*, generating new homoploid and alloploid taxa, and through the introgression of important phenotypic traits. *Senecio squalidus* is a homoploid hybrid between *S. aethnensis* and *S. chrysanthemifolius*, which has formed within the last 300 years in a hybrid swarm in Sicily (James & Abbott, 2005). Since being introduced to the United Kingdom, *Senecio squalidus* has gone on to hybridize with *S. vulgaris* to give rise to new hybrid derivatives *S. vulgaris* var. *hibernicus* Syme. and the York radiate groundsel (Lowe & Abbott, 2000). Introgression of the *RAY* locus from *S. squalidus* into non-radiate *S. vulgaris* shows the importance of introgression for mediating rapid phenotypic changes (Kim *et al.*, 2008). Past introgression has also been supported in Mediterranean *Senecio* species, with *S. flavus* susp. *breviflorus* Kaderit and *S. rupestris* Waldst. & Kit. found to aquire ITS sequences and plastid haplotypes from related species in sympatry (Comes & Abbott, 2001). Similarly, *Senecio massaicus* Maire has either received genes through introgression, or may be a species of reticulate origin, as it contains genetic material from two divergent clades in a *Senecio* phylogenetic study (Pelser *et al.*, 2012).

At the very broad scale, the evolutionary processes proposed to promote diversification in large genera Sanderson & Wojciechowski (1996) are present in both *Begonia* and *Senecio*, including their predominantly herbaceous growth form, frequent polyploidy and multiple transitions between ecological states. However, of the four characteristics proposed to promote divergence in *Begonia* some characteristics directly contrast *Senecio*, such as seed dispersal distances, while others are generally in agreement, such as low pollen dispersal. Geographic structuring of populations is an important precursor to allopatric speciation, and this occurs in different ways in the two genera, through low seed and pollen dispersal in *Begonia*, and in most *Senecio* species just through restricted pollen flow. This geographic structuring of genetic diversity may be promoted by selfing in *Begonia* and those *Senecio* species that are self-compatible.

7.1.2. Genetic resources for future studies

In addition to empirical findings for *Begonia*, the molecular markers developed for this thesis will be valuable for future genetic studies in *Begonia*. The nuclear microsatellites were designed from transcriptome sequence data, and unlike the species-specific markers currently available for *Begonia* (Hughes *et al.*, 2002; Nakamura *et al.*, 2012), they amplify over a wide phylogenetic scope (Chapter 3). These will facilitate population genetic studies in a range of *Begonia* species, allowing direct comparisons of levels of diversity and genetic differentiation using a common set of markers. The plastid markers will be valuable for tracing the parentage of experimental crosses, and as a seed specific marker for future population biology studies. These markers add to the suit of genetic resources that are currently being developed for *Begonia*, which include: transcriptome sequences of three species (*B. conchifolia*, *B. plebeja*, Asian *B. venusta* King, Brennan *et al.*, submitted), plastid genomes of 16 species (Harrison, in prep.), BC genetic linkage maps (*B. plebeja* x *B. conchifolia*, Brennan *et al.*, submitted; Chapter 5), and the whole genome sequence of *B. conchifolia* (Kidner, ubpubl. data). These genetic markers will be used to answer some of the outstanding questions about speciation in *Begonia* that have been raised by this study, which are outlined below.

7.2. Future questions

7.2.1. What is the colonization history of *B. heracleifolia* and *B. nelumbiifolia* in Central America?

The phylogeographic scenario in Chapter 2, where *B. heracleifolia* populations persisted in situ during the Pleistocene, whereas *B. nelumbiifolia* was restricted to refugia of unknown location, was based on samples only from south Mexico and west Guatemala. Genotyping samples from across the species ranges with the same markers would shed light on their colonization histories. This would be of particular interest if the intraspecific patterns could be related to geographic structuring of a phylogeny of Central American *Begonia* (see below).

7.2.2. How rapidly has speciation occurred in Central American *Begonia*?

The lack of species cohesion, and the rapid accumulation of reproductive barriers (Chapters 4 & 5), raises the hypothesis that Central American *Begonia* has undergone rapid speciation. A species-level phylogeny, calibrated with a relative dimension of time, would be the ideal way to address this hypothesis. The main difficulty would be obtaining a comprehensive sample of the large number of species that occur in remote localities (Thomas, 2010). If species representative of major *Begonia* clades were sequenced, new Bayesian analytical approaches could be applied, where diversification rates are estimated with incomplete species-level samples (Moore & Donoghue, 2009; Silvestro *et al.*, 2011; Wertheim & Sanderson, 2011; Drummond *et al.*, in press). The second barrier to this approach is the scarcity of fossil *Begonia* material (Stults & Axsmith, 2011) for calibrating a dated phylogeny. Alternative approaches include the use of a calibration point from a broader phylogenetic study (i.e. a secondary calibration point, e.g. Thomas *et al.*, 2011a), calibration from a geographic event of known age (Plana *et al.*, 2003), or using mutation rates from other species (Richardson *et al.*, 2001).

7.2.3. Which genes contribute to the early stages of speciation in *Begonia*?

The search for speciation genes is an increasingly popular research direction for evolutionary biology (Rieseberg & Blackman, 2010). *Begonia* is an ideal system to study speciation genes, as populations within species differ markedly in their levels of divergence, and may represent different stages in the speciation process (Chapter 4). Genome scans for pairwise F_{ST} outliers between differentiated populations would be one way to identify genes under selection, and these would be candidate genes for adaptive differences (Scotti-Saintagne *et al.*, 2004; Hohenlohe *et al.*, 2010; Scascitelli *et al.*, 2010). Such an experiment is increasingly feasible with next-generation sequencing technologies. Alternatively, if differentiated populations have clearly distinguishable phenotypes that may contribute to isolation, a QTL analysis would allow the genomic location of these genes to be mapped, and would be the first stage towards fine-mapping and more detailed genetic characterization.

7.2.4. Why is the sister genus and related families so species poor?

Hughes (2002) raised the question: why is the sister genus, *Hillebrandia*, and related families such as Datiscaceae, so species poor in comparison to *Begonia*? Explanations could include any combination of high extinction/low speciation in related groups, or low extinction/high speciation in *Begonia*. An analysis of diversification rates that accounts for extinction (reviewed in Purvis, 2008) could be applied to samples of *Begonia* and related groups to test this hypothesis. This approach would have to use DNA sequences that resolve both the backbone and species-level relationships with confidence, over a broad phylogenetic scope. Another approach, along the lines explored in this thesis, would be to investigate ongoing population processes that predispose these groups to low species diversity. These may include, high intraspecific gene flow preventing allopatric speciation, or low effective population sizes preventing adaptation to changing conditions, which may cause frequent local extinction.

7.2.5. Has polyploidy promoted diversification in *Begonia*?

Genome duplication is common in large plant genera (Sanderson & Wojciechowski, 1996), and *Begonia* species vary ten-fold in their chromosome numbers (Legro & Doorenbos, 1969, 1971, 1973; Dewitte *et al.*, 2011). Recent transcriptome analysis suggests a whole genome duplication has occurred prior to diversification of *Begonia*, and polyploidy may have been an evolutionary stimulus in the group (Brennan *et al.*, submitted). The species investigated here were diploid, so the role that polyploidy has played in diversification could not be assessed. This could be addressed by comparing rates of diversification between diploid and polyploid clades in a phylogeny. However, this approach cannot identify polyploidy as the actual cause of elevated divergence rates. To understand the functional role duplicated genes play, expression levels of homeologous loci could be compared between recent polyploids and their diploid progenitors (Buggs *et al.*, 2011). This could use synthetic *Begonia* polyploids as a comparison (Dewitte *et al.*, 2009, 2010).

7.2.6. Does hybridization play an evolutionary important role in *Begonia*?

The hybrid swarm analysis (Chapter 5) shows introgression in young hybrid swarms is limited, but this may not prevent important evolutionary outcomes over longer time periods. A study of past hybridization could test whether introgression, or hybrid speciation (Buerkle *et al.*, 2000; Gross & Rieseberg, 2005; Chapman & Burke, 2007; Mallet, 2007), contributes to diversity in *Begonia*. Many techniques can be used to detect ancient hybrids, and these include comparing the topologies of species-level phylogenies constructed with unlinked markers, or comparative genome scans to identify shared alleles (reviewed in Twyford & Ennos, 2012, see Appendix 11).

7.2.7. Does ecological divergence promote speciation in *Begonia*?

The attributes of *Begonia* discussed in this thesis, particularly those which lead to geographic isolation of populations, go some way to explain why such species richness has accumulated in the genus. However, these attributes are found in many other taxa too, including genera that are not species-rich. While extant species richness reflects the stochastic nature of evolution, as well as the factors discussed above, many other factors are also likely to be involved in promoting divergence in *Begonia*. In particular, the ease at which colonizing species can adapt to new ecological conditions will be important. One explanation for rapid adaptation to new environments is pre-existing genetic variation (standing genetic variation), which will be a source for selection to act on in new habitats. The genetic signature of standing genetic variation can be distinguished from new mutations using population genomic approaches (review in Barrett & Schluter, 2008). QTL analyses of ecologically important traits will also be useful in understanding the genetics of adaptation in *Begonia*.

7.3. Conclusion

Begonia is an emerging model system to study the mechanisms that promote diversification in large plant genera. Through genetic analysis, the population biology of *Begonia* has been shown to have profound consequences for speciation. Future genetic and genomic studies of *Begonia*, when integrated in a broader phylogenetic context, will continue to teach us much more about the fascinating and intricate evolutionary history of speciose plant genera.

8. REFERENCES

Abbott, R.J., Ashton, P.A. & Forbes, D.G. (1992) Introgressive origin of the radiate groundsel, *Senecio vulgaris* L. var. *hibernicus* syme: *Aat-3* evidence. *Heredity*, **68**, 425-435

Abbott, R.J., Ritchie, M.G. & Hollingsworth, P.M. (2008) Introduction. Speciation in plants and animals: pattern and process. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363**, 2965-2969

Ågren, J. & Schemske, D.W. (1991) Pollination by deceit in a neotropical monoecious herb, *Begonia involucrata*. *Biotropica*, **23**, 235-241

Ågren, J. & Schemske, D.W. (1993) Outcrossing rate and inbreeding depression in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata*. *Evolution*, **47**, 125-135

Ågren, J. & Schemske, D.W. (2012) Reciprocal transplants demonstrate strong adaptive differentiation of the model organisms *Arabidopsis thaliana* in its native range. *New Phytologist*, **194**, 1112-1122

Allard, R.W., Jain, S.K. & Workman, P.L. (1969) Genetics of inbreeding populations. *Advances in Genetics*, **14**, 55-131

Allen, A.M., Thorogood, C.J., Hegarty, M.J., Lexer, C. & Hiscock, S.J. (2011) Pollen-pistil interactions and self-incompatibility in the Asteraceae: new insights from studies of *Senecio squalidus* (Oxford ragwort). *Annals of Botany*, **108**, 687-698

Alvarez, J., Guli, C.L., Yu, X.-H. & Smyth, D.R. (1992) *terminal flower*: a gene affecting inflorescence development in *Arabidopsis thaliana*. *The Plant Journal*, **2**, 103-116

Anderson, E.C. & Thompson, E.A. (2002) A model-based method for identifying species hybrids using multilocus genetic data. *Genetics*, **160**, 1217-1229

Antonelli, A. & Sanmartín, I. (2011) Why are there so many plant species in the Neotropics? *Taxon*, **60**, 403-414

Arnaud-Haond, S. & Belkhir, K. (2007) GENCLONE: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. *Molecular Ecology Notes*, **7**, 15-17

Arnold, M.L. (1993) *Iris nelsonii* (Iridaceae): origin and genetic composition of a homoploid hybrid species. *American Journal of Botany*, **80**, 577-583

Arnold, M.L. (1997) *Natural hybridization and evolution*. Oxford University Press, New York.

Arnold, M.L. & Hodges, S.A. (1995) Are natural hybrids fit or unfit relative to their parents? *Trends in Ecology and Evolution*, **10**, 67-71

Arnold, M.L., Tang, S., Knapp, S.J. & Martin, N.H. (2010) Asymmetric introgressive hybridization among louisiana iris species. *Genes*, **1**, 9-22

Ashman, T.L. & Diefenderfer, C. (2001) Sex ratio represents a unique context for selection on attractive traits: consequence for the evolution of sexual dimorphism. *The American Naturalist*, **157**, 334-347

Ashman, T.-L. & Hitchens, M.S. (2000) Dissecting the causes of variation in intra-inflorescence allocation in a sexually polymorphic species, *Fragaria virginiana* (Rosaceae). *American Journal of Botany*, **87**, 197-204

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A. & Saunders, N.C. (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics*, **18**, 489-522

Bachmann, K. (1998) Species as units of diversity: an outdated concept. *Theory in Biosciences*, **117**, 213-230

Bacles, C.F.E., Lowe, A.J. & Ennos, R.A. (2006) Effective seed dispersal across a fragmented landscape. *Science*, **311**, 628

Baker, H.G. (1955) Self-compatability and establishment after 'long-distance' dispersal. *Evolution*, **9**, 347-349

Barraclough, T.G., Vogler, A.P. & Harvey, P.H. (1998) Revealing the factors that promote speciation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **353**, 241-249

Barrett, R.D.H. & Schluter, D. (2008) Adaptation from standing genetic variation. *Trends in Ecology & Evolution*, **23**, 38-44

Barrier, E., Velasquillo, L., Chavez, M. & Gaulon, R. (1998) Neotectonic evolution of the Isthmus of Tehuantepec (southeastern Mexico). *Tectonophysics*, **287**, 77-96

Barton, N.H. & Bengtsson, B.O. (1986) The barrier to genetic exchange between hybridising populations. *Heredity*, **57**, 357-376

Barton, N.H. & Hewitt, G.M. (1985) Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, **16**, 113-48

Bawa, K.S. (1992) Mating systems, genetic differentiation and speciation in tropical rain forest plants. *Biotropica*, **24**, 250-255

Bell, D., Long, D.G., Forrest, A.D., Hollingsworth, M.L., Blom, H.H. & Hollingsworth, P.M. (2012) DNA barcoding of European *Herbertus* (Marchantiopsida, Herbertaceae) and the discovery and description of a new species. *Molecular Ecology Resources*, **12**, 36-47

Bermingham, E. & Moritz, C. (1998) Comparative phylogeography: concepts and applications. *Molecular Ecology*, **7**, 367-369

Bohs, L. & Olmstead, R.O. (1997) Phylogenetic relationshipes in *Solanum* (Solanaceae) based on *ndh*F sequences. *Systematic Botany*, **22**, 5-17

Bomblies, K., Yant, L., Laitinen, R.A., Kim, S.-T., Hollister, J.D., Warthmann, N., Fitz, J. & Weigel, D. (2010) Local-scale patterns of genetic variability, outcrossing, and spatial structure in natural stands of *Arabidopsis thaliana*. *PLoS Genet*, **6**, e1000890

Bradley, D., Carpenter, R., Copsey, L., Vincent, C., Rothstein, S. & Coen, E. (1996) Control of inflorescence architecture in *Antirrhinum*. *Nature*, **379**, 791-797

Bremer, K. & Wanntorp, H.-E. (1978) Phylogenetic systematics in botany. *Taxon*, **27**, 317-329

Brochmann, C. (2002) Hybridization and distribution of *Argyranthemum coronopifolium* (Asteraceae – Anthemideae) in the Canary Islands. *Nordic Jounral of Botany*, **4,** 729-736

Brown, K.M., Burk, L.M., Henagan, L.M. & Noor, M.A.F. (2004) A test of the chromosomal rearrangement model of speciation in *Drosophila pseudoobscura*. *Evolution*, **58**, 1856-1860

Bryson, R.W., García-Vázquez, U.O. & Riddle, B.R. (2011) Phylogeography of Middle American gophersnakes: mixed responses to biogeographical barriers across the Mexican Transition Zone. *Journal of Biogeography*, **38**, 1570-1584

Buerkle, C.A., Morris, R.J., Assumesen, M.A. & Rieseberg, L.H. (2000) The likelihood of homoploid speciation. *Heredity*, **84**, 441-451

Buggs, R.J.A., Chamala, S., Wu, W.E.I., Gao, L.U., May, G.D., Schnable, P.S., Soltis, D.E., Soltis, P.S. & Barbazuk, W.B. (2010) Characterization of duplicate gene evolution in the recent natural allopolyploid *Tragopogon miscellus* by next-generation sequencing and sequenom iPLEX massARRAY genotyping. *Molecular Ecology*, **19**, 132-146

Buggs, R. J.A., Zhang, L., Miles, N., Tate, J.A., Gao, L., Wei, W., Schnable, P.S., Barbazuk, W.B., Soltis, P.S.. & Soltis, D.E. (2011) Transcriptomic shock generates evolutionary novelty in a newly formed, natural allopolyploid plant. *Current Biology*, **21**, 551-556

Burgarella, C., Lorenzo, Z., Jabbour-Zahab, R., Lumaret, R., Guichoux, E., Petit, R.J., Soto, A. & Gil, L. (2009) Detection of hybrids in nature: application to oaks (*Quercus suber* and *Q. ilex*). *Heredity*, **102**, 442-452

Burke, J.M. & Arnold, M.L. (2001) Genetics and the fitness of hybrids. *Annual Review of Genetics*, **35**, 31-52

Burt-Utley, K. (1985) A revision of Central American species of *Begonia* section *Gireoudia* (Begoniaceae). *Tulane studies in zoology and botany*, **25**, 1-131

Butlin, R. (2010) Population genomics and speciation. *Genetica*, **138**, 409-418

Bylesjö, M., Segura, V., Soolanayakanahally, R.Y., Rae, A.M., Trygg, J., Gustafsson, P., Jansson, S. & Street, N.R. (2008) LAMINA: a tool for rapid quantification of leaf size and shape parameters. *BMC Plant Biology*, **8**, 82

Cárdenas, M.L., Gosling, W.D., Sherlock, S.C., Poole, I., Pennington, R.T. & Mothes, P. (2011) The response of vegetation on the Andean flank in western amazonia to Pleistocene climate change. *Science*, **331**, 1055-1058

Cardoso, M.A., Provan, J., Powell, W., Ferreira, P.C.G. & De Oliveira, D.E. (1998) High genetic differentiation among remnant populations of the endangered *Caesalpinia echinata* lam. (Leguminosae – Caesalpinioideae). *Molecular Ecology*, **7**, 601-608

Carney, S.E., Cruzan, M.B. & Arnold, M.L. (1994) Reproductive interactions between hybridizing irises: analysis of pollen-tube gorwth and fertilization success. *American Journal of Botany*, **81**, 1169-1175

Case, A.L. & Willis, J.H. (2008) Hybrid male sterility in *Mimulus* (Phryaceae) is associated with a geographically restricted mitochondrial rearrangment *Evolution*, **62-5**, 1026-1039

Castillo, R., Caballero, H., Boege, K., Fornoni, J. & Domínguez, C. (2012) How to cheat when you cannot lie? Deceit pollination in *Begonia gracilis*. *Oecologia*, **3**, 1-10

Castillo, R.A., Cordero, C. & Domínguez, C.A. (2002) Are reward polymorphisms subject to frequency- and density-dependent selection? Evidence from a monoecious species pollinated by deceit. *Journal of Evolutionary Biology*, **15**, 544-552

Castoe, T.A., Daza, J.M., Smith, E.N., Sasa, M.M., Kuch, U., Campbell, J.A., Chippindale, P.T. & Parkinson, C.L. (2009) Comparative phylogeography of pitvipers suggests a consensus of ancient Middle American highland biogeography. *Journal of Biogeography*, **36**, 88-103

Cavers, S., Navarro, C. & Lowe, A.J. (2003) Chloroplast DNA phylogeography reveals colonization history of a neotropical tree, *Cedrela odorata* L., in Mesoamerica. *Molecular Ecology*, **12**, 1451-1460

Ceplitis, A.L.F., Su, Y. & Lascoux, M. (2005) Bayesian inference of evolutionary history from chloroplast microsatellites in the cosmopolitan weed *Capsella bursa-pastoris* (Brassicaceae). *Molecular Ecology*, **14**, 4221-4233

Chan, E.Y. (2008) Next-generation sequencing methods: impact of sequencing accuracy on SNP discovery. *Methods in Molecular Biology,* **578**, 95-111

Chan, K.M.A. & Levin, S.A. (2005) Leaky prezygotic isolation and porous genomes: rapid introgression of maternally inherited DNA. *Evolution*, **59**, 720-729

Chapman, M.A. & Abbott, R.J. (2010) Introgression of fitness genes across a ploidy barrier. *New Phytologist*, **186**, 63-71

Chapman, M.A. & Burke, J.M. (2007) Genetic divergence and hybrid speciation. *Evolution*, **61**, 1773-1780

Charlesworth, B. (1991) The evolution of sex chromosomes. *Science*, **251**, 1030-1033

Charlesworth, B. (2009) Effective population size and patterns of molecular evolution and variation. *Nature Reviews Genetics*, **10**, 195-205

Charlesworth, D. (2002) Plant sex determination and sex chromosomes. *Heredity*, **88**, 94-101

Charlesworth, D. (2003) Effects of inbreeding on the genetic diversity of populations. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **358**, 1051-1070

Charlesworth, D. (2006) Evolution of plant breeding systems. *Current Biology*, **16**, 726-735

Charlesworth, D., Charlesworth, B. & Marais, G. (2005) Steps in the evolution of heteromorphic sex chromosomes. *Heredity*, **95**, 118-128

Charlesworth, D. & Morgan, M.T. (1991) Allocation of resources to sex functions in flowering plants. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **332**, 91-102

Chazdon, R.L. & Fletcher, N. (1984) Photosynthetic light environments in a lowland tropical rain forest in Costa Rica. *Journal of Ecology*, **72**, 553-564

Chung, K.-S., Weber, J.A. & Hipp, A.L. (2011) Dynamics of chromosome number and genome size variation in a cytogenetically variable sedge (*Carex scoparia* var. *scoparia*, Cyperaceae). *American Journal of Botany*, **98**, 122-129

Clark, S.E., Running, M.P. & Meyerowitz, E.M. (1993) CLAVATA1, a regulator of meristem and flower development in *Arabidopsis*. *Development*, **119**, 397-418

Clayton, W.D. (1974) The logarithmic distribution of angiosperm families. *Kew Bulletin*, **29**, 271-279

Clement, W.L., Tebbit, M.C., Forrest, L.L., Blair, J.E., Brouillet, L., Eriksson, T. & Swenser, S.M. (2004) Phylogenetic position and biogeography of *Hillebrandia sandwicensis* (Begoniaceae): a rare Hawaiian relict. *American Journal of Botany*, **91**, 905-917

Coleman, M. & Abbott, R.J. (2003) Possible causes of morphological variation in an endemic Moroccan groundsel (*Senecio leucanthemifolius* var. *casablancae*): evidence from chloroplast DNA and random amplified polymorphic DNA markers. *Molecular Ecology*, **12**, 423-434

Colinvaux, P.A., De Oliveira, P.E. & Bush, M.B. (2002) Amazonian and Neotropical plant communities on glacial timescales: the failure of the aridity and refuge hypothesis. *Quaternary Science Reviews*, **19**, 141-169

Comes, H.P. & Abbott, R.J. (1998) The relative importance of historical events and gene flow on the population structure of a Mediterranean ragwort, *Senecio gallicus*. *Evolution*, **52**, 355-367

Comes, H.P. & Abbott, R.J. (1999) Population genetic structure and gene flow across arid versus mesic environments: a comparative study of two parapatric *Senecio* species from the Near East. *Evolution*, **53**, 36-54

Comes, H.P. & Abbott, R.J. (2001) Molecular phylogeography, reticulation, and lineage sorting in mediterranean *Senecio* sect. *Senecio* (Asteraceae). *Evolution*, **55**, 1943-1962

Conti, L. & Bradley, D. (2007) TERMINAL FLOWER1 is a mobile signal controlling *Arabidopsis* architecture. *The Plant Cell Online*, **19**, 767-778

Coombs, J.A., Letcher, B.H. & Nislow, K.H. (2008) CREATE: a software to create input files from diploid genotypic data for 52 genetic software programs. *Molecular Ecology Resources*, **8**, 578-580

Corander, J., Marttinen, P., Siren, J. & Tang, J. (2008) Enhanced bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics*, **9**, doi:10.1186/1471-2105-9-539

Corlett, R. & Primack, R.B. (2010) *Tropical rainforests: an ecological and biogeographic comparison*. Wiley-Blackwell, Malden, MA.

Cornuet, J.M. & Luikart, G. (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001-2014

Corriveau, J.L. & Coleman, A.W. (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. *American Journal of Botany*, **75**, 1443-1458

Costa, F.R.C. (2004) Structure and composition of the ground-herb community in a Terra-Firme Central Amazonian forest. *Acta Amazonica*, **34**, 53-59

Coyne, J.A. & Orr, A.O. (1989) Patterns of speciation in *Drosophila*. *Evolution*, **43**, 362-381

Coyne, J.A. & Orr, A.O. (2004) *Speciation*. Sinauer Associates, Sunderland.

Cozza, J. (2008) Sex expression in a rainforest understory herb, *Begonia urophylla*. PhD Thesis, University of Miami

Crawford, N.G. (2010) SMOGD: software for the measurement of genetic diversity. *Molecular Ecology Resources*, **10**, 556-557

Cronn, R., Liston, A., Parks, M., Gernandt, D.S., Shen, R. & Mockler, T. (2008) Multiplex sequencing of plant chloroplast genomes using solexa sequencing-by-synthesis technology. *Nucleic Acids Research*, **36**, e122

Cruzan, M.B. & Arnold, M.L. (1993) Ecological and genetic associations in an iris hybrid zone. *Evolution*, **47**, 1432-1445

Currat, M., Ruedi, M., Petit, R.J. & Excoffier, L. (2008) The hidden side of invasions: massive introgression of local genes. *Evolution*, **62**, 1908-1920

Darwin, C. (1877) *The various contrivances by which orchids are fertilised by insects*. University of Chicago Press, Chicago.

David, P., Pujol, B., Viard, F., Castella, V. & Goudet, J. (2007) Reliable selfing rate estimates from imperfect population genetic data. *Molecular Ecology*, **16**, 2474-2487

Davies, T.J. & Barraclough, T.G. (2007) The diversification of flowering plants through time and space: key innovations, climate and chance. *Reconstructing the tree of life:*

taxonomy and systematics of species rich taxa. (ed. by T. Hodkinson, Parnell, J. And Waldren, S), pp. 149-164. CRC Press.

Delph, L. & Lloyd, D.G. (1991) Environmental and genetic control of gender dimorphic shrub *Hebe subalpina*. *Evolution*, **45**, 1957-1964

Devitt, T.J. (2006) Phylogeography of the western lyresnake (*Trimorphodon biscutatus*): testing aridland biogeographical hypotheses across the Nearctic–Neotropical transition. *Molecular Ecology*, **15**, 4387-4407

Dewey, R.E., Timothy, D.H. & Levings, C.S. (1987) A mitochondrial protein associated with cytoplasmic male sterility in the cytoplasm of maize. *Proceedings of the National Academy of Sciences*, **84**, 5374-5378

Dewitte, A., Eeckhaut, T., Van Huylenbroeck, J. & Van Bockstaele, E. (2009a) Occurrence of viable unreduced pollen in a *Begonia* collection. *Euphytica*, **168**, 81-94

Dewitte, A., Eeckhaut, T., Van Huylenbroeck, J. & Van Bockstaele, E. (2010) Induction of 2n pollen formation in *Begonia* by trifluralin and N_2O treatments. *Euphytica*, **171**, 283-293

Dewitte, A., Twyford, A.D., Thomas, D.C., Kidner, C.A. & Van Huylenbroeck, J. (2011) The origin of diversity in *Begonia*: genome dynamism, population processes and phylogenetic patterns. *The dynamical processes of biodiversity - case studies of evolution and spatial distribution* (ed. by O. Grillo and G. Venora). InTech.

Dick, C.W., Hardy, O.J., Jones, F.A. & Petit, R.J. (2008) Spatial scales of pollen and seed-mediated gene flow in tropical rain forest trees. *Tropical plant biology*, **1**, 20-33

Dobzhansky, T. (1937) *Genetics and the origin of species*. Columbia University Press, New York.

Domínguez Lozano, F. & Schwartz, M.W. (2005) Patterns of rarity and taxonomic group size in plants. *Biological Conservation*, **126**, 146-154

Doorenbos, J., Sosef, M.S.M. & De Wilde, J.J.F.E. (1998) *The sections of Begonia – studies in Begoniaceae* Backhuys.

Dorken, M.E. & Barrett, S.C.H. (2003) Gender plasticity in *Sagittaria sagittifolia* (Alistmataceae), a monoecious aquatic species. *Plant systematics and evolution*, **237**, 99-106

Doyle, J.J. (1992) Gene trees and species trees: molecular systematic as one-character taxonomy. *Systematic Botany*, **17**, 144-163

Drummond, A., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T. & Wilson, A. (2011) Geneious v5.4. Available from http://www.Geneious.Com/.

Drummond, C.S., Eastwood, R.J., Miotto, S.T.S. & Hughes, C.E. (in press) Multiple continental radiations and correlates of diversification in *Lupinus* (Leguminosae): testing for key innovation with incomplete taxon sampling. *Systematic Biology*

Duchesne, P. & Turgeon, J. (2009) FLOCK: a method for quick mapping of admixture without source samples. *Molecular Ecology Resources*, **9**, 1333-1344

Duminil, J., Hardy, O. & Petit, R. (2009) Plant traits correlated with generation time directly affect inbreeding depression and mating system and indirectly genetic structure. *BMC Evolutionary Biology*, **9**, doi:10.1186/1471-2148-9-177

Dupanloup, I., Schneider, S. & Excoffier, L. (2002) A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, **11**, 2571-2581

Ebert, D. & Peakall, R. (2009) Chloroplast simple sequence repeats (cpSSRs): technical resources and recommendations for expanding cpSSR discovery and application to a wide array of plant species. *Molecular Ecology Resources*, **9**, 673-690

Edelaar, P.I.M., Burraco, P. & Gomez-Mestre, I. (2011) Comparisons between Q_{ST} and F_{ST} — how wrong have we been? *Molecular Ecology*, **20**, 4830-4839

Edwards, C.E. & Weinig, C. (2011) The quantitative-genetic and QTL architecture of trait integration and modularity in *Brassica rapa* across simulated seasonal settings. *Heredity*, **106**, 661-677

Ehrlich, P.R. & Raven, P.H. (1969) Differentiation of populations. *Science*, **165**, 1228-1232

Ekblom, R. & Galindo, J. (2011) Applications of next generation sequencing in molecular ecology of non-model organisms. *Heredity*, **107**, 1-15

Emms, S.K. & Arnold, M.L. (1997) The effect of habitat on parental and hybrid fitness: transplant experiments with Louisiana irises. *Evolution*, **51**, 1112-1119

Emms, S.K. & Arnold, M.L. (2000) Site-to-site differences in pollinator visitation patterns in a Louisiana iris hybrid zone. *Oikos*, **91**, 568-578

Endersby, J. (2009) Lumpers and splitters: Darwin, hooker, and the search for order. *Science*, **326**, 1496-1499

Endress, P.K. & Doyle, J.A. (2009) Reconstructing the ancestral angiosperm flower and its initial specializations. *American Journal of Botany*, **96**, 22-66

Ennos, R.A. (1994) Estimating the relative rates of pollen and seed migration among plant populations. *Heredity*, **72**, 250-259

Ennos, R.A., French, G.C. & Hollingsworth, P.M. (2005) Conserving taxonomic complexity. *Trends in Ecology & Evolution*, **20**, 164-168

Ennos, R.A., Sinclair, W.T., Hu, X.-S. & Langdon, A. (1999) Using organelle markers to elucidate the history, ecology and evolution of plant populations. *Molecular systematics and plant evolution* (ed. by P.M. Hollingsworth, R.M. Bateman and R.J. Gornall), pp. 1-19. Taylor and Francis Group, London.

Erkens, R.H.J., Chatrou, L.W., Maas, J.W., Van Der Niet, T. & Savolainen, V. (2007) A rapid diversification of rainforest trees (*Guatteria*, Annonaceae) following dispersal from central into South America. *Molecular Phylogenetics and Evolution*, **44**, 399-411

Ersts PJ. Geographic Distance Matrix Generator (version 1.2.3). American Museum of Natural History, Center for Biodiversity and Conservation. Available at: http:// biodiversityinformatics.amnh.org/open_source/gdmg (Accessed on 1 November 2011).

Escalante, T., Rodríguez, G., Cao, N., Ebach, M. & Morrone, J. (2007) Cladistic biogeographic analysis suggests an early Caribbean diversification in Mexico. *Naturwissenschaften*, **94**, 561-565

Escudero, M., Hipp, A.L., Waterway, M.J. & Valente, L.M. (2012) Diversification rates and chromosome evolution in the most diverse angiosperm genus of the temperate zone (*Carex*, Cyperaceae). *Molecular Phylogenetics and Evolution*, 650-655

Estoup, A., Jarne, P. & Cornuet, J.-M. (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular Ecology*, **11**, 1591-1604

Evanno, G., Regnaut, S. & Goudet, J. (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, **14**, 2611-2620

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47-50

Fazekas, A.J., Steeves, R. & Newmaster, S.G. (2010) Improving sequencing quality from PCR products containing long mononucleotide repeats. *BioTechniques*, **48**, 277-285

Falconer, D.S. & Mackay, T. (1996) *Introduction to quantitative genetics*, 4th edn. Longmans Green, Harlow, Essex, UK.

Feder, J.L. & Nosil, P. (2010) The efficacy of divergence hitchhiking in generating genomic islands during ecological speciation. *Evolution*, **64**, 1729-1747

Ferrer, M.M. & Good-Avila, S.V. (2007) Macrophylogenetic analyses of the gain and loss of self-incompatibility in the Asteraceae. *New Phytologist*, **173**, 401-414

Fishman, L. & Willis, J.H. (2001) Evidence for Dobzhansky-Muller incompatabilities contributing to the sterility of hybrids between *Mimulus guttatus* and *M. nasutus*. *Evolution*, **55**, 1932-1942

Fishman, L. & Willis, J.H. (2007) A cytonuclear incompatability causes anther sterility in *Mimulus* hybrids. *Evolution*, **60**, 1372-1381

Fontaine, C., Lovett, P.N., Sanou, H., Maley, J. & Bouvet, J.M. (2004) Genetic diversity of the shea tree (*Vitellaria paradoxa* c.F. Gaertn), detected by RAPD and chloroplast microsatellite markers. *Heredity*, **93**, 639-648

Forrest, L.L. (2000) A phylogeny of the Begoniaceae Bercht. & J. Presl. PhD Thesis, University of Glasgow

Forrest, L.L. & Hollingsworth, P.M. (2003) A recircumscription of *Begonia* based on nuclear ribosomal sequences. *Plant systematics and evolution*, **241**, 193-211

Frajman, B. & Schönswetter, P. (2011) Giants and dwarfs: molecular phylogenies reveal multiple origins of annual spurges within *Euphorbia* subg. *Esula*. *Molecular Phylogenetics and Evolution*, **61**, 413-424

Freeland, J.R., Biss, P. & Silvertown, J. (2011) Structure of sweet vernal grass (*Anthoxanthum odoratum*) populations. *Journal of Heredity*, **103**, 28-35

French, G.C. (2003) Conservation genetics of British *Euphrasia* L. PhD Thesis, University of Edinburgh and Royal Botanic Garden Edinburgh

Frodin, D.G. (2004) History and concepts of big plant genera. *Taxon*, **53**, 753-776

Funk, D.J., Nosil, P. & Etges, W.J. (2006) Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 3209-3213

Gao, H., Williamson, S. & Bustamante, C.D. (2007) A Markov Chain Monte Carlo approach for joint inference of population structure and inbreeding rates from multilocus genotype data. *Genetics*, **176**, 1635-1651

Gentry, A. (1992) Tropical forest biodiversity: distributional patterns and their conservational significance. *Oikos*, **63**, 19-28

Gitzendanner, M.A. & Soltis, P.S. (2000) Patterns of genetic variation in rare and widespread congeners. *American Journal of Botany*, **87**, 783-792

Gleason, J.M. & Ritchie, M.G. (1998) Evolution of courtship song and reproductive isolation in the *Drosophila willistoni* species complex: do sexual signals diverge the most quickly? *Evolution*, **52**, 1493-1500

Goodall-Copestake, W., Pérez-Espona, S., Harris, D.J. & Hollingsworth, P.M. (2010) The early evolution of the mega-diverse genus *Begonia* (Begoniaceae) inferred from organelle DNA phylogenies. *Biological Journal of the Linnean Society*, **101**, 243-250

Goodall-Copestake, W.P., Harris, D.J. & Hollingsworth, P.M. (2009) The origin of a mega-diverse genus: dating *Begonia* (Begoniaceae) using alternative datasets, calibrations and relaxed clock methods. *Botanical Journal of the Linnean Society*, **159**, 363-380

Goudet, J. (1995) FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity*, **86**, 485-486

Goulson, D. (2000) Are insects flower constant because they use search images to find flowers? *Oikos*, **88**, 547-552

Gross, B.L. & Rieseberg, L.H. (2005) The ecological genetics of homoploid hybrid speciation. *Journal of Heredity*, **96**, 241-252

Grundt, H.H., Kjølner, S., Borgen, L., Rieseberg, L.H. & Brochmann, C. (2006) High biological diversity in the arctic flora. *Proceedings of the National Academy of Science*, **103**, 972-975

Guan, B.-C., Fu, C.-X., Qiu, Y.-X., Zhou, S.-L. & Comes, H. (2010) Genetic structure and breeding system of a rare understory herb, *Dysosma versipellis* (Berberidaceae), from temperate deciduous forests in china. *American Journal of Botany*, **97**, 111-122

Guichoux, E., Lagache, L., Wagner, S., Chaumeil, P., Léger, P., Lepais, O., Lepoittevin, C., Malausa, T., Revardel, E., Salin, F. & Petit, R.J. (2011) Current trends in microsatellite genotyping. *Molecular Ecology Resources*, **11**, 591-611

Guo, S., Zheng, Y., Joung, J.-G., Liu, S., Zhang, Z., Crasta, O., Sobral, B., Xu, Y., Huang, S. & Fei, Z. (2010) Transcriptome sequencing and comparative analysis of cucumber flowers with different sex types. *BMC Genomics*, **11**, doi: 10.1186/1471-2164-11-384

Gutiérrez-Rodríguez, C., Ornelas, J.F. & Rodríguez-Gómez, F. (2011) Chloroplast DNA phylogeography of a distylous shrub (*Palicourea padifolia*, Rubiaceae) reveals past fragmentation and demographic expansion in Mexican cloud forests. *Molecular Phylogenetics and Evolution*, **61**, 603-615

Haldimann, P., Steinger, T. & Müller-Schärer, H. (2003) Low genetic differentiation among seasonal cohorts in *Senecio vulgaris* as revealed by amplified fragment length polymorphism analysis. *Molecular Ecology*, **12**, 2541-2551

Haffer, J. (1969) Speciation in amazonian forest birds. *Science*, **165**, 131-137

Hall, M.C., Basten, C.J. & Willis, J.H. (2006) Pleiotropic quantitative trait loci contribute to population divergence in traits associated with life-history variation in *Mimulus guttatus*. *Genetics*, **172**, 1829-1844

Hamrick, J. & Godt, M. (1996) Effects of life history traits on genetic diversity in plants. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **351**, 1291-1298

Hanano, S. & Goto, K. (2011) *Arabidopsis* TERMINAL FLOWER 1 is involved in the regulation of flowering time and inflorescence development through transcriptional repression. *The Plant Cell*, **23**, 3172-3184

Hans Köhler, R., Horn, R., Lössl, A. & Zetsche, K. (1991) Cytoplasmic male sterility in sunflower is correlated with the co-transcription of a new open reading frame with the *atp*A gene. *Molecular and General Genetics*, **227**, 369-376

Harder, L.D. & Barrett, S.C.H. (1995) Mating costs of large floral displays in hermaphrodite plants. *Nature*, **373**, 512-515

Harder, L.D. & Barrett, S.C.H. (2000) The mating consequences of sexual segregation within inflorescences of flowering plants. *Proceedings of the Royal Society B*, **267**, 315-320

Hardy, O.J. & Vekemans, X. (2002) SPAGEDI: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*, **2**, 618-620

Hausdorf, B. (2011) Progress towards a general species concept. *Evolution*, **65**, 923-931

Hedrick, P.W. (2005) A standardized genetic differentiation measure. *Evolution*, **59**, 1633-1638

Hegarty, M.J., Barker, G.L., Brennan, A.C., Edwards, K.J., Abbott, R.J. & Hiscock, S.J. (2008) Changes to gene expression associated with hybrid speciation in plants: further insights from transcriptomic studies in *Senecio*. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363**, 3055-3069

Hennig, W. (1966) *Phylogenetic systematics*, English translation edn, Urbana: University of Illinios Press.

Heuertz, M., Vekemans, X., Hausman, M., Palada, M. & Hardy, O.J. (2003) Estimating seed vs. pollen dispersal from spatial genetic structure in the common ash. *Molecular Ecology*, **12**, 2483-2495

Hewitt, G.M. (2000) The genetic legacy of the quaternary ice ages. *Nature*, **405**, 907-913

Hey, J & Machado, (2003) The study of structured populations – new hope for a difficult divided science. *Nature Reviews Genetics*, **4**, 535-543

Hey, J. & Pinho, C. (2012) Population genetics and objectivity in species diagnosis. *Evolution*

Heywood, J. (1991) Spatial analysis of genetic variation in plant populations. *Annual Review of Ecology and Systematics*, **22**, 335-355

Heywood, V.H., Brummit, R.K., Culham, A. & Seberg, O. (2007) *Flowering plant families of the world*. Royal Botanic Gardens, Kew, Richmond.

Hipp, A., Rothrock, P. & Roalson, E. (2009) The evolution of chromosome arrangements in *Carex* (Cyperaceae). *The Botanical Review*, **75**, 96-109

Hiscock, S.J. (2000) Genetic control of self-incompatability in *Senecio squalidus* L. (Asteraceae): a successful colonizing species. *Heredity*, **85**, 10-19

Hodges, S.A. & Arnold, M.L. (1994) Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings of the National Academy of Sciences*, **91**, 2493-2496

Hoffmann, A. & Rieseberg, L.H. (2008) Revisiting the impact of inversions in evolution: from population genetic markers to drivers of adaptive shifts and speciation? *Annual Review of Ecology, Evolution and systematics*, **39**, 21-42

Hohenlohe, P.A., Bassham, S., Etter, P.D., Stiffler, N., Johnson, E.A. & Cresko, W.A. (2010) Population genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. *PLoS Genet*, **6**, e1000862

Hoover, S. (1979) Notes on the spatial distribution patterns for three Mexican species of *Begonia*. *Phytologia*, **34**, 107-132

Horn, J.W., Van E. B.W., Morawetz, J.J., Riina, R., Steinmann, V.W., Berry, P.E. & Wurdack, K.J. (2012) Phylogenetics and the evolution of major structural characters in the giant genus *Euphorbia* L. (*Euphorbiaceae*). *Molecular Phylogenetics and Evolution*, **63**, 305-326

Hubisz, M.J., Falush, D., Stephens, M. & Pritchard, J.K. (2009) Inferring weak population structure with the assistance of sample group information. *Molecular Ecology Resources*, **9**, 1322-1332

Hughes, C. & Eastwood, R. (2006) Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. *Proceedings of the National Academy of Sciences*, **103**, 10334-10339

Hughes, M. (2002) Population structure and speciation in *Begonia* L., PhD thesis, University of Glasgow, Glasgow

Hughes, M. (2008) *An annotated checklist of southeast asian Begonia*. Royal Botanic Garden Edinburgh, Edinburgh.

Hughes, M. & Hollingsworth, P.M. (2003) Population structure in the endemic *Begonia* of the Socotra Archipelago. *Biological Conservation*, **113**, 277-284

Hughes, M. & Hollingsworth, P.M. (2008) Population genetic divergence corresponds with species-level biodviersity patterns in the large genus *Begonia*. *Molecular Ecology*, **17**, 2643-2651

Hughes, M., Russell, J. & Hollingsworth, P.M. (2002) Polymorphic microsatellite markers for the Socotran endemic herb *Begonia socotrana*. *Molecular Ecology Resources*, **2**, 159-160

Jakobsson, M. & Rosenberg, N.A. (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics*, **23**, 1801-1806

Jakobsson, M., Säll, T., Lind-Halldán, C. & Halldén, C. (2007) Evolution of chloroplast mononucleotide microsatellites in *Arabidopsis thaliana*. *Theoretical and Applied Genetics*, **114**, 223-235

James, J.K. & Abbott, R.J. (2005) Recent, allopatric, homoploid hybrid speciation: The origin of *Senecio squalidus* (Asteraceae) in the British Isles from a hybrid zone on Mount Etna, Sicily. *Evolution*, **59**, 2533-2547

Jarne, P. & David, P. (2008) Quantifying inbreeding in natural populations of hermaphroditic organisms. *Heredity*, **100**, 431-439

Jensen, J., Bohonak, A. & Kelley, S. (2005) Isolation By Distance Web Service. *BMC Genetics*, **6**, 13

Jiggins, C.D. & Mallet, J. (2000) Bimodal hybrid zones and speciation. *Trends in Ecology & Evolution*, **15**, 250-255

Joehanes, R. & Nelson, J.C. (2008) Qgene 4.0, an extensible java QTL-analysis platform. *Bioinformatics*, **24**, 2788-2789

Jordan, C.Y. & Harder, L.D. (2006) Manipulation of bee behavior by inflorescence architecture and its consequences for plant mating. *The American Naturalist*, **167**, 496-506

Jost, L. (2008) G_{ST} and its relatives do not measure differentiation. *Molecular Ecology*, **17**, 4015-4026

Kater, M.M., Colombo, L., Franken, J., Busscher, M., Masiero, S., Van Lookeren Campagne, M.M. & Angenent, G.C. (1998) Multiple AGAMOUS homologs from cucumber and petunia differ in their ability to induce reproductive organ fate. *The Plant Cell*, **10**, 171-182

Kettle, C.J., Hollingsworth, P.M., Jaffré, T., Moran, B. & Ennos, R.A. (2007) Identifying the early genetic consequences of habitat degradation in a highly threatened tropical conifer, *Araucaria nemorosa* Laubenfels. *Molecular Ecology*, **16**, 3581-3591

Kiew, R., Teo, L.L. & Gan, Y.Y. (2003) Assessment of the hybrid status of some Malesian plants using amplified fragment length polymorphism. *Telopea*, **10**, 225-233

Kim, M., Cui, M.-L., Cubas, P., Gillies, A., Lee, K., Chapman, M.A., Abbott, R.J. & Coen, E. (2008) Regulatory genes control a key morphological and ecological trait transferred between species. *Science*, **322**, 1116-1119

Kim, S.-C. & Rieseberg, L.H. (1999) Genetic architecture of species differences in annual sunflowers: implications for adaptive trait introgression. *Genetics*, **153**, 965-977

Kimura, M. (1955) Solution of a process of random genetic drift with a continuous model. *Proceedings of the National Academy of Science*, **41**, 144-150

Kimura, M. & Weiss, G. (1964) The stepping stone model of population structure and the decrease of genetic correlation with distance. *Genetics*, **49**, 561-576

Kirk, H., Máčel, M., Klinkhamer, P.G.L. & Vrieling, K. (2004) Natural hybridization between *Senecio jacobaea* and *Senecio aquaticus*: molecular and chemical evidence. *Molecular Ecology*, **13**, 2267-2274

Kirk, H., Vrieling, K. & Klinkhamer, P.G.L. (2005a) Maternal effects and heterosis influence the fitness of plant hybrids. *New Phytologist*, **166**, 685-694

Kirk, H., Vrieling, K. & Klinkhamer, P.G.L. (2005b) Reproductive fitness of hybrids between *Senecio jacobaea* and *S. aquaticus* (Asteraceae). *American Journal of Botany*, **92**, 1467-1473

Kisel, Y. & Barraclough, T.G. (2010) Speciation has a spetial scale that depends on levels of gene flow. *The American Naturalist*, **175**, 316-334

Knott, S.A. (2005) Regression-based quantitative trait loci mapping: robust, efficient and effective. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **360**, 1435-1442

Lai, Z., Nakazato, T., Salmaso, M., Burke, J.M., Tang, S., Knapp, S.J. & Rieseberg, L.H. (2005) Extensive chromosomal repatterning and the evolution of sterility barriers in hybrid sunflower species. *Genetics*, **171**, 291-303

Lasso, E., Dalling, J.W. & Bermingham, E. (2011) Strong spatial genetic structure in five tropical *Piper* species: should the Baker-Fedorov hypothesis be revived for tropical shrubs. *Ecology and Evolution*, **1**, 502-516

Laughnan, J.R. & Gabay-Laughnan, S. (1983) Cytoplasmic male sterility in maize. *Annual Review of Genetics*, **17**, 27-48

Laver, H.K., Reynolds, S.J., Moneger, F. & Leaver, C.J. (1991) Mitochondrial genome organization and expression associated with cytoplasmic male sterility in sunflower (*Helianthus annuus*). *The Plant Journal*, **1**, 185-193

Le Gac, M., Hood, M.E., Giraud, T. & Funk, D. (2007) Evolution of reproductive isolation within a parasitic fungal species complex. *Evolution*, **61**, 1781-1787

Lee, D.W., Bone, R.A., Tarsis, S.L. & Storch, D. (1990) Correlates of leaf optical properties in tropical forest sun and extreme-shade plants. *American Journal of Botany*, **77**, 370-380

Legro, R.A.H. & Doorenbos, J. (1969) Chromosome numbers in *Begonia*. *Netherlands Journal of Agricultural Science*, **17**, 189-202

Legro, R.A.H. & Doorenbos, J. (1971) Chromosome numbers in *Begonia* 2. *Netherlands Journal of Agricultural Science*, **19**, 176-183

Legro, R.A.H. & Doorenbos, J. (1973) Chromosome numbers in *Begonia* 3. *Netherlands Journal of Agricultural Science*, **21**, 167-170

Lepais, O. & Bacles, C.F.E. (2011) Comparison of random and SSR-enriched shotgun pyrosequencing for microsatellite discovery and single multiplex PCR optimization in *Acacia harpophylla*. F. Muell ex Benth. *Molecular Ecology Resources*, **11**, 711-724

Lepais, O., Petit, R.J., Guichoux, E., Lavabre, J.E., Alberto, F., Kremer, A. & Gerber, S. (2009) Species relative abundance and direction of introgression in oaks. *Molecular Ecology*, **18**, 2228-2242

Levin, D.A. (1983) Polyploidy and novelty in flowering plants. *The American Naturalist*, **122**, 1-25

Levin, D.A., Francisco-Ortega, J. & Jansen, R.K. (1996) Hybridization and the extinction of rare plant species. *Conservation Biology*, **10**, 10-16

Lexer, C., Fay, M.F., Joseph, J.A., Nica, M.-S. & Heinze, B. (2005) Barrier to gene flow between two ecologically divergent *Populus* species, *P. alba* (white poplar) and *P. tremula* (European aspen): the role of ecology and life history in gene introgression. *Molecular Ecology*, **14**, 1045-1057

Li, X., Ding, X., Chu, B., Zhou, Q., Ding, G. & Gu, S. (2008) Genetic diversity analysis and conservation of the endangered chinese endemic herb *Dendrobium officinale* (Orchidaceae) based on AFLP. *Genetica*, **133**, 159-166

Liljegrena, S.J., Gustafson-Browna, C., Pinyopicha, A., Dittaa, G.S. & Yanofskya, M.F. (1999) Interactions among APETELA1, LEAFY, and TERMINAL FLOWER1 specify meristem fate. *Plant Cell*, **11**, 1007-1018

Linder, H.P. (2008) Plant species radiations: where, when, why? *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363**, 3097-3105

Linhart, Y. & Grant, M. (1996) Evolutionary significance of local genetic differentiation in plants. *Annual Review of Ecology and Systematics*, **27**, 237-277

Lloyd, D.G. (1979) Self reproductive factors affecting the selection of self-fertilization in plants. *The American Naturalist*, **113**, 67-79

Lopéz, S. & Domínguez, C.A. (2003) Sex choice in plants: facultative adjustment of the sex ratio in the perennial herb *Begonia gracilis*. *Journal of Evolutionary Biology*, **16**, 1177-1185

Louthan, A. & Kay, K. (2011) Comparing the adaptive landscape across trait types: larger QTL effect size in traits under biotic selection. *BMC Evolutionary Biology*, **11**, doi:10.1186/1471-2148-11-60

Loureiro, J., Rodriguez, E., Doležel, J., Santos, C. (2007) Two new nuclear isolation buffers for plant DNA flow cytometry: a test with 37 species. *Annals of Botany*, **100**, 875-888.

Lowe, A.J. & Abbott, R.J. (2000) Routes of origin of two recently evolved hybrid taxa: *Senecio vulgaris* var. *hibernicus* and York radiate groundsel (Asteraceae). *American Journal of Botany*, **87**, 1159-1167

Lowe, A.J. & Abbott, R.J. (2004) Reproductive isolation of a new hybrid species, *Senecio eboracensis* Abbott & Lowe (Asteraceae). *Heredity*, **92**, 386-395

Mable, B.K. & Adam, A. (2007) Patterns of genetic diversity in outcrossing and selfing populations of *Arabidopsis lyrata*. *Molecular Ecology*, **16**, 3565-3580

Machado, C.A. & Hey, J. (2003) The causes of phylogenetic conflict in a classic *Drosophila* species group. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **270**, 1193-1202

Mallet, J. (1995) A species definition for the modern synthesis. *Trends in Ecology & Evolution*, **10**, 294-299

Mallet, J. (2007) Hybrid speciation. *Nature*, **446**, 279-283

Malone, J.H. & Fontenot, B.E. (2008) Patterns of reproductive isolation in toads. *PLoS ONE*, **3**, e3900

Marazzi, B. & Sanderson, M.J. (2010) Large-scale patterns of diversification in the widespread legume genus *Senna* and the evolutionary role of extrafloral nectaries. *Evolution*, **64**, 3570-3592

Marshall, D.F. & Abbott, R.J. (1982) Polymorphism for outcrossing frequency at the ray floret locus in *Senecio vulgaris* L. I. Evidence. *Heredity*, **48**, 227-235

Marshall, J.L., Arnold, M.L. & Howard, D.J. (2002) Reinforcement: the road not taken. *Trends in Ecology & Evolution*, **17**, 558-563

Martin, N.H., Bouck, A.C. & Arnold, M.L. (2007) The genetic architecture of reproductive isolation in Louisiana irises: flowering phenology. *Genetics*, **175**, 1803-1812

Martin, N.H. & Willis, J.H. (2010) Geographical variation in postzygotic isolation and its genetic basis within and between two *Mimulus* species. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **365**, 2469-2478

Martins, W.S., Soares Lucas, D.C., De Souza Neves, K.F. & Bertioli, D.J. (2009) Websat - a web software for microsatellite marker development. *Bioinformation*, **3**, 282–283

Martinsen, G.D., Whitham, T.G., Turek, R.J. & Keim, P. (2001) Hybrid populations selectively filter gene introgression between species. *Evolution*, **55**, 1325-1335

Matolweni, L.O., Balkwill, K. & Mclellan, T. (2000) Genetic diversity and gene flow in the morphologically variable, rare endemic *Begonia dregei* and *Begonia homonyma* (Begoniaceae). *American Journal of Botany*, **87**, 431-439

Matthews, A.C. (2007) Cytological investigation of species barriers in *Begonia* section *Gireoudia*. Undergraduate thesis, University of Edinburgh

Matzke, E.B. (1938) Inflorescence patterns and sexual expression in *Begonia semperflorens*. *American Journal of Botany*, **25**, 465-478

Mayr, E. (1942) *Systematics and the origin of species*. Columbia University Press, New York.

Mayr, E. (1969) The biological meaning of species. *Biological Journal of the Linnean Society*, **1**, 311-320

Mayr, E. & O'hara, R.J. (1986) The biogeographic evidence supporting the Pleistocene forest refuge hypothesis. *Evolution*, **1**, 55-67

Mccauley, D.E. (1995) The use of chloroplast DNA polymorphism in studies of gene flow in plants. *Trends in Ecology & Evolution*, **10**, 198-202

Mcevoy, P.B. & Cox, C.S. (1987) Wind dispersal distances in dimorphic achenes of ragwort, *Senecio jacobaea*. *Ecology*, **68**, 2006-2015

Mckone, M., Ostertag, R., Rauscher, J., Heiser, D. & Russell, F.L. (1995) An exception to Darwin's syndrome: floral position, protogyny, and insect visitation in *Besseya bullii* (Scrophulariaceae). *Oecologia*, **101**, 68-74

Meirmans, P.G. (2006) Using the AMOVA framework to estimate a standardized genetic differentiation measure. *Evolution*, **60**, 2399-2402

Meirmans, P.G. & Hedrick, P.W. (2011) Assessing population structure: F_{ST} and related measures. *Molecular Ecology Resources*, **11**, 5-18

Mendelson, T.C. (2003) Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae: Etheostoma). *Evolution*, **57**, 317-327

Metcalfe, S.E., O'hara, S.L., Caballero, M. & Davies, S.J. (2000) Records of late Pleistocene-Holocene climatic change in Mexico - a review. *Quaternary Science Reviews*, **19**, 699-721

Metzker, M.L. (2010) Sequencing technologies - the next generation. *Nat Rev Genet*, **11**, 31-46

Meudt, H.M. & Clarke, A.C. (2007) Almost forgotten or latest practice? AFLP applications, analyses and advances. *Trends in Plant Science*, **12**, 107-117

Micheneau, C., Duffy, K.J., Smith, R.J., Stevens, L.J., Stout, J.C., Civeyrel, L., Cowan, R.S. & Fay, M.F. (2010) Plastid microsatellites for the study of genetic variability in the widespread *Cephalanthera longifolia*, *C. damasonium* and *C. rubra* (Neottieae, Orchidaceae), and cross-amplification in other *Cephalanthera* species. *Botanical Journal of the Linnean Society*, **163**, 181-193

Milne, R.I. & Abbott, R.J. (2008) Reproductive isolation among two interfertile *Rhododendron* species: low frequency of post-F1 hybrid genotypes in alpine hybrid zones. *Molecular Ecology*, **17**, 1108-1121

Milne, R.I., Terzioglu, S. & Abbott, R.J. (2003) A hybrid zone dominated by fertile F1s: maintenance of species barriers in *Rhododendron*. *Molecular Ecology*, **12**, 2719-2729

Minder, A.M., Rothenbuehler, C. & Widmer, A. (2007) Genetic structure of hybrid zones between *Silene latifolia* and *Silene dioica* (Caryophyllaceae): evidence for introgressive hybridization. *Molecular Ecology*, **16**, 2504-2516

Mishler, B.D. & Donoghue, M.J. (1982) Species concepts: a case for pluralism. *Systematic Zoology*, **31**, 491-503

Mittelbach, G.G., Schemske, D.W., Cornell, H.V., Allen, A.P., Brown, J.M., Bush, M.B., Harrison, S.P., Hurlbert, A.H., Knowlton, N., Lessios, H.A., Mccain, C.M., Mccune, A.R., Mcdade, L.A., Mcpeek, M.A., Near, T.J., Price, T.D., Ricklefs, R.E., Roy, K., Sax, D.F., Schluter, D., Sobel, J.M. & Turelli, M. (2007) Evolution and the latitudinal diversity gradient: speciation, extinction and biogeography. *Ecology Letters*, **10**, 315-331

Moore, B.R. & Donoghue, M.J. (2009) A bayesian approach for evaluating the impact of historical events on rates of diversification. *Proceedings of the National Academy of Sciences*, **106**, 4307-4312

Moran, P.A.P. (1975) Wandering distributions and the electrophoretic profile. *Theoretical Population Biology*, **8**, 318-330

Morrone, J.J. (2006) Biogeographic areas and transition zones of Latin America and the Carribbean Islands based on panbiogeographic and cladistic analyses of the entomofauna. *Annual Review of Entomology*, **51**, 467-494

Morgante, M. & Olivieri, A.M. (1993) PCR-amplified microsatellites as markers in plant genetics. *The Plant Journal*, **3**, 175-182

Morjan, C.L. & Rieseberg, L.H. (2004) How species evolve collectively: implications of gene flow and selection for the spread of advantageous alleles. *Molecular Ecology*, **13**, 1341-1356

Mousadik, A. & Petit, R.J. (1996) High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (l.) skeels] endemic to Morocco. *Theoretical and Applied Genetics*, **92**, 832-839

Moyle, L.C., Olson, M.S., Tiffin, P. & Baum, D. (2004) Patterns of reproductive isolation in three angiosperm genera. *Evolution*, **58**, 1195-1208

Mulcahy, D.G., Morrill, B.H. & Mendelson, J.R. (2006) Historical biogeography of lowland species of toads (*Bufo*) across the trans-mexican neovolcanic belt and the Isthmus of Tehuantepec. *Journal of Biogeography*, **33**, 1889-1904

Müller-Schärer, H. & Fischer, M. (2001) Genetic structure of the annual weed *Senecio vulgaris* in relation to habitat type and population size. *Molecular Ecology*, **10**, 17-28

Muller, H.J. (1942) Isolating mechanisms, evolution, and temperature. *Biological Symposia*, **6**, 71-125

Myers, N., Mittermeier, R.A., Mittermeier, C.G., De Fonseca, G.A.B. & Kent, J. (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853-858

Nakamura, K., Huang, C., Jr., Rubite, R.R., Leong, W.-C., Kono, Y., Yang, H.-A. & Peng, C.-I. (2012) Isolation of compound microsatellite markers in *Begonia fenicis* (Begoniaceae) endemic to East and Southeast Asian islands. *American Journal of Botany Primer Notes & Protocols in the Plant Sciences*, **99**, e20-e23

Nason, J.D., Ellstrand, N.C. & Arnold, M.L. (1992) Patterns of hybridization and introgression in populations of oaks, manzanitas, and irises. *American Journal of Botany*, **79**, 101-111

Neale, S., Goodall-Copestake, W. & Kidner, C.A. (2006) The evolution of diversity in *Begonia*. *Floriculture, ornamental and plant biotechnology volume iv*. Global Science Books.

Nei, M. (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583-590

Nei, M. (1987) *Molecular evolutionary genetics*. Columbia University Press, New York.

Nei, M., Tajima, F. & Tateno, Y. (1983) Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution*, **19**, 153-170

Nei, M., Maruyama, T. & Chakraborty, R. (1975) The bottleneck effect and genetic variability in populations. *Evolution*, **29**, 1-10

Nielsen, E.E., Bach, L.A. & Kotlicki, P. (2006) HYBRIDLAB (version 1.0): a program for generating simulated hybrids from population samples. *Molecular Ecology Notes*, **6**, 971-973

Noor, M.A. (1999) Reinforcement and other consequences of sympatry. *Heredity*, **83**, 503-508

Nosil, P. (2008) Speciation with gene flow could be common. *Molecular Ecology*, **17**, 2103-2106

Nosil, P. & Feder, J.L. (2012) Genomic divergence during speciation: causes and consequences. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **367**, 332-342

Ohta, T. & Kimura, M. (1973) A model of mutation appropriate to estimate the number of electrophoretically detectable alleles in a finite population. *Genetical Research*, **22**, 201-204

Ornelas, J.F., Ruiz-Sánchez, E. & Sosa, V. (2010) Phylogeography of *Podocarpus matudae* (Podocarpaceae): Pre-Quaternary relicts in northern Mesoamerican cloud forests. *Journal of Biogeography*, **37**, 2384-2396

Ortiz-Barrientos, D., Counterman, B.A. & Noor, M.A. (2012) The genetics of speciation by reinforcement. *PLOS Biology*, **2**

Ortiz, P.L., Berjano, R., Talavera, M.A. & Arista, M. (2009) The role of resources and architecture in modeling floral variability for the monoecious amphicarpic *Emex spinosa* (Polygonaceae). *American Journal of Botany*, **96**, 2062-2073

Palma-Silva, C., Lexer, C., Paggi, G., Barbará, T., Bered, F. & Bodanese-Zanettini, M. (2009) Range-wide patterns of nuclear and chloroplast DNA diversity in *Vriesea gigantea* (Bromeliaceae), a Neotropical forest species. *Heredity*, **103**, 503-512

Park, K.-R. (2004) Comparisons of allozyme variation of narrow endemic and widespread species of Far East *Euphorbia* (Euphorbiaceae). *Botanical Bulletin of Academia Sinica*, **45**, 221-228

Park, S. (2001) Trypanotolerance in west african cattle and the population genetic effects of selection. PhD thesis, University of Dublin

Park, S.J., Jiang, K., Schatz, M.C. & Lippman, Z.B. (2012) Rate of meristem maturation determines inflorescence architecture in tomato. *Proceedings of the National Academy of Sciences*, **109**, 639-644

Parks, J.C. & Werth, C.R. (1993) A study of spatial features of clones in a population of bracken fern, *Pteridium aquilinum* (Dennstaedtiaceae). *American Journal of Botany*, **80**, 537-544

Pelser, P.B., Abbott, R.J., Comes, H.P., Milton, J.J., Möller, M., Looseley, M.E., Cron, G.V., Barcelona, J.F., Kennedy, A.H., Watson, L.E., Barone, R., Hernández, F. & Kadereit, J.W. (2012) The genetic ghost of an invasion past: colonization and extinction revealed by historical hybridization in *Senecio*. *Molecular Ecology*, **21**, 369-387

Pelser, P.B., Gravendeel, B. & Van Der Meijden, R. (2002) Tackling speciose genera: species composition and phylogenetic position of *Senecio* sect. *Jacobaea* (Asteraceae) based on plastid and nrDNA sequences. *American Journal of Botany*, **89**, 929-939

Pelser, P.B., Nordenstam, B., Kadereit, J.W. & Watson, L.E. (2007) An ITS phylogeny of tribe Senecioneae (Asteraceae) and a new delimitation of *Senecio* L. *Taxon*, **56**, 1077-1104

Pemberton, J.M., Slate, J., Bancroft, D.R. & Barrett, J.A. (1995) Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology*, **4**, 249-252

Peng, C.-I. & Chen, Y.-K. (1991) Hybridity and parentage of *Begonia buimontana* Yamamoto (Begoniaceae) from Taiwan. *Ann, Missouri Bot. Gard.*, **78**, 995-1001

Peng, C.-I. & Chiang, T.-Y. (2000) Molecular confirmation of unidirectional hybridization in *Begonia* x *taipeiensis* peng (Begoniaceae) from Taiwan. *Ann, Missouri Bot. Gard.*, **87**, 273-285

Peng, C.-I., Liu, Y., Shin-Ming, K.U., Yoshiko, K. & Chung, K.-F. (2010) *Begonia* × *breviscapa* (Begoniaceae), a new intersectional natural hybrid from limestone areas in Guangxi, China. *Botanical Studies*, **51**, 107-117

Peng, C.-I. & Shin-Ming, K.U. (2009) *Begonia* × *chungii* (Begoniaceae), a new natural hybrid in Taiwan. *Botanical Studies*, **50**, 241-250

Peng, C.-I. & Sue, C.-Y. (1991) *Begonia* × *taipeiensis* (Begoniaceae), a new natural hybrid in Taiwan. *Botanical Bulletin of Academia Sinica*, **41**, 151-158

Pennington, R.T., Lavin, M., Oliveira-Filho, A., (2009) Woody plant diversity, evolution, and ecology in the tropics: perspectives from seasonally dry tropical forests. *Annual Review of Ecology, Evolution, and Systematics*, **40**, 437-457

Petit, R.J., Aguinagalde, I., De Beaulieu, J.-L., Bittkau, C., Brewer, S., Cheddadi, R., Ennos, R., Fineschi, S., Grivet, D., Lascoux, M., Mohanty, A., Müller-Starck, G., Demesure-Musch, B., Palmé, A., Martín, J.P., Rendell, S. & Vendramin, G.G. (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. *Science*, **300**, 1563-1565

Petit, R.J., Duminil, J., Fineschi, S., Hampe, A., Salvini, D. & Vendramin, G.G. (2005) Comparative organiszation of chloroplast, mitochondrial, and nuclear diversity in plant populations. *Molecular Ecology*, **14**, 689-701

Plana, V., Gascoigne, A., Forrest, L.L., Harris, D.J. & Pennington, R.T. (2003) Pleistocene and pre-Pleistocene *Begonia* speciation in Africa. *Molecular Phylogenetics and Evolution*, **31**, 449-461

Poelchau, M., Hamrick, J. (2012) Palaeodistribution modeling does not support disjunct Pleistocene refugia in several Central American plant taxa. *Journal of Biogeography* DOI: 10.1111/j.1365-2699.2011.02648.x.

Pons, O. & Petit, R.J. (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. *Genetics*, **144**, 1237-1245

Prance, G.T. (1994) A comparison of the efficacy of higher taxa and species numbers in the assessment of biodiversity in the Neotropics. *Philosophical Transactions: Biological Sciences*, **345**, 89-99

Presgraves, D.C., Balagopalan, L., Abmayr, S.M. & Orr, A.O. (2003) Adaptive evolution drives divergence of a hybrid inviability gene between two species of *Drosophila*. *Nature*, **423**, 715-719

Price, T.D. & Bouvier, M.M. (2002) The evolution of F1 postzygotic incompatabilities in birds. *Evolution*, **56**, 2083-2089

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945-959

Provan, J., Powell, W. & Hollingsworth, P.M. (2001) Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends in Ecology & Evolution*, **16**, 142-147

Provan, J., Soranzo, N., Wilson, N.J., Goldstein, D.B. & Powell, W. (1999) A low mutation rate for chloroplast microsatellites. *Genetics*, **153**, 943-947

Prusinkiewicz, P., Erasmus, Y., Lane, B., Harder, L.D. & Coen, E. (2007) Evolution and development of inflorescence architectures. *Science*, **316**, 1452-1456

Pryer, K.M., Schuettpelz, E., Wolf, P.G., Schneider, H., Smith, A.R. & Cranfill, R. (2004) Phylogeny and evolution of ferns (Monilophytes) with a focus on the early leptosporangiate divergences. *American Journal of Botany*, **91**, 1582-1598

Pujol, B., Wilson, A.J., Ross, R.I.C. & Pannell, J.R. (2008) Are Q_{ST} – F_{ST} comparisons for natural populations meaningful? *Molecular Ecology*, **17**, 4782-4785

Purvis, A. (2008) Phylogenetic approaches to the study of extinction. *Annual Review of Ecology, Evolution, and Systematics*, **39**, 301-319

Quinn, J.F. & Harrison, S.P. (1988) Effects of habitat fragmentation and isolation on species richness: evidence from biogeographic patterns. *Oecologia*, **75**, 132-140

Ramakrishnan, A.P., Meyer, S.E., Fairbanks, D.J. & Coleman, C.E. (2006) Ecological significance of microsatellite variation in western North American populations of *Bromus tectorum*. *Plant Species Biology*, **21**, 61-73

Rambaut, A. Figtree: Tree figure drawing tool, version 1.2.2. available from: http://tree.bio.ed.ac.uk/software/figtree/

Rand, D.M., Haney, R.A. & Fry, A.J. (2004) Cytonuclear coevolution: the genomics of cooperation. *Trends in Ecology & Evolution*, **19**, 645-653

Rice, P., Longden, I. & Bleasby, A. (2000) The European molecular biology open source suite. *Trends in Genetics*, **16**, 276-277

Richardson, J.E., Pennington, R.T., Pennington, T.D. & Hollingsworth, P.M. (2001) Rapid diversification of a species-rich genus of Neotropical rain forest trees. *Science*, **293**, 2242-2245

Rieseberg, L.H. (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae) evidence from ribosomal genes. *American Journal of Botany*, **78**, 1218-1237

Rieseberg, L.H. (2000) Crossing relationships among ancient and experimental sunflower hybrid lineages. *Evolution*, **54**, 859-865

Rieseberg, L.H. (2001) Chromosomal rearrangements and speciation. *Trends in Ecology & Evolution*, **16**, 351-358

Rieseberg, L.H. & Blackman, B.K. (2010) Speciation genes in plants. *Annals of Botany*, **106**, 439-455

Rieseberg, L.H. & Burke, J.M. (2001) The biological reality of species: gene flow, selection, and collective evolution. *Taxon*, **50**, 47-67

Rieseberg, L.H. & Ellstrand, N.C. (1993) What can molecular and morphological markers tells us about plant hybridization? *Critical reviews in plant sciences*, **12**, 213-241

Rieseberg, L.H., Whitton, J. & Gardner, K. (1999) Hybrid zones and the genetic architecture of a barrier to gene flow between two sunflower species. *Genetics*, **152**, 713-727

Rieseberg, L.H., Widmer, A., Arntz, A.M. & Burke, J.M. (2003) The genetic architecture necessary for transgressive segregation is common in both natural and domesticated populations. *Philos Trans R Soc Lond B Biol Sci.*, **358**, 1141-1147

Rieseberg, L.H. & Willis, J.H. (2007) Plant speciation. *Science*, **317**, 910-914

Rieseberg, L.H., Wood, T.E. & Baack, E.J. (2006) The nature of plant species. *Nature*, **440**, 524-527

Rosenberg, N.A. (2004) DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes*, **4**, 137-138

Rousset, F. (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, **145**, 1219-1228

Rubino, D.B. & Wehner, T.C. (1986) Effect of inbreeding on horticultural performance of lines developed from an open-pickling cucumber population. *Euphytica*, **35**, 459-464

Ruhsam, M., Hollingsworth, P.M. & Ennos, R.A. (2010) Early evolution in a hybrid swarm between outcrossing and selfing lineages in *Geum*. *Heredity*, **107**, 246-255

Sanderson, M.J. & Wojciechowski, M.F. (1996) Diversification rates in a temperate legume clade: why are there "so many species" of *Astragalus* (Fabaceae). *American Journal of Botany*, **83**, 1488-1502

Sasa, M.M., Chippindale, P.T. & Johnson, N.A. (1998) Patterns of postzygotic isolation in frogs. *Evolution*, **52**, 1811-1820

Scascitelli, M., Whitney, K.D., Randell, R.A., King, M., Buerkle, C.A. & Rieseberg, L.H. (2010) Genome scan of hybridizing sunflowers from texas (*Helianthus annuus* and *H. debilis*) reveals asymmetric patterns of introgression and small islands of genomic differentiation. *Molecular Ecology*, **19**, 521-541

Schaefer, H. & Renner, S.S. (2011) Phylogenetic relationships in the order cucurbitales and a new classification of the gourd family (Cucurbitaceae). *Taxon*, **60**, 122-138

Schemske, D.W., Ågren, J., Le Corff, J., Lloyd, D.G. & Barrett, S.C.H. (1996) Deceipt pollination in the monoecious, neotropical herb *Begonia oaxacana* (Begoniaceae). *Floral biology: studies of floral evolution in animal-pollinated plants* (ed. by D.G. Lloyd and S.C.H. Barrett), pp. 292-318. Chapman & Hall, New York.

Schemske, D.W. & Bradshaw, H.D. (1999) Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 11910-11915

Schmitt, J. (1980) Pollinator foraging behavior and gene dispersal in *Senecio* (Compositae). *Evolution*, **34**, 934-943

Schoen, D.J. & Brown, A.H.D. (1991) Intraspecific variation in population gene diversity and effective population size correlates with the mating system in plants. *Proceedings of the National Academy of Science*, **88**, 4494-4497

Schoen, D.J. & Dubuc, M. (1990) The evolution of inflorescence size and number: a gamete-packaging strategy in plants. *The American Naturalist*, **135**, 841-857

Schuelke, M. (2000) An economic method for the fluorescent labeling of PCR fragments. *Nat Biotech*, **18**, 233-234

Scopece, G., Lexer, C., Widmer, A. & Cozzolino, S. (2010) Polymorphism of postmating reproductive isolation within plant species. *Taxon*, **59**, 1367-1374

Scopece, G., Musacchio, A., Widmer, A. & Cozzolino, S. (2007) Patterns of reproductive isolation in Mediterranean deceptive orchids. *Evolution*, **61**, 2623-2642

Scotti-Saintagne, C., Mariette, S., Porth, I., Goicoechea, P.G., Barreneche, T., Bodenes, C., Burg, K. & Kremer, A. (2004) Genome scanning for interspecific differentiation between two closely related oak species [*Quercus robur* L. and *Q. petraea* (Matt.) Liebl.]. *Genetics*, **168**, 1615-1626

Seeb, J.E., Carvalho, G., Hauser, L., Naish, K., Roberts, S. & Seeb, L.W. (2011) Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in nonmodel organisms. *Molecular Ecology Resources*, **11**, 1-8

Shaffer, H.B. & Thomson, R.C. (2007) Delimiting species in recent radiations. *Systematic Biology*, **56**, 896-906

Shrestha, M.K., Golan-Goldhirsh, A. & Ward, D. (2002) Population genetic structure and the conservation of isolated populations of *Acacia raddiana* in the Negev Desert. *Biological Conservation*, **108**, 119-127

Silvestro, D., Schnitzler, J. & Zizka, G. (2011) A bayesian framework to estimate diversification rates and their variation through time and space. *BMC Evolutionary Biology*, **11**, 311

Siol, M., Prosperi, J.M., Bonnin, I. & Ronfort, J. (2008) How multilocus genotypic pattern helps to understand the history of selfing populations: a case study in *Medicago truncatula*. *Heredity*, **100**, 517-525

Sites, J.W. & Marshall, J.C. (2004) Operational criteria for delimiting species. *Annual Review of Ecology, Evolution and systematics*, **35**, 199-277

Slatkin, M. (1977) Gene flow and genetic drift in a species subject to frequent local extinctions. *Theoretical Population Biology*, **12**, 253-262

Slatkin, M. (1987) Gene flow and the geographic structure of natural populations. *Science*, **236**, 787-792

Slatkin, M. (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457-462

Smith, A.B. (2009) *Systematics and the fossil record*. Blackwell Science Ltd.

Soltis, D.E., S. Soltis, P., Pires, J.C., Kovarik, A., Tate, J.A. & Mavrodiev, E. (2004) Recent and recurrent polyploidy in *Tragopogon* (Asteraceae): cytogenetic, genomic and genetic comparisons. *Biological Journal of the Linnean Society*, **82**, 485-501

Soltis, P.S. & Soltis, D.E. (2009) The role of hybridization in plant speciation. *Annual Review of Plant Biology*, **60**, 561-588

Spitze, K. (1993) Population structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. *Genetics*, **135**, 367-74

Squirrell, J., Hollingsworth, P.M., Woodhead, M., Russell, J., Lowe, A.J., Gibby, M. & Powell, W. (2003) How much effort is required to isolate nuclear microsatellites from plants? *Molecular Ecology*, **12**, 1339-1348

Squirrell, J., Hollingsworth, P.M., Bateman, R.M., Dickson, J.H., Light, M.H.S., MacConaill, M., Tebbitt, M.C. (2001) Partitioning and geneticdiversity of nuclear and organelle markers in native and introduced populations of *Epipactis helleborine* (Orchidaceae). *American Journal of Botany*, **88**, 1409 - 1418

Stace, C.A. (1975) *Hybridization and the flora of the British Isles*. Academic Press, London.

Stace, C.A. (1989) *Plant taxonomy and biosystematics*, 2nd Ed edn. Cambridge University Press, Cambridge.

Steane, D., Conod, N., Jones, R., Vaillancourt, R. & Potts, B. (2006) A comparative analysis of population structure of a forest tree, *Eucalyptus globulus* (Myrtaceae), using microsatellite markers and quantitative traits. *Tree Genetics & Genomes*, **2**, 30-38

Stebbins, G.L. (1956) Taxonomy and the evolution of genera, with special reference to the family Gramineae. *Evolution*, **10**, 235-245

Stebbins, G.L. (1973) Evolutionary trends in the inflorescence of angiosperms. *Flora*, **162**, 501-528

Stebbins, G.L. (1974) *Flowering plants: Evolution above the species level*. The Belknap Press of Harvard University Press, Cambridge, Ma, C.

Stehlik, I., Friedman, J. & Barrett, S.C.H. (2008) Environmental influence on primary sex ratio in a dioecious plant. *Proceedings of the National Academy of Science*, **105**, 10847-10852

Steinger, T., Haldimann, P., Leiss, K.A. & Müller-Schärer, H. (2002) Does natural selection promote population divergence? A comparative analysis of population structure using amplified fragment length polymorphism markers and quantitative traits. *Molecular Ecology*, **11**, 2583-2590

Strasburg, J.L., Sherman, N.A., Wright, K.M., Moyle, L.C., Willis, J.H. & Rieseberg, L.H. (2012) What can patterns of differentiation across plant genomes tell us about adaptation and speciation? *Philosophical Transactions of the Royal Society B: Biological Sciences*, **367**, 364-373

Stults, D.Z. & Axsmith, B.J. (2011) First macrofossil record of *Begonia* (Begoniaceae). *American Journal of Botany*, **98**, 150-153

Sullivan, J., Arellano, E. & Rogers, D.S. (2000) Comparative phylogeography of mesoamerican highland rodents: concerted versus independent response to past climatic fluctuations. *The American Naturalist*, **155**, 755-768

Swarbreck, D., Wilks, C., Lamesch, P., Berardini, T.Z., Garcia-Hernandez, M., Foerster, H., Li, D., Meyer, T., Muller, R., Ploetz, L., Radenbaugh, A., Singh, S., Swing, V., Tissier, C., Zhang, P. & Huala, E. (2008) The *Arabidopsis* information resource (TAIR): gene structure and function annotation. *Nucleic Acids Research*, **36**, D1009-D1014

Sweigart, A.L., Fishman, L. & Willis, J.H. (2006) A simple genetic incompatibility causes hybrid male sterility in *Mimulus*. *Genetics*, **172**, 2465-2479

Takezaki, N., Nei, M. & Tamura, K. (2010) POPTREE2: Software for constructing population trees from allele frequency data and computing other population statistics with windows interface. *Molecular Biology and Evolution*, **27**, 747-752

Teacher, A.G.F. & Griffiths, D.J. (2011) HapStar: automated haplotype network layout and visualization. *Molecular Ecology Resources*, **11**, 151-153

Tao, Y. & Hartl, D.L. (2003) Genetic dissection of hybrid incompatabilities between *Drosophila simulans* and *D. mauritiana*: III. Heterogeneous accumulation of hybrid incompatabilties, degree of dominance, and implications for Haldane’s rule. *Evolution*, **57**, 2580-2598

Taylor, E.B., Boughman, J.W., Groeneboom, M., Sniatynski, M., Schluter, D. & Gow, L. (2006) Speciation in reverse: morphological and genetic evidence of the collapse of a three-spined stickleback (*Gasterosteus aculeatus*) species pair. *Molecular Ecology*, **15**, 343-355

Tebbit, M.C. (2005) *Begonias: cultivation, natural history, and identification*. Timber Press, Portland.

Teo, L.-L. & Kiew, R. (1999) First record of a natural *Begonia* hybrid in Malaysia. *Gardens' Bulletin Singapore*, **51**, 103-118

Thomas, D.C. (2010) Phylogenetics and historical biogeography of South East Asian *Begonia* L. (Begoniaceae). PhD thesis, University of Glasgow, Glasgow

Thomas, D.C., Hughes, M., Phutthai, T., Ardi, W.H., Rajbhandary, S., Rubite, R., Twyford, A.D. & Richardson, J.E. (2011a) West to east dispersal and subsequent rapid diversification of the mega-diverse genus *Begonia* (Begoniaceae) in the Malesian Archipelago. *Journal of Biogeography*, **39**, 98-113

Thomas, D.C., Hughes, M., Phutthai, T., Rajbhandary, S., Rubite, R., Ardi, W.H. & Richardson, J.E. (2011b) A non-coding plastid DNA phylogeny of asian *Begonia* (Begoniaceae): evidence for morphological homoplasy and sectional polyphyly. *Molecular Phylogenetics and Evolution*, **60**, 428-444

Tilley, S.G., Verrell, P.A. & Arnold, S.J. (1990) Correspondence between sexual isolation and allozyme differentiation: a test in the salamander *Desmognathus ochrophaeus*. *Proceedings of the National Academy of Sciences*, **87**, 2715-2719

Toledo, V.M. (1982) Pleistocene changes of vegetation in tropical Mexico. *Biological diversification in the tropics: Proceedings of the firth international symposium of the association for tropical biology, caracas.* (ed. by G.T. Prance), pp. 93-111. Columbia University Press, New York.

Travis, S.E., Maschinski, J. & Keim, P. (1996) An analysis of genetic variation in *Astragalus cremonophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. *Molecular Ecology*, **5**, 735-745

Tucker, S. (1999) The inflorescence: introduction. *The Botanical Review*, **65**, 303-316

Turner, T.L., Hahn, M.W. & Nuzhdin, S.V. (2005) Genomic islands of speciation in *Anopheles gambiae*. *PLoS Biol*, **3**, e285

Twyford, A.D. & Ennos, R.A. (2012) Next-generation hybridization and introgression. *Heredity*, **108**, 179-189

Ungerer, M.C., Baird, S.J.E., Pan, J. & Rieseberg, L.H. (1998) Rapid hybrid speciation in wild sunflowers. *Proceedings of the National Academy of Sciences*, **95**, 11757-11762

Vähä, J.P. & Primmer, C.R. (2006) Efficiency of model-based bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology*, **15**, 63-72

Valdes, A.M., Slatkin, M. & Freimer, N.B. (1993) Allele frequencies at microsatellite loci: the stepwise mutation model revisited. *Genetics*, **133**, 737-49

Valente, L.M., Savolainen, V. & Vargas, P. (2010) Unparalleled rates of species diversification in Europe. *Proceedings of the royal society B Biological Sciences*, **277**, 1489-1486

Vallone, P.M. & Butler, J.M. (2004) AutoDimer: a screening tool for primer-dimer and hairpin structures. *BioTechniques*, **37**, 226-231

Vamosi, J.C. & Dickinson, T.A. (2006) Polyploidy and diversification: a phylogenetic investigation in Rosaceae. *International Journal of Plant Sciences*, **167**, 349-358

Varshney, R.K., Graner, A. & Sorrells, M.E. (2005) Genic microsatellite markers in plants: features and applications. *Trends in Biotechnology*, **23**, 48-55

Vellend, M.V. & Geber, M.A. (2005) Connections between species diversity and genetic diversity. *Ecology Letters*, **8**, 767-781

Vendramin, G.G., Fady, B., González-Martínez, S.C., Hu, F.S., Scotti, I., Sebastiani, F., Soto, Á. & Petit, R.J. (2008) Genetically depauperate but widespread: the case of an emblematic Mediterranean pine. *Evolution*, **62**, 680-688

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T.V.D., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M. & Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**, 4407-4414

Waldmann, P. & Andersson, S. (1999) Multilocus and multitrait differentiation of populations of the locally rare plant *Scabiosa canescens* and the more common *S. columbaria*. *Hereditas*, **130**, 341-343

Walters, S.M. (1986) The name of a rose: a review of ideas on the european bias in angiosperm classification. *New Phytologist*, **104**

Wang, D.-H., Li, F., Duan, Q.-H., Han, T., Xu, Z.-H. & Bai, S.-N. (2010) Ethylene perception is involved in female cucumber flower development. *The Plant Journal*, **61**, 862-872

Weir, B.S. & Cockerham, C.C. (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358-1370

Weir, J.T. & Schluter, D. (2007) The latitudinal gradient in recent speciation and extinction rates of birds and mammals. *Science*, **315**, 1574-1576

Wertheim, J.O. & Sanderson, M.J. (2011) Estimating diversification rates: how useful are divergence times? *Evolution*, **65**, 309-320

Whitney, K.D., Randell, R.A. & Rieseberg, L.H. (2010) Adaptive introgression of abiotic tolerance traits in the sunflower *Helianthus annuus*. *New Phytologist*, **187**, 230-239

Whittall, J.B., Syring, J., Parks, M., Buenrostro, J., Dick, C., Liston, A. & Cronn, R. (2010) Finding a (pine) needle in a haystack: chloroplast genome sequence divergence in rare and widespread pines. *Molecular Ecology*, **19**, 100-114

Widmer, A., Lexer, C. & Cozzolino, S. (2009) Evolution of reproductive isolation in plants. *Heredity*, **102**, 31-38

Willi, Y. & Määttanen, K. (2010) The relative importance of factors determining genetic drift: mating system, spatial genetic structure, habitat and census size in *Arabidopsis lyrata*. *New Phytologist*, **189**, 1200-1209

Willis, J.C. (1922) *Age and area*. Cambridge University Press, Cambridge.

Willyard, A., Cronn, R., Liston, A., (2009) Reticulate evolution and incomplete lineage sorting among the ponderosa pines. *Molecular Phylogenetics and Evolution*, **52**, 498-511

Worth, J.R.P., Marthick, J.R., Jordan, G.J. & Vaillancourt, R.E. (2011) Low but structured chloroplast diversity in *Atherosperma moschatum* (Atherospermataceae) suggests bottlenecks in response to the Pleistocene glacial. *Annals of Botany* **108**, 1247-1256

Wright, I.J. (1931) Evolution in Mendelian populations. *Genetics*, **16**

Wright, S. (1943) Isolation by distance. *Genetics*, **28**, 114-138

Wright, S.I., Nano, N., Foxe, J.P. & Dar, V.N. (2008) Effective population size and tests of neutrality at cytoplasmic genes in *Arabidopsis*. *Genetics Research*, **90**, 119-128

Wu, C.-I. (2001a) The genic view of the process of speciation. *Journal of Evolutionary Biology*, **14**, 851-865

Wu, C.-I. & Ting, C.-T. (2004) Genes and speciation. *Nature Reviews Genetics*, **5**, 114-122

Wu, C.A., Lowry, D.B., Cooley, A.M., Wright, K.M., Lee, Y.W. & Willis, J.H. (2008) *Mimulus* is an emerging model system for the intergration of ecological and genomic strudies. *Heredity*, **100**, 220-230

Wu, T., Qin, Z., Zhou, X., Feng, Z. & Du, Y. (2010) Transcriptome profile analysis of floral sex determination in cucumber. *Journal of Plant Physiology*, **167**, 905-913

Wyatt, G.E. & Sazima, M. (2011) Pollination and reproductive biology of thirteen species of *Begonia* in the Serra do Mar State Park, São Paulo, Brazil. *Journal of Pollination Ecology*, **6**, 95-107

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. & Meyerowitz, E.M. (1990) The protein encoded by the *Arabidopsis* homeotic gene agamous resembles transcription factors. *Nature*, **346**, 35-39

Yatabe, Y., Kane, N.C., Scotti-Saintagne, C. & Rieseberg, L.H. (2007) Rampant gene exchange across a strong reproductive barrier between the annual sunflowers, *Helianthus annuus* and *H. petiolaris*. *Genetics*, **175**, 1883-1893

Zane, L., Bargelloni, L., Patarenllo, T. (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology*, **11**, 1-1

9. APPENDICES

The CD-ROM that accompanies the thesis includes the content listed in the table below. Full description of the data is included in each file.

File name	Chapter	Contents
Appendix 1 – *Begonia heracleifolia* plastid haplotypes.txt	2	Plastid microsatellite genotypes at 7 loci for 18 populations of *B. heracleifolia* (263 individuals)
Appendix 2 - *Begonia heracleifolia* nuclear microsatellite data.txt	4	Nuclear microsatellite genotypes at 9 loci for 13 populations of *B. heracleifolia* (306 individuals)
Appendix 3 - *Begonia nelumbiifolia* nuclear microsatellite data.txt	4	Nuclear microsatellite genotypes at 9 loci for 7 populations of *B. nelumbiifolia* (177 individuals)
Appendix 4 – Genetic clustering of *B. heracleifolia* and *B. nelumbiifolia* individuals.pdf	4	Genetic clustering results from InStruct and STRUCTURE for *B. heracleifolia* and *B. nelumbiifolia*, over all K values tested. See text for details
Appendix 5 - Nuclear microsatellite genotpes for hybrid swarm 1 (HS1).txt	5	Nuclear microsatellite genotypes at 12 loci for 61 individuals in a hybrid swarm between *B. heracleifolia* and *B. nelumbiifolia*
Appendix 6 - Nuclear microsatellite genotpes for HS2.txt	5	Nuclear microsatellite genotypes at 12 loci for 71 individuals in a hybrid swarm between *B. heracleifolia* and *B. nelumbiifolia*
Appendix 7 - Nuclear microsatellite genotpes for HS3.txt	5	Nuclear microsatellite genotypes at 12 loci for 61 individuals in a hybrid swarm between *B. heracleifolia* and *B. nelumbiifolia*
Appendix 8 - Nuclear microsatellite genotpes for HS4.txt	5	Nuclear microsatellite genotypes at 12 loci for 100 individuals in a hybrid swarm between *B. heracleifolia* and *B. sericoneura*
Appendix 9 – PBC QTL.txt	6	Genetic map and trait variation for QTL analysis in the *Begonia plebeja* backcross mapping family. (Genotype data from Brennan *et al.*, submitted)
Appendix 10 – CBC QTL.txt	6	Genetic map and trait variation for QTL analysis in the *Begonia conchifolia* backcross mapping family. (Genotype data from Brennan *et al.*, submitted)
Appendix 11 - Twyford and Ennos (2012) Next-generation hybridization and introgression.pdf	n/a	Publication in Heredity during the PhD