

The Mating System Evolution of *Ipomoea lacunosa*

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

The evolution of selfing from outcrossing is one of the most frequent mating system transitions in angiosperms. Plants that are highly selfing typically exhibit a suite of morphological traits termed a “selfing syndrome,” including reduced corollas and reproductive structures, loss of corolla pigmentation, little anther-stigma separation, and a low pollen/ovule ratio. The overall consensus among scientist is that the morphological changes that accompany the transition to selfing are adaptive and thus a product of natural selection. Few attempts, however, have been made to determine whether traits of the selfing syndrome are truly an operation of natural selection or if genetic drift could be the acting force. My dissertation examines the roles that natural selection and genetic drift played in the evolution of the selfing syndrome in *Ipomoea lacunosa*. With the use of field observations, crossing data, and molecular analyses, I show that *I. lacunosa* has evolved increased selfing ability, decreased anther-stigma distance and smaller, white flowers, compared to its closest relative *I. cordatotriloba*. Furthermore, using a standard $Q_{ST} - F_{ST}$ comparison, I evaluated the relative importance of selection and drift in the evolution of the selfing syndrome in *I. lacunosa*. I also identified the genetic basis of flower color divergence between *I. lacunosa* (white) and *I. cordatotriloba* (purple) and examined patterns of variation to determine if selection or genetic drift caused the divergence. Analyses revealed that the traits of *I. lacunosa* characteristic of the selfing syndrome have evolved as a product of natural selection, not genetic drift.

Dedication

To Chris and our baby girl. We can't wait to meet you!

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Introduction

“The close proximity of the anthers to the stigma in a multitude of species favours, and often leads, to self-fertilisation...[flowers] are minute... producing an extremely small quantity of pollen. The flowers of the other kind produce much pollen...bear larger much more conspicuous flowers plainly constructed for cross-fertilisation by the aid of insects; and without their aid these produce no seeds.” -(Darwin, 1876)

It has long been observed that there are phenotypic traits associated with a plant's mating system. Autogamous, or self-pollinating, plants are often characterized by a “selfing syndrome,” which consists of having small, light-colored, scentless flowers, closer anther-stigma distance, and a decrease in pollen/ovule (P/O) ratio (Kalisz et al., 2012; Ornduff, 1969; Sicard & Lenhard, 2011). Plants that outcross contrast markedly from plants that self-pollinate, often having large colorful flowers that are rich in scent and produce copious amounts of pollen, all of which are thought to have evolved to attract pollinators (Ornduff, 1969). The association of selfing rate with flower traits has been shown in many genera, such as *Arenaria* (Wyatt, 1986), *Leavenworthia* (Lloyd, 1965), *Collinsia* (Armbruster et al., 2002), *Mimulus* (Ritland & Ritland, 1989) and *Ipomoea* (McDonald et al., 2011). Furthermore, a recent study found an inverse relationship between flower size and selfing rate in angiosperms (Goodwillie et al., 2010). It is thought that the ability to self-pollinate is a derived characteristic. Selfing plants often encompass novel ecological niches, allowing them to quickly diverge morphologically as well as ecologically from closely related species (Ornduff, 1969). The evolutionary transition from outbreeding to selfing is one of the most recurring

changes in all angiosperms, with an estimated 20% of all flowering plants evolving autogamy as the predominant mode of reproduction (Barrett, 2002). This substantial number of transitions to autogamy is probably why it is the most extensively studied mating system change in flowering plants (Barrett, Morgan, & Husband, 1989).

Evolution of Autogamy- There are two widely accepted hypotheses on why autogamy so frequently evolves. The first theory is known as the “reassurance hypothesis” which states that self-pollination evolves to ensure production of seeds, such as when pollinator availability is low (Baker, 1955; Darwin, 1876; Stebbins, 1950). Support for this hypothesis comes from extensive work that has occurred on the California annuals *Clarkia Xantiana ssp. xantiana* which outcrosses, and *Clarkia Xantiana ssp. pariflora* which predominately selfs (Runions & Geber, 2000). An investigation of the two taxa in natural populations found that plant and flower densities were lowest for selfing populations, supporting the hypothesis that self-pollination arises in situations where there are fewer outcrossing opportunities (Fausto, Eckhart, & Geber, 2001). Moreover, pollinator visitation rates were significantly lower in completely selfing populations than in completely outcrossing populations (Fausto et al., 2001). This decreased trend in pollinator visitation to selfing plants has also been shown in *Impatiens* (Sato & Yahara, 1999).

Further support for the reassurance hypothesis has come from an influential paper in 1955, by H.G. Baker, where he noted similarities in colonization strategies

between plants and the Notostraca fresh water shrimp (Baker, 1955). Baker discussed how self-compatible organisms are more likely than self-incompatible organism to establish a colony after "long-distance" dispersal because they need no other individuals as mates. This idea later became known as Baker's Law (Stebbins, 1957). Empirical support for Baker's Law comes from studies of plants of the Galapagos Islands where the majority of plants studied were self-compatible (McMullen, 1987). Recently, a meta-analysis of published surveys on plant mating systems found that oceanic islands are much more likely to have self-compatible plants than mainland areas (Igic, Lande, & Kohn, 2008).

The second hypothesis for the evolution of autogamy is "automatic selection" which states that alleles that confer self-compatibility have a 3:2 transmission advantage compared to an allele promoting outcrossing (Fisher, 1941; Holsinger, 2000; Schoen, Morgan, & Bataillon, 1996). This transmission advantage is due to the fact that a self-pollinating plant can act as a pollen donor to its own ovule as well as a pollen donor to an outcrossing plant in a population. On the other hand, a plant that outcrosses will act as an ovule donor and a single pollen donor to an outcrossing plant. The automatic selection hypothesis, however, assumes that morphological changes that often accompany self-fertilization do not lower the plant's ability to outcross, which may not always be a reasonable assumption. "Pollen discounting" is the process by which selfing reduces pollen transmission to other plants (Loyd, 1979; Wells, 1979). The small flowers, short

anther-stigma position, and low pollen count often associated with the “selfing-syndrome” reduce transmission of the selfing allele by pollen. This is because small-flowered plants are visited less often by pollinators or less pollen is transmitted because the small amount of pollen produced by selfing pollinating plants is directly deposited onto its own anther and not onto other plants in a population (Charlesworth, 2006).

Evolution of the “Selfing Syndrome”- Like the evolution of autogamy, there are multiple hypotheses on how the selfing syndrome evolves. The first explanation of the evolution of the selfing syndrome centers around sex allocation theory which is based on the assumption that plants have limited resources to invest in reproduction (Charlesworth & Charlesworth, 1981; Charlesworth & Morgan, 1991). Selfing species, therefore, are predicted to invest fewer resources to male function than in female function (Charnov, 1987; de Jong, 1999). Support for this prediction comes from the numerous observations in which the P/O ratio is found to be lower in selfing plants compared to outcrossing plants (Cruden, 1977; McKone, 1987; Plitmann & Levin, 1990; Ritland & Ritland, 1989; Schoen, 1982). It is thought that pollen production represents a substantial investment to a plant and that selection acts to lower this cost (Sicard & Lenhard, 2011). Additionally, research studies have shown that there is often a trade-off between floral size and the number of flowers produced, with small-flowered plants producing more flowers per plant than plants that produce large flowers (Caruso, 2004; Goodwillie et al., 2010; Sargent et al, 2007; Sato & Yahara, 1999; Wyatt, 1986).

The second explanation for the evolution of the selfing syndrome is that natural selection acts to increase self-pollination efficiency. Close anther-stigma distance has been shown in a number of systems to increase selfing ability (Chang & Rausher, 1998; Schueller, 2004; Takebayashi, Wolf, & Delph, 2006). Interestingly, QTLs have found that underlie both anther-stigma distance and flower size (Fishman, Kelly, & Willis, 2002). Selection for these genes could create an increase in self-pollination as well as the characteristically small flowers of plants that self-pollinate.

The third proposed idea for the evolution of selfing syndrome is the “time limitation” hypothesis, which stems from the observation that most plants that self-pollinate are annuals and live in marginal areas (Aarssen, 2003; Arroyo, 1973). In marginal areas, selection is thought to favor individuals that finish reproduction early because the challenging environment may shorten the time a plant has to set seed (Snell & Aarssen, 2005). Therefore, selection favors self-pollination because it decreases the time it takes for an ovule to be pollinated. Additionally, small flowers with decreased anthers stigma distance are favored in challenging environment because they take less time to produce as well as facilitate autogamy (Aarssen, 1989).

A final hypothesis that is seldom discussed is the possibility that many selfing-syndrome traits were not actively selected but arose through genetic drift. Because autogamous plants do not need to attract pollinators their flowers are freed from selective constraints and accumulate random mutations by genetic drift which could disrupt the developmental processes responsible for producing floral traits. The lower effective

population size associated with autogamy greatly enhances the effect of genetic drift, making this hypothesis plausible. However, little attention has been paid to the role the effect genetic drift plays in shaping the selfing syndrome.

Autogamy's Effect on Genome Evolution- Like the evolution of autogamy and the selfing syndrome, the distinctive ways in which autogamy effects the overall genetic diversity of a species are rich in evolutionary theory and empirical data (Charlesworth & Wright, 2001). Neutral loci of autogamous plants show an increase in homozygosity, while species that outcross have allele frequencies in Hardy-Weinberg proportions unless gene flow is inhibited between populations (Charlesworth & Wright, 2001). The excess homozygosity found in self-pollinating plants lowers their effective population size (N_e) and increases the effect of genetic drift (Pollak, 1987). Complete inbreeding reduces N_e by half (Nordborg, 1999). The increased homozygosity enhances the expression of deleterious recessive mutations which reduces the survival of a plant, a process known as “inbreeding depression.” Over time, however, the reduced survival of individuals with slight deleterious alleles will be eventually purged from populations, and the detrimental effects of inbreeding depression is lowered (Byers & Waller, 1999). Conversely, inbreeding can also have an advantageous effect by increasing the expression of beneficial alleles that would not be seen in outcrossing populations because they are recessive. In highly selfing populations a greater number of mutations are therefore effected by selection (Charlesworth, 1992).

Genetic diversity is typically very low in inbreeding populations. Meiosis and recombination occur in highly inbred populations. However, the high level of homozygosity and genetic similarity among highly selfing individuals make the effective recombination rate extremely low (Nordborg, 1999). The lower effective recombination rate as well as the small effective population size greatly reduce the overall genetic diversity in self-pollinating plants (Charlesworth, 2006). Furthermore, populations of highly selfing plants are often more isolated than populations of outcrossing plants. This is because autogamous plants tend to produce less pollen as well as self-fertilize as soon as a flower matures, which decreases the level of gene flow through pollen (Charlesworth & Wright, 2001). Genetic diversity is expected to be greater between populations rather than within populations in self-fertilizing plants (Charlesworth, 2003). A meta-analysis of the genetic diversity of seed plants found that the genetic difference between populations is greatest for selfing and lowest for outcrossing species (Hamrick & Godt, 1996).

Summary- The transition to autogamy is rich in evolutionary theory and empirical data that dates back over 130 years with observations made by Darwin. To date, it remains an active area of research, and with modern technology the selfing syndrome is a wonderful system to answer further evolutionary questions. For example, the numerous parallel transitions from outcrossing to selfing throughout angiosperms make it an ideal system to study the constraints that underlie morphological evolution of plants. Only recently can questions be addressed such as: Are the same genes causing the

phenotypic transitions to the selfing syndrome? Are coding or expression changes creating the phenotypic change? How are selection and drift maintaining selfing syndrome traits at the molecular level? The only way to address these questions is to find the genes underlying these traits for multiple taxa with differing mating systems. Ideally, the taxa will be recently diverged, giving a clearer understanding of the evolutionary forces that created floral transitions.

Dissertation Focus- My dissertation is a first step in characterizing and understanding the evolutionary forces driving the “selfing syndrome” in closely related taxa of *Ipomoea*. Field observations, crossing data, and molecular analyses were used to understand the relatedness of *I. cordatotriloba*, *I. X leucantha*, *I. austinii*, and *I. lacunosa*. Aspects of genetic structure of *I. cordatotriloba* and *I. lacunosa* were analyzed to understand each plant’s mating system. Furthermore, molecular analyses were conducted to understand the role that genetic drift and natural selection played in creating the divergent floral morphology of the two taxa, with *I. cordatotriloba* having large purple flowers and *I. lacunosa* the small white flowers characteristic of a highly selfing plant. The close relatedness of the taxa makes them ideal for studying the evolution of floral transition.

In chapter 1, I quantified morphological and genetic divergence among *I. cordatotriloba*, *I. lacunosa*, *I. austinii*, and *I. X leucantha* in North and South Carolina to understand how interspecific gene flow is shaping species boundaries among the taxa. I censused populations of each taxa growing in NC and SC and conducted analyses of

genetic structure, using microsatellite markers, to determine the relationships among the plants. I also determined if reproductive incompatibilities exist between the taxa by conducting crosses.

In chapter 2, I quantified patterns of genetic variation across the geographic range of *I. lacunosa* and *I. cordatotriloba* to determine whether genetic drift can explain the divergence in floral morphological traits between the *I. lacunosa* and *I. cordatotriloba*. I performed an F_{st} - Q_{st} analysis to determine whether morphological differences between *I. cordatotriloba* and *I. lacunosa* could be explained by genetic drift.

In chapter 3, I identified the genetic basis of flower color divergence between *I. lacunosa* (white) and *I. cordatotriloba* (purple) and examine patterns of variation to determine if selection or genetic drift caused divergence. I conducted co-segregation analyses and expression assays on anthocyanin genes in which mutations are known to produce white flowers. After identifying the gene responsible for white flower color, I used standard population-genetic analyses of sequence variation to detect whether evolution of this locus departs from neutral expectations.

Chapter 1. The effects of interspecific gene flow on species boundaries between closely related taxa in the *Ipomoea batatas* complex

1.1 Introduction

Decades of research have been spent characterizing the relationships among taxa of the *Ipomoea* series *Batatas*, which includes the economically important sweet potato as well as 12 other recognized species and at least one species believed to have arisen through homoploid hybridization (Austin, 1991, 1988; 1978; Austin & Bianchini, 1998; Austin, Delapuenta, & Contreras, 1991; Austin & Huaman, 1996; Ooststroom, 1953; Verdcourt, 1967). Nevertheless, the relationships among these taxa remain largely unresolved (Nimmakayala, Vajja, & Reddy, 2011). Phylogenetic reconstruction based on morphology has proven unreliable because many of the taxa share overlapping traits (Austin, 1988). In addition, high levels of shared genetic variation among the taxa complicate molecular phylogenetic analyses, creating conflicting or uninformative species relationships (Rajapakse et al., 2004). Recent common ancestry, homoplasy, and interspecific hybridization are all thought to be homogenizing character states and the genetic architecture in this group, making a complete phylogenetic understanding difficult (Jarret, Gawel, & Whittemore, 1992).

Within this group, *I. cordatotriloba*, *I. lacunosa*, and *I. X leucantha* are a closely related group of taxa that are frequently misclassified because of morphological similarities (Austin, 1978). Field surveys of natural populations of *I. cordatotriloba* and

I. lacunosa have shown that morphological traits within in each taxa exhibit wide variation (Abel & Austin, 1981). Furthermore, offspring generated by artificial crosses between the two taxa revealed a diverse array of morphologies which were similar to the variation found in nature (Abel & Austin, 1981). From these field observations, as well as crossing data, it has been hypothesized that introgressive gene flow is occurring between *I. cordatotriloba* and *I. lacunosa* and that *I. X leucantha* is a stable homoploid hybrid of these two taxa (Abel & Austin, 1981). Other variants of *I. X leucantha* can be found in nature but are thought to be insufficiently stabilized to be given a formal species name (Abel & Austin, 1981; Austin, 1978). Although additional studies have been conducted to characterize the macromorphological features of *I. cordatotriloba*, *I. lacunosa*, and *I. X leucantha* (Bryson, Reddy, & Burke, 2008; Stephenson, Oliver, Burgos, & Gbur, 2006), no genetic analysis has been conducted on natural populations to determine whether gene flow is restricted between any of these taxa

Genetic methods such as restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), and inter-simple sequence repeat (ISSR) have been employed to understand the species relationships in the *Batatas* series (Huang & Sun, 2000; Jarret & Austin, 1994; Jarret et al., 1992). Unfortunately, the resolution of these markers has been insufficient to draw distinct conclusions about taxon relationships or about gene flow. Recently, microsatellite markers have been developed for taxa in the *Batatas* series (Hu, Nakatani, Lalusin, & Fujimura, 2004). Microsatellites

are repetitive motifs of DNA that have been found to be extremely variable both within and between species (Tautz, Trick, & Dover, 1986). The variability of microsatellites is generated by DNA slippage during replication and unequal crossing over during meiosis (Levinson & Gutman, 1987). When comparing the ability of RFLP, RAPD, and microsatellites to capture genetic diversity within soybeans, the microsatellites were able to capture the highest level of heterozygosity (Powell et al., 1996), which is critical to quantifying gene flow between closely related taxa in natural populations.

The purpose of the study described here was to quantify the level of morphological and genetic variation within as well as differentiation among populations of *I. lacunosa*, *I. cordatotriloba* and *I. X leucantha*. Specifically, the study asked 1) Are morphological traits significantly differentiated among taxa? 2) Are the taxa genetically differentiated? 3) Is there substantial gene flow among any of the taxa? and 4) Do reproductive incompatibilities exist that could impede gene exchange between the taxa?

To answer these questions I conducted a survey of all three taxa in North Carolina (NC) and South Carolina (SC) because all three plants had previously been described to co-occur in those areas (Abel & Austin, 1981). During the course of the investigation, I identified a substantial number of populations that appeared to belong to the *I. batatas* complex but also appeared to be morphologically distinct from the other three taxa. Based on morphological examination, plants from these populations have been identified by Dr. D. Austin, as another, unstable, hybrid between *I. cordatotriloba* and *I. lacunosa*

that I tentatively term "*I. austinii*." I included *I. austinii* in my analyses to further understand the effects of gene flow and homoploid hybridization between these closely related group of plants.

1.2 Materials and Methods

Study organisms- *Ipomoea lacunosa* and *I. cordatotriloba* are noxious weeds indigenous to the southeastern United States (Jones & Deonier, 1965). *I. cordatotriloba* and *I. lacunosa* can cross and make viable offspring, and extensive gene flow is thought to be occurring between these taxa in nature (Abel & Austin, 1981; Diaz, Schmiediche, & Austin, 1996; Jones & Deonier, 1965). It has been hypothesized that *I. X leucantha* is a stable hybrid between *I. lacunosa* and *I. cordatotriloba* (Abel & Austin, 1973, 1981). Additionally, *I. austinii* is a taxon thought to be derived from *I. cordatotriloba* and *I. lacunosa* but insufficiently distinct to be given a formal species name (D. Austin, *personal communication*). I found that *I. austinii* was true breeding when grown in a greenhouse. All four taxa are found predominately along roadsides, in agricultural fields and in other disturbed areas. In NC and SC the plants germinate in late May and begin to flower in August or early September (*personal observations*). Flowering ceases sometime in mid to late fall and plants die at the first hard frost. Plants of each taxon are self-compatible and are highly autogamous in the greenhouse. Because species in the *Batatas* series are often misidentified, I had Dr. D. Austin verify that exemplars of each

taxon were properly classified in the study (D. Austin, *personal communication*).

Voucher specimens have been deposited in the Duke University herbarium.

Species survey and floral measurements- In August and September of 2010, a large census was conducted on populations of *I. lacunosa*, *I. cordatotriloba*, *I. X leucantha*, and *I. austinii* throughout NC and SC. For each of the 154 sites visited, GPS coordinates were recorded (Appendix A). During the survey morphological measurements of corolla shape, anther color, anther-stigma position, flower width, flower length, flower width-length ratio, stigma length, number of flowers on the cymes, leaf length, leaf width, and leaf width-length ratio were performed on a subset of populations throughout the census range. Many of these traits were measured in a previous examination of morphological variation within and between *I. cordatotriloba* and *I. lacunosa* (Abel & Austin, 1981). In total, I surveyed 896 plants from 39 populations.

Analysis of morphological data- To determine whether the taxa were morphologically differentiated, I performed a discriminant analysis using the four taxa. I then performed a multivariate analysis of variance (MANOVA) on the discriminant scores to determine the significance of morphological differences among the taxa (Timm, 1975). This analysis contained two independent variables: taxon and population nested within taxon. Both analyses were performed using SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA). Leaf length and width were not included in the analysis because the measurements did not differentiate the species.

Tissue collection and DNA extraction- During the survey, leaf tissue was collected for genotyping from 5-30 individuals from each of 7 *I. cordatotriloba*, 6 *I. X leucantha*, 5 *I. austinii*, and 8 *I. lacunosa* populations. Leaf tissue was collected from plants at least 3-5 meters apart to minimize sampling of related individuals. Leaf tissue was collected from 288 individuals throughout the census range.

DNA was extracted using a CTAB protocol (Doyle & Doyle, 1981). I used primers that had been developed for *I. trifidia* but were also reported to amplify microsatellite regions in *I. lacunosa* (Hu et al., 2004). Out of the 8 microsatellites reported to amplify in *I. lacunosa*, I found that only 4 amplified and contained sufficient variability to distinguish among the study taxa (Table 1). Each of the 4 microsatellite markers was amplified with Hex or Fam fluorescently labeled primers, using KAPA taq (Kapa Biosystems, Woburn, Massachusetts, USA), and fragment analysis was conducted on a ABI 3730 x 1 DNA Analyzer. Each marker was visually scored using the software GENEMARKER (SoftGenetics, 2005, State College, Pennsylvania, USA).

Microsatellite data analysis- To interpret the microsatellite data, several approaches were used. First, I determined the genetic relationship between *I. lacunosa*, *I. cordatotriloba*, *I. X leucantha* and *I. austinii*, using the program Structure 2.3.3 (Pritchard, Stephens, & Donnelly, 2000). Twenty replicates of a model were run assuming four populations (k=4) with admixture and independent allele frequencies as well as a burn-in of 10,000 Markov Chain Monte Carlo (MCMC) steps followed by

50,000 iterations. Additionally, I determined the relatedness between different subsets of the taxa (Analysis 1: *I. cordatotriloba*, *I. lacunosa* (k=2); Analysis 2: *I. cordatotriloba*, *I. lacunosa*, *I. X leucantha* (k=2); Analysis 3: *I. cordatotriloba*, *I. lacunosa*, *I. austinii* (k=2)) by running multiple STRUCTURE models that used the same parameters as described above but assumed two populations. Each of the twenty replicates of the STRUCTURE analyses was consolidated using the program CLUMPP (Jakobsson & Rosenberg, 2007), and the output was visualized using the program DISTRUCT (Rosenberg, 2004).

To visualize the patterns of genetic variation contained within the microsatellite data, a principal coordinate analysis (PCA) was performed in the program GenAlEx on the pairwise population matrix of the mean species genotypic genetic distance (Peakall & Smouse, 2006). Additionally, to test if there was significant genetic differentiation that distinguished the four taxa, I ran an analysis of molecular variance (AMOVA) and conducted pairwise comparisons of the four taxa in the program Arlequin 3.5 (Excoffier & Lischer, 2010).

Genomic size of species- To confirm that all four taxa have similar genome sizes, and thus are of similar ploidy, I used flow cytometry. I examined one individual from each taxon using a modified version (Modliszewski & Willis, 2012) of a previously published protocol (Dart, Kron, & Mable, 2004). Plant tissue was analyzed on a Partec

flow cytometer (Partec, Münster, Germany), and each sample was run with *Petunia × hybrida* as an internal control.

Isolation by distance- I tested for isolation by distance for each species pair by conducting a Mantel test with 9,999 permutations between a pair-wise F_{ST} matrix and a geographic-distance matrix in GenAlEx (Peakall & Smouse, 2006). The F_{ST} matrix was created with the microsatellite data in the program Arlequin 3.5. The geographic-distance matrix was created using the GPS coordinates of the populations where the genetic data was obtained using the web program Geographic Distance Matrix (Ersts, 2011).

Crossability of taxa- To determine if there were post-mating incompatibilities among the taxa, I reciprocally crossed all pairs of taxa, except that *I. X leucantha* was not used as pollen recipient because of damage that occurred during anther removal that led to inconsistent results. Three individuals of each taxon collected throughout NC and SC were grown in a greenhouse at Duke University. The night before a plant flowered, the anthers from one of its flowers were removed. Approximately 12 hours after the removal of the anthers, the emasculated flower was manually pollinated using pollen either from another flower on the same plant, from another plant of the same taxon, or from a plant of a different taxon. Each experimental cross was repeated 1-3 times depending on flower availability (average = 1.9 flowers per pollen recipient; 84 crosses total). The average

number of seeds generated from a cross was compared by ANOVA using the SAS statistical software (SAS Institute Inc., Cary, NC, USA).

Reproductive incompatibilities in F₁ (I. X leucantha X I. lacunosa)- In this experiment I examined reproductive incompatibilities of three individuals that were F₁ hybrids between *I. lacunosa* and *I. X leucantha* (F₁(lexla)). As controls, three F₁ individuals were created by crossing purple-flowered *I. lacunosa* with white-flowered *I. lacunosa* (F₁(control)). Each of these plants was crossed as pollen recipient to each of 3 F₁(lexla), 3 F₁(control), 5 white-flowered *I. lacunosa*, 3 purple-flowered *I. lacunosa*, and 4 *I. X leucantha* individuals acting as pollen donors in a greenhouse at Duke University. Three flowers recipient flowers were used for each of these crosses (total number of flowers =306). In addition, 20 flowers on each F₁ plant were allowed to self-pollinate. Experimental plants were not emasculated during the experiment, so seed set could be a product of self or crossed pollen. Consequently, failure to produce seed indicates crossing incompatibility, whereas normal seed production is ambiguous. The average number of seeds produced per capsule was compared by ANOVA using the SAS statistical software (SAS Institute Inc., Cary, NC, USA).

1.3 Results

Geographic distributions- The survey of NC and SC revealed that the four study taxa have separate but overlapping geographical ranges. *I. cordatotriloba* grows primarily along the coast, while *I. X leucantha* and *I. austinii* grow slightly more inland.

I. lacunosa has the widest range. Although it is found primarily inland, some populations are found near the coast. In general, the range of *I. lacunosa* overlaps with all of the other taxa (Figure 1). Consistent with the hypothesis that *I. X leucantha* and *I. austinii* resulted from hybridization between *I. lacunosa* and *I. cordatotriloba*, they occupy locations that lie on the boundary between the other two taxa.

Morphological comparison- During the survey I discovered that the majority of *I. lacunosa* had white flower color; however, a few purple-flowered individuals could be found in some populations in NC. Preliminary comparison of white and purple *I. lacunosa* revealed no difference in morphology (color effect of MANOVA on first 4 principal components of morphology, which account for 90% of the variation: $P = 0.16$); consequently, I pooled data from white and purple *I. lacunosa* for subsequent analyses.

The four taxa had some traits whose means were distinct while the trait means of others were overlapping (Table 2). To examine morphological differentiation of the four taxa I performed a discriminant analysis (Figure 2). The first two canonical variates accounted for 97% of the morphological variation. Canonical axis 1 corresponded primarily to anther color while axis 2 corresponded to a combination of corolla size and shape and stigma length (Table 3).

A four-group discriminant function analysis correctly classified *I. cordatotriloba* and *I. austinii* individuals with 93% or greater accuracy, indicating that these two taxa are morphologically distinct from the other taxa (Table 4). *I. cordatotriloba*, *I. X leucantha*,

and *I. lacunosa* are distinguished from *I. austinii* along the first canonical axes, indicating that *I. austinii* has white anthers while the other taxa have predominately purple anthers. *I. cordatotriloba* is distinguished from *I. lacunosa*, *I. X leucantha*, *I. austinii* along canonical axis 2, indicating that it has a longer, wider corolla and longer stigma than those taxa (Figure 2).

I. X leucantha and *I. lacunosa* were correctly classified with 67% and 92% accuracy, respectively (Table 4). The reduced accuracy for *I. X leucantha* is largely due to substantial morphological overlap between these two taxa, which are morphologically much more similar to each other than to either of the other taxa. However, although I did not include corolla pigmentation in my analysis, these two taxa can be distinguished with very high accuracy by this trait: *I. X leucantha* individuals universally have pigmented corollas, while all but a small proportion of *I. lacunosa* individuals have white corollas lacking pigments.

An overall MANOVA on the canonical variates that included all four taxa was highly significant for the taxon effect, confirming that there was significant morphological differentiation among the four taxa. This overall effect was broken down into three orthogonal independent contrasts: (1) between *I. lacunosa* and *I. X leucantha*; (2) between *I. cordatotriloba* and *I. austinii* ; and (3) between the mean of *I. cordatotriloba* and *I. austinii* and the mean of *I. lacunosa* and *I. X leucantha*. The

contrasts were highly significant for both canonical variates 1 and 2 (Table 5), which supports the inference that all four species were morphologically distinct.

Genetic differentiation- Analysis of microsatellite markers exhibits two main patterns. The first is that *I. lacunosa* and *I. cordatotriloba* are genetically very similar. Averaged across populations, the allele with highest frequency is the same for both taxa at three out of the four microsatellite loci (Table 6). Additionally, at the fourth locus the highest frequency allele in *I. cordatotriloba* is the allele with the second highest frequency (0.31) in *I. lacunosa*, with the most common allele having only a slightly higher frequency (0.34) (Table 6). A STRUCTURE analysis that grouped the microsatellite variation into k=4 groups exhibits a very similar pattern for these two species (Figure 3a), as does a similar analysis when these two taxa are considered alone with k=2 (Figure 4a). In addition, the means of these two species exhibit minimal separation in a plot of the first two principal components of genetic variation, which accounts for 61% of the genetic variation (Figure 3b). This separation is not statistically significant, as judged by AMOVA (Table 7). These results indicate that *I. lacunosa* and *I. cordatotriloba* have diverged little at neutral markers and suggests that substantial gene flow may be occurring between the two species.

The second pattern is that *I. X leucantha* and *I. austinii* exhibit substantial genetic differentiation from each other and from *I. lacunosa* and *I. cordatotriloba*. For each taxon, the most common allele at three of the four microsatellite loci differs from that in

I. lacunosa and *I. cordatotriloba* (Table 6). This differentiation is reflected in their unique genotypic composition revealed by the STRUCTURE analysis (Figure 3a) and by their pronounced separation from the other taxa in the PCA analysis (Figure 3b). This differentiation is maintained in STRUCTURE analyses with $k=2$ in which either *I. X leucantha* or *I. austinii* is compared with *I. cordatotriloba* and *I. lacunosa* (Figure 4b, c). Finally, pairwise AMOVAs corrected for multiple comparisons indicate that *I. X leucantha* and *I. austinii* are genetically distinct from both each other and from *I. cordatotriloba* and *I. X leucantha* (Table 8).

Genome size- A common barrier to gene flow among taxa is difference in ploidy, which would be reflected as differences in genome size. I therefore used flow cytometry to assess the genome size of all the four taxa. The four taxa have approximately similar genome sizes (Table 9), providing no evidence that differential ploidy or genome size might create barriers to gene flow among the taxa.

Isolation by distance- In the absence of reproductive isolating mechanisms, populations can diverge genetically if they are sufficiently isolated geographically. Although the ranges of the four taxa overlap, there are also extensive areas, particularly for *I. cordatotriloba* and *I. lacunosa*, in which each taxon grows by itself. Divergence in these areas could then account for the overall genetic divergence among the taxa, even if reproductive isolation is not high where their ranges overlap.

I examined this possibility by estimating isolation by distance within each species, which would be reflected by a positive correlation between genetic distance and geographic distance. However, the correlation coefficients for all four taxa are less than 0.05 and non-significant (Table 10), indicating that isolation by distance is essentially absent.

Reproductive incompatibilities between and among taxa- Gene flow between the taxa is likely to be inhibited because of their ability to produce seed by self-pollination. However, there seem to be reproductive incompatibilities between all pairs of taxa that would further impede gene flow. Within-taxon pollination produces approximately as many seeds as autogamy (Table 11). Compared to these controls, however, interspecific pollination resulted in a 35% to 95% reduction in seed set. These reductions are highly statistically significant in most cases and indicate the presence of strong, though not complete, post-mating isolation among all four taxa. These barriers are weakest between *I. lacunosa* and *I. cordatotriloba* (average number of seeds per reciprocal crosses = 2.0 ± 0.5 = 50% of control) and somewhat stronger between *I. lacunosa* and *I. X austinii* (average number of seeds per reciprocal crosses = 1.2 ± 0.7 = 30% of control) and between *I. cordatotriloba* and *I. austinii* (average number of seeds per reciprocal crosses = 1.5 ± 0.6 seeds = 39.5% of control). Although I did not score crossability of *I. X leucantha* acting as pollen recipient to the other species, it shows the lowest crossabilities

as pollen parent to the other three taxa (average number of seeds per reciprocal crosses = $0.2 - 0.5 = 5 - 12.5\%$ of controls).

I. X leucantha X I. lacunosa hybrid fertility- Although *I. X leucantha* and *I. lacunosa* are similar morphologically, they are sharply differentiated genetically even though they sometimes grow in close proximity within the same field. This pattern suggests there are barriers to gene exchange. Above I found that crossability of *I. X leucantha* as a pollen donor to the other taxa is extremely low. In this experiment I assessed the ability of *I. X leucantha* to cross as a pollen recipient. Non-emasculated hybrids were pollinated by self-pollen, pollen from another hybrid, from F₁ individuals resulting from a cross between a purple-flowered and white-flowered *I. lacunosa*, or from individuals of *I. lacunosa* or *I. X leucantha*. As controls, I crossed the same pollen donors to the *I. lacunosa* F₁ individuals. Regardless of the sire used, the *I. X leucantha X I. lacunosa* hybrids set essentially no seeds (Table 12). By contrast, the control crosses averaged between 2.5 and 3.6 seeds per flower, and these differences were highly significant. It thus appears that there is almost complete hybrid female sterility in the offspring of crosses between *I. lacunosa* and *I. X leucantha*, a strong barrier to gene exchange between these two species. Because I did not test the ability of hybrid pollen to fertilize *I. lacunosa*, I do not know whether hybrid male sterility serves as an additional barrier.

In order to further assess the degree of reproductive isolation between *I. X leucantha* and *I. lacunosa*, I asked whether the two taxa remain genetically distinct even when they grow in close proximity. I chose one locality in SC where the two taxa have co-occurred for at least 3 years and assessed their genetic distinctness. An AMOVA indicated that approximately 82% of the genetic variation at the site occurred between the two taxa (Table 13), a value that is actually greater than the average F_{CT} of 0.71 between *I. lacunosa* and *I. X leucantha*. This result suggests that substantial reproductive isolation prevents genetic admixture of the two taxa when they grow together.

1.4 Discussion

The status of taxa in the *Batatas* section of *Ipomoea* has long been confusing (Nimmakayala et al., 2011). This is particularly true of taxa closely related to *I. lacunosa*, i.e. *I. cordatotriloba* and *I. X leucantha*. Although attempts have been made to understand the relationships among these taxa (Abel & Austin, 1973, 1981), little progress has been made. Based on morphological characteristics, it has been suggested that these three taxa are undergoing extensive genetic exchange and that they may represent a syngameon with no distinct species (Abel & Austin, 1981). In order to resolve this issue, I undertook an examination of genetic differentiation among the taxa and an analysis of reproductive isolation. Based on this analysis, I draw two conclusions: (1) *I. cordatotriloba* and *I. lacunosa*, although morphologically distinct, are genetically indistinguishable, exhibit only partial crossing incompatibility, and may experience gene

exchange in nature; and (2) *I. X leucantha* and *I. austinii* are each genetically distinct, experience little gene flow from the other taxa, exhibit substantial reproductive isolation, and should be considered separate species. I discuss each of these conclusions below.

Introgressive gene flow between I. cordatotriloba and I. lacunosa- I.

cordatotriloba and *I. lacunosa* are morphologically very distinct, with little overlap in floral characteristics, as indicated by discriminant analysis. Nevertheless, genetic differentiation between these two taxa is weak if not absent, which may be due to either continuing gene flow or recent divergence. Partially reduced crossability of the two species, compared to intra-specific crossability, indicates that there is some reproductive isolation between the two species. However, this degree of isolation does not preclude the possibility of continued introgression. Moreover, the low isolation by distance in both species suggests that distance is not a substantial barrier to introgression.

The substantial divergence in floral morphology between the two taxa contrasts markedly with the genetic similarity indicated by the microsatellite markers. Regardless of whether this similarity is due to continued gene flow or recent separation, this pattern suggests that floral morphological divergence was due to natural selection. Confirmation of this possibility, however, will require a formal analysis of selection on the floral characters.

Lack of introgression in I. X leucantha and I. austinii- It appears that gene flow from the other taxa is minimal for both *I. X leucantha* and *I. austinii* and does not seem to

have prevented genetic divergence by either taxon. These taxa are genetically distinct from each other and from *I. cordatotriloba* and *I. lacunosa*, suggesting that each is a true species. *I. austinii* should therefore be recognized as a new species in the *Batatas* complex, and needs to be formally classified. Additionally, the identification of *I. X leucantha* as a true species agrees with Able and Austin's (Able & Austin, 1981) suggestion that it is a stable hybrid between *I. lacunosa* and *I. cordatotriloba*. For *I. X leucantha*, strong crossability barriers appear to impede genetic exchange that would prevent genetic divergence. This species exhibits an almost complete inability to cross as a pollen donor to the other three taxa. In addition, hybrids produced from crossing with *I. lacunosa* appear to be almost completely female-sterile. These barriers are sufficient to explain lack of gene flow between *I. X leucantha* and the other species, even when they grow at the same sites. Similarly, crossability of *I. austinii* to *I. lacunosa* and *I. cordatotriloba* is low and would impede gene exchange, although not to the extent as in *I. X leucantha*. There are likely other isolating mechanisms that have not yet been examined that allow *I. austinii* to remain genetically distinct.

Origins of the taxa- It has been suggested that *I. X leucantha* represents a homoploid hybrid species formed from hybridization between *I. lacunosa* and *I. cordatotriloba* (Able & Austin, 1981). Daniel Austin has also suggested that *I. austinii* may have originated in a similar fashion (*personal communication*). Unfortunately, the genetic data do not allow assessment of these hypotheses. Previous genetic

investigations of homoploid hybrid species have demonstrated that genetically they are mosaics, with some loci similar to homologous loci in one parental species, and others similar to homologous loci in the other parental species (Arnold et al., 2010). This pattern is not exhibited by either *I. X leucantha* or *I. austinii*. In both *I. X leucantha* and *I. austinii*, the predominant allele is one that is either absent or rare in both purported parental taxa at three of the four microsatellite loci. More importantly, because the allele with the highest frequency at three of the microsatellite loci is the same in *I. cordatotriloba* and *I. lacunosa* and there is substantial shared variation between the two taxa at the fourth loci, it is impossible to ascribe either parent to any of the alleles present in the purported hybrids. However, the data also does not preclude the possibility of hybrid origin for these taxa. If gene flow, even at a low level, occurs between *I. cordatotriloba* and *I. lacunosa*, introgression after hybridization may have erased differences that existed at the time of hybridization. Another possibility is that loci in the purported hybrids may have diverged after hybridization from the common allele present in the purported parental taxa. In a situation like this, the loci most likely to be informative on possible hybrid origin will be those associated with the divergent characters that distinguish *I. cordatotriloba* and *I. lacunosa*. Such loci will presumably have diverged in sequence, affording the opportunity to determine whether at different loci the parental origins of alleles in the purported hybrids differ, indicating a hybrid

origin. This type of analysis thus awaits characterization of the appropriate floral morphology genes.

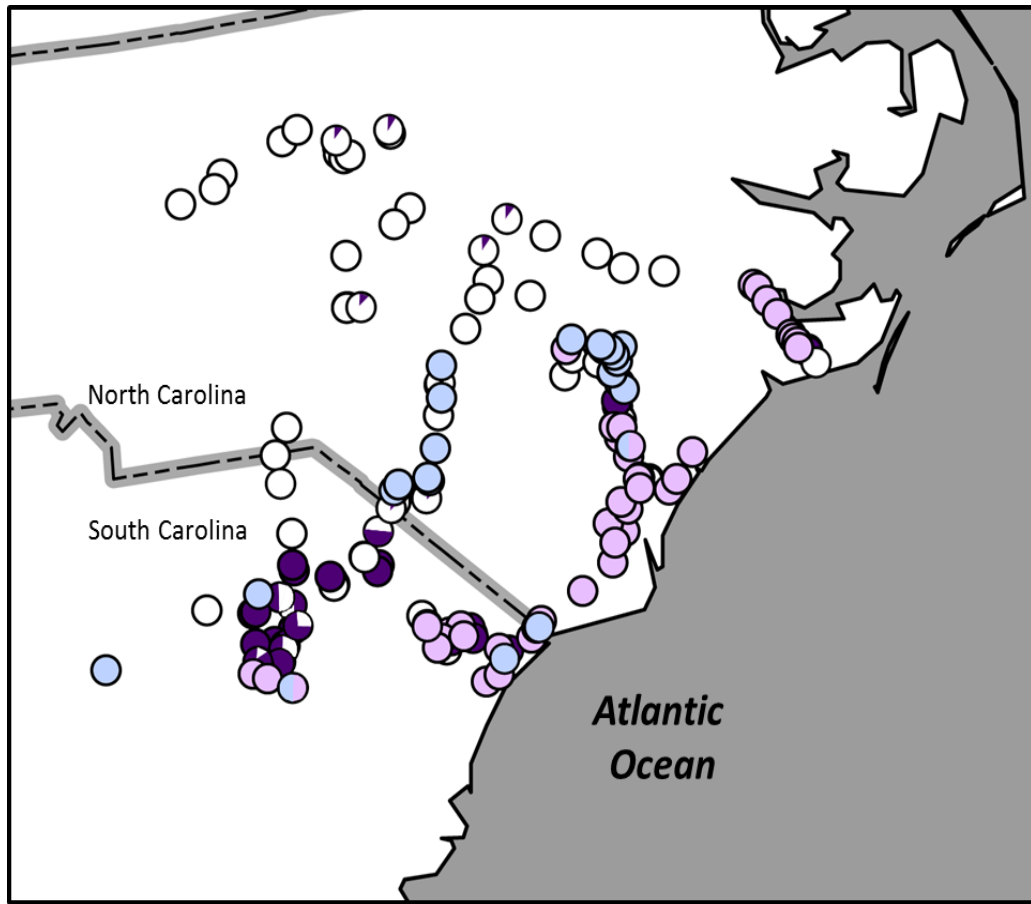


Figure 1: Geographic distributions of taxa.

Circles represent individual sites. The color of the circle indicates which species can be found in the population: white=white flowered *I. lacunosa* (the small purple sectors in NC populations indicate rare purple-flowered *I. lacunosa*); purple = *I. X leucantha*; blue= *I. austinii*; pink=*I. cordatotriloba*.

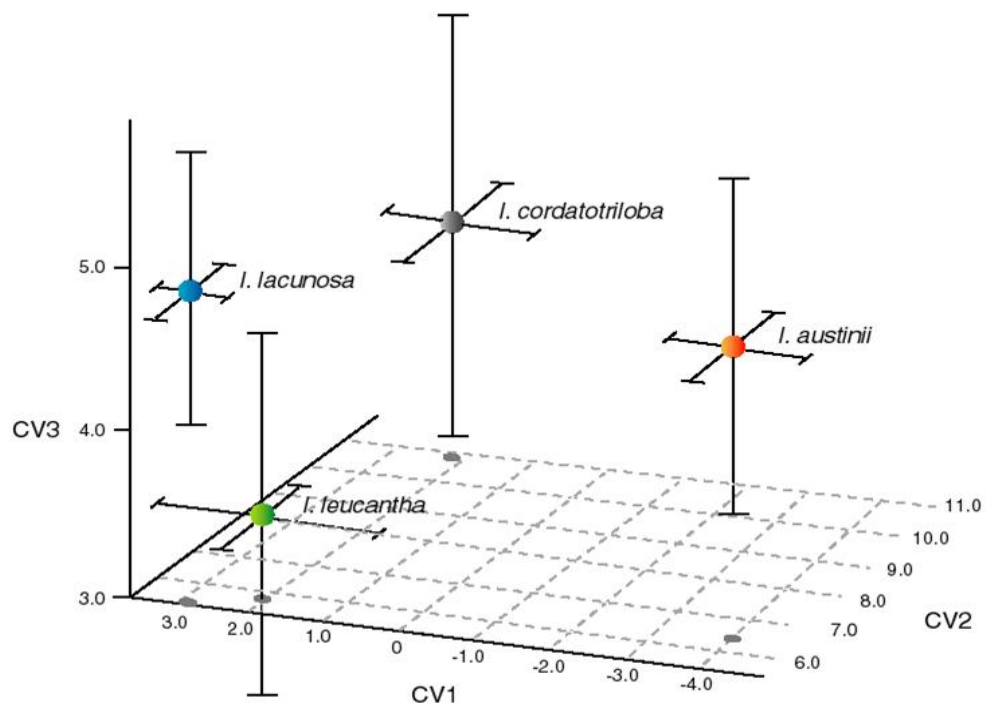


Figure 2: Morphological differentiation among the four taxa.

Means \pm SE of individuals for the two canonical variates are shown. Figure represents data on 8 floral traits of 896 individuals from 39 populations throughout NC and SC. A total of 97% of the overall variation is explained by the first two canonical axes (CAN1= 53%;CAN2=44%).

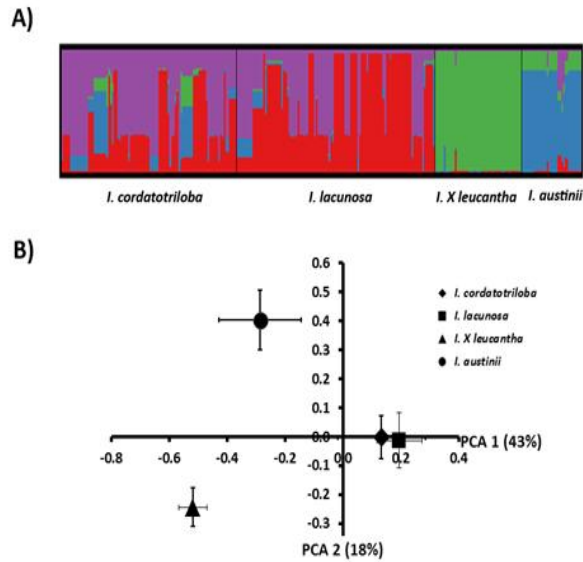


Figure 3: Genetic differentiation among taxa.

A) Structure analysis of four neutral microsatellite loci. Number of classes is $k=4$. B) Plot of location of the four taxa on the first two axes of a principal components analysis of microsatellite markers (PCA1 and PCA2 account for 43% and 18% of the variation, respectively).

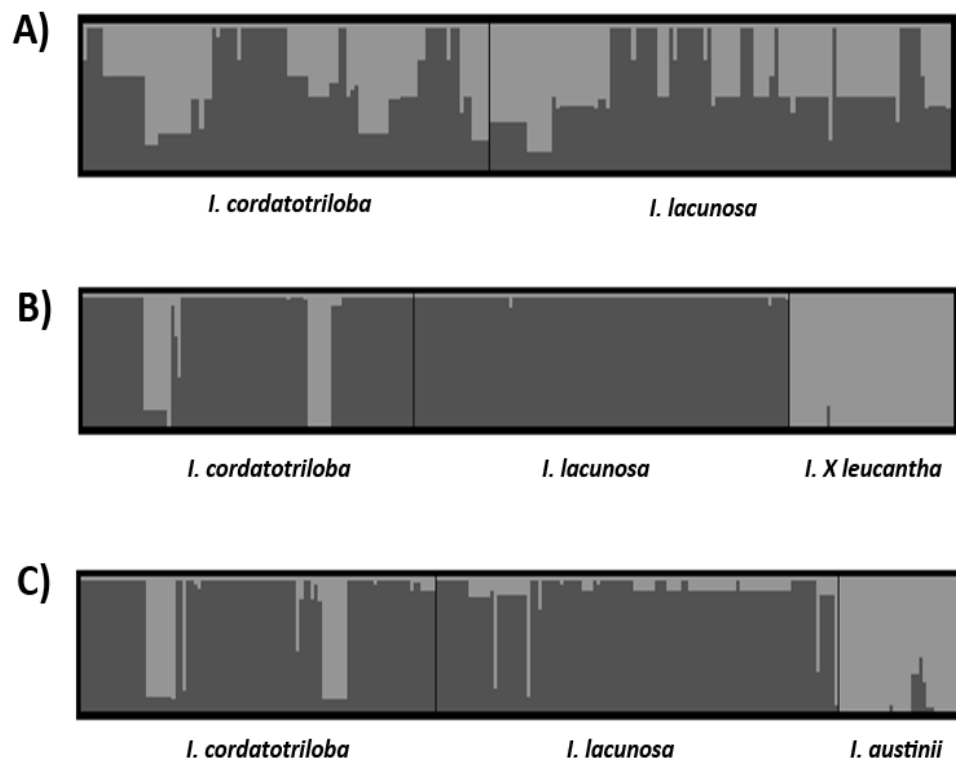


Figure 4: STRUCTURE analyses assuming 2 groups.

A) Analysis of *I. cordatotriloba* and *I. lacunosa* with $k=2$ B) Analysis of *I. X leucantha*, *I. cordatotriloba* and *I. lacunosa* with $k=2$. C) Analysis of *I. austinii*, *I. cordatotriloba* and *I. lacunosa* with $k=2$.

Table 1: Microsatellite primers used to determine populations structure of taxa.

Primers were developed and PCR optimized in Hu et. al (Hu et al., 2004).

	Forward Primer (5'→3')	Reverse Primer (5'→3')	Size (bp)	Tm (°C)
ITSSR 02	AGGTC AAGGTGGTTTGGTTCTG	TTGCCCTCCAACAAGCATTCCC	157-178	60
ITSSR04	GCGTAACACATAAAATTGACTG	GGAAGTTTGAAAAGGTTAAGCC	205-209	60
ITSSR07	CACCATACCCAATTTTACAGATGC	GATTGAATGAATGATGCGG	160-172	56
ITSSR14	CTCCATTCAAACAGCGTCTC	CGGGCAATTCAGTTGACTC	133-206	56

Table 2: Average measurement (mean \pm SE) for 8 morphological traits taken for taxa.

Species	<i>I. cordatotriloba</i>	<i>I. lacunosa</i>	<i>I. X leucantha</i>	<i>I. austinii</i>
N	166	431	196	103
Corolla Length (mm)	24.23 \pm 0.32	18.11 \pm 0.10	16.55 \pm 0.14	17.06 \pm 0.11
Corolla Width (mm)	28.94 \pm 0.41	16.23 \pm 0.10	16.25 \pm 0.17	17.11 \pm 0.31
Width / Length	1.20 \pm 0.01	0.90 \pm 0.004	0.98 \pm 0.008	1.02 \pm 0.01
Corolla Shape	0.80 \pm 0.02	0.04 \pm .01	-0.02 \pm 0.02	0.53 \pm 0.04
Num. of Flowers on Cyme	2.21 \pm 0.11	1.44 \pm 0.03	2.44 \pm 0.08	4.60 \pm 0.21
Anther Color	0.96 \pm 0.01	1.00 \pm 0.00	0.90 \pm 0.02	0.01 \pm 0.01
Anther- Stigma Position	0.30 \pm 0.04	0.03 \pm 0.10	0.34 \pm 0.03	0.05 \pm 0.03
Stigma Length (mm)	16.03 \pm 0.17	10.33 \pm 0.05	10.22 \pm 0.09	9.70 \pm 0.14

Table 3: Standardized scoring coefficients from the discriminant analysis.

	% variation	Corolla Length	Corolla Width	Corolla W/L	Corolla Shape	flow. on chy.	anther color	anther stigma pos.	stigma length
Canon1	53	-0.35	0.24	-0.36	-0.20	-0.36	0.90	0.02	0.25
Canon2	44	-0.31	0.56	0.08	0.47	-0.02	0.04	-0.08	0.54

Table 4: Classification of the taxa based on a discriminant function analysis.

Actual Taxa	Predicted Taxa			
	<i>I. cordatotriloba</i>	<i>I. lacunosa</i>	<i>I. X leucantha</i>	<i>I. austinii</i>
<i>I. cordatotriloba</i>	157	3	2	6
<i>I. lacunosa</i>	0	396	35	0
<i>I. X leucantha</i>	0	46	131	19
<i>I. austinii</i>	0	1	0	102

Table 5: Canonical varieties 1 and 2 are statistically different for each contrasts.

	Contrast	DF	F Value	p-value
Canonical				
Variate 1				
	<i>I. lacunosa</i> vs. <i>I. X austinii</i>	1	1160.52	<0.001
	<i>I. lacunosa</i> vs. <i>I. X leucantha</i>	1	48.53	<0.001
	<i>I. lacunosa</i> vs. <i>I. cordatotriloba</i>	1	5649.25	<0.001
	<i>I. X austinii</i> vs. <i>I. X leucantha</i>	1	691.02	<0.001
	<i>I.X austinii</i> vs. <i>I. cordatotriloba</i>	1	644.82	<0.001
	<i>I. X leucantha</i> vs. <i>I. cordatotriloba</i>	1	3724.55	<0.001
Canonical				
Variate 2				
	<i>I. lacunosa</i> vs. <i>I. X austinii</i>	1	4018.45	<0.001
	<i>I. lacunosa</i> vs. <i>I. X leucantha</i>	1	263.23	<0.001
	<i>I. lacunosa</i> vs. <i>I. cordatotriloba</i>	1	176.68	<0.001
	<i>I. X austinii</i> vs. <i>I. X leucantha</i>	1	2163.91	<0.001
	<i>I.X austinii</i> vs. <i>I. cordatotriloba</i>	1	4518.53	<0.001
	<i>I. X leucantha</i> vs. <i>I. cordatotriloba</i>	1	665.61	<0.001

Table 6: Frequency of average microsatellite allele across population.

	n	ITSSR 02	ITSSR 04	ITSSR 07	ITSSR 14
<i>I. cordatotriloba</i>	97	157 (19%)	205(10%)	164(14%)	133 (6%)
		173(81%)	207(60%)	166(86%)	169 (6%)
			209(30%)		174(13%)
					184(31%)
					192 (30%)
					196(1%)
					203 (6%)
<i>I. lacunosa</i>	110	173(100%)	207(81%)	150(3%)	184(30%)
			209(9%)	166(94%)	199 (9%)
				172(3%)	203 (34%)
					206 (26%)
					212 (1%)
					217 (3%)
<i>I. X leucantha</i>	48	154 (2%)	205(100%)	150(3%)	133 (100%)
		178(96%)		166(94%)	
<i>I. X austinii</i>	33	154 (2%)	205(100%)	172(3%)	
		173 (58%)		160(2%)	184 (8%)
		206 (40%)		166(10%)	196 (42%)
				170(2%)	206 (50%)
			172(86%)		

Table 7: AMOVA results for *I. lacunosa* and *I. cordatotriloba*.

Source of Variation	d.f.	Sum of Squares	Variance Components	Percentage of Variation	P
Between <i>I. lacunosa</i> and <i>I. cordatotriloba</i>	1	19.068	0.032	4.24	0.13
Among populations within <i>I. lacunosa</i> and <i>I. cordatotriloba</i>	13	128.003	0.35	45.10	0
Among individuals within populations	192	118.47	0.23	29.61	0
Within individuals	207	33.50	0.16	21.05	0

Table 8: F_{CT} values between species pairs.

	<i>I. cordatotriloba</i>	<i>I. X leucantha</i>	<i>I. austinii</i>
<i>I. cordatotriloba</i>			
<i>I. X leucantha</i>	0.61**		
<i>I. austinii</i>	0.46**	0.70**	
<i>I. lacunosa</i>	0.04*	0.71**	0.56**

* $P=0.13$, ** $P<0.01$

Table 9: Genome sizes as determined by flow cytometry.

Taxa	Genome Size (Mb)
<i>I. cordatotriloba</i>	525
<i>I. lacunosa</i>	497
<i>I. X leucantha</i>	460
<i>I. austinii</i>	501

Table 10: Slope of genetic distance on physical distance for each species.
P is the probability that the slope differs from 0.

Species	R_{xy}	P
<i>I. lacunosa</i>	-0.36	0.11
<i>I. cordatotriloba</i>	0.03	0.44
<i>I. X leucantha</i>	-0.14	0.26
<i>I. austinii</i>	0.02	0.42

Table 11: Mean \pm SE of seeds produced per flower in crosses between the various taxa.

Sire = pollen parent. Dam = female pollen recipient. n = # of crosses.
 *interspecific crosses produced less seeds than selfed and intraspecific crosses (P<0.05);
 interspecific crosses produced less seeds than selfed (P<0.05) and intraspecific crosses (P=0.07); *interspecific crosses produced less seeds than selfed (P<0.05) and intraspecific crosses (P=0.06).

	DAM	<i>I. lacunosa</i>	<i>I. cordatotriloba</i>	<i>I. austinii</i>
SIRE	Self	4.0 \pm 0.0 (6)	4.0 \pm 0.0 (6)	4.0 \pm 0.0 (6)
	<i>I. lacunosa</i>	4.0 \pm 0.0 (6)	1.4 \pm 0.3 (7)*	1.2 \pm 0.6 (5)*
	<i>I. cordatotriloba</i>	2.8 \pm 0.5(5)	4.0 \pm 0.0 (4)	1.0 \pm 0.4 (6)*
	<i>I. austinii</i>	1.2 \pm 0.4 (6)*	2.0 \pm 0.5 (6)***	3.8 \pm 0.2 (5)
	<i>I. X leucantha</i>	0.2 \pm 0.1 (7)*	0.5 \pm 0.2 (5)*	0.3 \pm 0.2 (4)*

Table 12: Mean \pm SE of seeds produced per flower in crosses between various taxa and F₁ hybrids.

Sire indicates the type of individual used as pollen parent and dam is female parent.
I. lacunosa(p)= purple flowered *I. lacunosa*; *I. lacunosa*(w) = white flowered *I. lacunosa*;
 F1(leXla)=*I. X leucantha* X *I. lacunosa* hybrid; F₁(control)= *I. lacunosa*(p) x *I. lacunosa*(w) hybrid.

DAM	SIRE					
	Selfing	F1 (lexla)	F1 (control)	<i>I. lacunosa</i> (p)	<i>I. lacunosa</i> (w)	<i>I. X leucantha</i>
F1 (lexla)	0.00 \pm 0.00	0.00 \pm 0.00	0.04 \pm 0.11	0.15 \pm 0.21	0.13 \pm 0.31	0.17 \pm 0.10
F1 (control)	2.92 \pm 0.57	3.58 \pm 0.43	3.33 \pm 0.33	3.44 \pm 0.55	3.37 \pm 0.41	3.22 \pm 0.55

Table 13: AMOVA results for *I. lacunosa* and *I. X leucantha*.

Source of Variation	d.f.	Sum of Squares	Variance Components	% of Variation
Between <i>I. lacunosa</i> and <i>I. X leucantha</i>	1	51.91	1.27	81.75
Among <i>I. lacunosa</i> and <i>I. X leucantha</i> individuals	44	21.16	0.20	12.66
Within individuals	46	4.00	1.87	5.59

Chapter 2: The evolution of mating systems between sister species *Ipomoea cordatotriloba* and *Ipomoea lacunosa*

2.1 Introduction

The evolutionary transition from outbreeding to selfing is one of the most common changes in angiosperms, with an estimated 20% of all flowering plants having evolved autogamy as the predominant mode of reproduction (Barrett, 2002).

Autogamous plants are typically characterized by a “selfing syndrome”, which consists of having small, scentless, often white flowers, reduced anther-stigma distance, and a decreased pollen/ovule (P/O) ratio (Kalisz et al., 2012; Ornduff, 1969; Sicard & Lenhard, 2011). These traits are thought to evolve under circumstances in which seed production needs to be ensured, such as when pollinator availability is low or potential mates are commonly absent (Baker, 1955; Darwin, 1876; Stebbins, 1950). Because autogamous plants are not constrained by the need for pollinators, they tend to have a larger ranges compared to outcrossing plants (Ornduff, 1969). Traditionally, characteristics of the selfing syndrome are thought to have evolved due to natural selection. Moreover, several hypotheses have been put forth regarding the benefits of these traits: redirecting resources to other fitness-enhancing functions, increasing efficiency of self-pollination, shortening reproduction time, and reducing florivory (reviewed in Sicard & Lenhard, 2011). An alternative, seldom-considered explanation for the evolution of these characters, however, is that because autogamous plants do not need to attract pollinators their flowers are freed

from selective constraints and accumulate random mutations by genetic drift which disrupts the developmental processes responsible for producing floral traits.

Beyond the characteristic phenotype associated with self-pollination, there are distinctive ways in which autogamy effects the overall genetic diversity of a species. Neutral loci of autogamous plants often show an increase in homozygosity, while species that outcross typically exhibit Hardy-Weinberg genotype frequencies (Charlesworth & Wright, 2001). Excess homozygosity lowers the effective population size (N_e) and increases the effect of genetic drift in selfing plants (Pollak, 1987). This increase in homozygosity is often reflected by Wright's F statistic, which reflects the level of inbreeding in a population (Wright, 1951): in highly selfing plants F is close to 1, whereas in plants that solely outcross it is approximately 0 (Clauss & Mitchell-Olds, 2006).

In this study, I examine the roles of natural selection and genetic drift in the evolution of the selfing syndrome in *Ipomoea lacunosa*. This species has evolved increased selfing, decreased anther-stigma distance, smaller, white flowers, and a decreased pollen/ovule ratio compared to its recent ancestors (McDonald et al., 2011). In chapter 1 I showed that *I. cordatotriloba* is the sister species to *I. lacunosa*. *I. cordatotriloba* differs markedly from *I. lacunosa* in having large, purple flowers, a greater anther-stigma separation, and a mixed mating system (Austin, 1978; McDonald et al., 2011). An analysis of the causes of divergence between these two species is thus

appropriate for determining whether natural selection or genetic drift is the predominant cause of the evolution of the selfing syndrome in *I. lacunosa*.

In this report I first verify, using microsatellite markers, that selfing rate is substantially higher for *I. lacunosa* than for *I. cordatotriloba*. I then report results of a comparison of presumably neutral microsatellite divergence to divergence in selfing-syndrome traits to understand the roles that natural selection and drift play in shaping the classic selfing syndrome of *I. lacunosa*.

2.2 Materials and Methods

Study system- *Ipomoea lacunosa* and *Ipomoea cordatotriloba* are noxious weeds that are indigenous to the southeastern United States (Jones & Deonier, 1965). The two plants have different floral morphologies, with *Ipomoea lacunosa* typically having small white flowers, and *I. cordatotriloba* normally having larger, purple flowers (Abel & Austin, 1981). My analysis of shared genetic variation in chapter 1 suggests that *I. lacunosa* and *I. cordatotriloba* are sister species because they are genetically indistinguishable at neutral markers. Reflecting this genetic similarity, I also showed that two species can cross and produce viable offspring. My survey of NC and SC the ranges of the two species overlap, with *I. lacunosa* growing along the coast as well as in the central area of the two states, while *I. cordatotriloba* is found predominately along the coast. In NC and SC the plants germinate in late May and begin to flower in August or early September. Flowering ceases sometime in mid to late fall and plants die at the first hard frost. Plants of each taxon are self-compatible; however, *I. cordatotriloba* has been

described as having a mixed mating system (selfing and outcrossing), while *I. lacunosa* is thought to reproduce largely by self-pollination (McDonald et al., 2011).

Estimating selfing rates- Although I did not estimate selfing rates directly, selfing rates can be estimated indirectly from neutral markers (microsatellites). In particular, the sampling of populations and individuals allows me to estimate selfing rates from observed heterozygosity, H . Selfing rate is related the inbreeding coefficient, F , by

$$s = 2 F / (1 + F)$$

(Hartl & Clark, 1997). In turn, the inbreeding coefficient is related to heterozygosity by

$$F = (H_0 - H) / H_0$$

where H_0 is the expected heterozygosity based on allele frequencies and random mating (Hartl & Clark, 1997). Combining these two equations yields the relationship between selfing rate and heterozygosity:

$$s = 2 (H_0 - H) / (2H_0 - H)$$

Estimation of selfing rates for individual populations were based on four microsatellite loci described in chapter 1, that were sampled from 97 *I. cordatotriloba* individuals from 7 populations and 110 *I. lacunosa* individuals from 8 populations. Observed and expected heterozygosity were calculate using the program Arlequin 3.5 (Excoffier & Lischer, 2010).

Morphological trait divergence- Morphological traits for flower length, flower width, flower length-width ratio, stigma length, anther-stigma distance, leaf width, leaf length, and leaf length-width ratio were measured on individuals from 7 populations of *I.*

cordatotriloba and 5 populations of *I. lacunosa* for which I had concordant microsatellite data. Methods for morphological trait measurements were described in chapter 1. Anther-stigma distance, however, was measured slightly differently than previously reported. This character was quantified as 0 if all anthers touched the stigma, 0.5 if at least one, but no more than 4, touched the stigma, and as 1 if no anthers touched the stigma. Correlations among traits were calculated using JMP[®], Version 9. SAS Institute Inc., Cary, NC, 1989-2007.

To determine whether trait divergence between the two species is larger than expected under neutrality, I used a modified $F_{ST} - Q_{ST}$ approach (Whitlock & Gilbert, 2012). The standard $F_{ST} - Q_{ST}$ approach compares populations at a single spatial level (e.g. Leinonen et al, 2008; Whitlock, 2008). However, in my analysis populations are nested within species and I am primarily concerned with examining divergence between species. There are thus two hierarchical “spatial” levels.

For each morphological trait measured, I calculated the summary statistic Q_{CT} using the method developed by Whitlock and Gilbert (Whitlock & Gilbert, 2012). Q_{CT} describes the magnitude of variation for a morphological trait between *I. lacunosa* and *I. cordatotriloba*. Under the assumption that trait divergence is due to genetic drift, the expected value of Q_{CT} is

$$Q_{CT} = V_C / (V_C + V_P + 2V_I) = F_{CT}$$

where V_C , V_P , and V_I are the genetic variances among species, among populations, and among individuals within populations, respectively (Whitlock & Gilbert, 2012) and F_{CT} is the between-species F-statistic for the microsatellite loci.

To test whether natural selection contributed to trait divergence between the two species, I tested whether the null hypothesis of no selection, corresponding to the above equation, could be rejected in favor of the hypothesis that $Q_{CT} > F_{CT}$. Using bootstrap sampling, I compared the distributions of Q_{CT} and F_{CT} . To generate a bootstrap sample from one of the species, I first randomly chose a population from that species. I then drew n individuals randomly with replacement from those in that population, where n was the actual number of individuals scored in that population. I continued sampling in this way until the number of populations in the sample was equal to the number actually scored. For each species, I formed 1000 bootstrap samples.

For each bootstrap sample, I calculated Q_{CT} or F_{CT} from the variance components from a standard nested ANOVA. Because V_I is the additive genetic variance within populations and could not be calculated for the traits from the data, I instead examined two extremes: heritability = 1 and heritability = 0. For heritability = 1, V_I was set equal to the within-population component of variation from the nested ANOVA for the trait (equal to the within-population phenotypic variance); for heritability = 0, I set $V_I = 0$. Because both approaches led to similar results, I report only the results for $V_I =$ within population variance component. This approach is conservative because it produces a smaller Q_{CT} .

2.3 Results

Selfing rates- The eight populations of *I. lacunosa* I sampled uniformly exhibited very high selfing rates (mean $s = 0.955 \pm 0.018$; Table 14). By contrast, *I. cordatotriloba* populations were more variable across populations (mean $s = 0.511 \pm 0.182$ (negative estimates considered 0); Table 14). This difference is statistically significant (one-tailed T-test: $t = 2.43$, d.f. = 13, $P < 0.025$). It thus appears that selfing rate is on average substantially higher for *I. lacunosa* than for *I. cordatotriloba*.

One factor likely contributing to the higher selfing rate of *I. lacunosa* is reduced anther-stigma distance. The mean distance across populations for *I. cordatotriloba* was 0.70 ± 0.13 , while that for *I. lacunosa* was 0.048 ± 0.03 (Table 15), and this difference is highly significant (one-tailed T-test: $t = 5.17$, d.f. = 10, $P < 0.001$).

Selection on divergent traits- To test whether divergence in floral morphological traits between *I. cordatotriloba* and *I. lacunosa* is best explained by genetic drift or natural selection, I conducted a hierarchical $Q_{CT} - F_{CT}$ analysis. If $Q_{CT} > F_{CT}$, divergence in the trait is inferred to be caused by selection. By contrast, if $Q_{CT} = F_{CT}$, then the data is consistent with divergence by genetic drift. I performed a comparison of Q_{CT} vs. F_{CT} for the measured floral traits, as well as for leaf traits that serve as a control.

Q_{CT} values calculated for flower width, flower length, and flower length-width ratio are extremely differentiated from F_{CT} values (Figure 5). For flower width and length, the distribution of bootstrap values of F_{CT} does not overlap the distribution for Q_{CT} , while for the ratio of floral length/width, there is only minimal overlap. In all three

cases the significance of the difference is $P < 0.001$, indicating selection was largely responsible for their divergence between the two species. Because these traits are highly correlated (Table 16), these tests are not independent and divergence in these traits likely reflects selection on a single developmental character. Stigma height and anther-stigma distance are less correlated with corolla length and width and with each other than are corolla length and width to each other (Table 16). Thus, stigma height and anther-stigma distance presumably represent separate developmental modules, while corolla length and width do not. Again, however, for these characters there is little or no overlap between the bootstrap distributions of Q_{CT} and F_{CT} (Figure 6). Both of these differences are significant (stigma, $p < 0.001$; anther-stigma distance, $p = 0.002$), again indicating selection was primarily responsible for divergence.

By contrast with the floral characters, distributions of Q_{CT} and F_{CT} values broadly overlap for leaf length, leaf width, leaf length-width ratio (Figure 7) and differences are not statistically significant (leaf length, $P = 0.80$; leaf width, $P = 0.64$; leaf length-width ratio, $P = 0.44$). This pattern provides no evidence for selection causing divergence in these traits and indicates they are diverging neutrally.

2.4 Discussion

Selfing rates- My results verify that *I. lacunosa* is highly selfing whereas *I. cordatotriloba* appears to have a mixed mating system. This difference in selfing rates is explainable by a much reduced anther-stigma distance in *I. lacunosa*, which in other plant

species increases autogamy (Chang & Rausher, 1998; Schueller, 2004; Takebayashi et al., 2006).

I. cordatotriloba populations are much more variable in estimated selfing rate than *I. lacunosa* populations. Some populations exhibit selfing rates nearly as high as those in *I. lacunosa*, whereas others are estimated to be largely outcrossing (Table 14). Some of these differences may reflect the small number of individuals from individual populations used to estimate selfing rates, and may thus reflect noise rather than true differences between populations. It probably does not reflect differences among *I. cordatotriloba* populations in anther-stigma separation. The mean separation for the three populations with high selfing rates is 0.88 ± 0.06 , whereas for the four populations with lower selfing rates it is 0.57 ± 0.19 (Table 14). This difference is not significant ($t = 1.51$, d.f. = 5, $P > 0.3$) and is driven by the single population in the latter group that has a separation of 0. Finally, it is possible that this variation is due to differences in ecological conditions, e.g. presence vs. absence of pollinators. Regardless of the cause of this variation, on average *I. cordatotriloba* selfs substantially less than *I. lacunosa*.

Selection drives evolution of “selfing syndrome”- A large study of transition to autogamy in *Ipomoea* showed that self-fertilizing plants have similar traits such as small, light-colored corollas, short anther- stigma distance, and low P/O ratio (McDonald et al., 2011), which collectively have been termed the “selfing syndrome” (Sicard & Lenhard, 2011). These repeated convergent transitions are often thought to be caused by natural selection (Sicard & Lenhard, 2011). However, it is not unreasonable to believe that traits

not directly associated with increased self-pollination, such as flower size and light coloration, could have arisen convergently by genetic drift. The lower effective population size associated with autogamy greatly enhances the effect of genetic drift, making the alternative hypothesis plausible. Yet the possibility that evolution of selfing-syndrome traits may reflect the operation of drift rather than selection has seldom been examined. The results demonstrate that genetic drift can be ruled out as a major factor in the evolution of selfing-syndrome traits in *I. lacunosa*.

A major factor contributing to the high selfing rate of *I. lacunosa* is the close proximity of the anthers to the stigma, which has been shown to facilitate autogamy as in other species (Chang & Rausher, 1998; Schueller, 2004; Takebayashi et al., 2006). The comparison of Q_{ST} for anther-stigma distance with F_{ST} indicates that increased autogamy was caused by natural selection. This result implies that increased selfing itself was favored by selection in the *I. lacunosa* lineage. Although a number of advantages have been suggested for increased selfing, including pollinator uncertainty, avoidance of interspecific hybridization, and increased genetic transmission (Fisher, 1941; R. A. Smith & Rausher, 2007), I currently have no information that would allow me to distinguish among these possibilities. However, it is likely that the evolution of high levels of selfing set the stage for the subsequent evolution of other selfing-syndrome characters.

In *I. lacunosa*, such characters include reduction in corolla size, reduction in stigma height, loss of pigmentation and a reduction in pollen/ovule ratio (McDonald et al., 2011). Here I have examined two of these changes, reduction in corolla size and

reduction in stigma height, and have found both to be driven by selection. A conventional explanation for such a change is that by reducing these structures, individuals can divert saved resources to other fitness-enhancing structures (Brunet, 1992). However, an alternative explanation, at least for corolla size reduction, is that it may reflect the same direct selection for reduced outcrossing that drove the reduction in anther-stigma separation. Such selection might arise, for example, if smaller corollas reduce pollinator attraction, thereby reducing outcross pollination. Distinguishing between these possibilities will require future experiments aimed at identifying the sources of selection on floral characters in *I. lacunosa*.

Even though floral size and mating systems have been found to be extremely correlated, a systematic survey of outcrossing and selfing species found that there is a much weaker correlation between flower size and vegetative tissues, such as leaf size (Ashman & Majetic, 2005). This weak correlation between flower size and vegetative tissue is consistent with the F_{ST} - Q_{ST} analyses of leaf length, leaf width, and leaf length-width ratio which revealed that these traits are evolving neutrally. Although genes have been described that control both flower and leaf development (Kim et al., 1999; Lee et al., 2006), it has been hypothesized that genes underlying these traits have evolved to remain largely developmentally isolated to reduce pleiotropic consequences of genetic alteration (Ashman & Majetic, 2005). More information, however, on the genetics underlying these traits needs to be elucidated to determine if this hypothesis is correct.

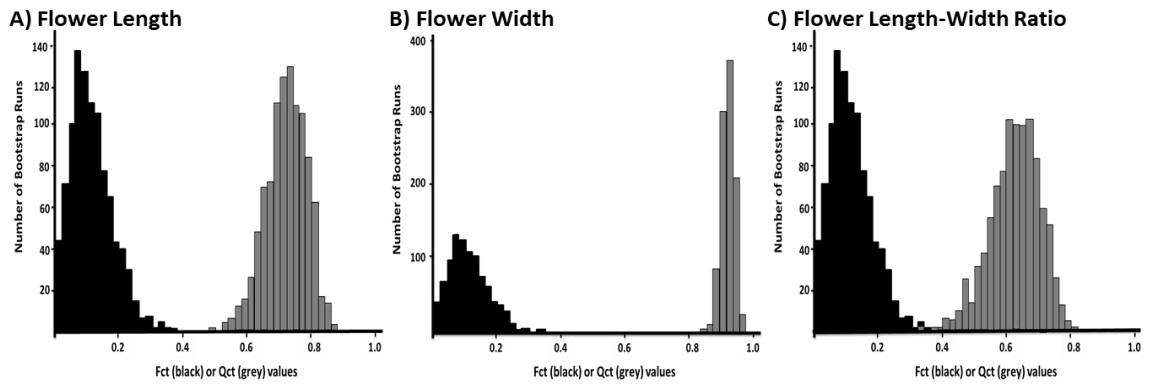


Figure 5: F_{CT} vs. Q_{CT} analyses A) Flower Length ($p < 0.001$) B) Flower Width ($p < 0.001$) C) Flower Length-Width Ratio ($p < 0.001$)

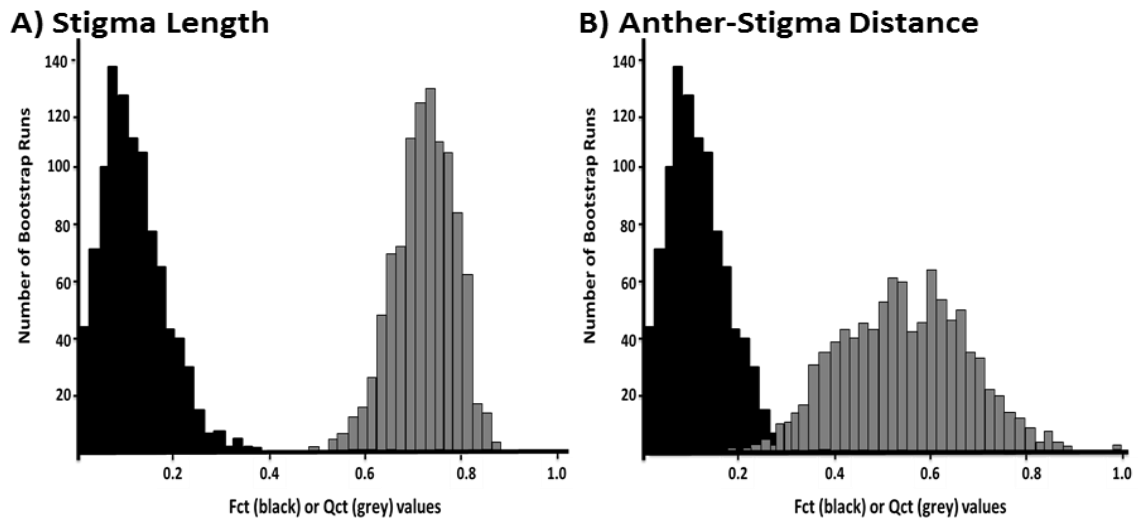


Figure 6: F_{CT} vs. Q_{CT} analyses A) Stigma Length ($p < 0.001$) B) Anther-Stigma Distance ($p = 0.002$)

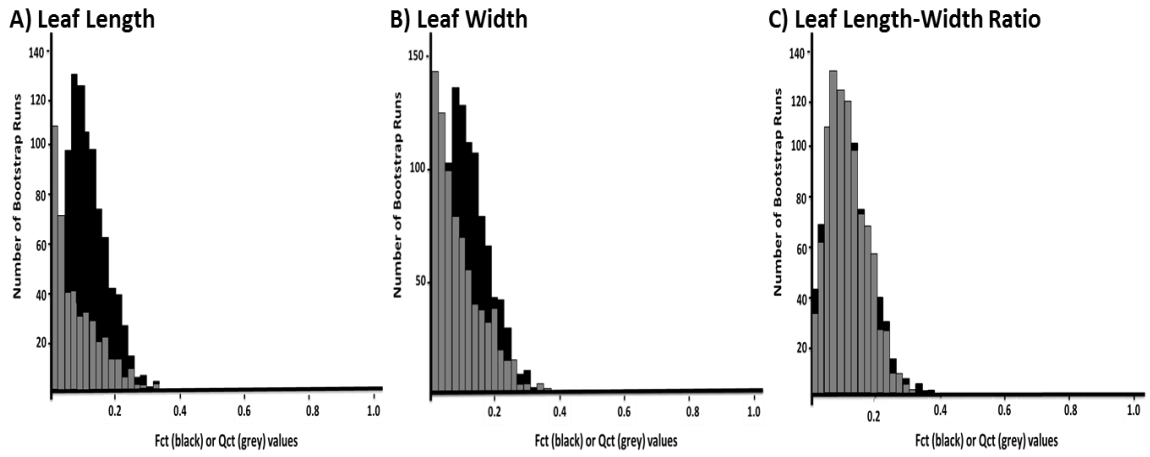


Figure 7: F_{CT} vs. Q_{CT} analyses A) Leaf Length ($p=0.80$) B) Leaf Width ($p=0.64$) C) Leaf Length-Width Ratio ($p=0.44$)

Table 14: Measures of genetic diversity and selfing rates for 7 populations of *I. cordatotriloba* and 8 populations of *I. lacunosa*.

N= sample size; NPL = number of polymorphic loci; A= mean (s.d) number of alleles;
 Ho=observed heterozygosity; He= expected heterozygosity, s = selfing rate

	population	N	NPL	A	Ho	He	s
<i>I. cordatotriloba</i>							
	cle1	18	2	2.0 (0.0)	0.03	0.33	0.95
	c7	13	4	3.0 (1.4)	0.61	0.55	-0.24
	c13	23	2	2.5 (0.7)	0.04	0.27	0.92
	cl4	12	3	2.7 (1.2)	0.06	0.43	0.93
	c22	8	4	2.5 (0.6)	0.50	0.43	-0.39
	c29	6	1	2.0 (0.0)	0.30	0.30	0.00
	clela3	17	2	2.0 (0.0)	0.18	0.50	0.78
<i>I. lacunosa</i>							
	la3	14	1	2.0 (0.0)	0.00	0.50	1.00
	la15	14	2	4.0 (0.0)	0.04	0.37	0.94
	lela8	31	2	2.5 (0.7)	0.05	0.26	0.89
	la43	8	2	2.0 (0.0)	0.00	0.38	1.00
	la30	14	2	2.0 (0.0)	0.04	0.20	0.89
	la35	16	2	3.0 (1.4)	0.03	0.20	0.92
	la7	6	1	2.0 (0.0)	0.00	0.30	1.00
	ula7	6	1	3.0 (0.0)	0.00	0.55	1.00

Table 15: Mean \pm SD for morphological measurements for populations of *I.*

population	Species	n	Corolla Length (mm)	Corolla Width (mm)	Corolla L/W Ratio	Anther Stamen Distance	Stigma Length (mm)	Leaf Length (mm)	Leaf Width (mm)	Leaf L/W Ratio	
1	cle1	I. cordatotriloba	10	25.50 \pm	29.00 \pm	0.88 \pm	1.00 \pm	16.20 \pm	41.40 \pm	43.60 \pm	1.01 \pm
				1.96	1.18	0.06	0.00	0.75	10.97	17.28	0.18
2	c7	I. cordatotriloba	5	21.60 \pm	29.80 \pm	0.73 \pm	0.00 \pm	16.80 \pm	25.20 \pm	24.20 \pm	1.10 \pm
				0.49	.33	0.03	0.00	0.75	6.52	6.52	0.25
3	c13	I. cordatotriloba	20	21.60 \pm	28.23 \pm	0.77 \pm	0.78 \pm	15.74 \pm	41.73 \pm	37.68 \pm	1.13 \pm
				1.81	2.99	0.08	0.40	1.56	10.64	10.68	0.20
4	cl4	I. cordatotriloba	14	26.71 \pm	32.57 \pm	0.83 \pm	0.86 \pm	16.21 \pm	50.07 \pm	38.79 \pm	1.30 \pm
				2.46	2.44	0.10	0.34	1.11	9.69	8.78	0.13
5	c22	I. cordatotriloba	6	27.57 \pm	32.07 \pm	0.86 \pm	0.86 \pm	17.50 \pm	39.71 \pm	39.00 \pm	1.04 \pm
				2.04	2.06	0.08	0.23	1.16	4.27	7.31	0.11
6	clela3	I. cordatotriloba	20	24.90 \pm	30.65 \pm	0.81	0.68 \pm	16.55 \pm	53.33 \pm	46.03 \pm	1.18 \pm
				2.78	2.46	\pm 0.08	0.40	1.0	14.28	11.77	0.26
7	c29	I. cordatotriloba	8	32.63 \pm	36.63 \pm	0.89 \pm	0.75 \pm	19.88	52.44 \pm	47.94 \pm	1.13
				1.88	2.06	0.07	0.43	\pm 0.93	11.94	12.42	\pm 0.24
8	la3	I. lacunosa	19	15.44 \pm	12.94 \pm	1.20 \pm	0.00 \pm	8.17 \pm	48.17 \pm	37.50 \pm	1.30 \pm
				1.21	1.03	0.09	0.00	0.50	9.31	8.67	0.10
9	la15	I. lacunosa	20	17.48 \pm	14.85 \pm	1.18 \pm	0.00 \pm	10.02 \pm	31.65 \pm	24.45 \pm	1.38 \pm
				1.12	1.19	0.08	0.00	0.53	11.93	11.35	0.24
10	lela8	I. lacunosa	16	15.56 \pm	15.00 \pm	1.04 \pm	0.09 \pm	9.25	34.72 \pm	28.22 \pm	1.30 \pm
				1.94	1.70	0.11	0.21	\pm 0.66	11.73	10.73	0.28
11	la43	I. lacunosa	29	18.14 \pm	16.17 \pm	1.23 \pm	0.00 \pm	10.54 \pm	56.86 \pm	43.76 \pm	1.42 \pm
				1.88	1.34	0.14	0.00	1.08	25.42	20.87	0.72
12	ula7	I. lacunosa	18	18.34 \pm	16.68 \pm	1.11 \pm	0.15 \pm	11.16 \pm	35.11 \pm	26.10 \pm	1.38 \pm
				1.70	1.75	0.10	0.23	1.18	14.16	12.18	0.21

cordatotriloba and *I. lacunosa* with concordant microsatellite data.

Table 16: Correlations among measured traits.

	Corolla Length	Corolla Width	Corolla Length/Width	Anther-Stigma Position	Stigma Length	Leaf Length	Leaf Width
Corolla Length							
Corolla Width	0.36 ± 0.23						
Corolla Length/Width	0.56 ± 0.20	-0.43 ± 0.36					
Anther-Stigma Position	-0.03 ± 0.16	-0.03 ± 0.29	0.00 ± 0.23				
Stigma Length	0.20 ± 0.33	0.28 ± 0.25	0.04 ± 0.33	0.07 ± 0.19			
Leaf Length	-0.02 ± 0.28	0.08 ± 0.36	-0.10 ± 0.38	0.08 ± 0.28	-0.04 ± 0.35		
Leaf Width	-0.04 ± 0.32	0.17 ± 0.28	-0.15 ± 0.23	0.07 ± 0.31	-0.10 ± 0.44	0.79 ± 0.22	
Leaf Length/Width	-0.10 ± 0.20	-0.05 ± 0.36	0.04 ± 0.26	0.00 ± 0.23	0.12 ± 0.36	-0.06 ± 0.38	0.43 ± 0.43

Chapter 3: *R2R3-Myb* responsible for divergent flower color between *Ipomoea cordatotriloba* and *Ipomoea lacunosa* shows molecular signature of selection

3.1 Introduction

The evolution of selfing from outcrossing is one of the most frequent mating system transitions in angiosperms and has occurred in most plant families (Kalisz et al., 2012; Sicard & Lenhard, 2011). Change in a suite of floral traits typically accompanies this transition, giving rise to a “selfing syndrome.” Typically, this syndrome includes reduction in traits associated with pollinator attraction (e.g. reduction in petal size, nectar and scent production and loss of pigmentation), decreased pollen/ovule ratios, and reduced distance between anthers and stigma (Kalisz et al., 2012; Ornduff, 1969; Sicard & Lenhard, 2011). These traits may be divided into two categories. On the one hand, there are traits that directly contribute to an increase in the rate and/or efficiency of selfing (“causal” traits). This category includes traits such as reduced physical and temporal separation of anthers and stigma and decreased pollen/ovule ratios. When selfing is favored, selection is expected to operate to alter these traits. On the other hand, there are selfing syndrome traits that do not necessarily contribute directly to enhanced selfing (“ancillary” traits). These traits are typically associated with reduced pollinator attraction and include reduced flower size, nectar and scent production, and sometimes pigmentation. In the early stages of the evolution of increased selfing, selection may favor these traits because they increase selfing by reducing visitation by pollinators. And

once a high selfing rate has evolved, selection may favor reallocation of resources away from these traits to other traits that enhance fitness (Brunet, 1992). However, it is also possible that these traits degenerate simply because they accumulate mutations that are not opposed by selection because pollinator attraction is no longer needed. Such degeneration would occur by genetic drift and is not necessarily adaptive.

The relative involvement of selection and drift in the evolution of ancillary traits contributing to the selfing syndrome is largely unknown. I am unaware of any studies that have attempted to address this issue. In this study, I address this issue by examining one ancillary character associated with the selfing syndrome in the morning glory *Ipomoea lacunosa*: white flowers (Figure 8A). *I. lacunosa* is a species in the *Batatas* clade of *Ipomoea* and has small, white flowers. Because the majority of species in the *Batatas* clade have purple flowers (Austin, 1978), the white color of *I. lacunosa* flowers is almost certainly a derived character. In chapter 2, I demonstrated that *I. lacunosa* is highly selfing and exhibits most of the characteristics typical of the selfing syndrome. Compared to its sister species *I. cordatotriloba* (Figure 8B), it exhibits loss of a reduced floral size, reduced distance between stigma and anthers, and a reduced pollen/ovule ratio (McDonald et al., 2011)

In the southeastern United States, *Ipomoea* species with purple flowers, including *I. cordatotriloba*, tend to be visited primarily by bumblebees (Brown & Clegg, 1984; Rausher, Augustine, & Vanderkooi, 1994; *personal observations*). Moreover, white-flowered mutants of *I. purpurea* are visited less often by bees than purple-flowered

individuals (Brown & Clegg, 1984; Fehr & Rausher, 2004; Fry & Rausher, 1997; Rausher, Augustine, & Vanderkooi, 1994), indicating that floral pigmentation is attractive to pollinators, which likely exert selection to maintain pigmentation. I do not know whether loss of floral pigmentation in *I. lacunosa* occurred prior to, concurrent with, or after the evolutionary decrease in anther-stigma separation and resultant shift to almost complete selfing. If prior or concurrent to the shift to self-pollination, natural selection favoring selfing may have favored the transition to white flowers. If after the shift to selfing, purifying selection on presence of pigmentation was likely relaxed because plants would no longer have needed to attract pollinators. In this situation, any costs associated with pigment production would have led to selection favoring loss of floral pigmentation. It is not known, however, whether there are costs associated with producing floral pigmentation. If present, they likely would have generated selection for loss of pigmentation. In the absence of such costs, by contrast, there presumably would have been no selection favoring pigment loss and white flowers would presumably have evolved by the fixation of loss-of-function mutations by genetic drift.

The objective of this investigation was to distinguish between these two possibilities. Specifically, I attempted to determine whether the evolution of white flowers in *I. lacunosa* was the result of selection or drift. To do so, I first characterized the genetic basis of white flowers and identified a major gene responsible for the transition to white flowers in *I. lacunosa*. Using this information, I compared divergence between *I. lacunosa* and its sister species *I. cordatotriloba* at presumably neutral markers

to divergence at the flower-color locus to determine whether color divergence is consistent with neutral expectations. Finally, I compared genetic diversity within the two species at the flower-color locus to determine whether I could detect a signal of a recent selective sweep.

3.2 Materials and Methods

Study organisms- *Ipomoea lacunosa* and *Ipomoea cordatotriloba* are noxious weeds that are indigenous to the southeastern United States (Jones & Deonier, 1965). The two plants have different floral morphologies, with *Ipomoea lacunosa* having smaller white flowers, while *I. cordatotriloba* typically has larger, purple (Abel & Austin, 1981). Even though both taxa have a characteristic flower color, white-flowered *I. cordatotriloba* and purple-flowered *I. lacunosa* individuals can be found in nature (*personal observation*). Although the phylogenetic relationships among these and related species in the *Batatas* section of *Ipomoea* have not been well characterized, I showed in chapter 1 that the shared genetic variation suggests that *I. lacunosa* and *I. cordatotriloba* are sister species: while the two species are morphologically distinct, there was no detectable genetic divergence at any of four microsatellite loci. Reflecting this genetic similarity, the two species can cross and produce viable offspring, and extensive gene flow is thought to be occurring between the plants in the field (Abel & Austin, 1981). Additionally, in chapter 1 I showed that in NC and SC, the ranges of the two species overlap. However, *I. lacunosa* grows along the coast as well as in the central area of the two states, while *I. cordatotriloba* is found predominately along the coast. A third taxon,

I. X leucantha, grows in the same region and is believed to be a stable hybrid formed by a cross between *I. lacunosa* and *I. cordatotriloba* (Abel & Austin, 1981). In NC and SC the plants germinate in late May and begin to flower in August or early September. Flowering ceases sometime in mid to late fall and plants die at the first hard frost. Plants of each taxon are self-compatible and are highly autogamous in the greenhouse.

Identification of anthocyanidins using HPLC- Anthocyaninidins, the direct precursor of anthocyanins, were extracted and identified using high-performance liquid chromatography using a previously described method (Streisfeld & Rausher, 2009) in the stem and flowers of 3 purple- and 3 white- flowered *I. lacunosa* as well as 2 purple flowered *I. cordatotriloba*.

Genetics of flower color- To determine how many genes contribute to the difference in floral pigmentation between the two species, I performed standard crosses. In 2007, 10 white- and 10 purple-flowered *I. lacunosa* individuals were used to create 10 F₁ hybrids. Each of these hybrids was allowed to self to produce S₂ individuals. In June 2008, approximately 75 S₂ seeds from each line were grown 1 meter apart in a field plot at Duke University. Flower color (purple vs. white) of the inner (tube) and outer (limb) corolla were recorded on each plant on three different occasions.

In a second experiment, F₁ hybrids were created by crossing 4 purple and 4 white flowered *I. lacunosa* parental plants. A total of 140 S₂ progeny were grown in greenhouse at Duke University. The outer corolla was phenotyped as light-, medium-, or dark-, and photographs were taken to ensure accuracy of results.

Identification of anthocyanin genes- Because absence of anthocyanin pigments in the white flowers is typically caused by disruption of the anthocyanin biosynthetic pathway, I focused on determining which anthocyanin genes were associated with absence of floral pigments in *I. lacunosa*. I first cloned several genes from this pathway. Partial coding sequences for genes coding for the anthocyanin enzymes dihydroflavonol reductase-B (*DFR-B*) and chalcone synthase-D (*CHS-D*) as well as for the *R2R3-Myb* anthocyanin transcription factor known to control their expression in the flowers of *Ipomoea* (FukadaTanaka et al., 1997; Inagaki et al., 1999; Morita, Saitoh, Hoshino, Nitasaka, & Iida, 2006) were amplified from *I. lacunosa* floral mRNA of using primers developed from closely related species of *Ipomoea* (Table 17). RNA was extracted using the SIGMA, Spectrum Total RNA extraction kit, and cDNA was produced using Invitrogen M-MLV reverse transcriptase. Each amplified sequence was Blasted against the National Center of Biotechnology Information protein database to confirm that it showed highest similarity to previously identified anthocyanin genes. Additionally, 345 b.p. of the 3'UTR region of *R2R3-Myb* gene was PCR amplified out of DNA of *I. lacunosa* using primers developed from *I. nil* (Table 17).

Quantifying gene expression levels- I used quantitative real-time PCR (qPCR) to compare relative expression of the anthocyanin genes between species and among S₂ individuals. Additionally, qPCR was conducted on 4 purple and 4 white *I. lacunosa* that had been field collected from seed and grown in the greenhouse. Flower buds were collected between 4-5pm the day before anthesis in an attempt to standardize the

developmental stage in which the flower tissue was collected. A 0.5 cm-long section of the inner corolla was flash-frozen in liquid nitrogen and stored at -80°C until RNA was extracted as described above.

For qPCR reactions, 300 ng of RNA were used to make 20 µl of cDNA for each sample. using methods described above. One microliter of cDNA was then used in a 20 µl SYBR green qPCR reaction. The reaction mix included 10 µl Dynamo SYBR green qPCR mix(REF), 0.2 µl primers, 0.4 µl Rox Passive Dye. Reactions were run on an ABI Prism 7000 Sequence Detection System using the following cycling protocol: 94 for 10 minutes, 40 cycles of 94 for 20 seconds, 55 for 30s, and 72 for 45 seconds. Q-PCR primers were designed to yield a product no larger than 200 bp (Table 18). Relative expression of the target gene was calculated using the method developed by Peirson et al. 2003 (Peirson, Butler, & Foster, 2003). One purple plant was used as the control to which all other expression levels were compared, and an ANOVA was used to determine whether there was a significant difference in expression of anthocyanin genes between purple and white individuals. All ANOVAs were conducted using JMP[®], Version 9. SAS Institute Inc., Cary, NC, 1989-2007.

Co-segregation analyses- A gene responsible for the color difference between purple- and white-flowered individuals will co-segregate with flower color in F₂ individuals. I therefore examined whether any of the anthocyanin genes I had identified co-segregated with flower color. Because I was unable to find genetic markers to differentiate anthocyanin genes from purple and white *I. lacunosa*, I created 3 F₁

individuals by crossing white-flowered *I. lacunosa* to its close relative *I. X leucantha*, which has purple flowers and scorable allelic differences. The F₁ plants produced very few seeds when allowed to self. Therefore, 41 F₂ seeds were generated by backcrossing the F₁ plants to white flowered *I. lacunosa*. Additionally, one purple flowered F₂ plant was backcrossed to white *I. lacunosa* and 12 F₃ plants were created.

DNA was extracted from the three parental *I. X leucantha* and *I. lacunosa*, as well as from 3 F₁ and their F₂ and F₃ backcrossed progeny using a CTAB protocol (Doyle & Doyle, 1981). Genetic markers were found to differentiate the parentals in the first intron region of *DFR-B* as well as the 3' UTR region of the *R2R3-Myb*. Both *DFR-B* and *R2R3-Myb* were amplified from the F₂ progeny using PCR, and the products were genotyped by restriction enzyme fragment analyses (Table 19).

Allele-specific expression- The above analyses revealed that the *R2R3-Myb* gene is down-regulated in white-flowered individuals. To determine if this down-regulation was due to a *cis*- or a *trans*-regulatory change, I used quantified allele-specific expression levels in heterozygotes (Wittkopp et al., 2004). The alleles are differentiated by a G/T polymorphism in the third exon of the gene (Figure 9). DNA was extracted and cDNA was generated using methods described above. PCR amplification was performed by pyrosequencing on four cDNA and four genomic DNA replicates for each of three F₁ individual as well as non-template and non-sequencing primer controls. Pyrosequencing reactions used PyroMARKTMQ961D (Qiagen) (Ahmadian et al., 2000; Wittkopp,

Haerum, & Clark, 2004). The DNA analysis provides a control for inherent differences in production of the two alleles by PCR.

Allele expression has been shown to be directly correlated to the peak sequencing height generated in a pyrosequencing reaction (Wittkopp et al., 2004). An ANOVA was used to detect if the purple and white allele showed significantly different proportional expression of the two alleles in cDNA and genomic DNA.

Flower color census- In the fall 2010, a large population census was conducted of *I. cordatotriloba* and *I. lacunosa*. During the census flower color frequency was taken on 50 populations of *I. cordatotriloba* growing in both NC and SC and 43 populations of *I. lacunosa* in NC (Appendix B). To measure the flower color frequency in a population, two transects were taken. Plants were sampled at 2 m intervals along transects to ensure that different individuals were scored. When flower color was fixed in the population, as determined by an initial visual inspection, I scored 100 flowers. However, when there was obvious variation in flower color in a population, 200 flowers were scored.

Frequency of flower color versus neutral genetic loci- To determine whether divergence in flower color between *I. lacunosa* and *I. cordatotriloba* is consistent with neutral expectations, I conducted a bootstrap analysis to compare gene frequency differences between the two species at the flower-color locus with differences in frequencies at the microsatellite loci. Because the census data did not permit me to determine whether purple-flowered individuals are homozygous or heterozygous, I estimated gene frequency in two ways. First, I assumed that there were no heterozygotes

and that all purple individuals were therefore homozygous (Method 1). The frequency of the white allele in this case is estimated as the frequency of white individuals in a population. Alternatively, I assumed that genotype frequencies at the flower-color locus are in Hardy-Weinberg equilibrium (Method 2). In this case, the frequency of the white allele is estimated as the square root of the proportion of white-flowered individuals. These two cases represent extreme possibilities that bracket the true proportions of heterozygotes in the population.

For the bootstrap analysis, I first generated for the microsatellite markers a distribution of between-species average difference in allele frequency, where the average was taken over loci. Each bootstrap sample was obtained in the following way: first populations were randomly sampled with replacement within a species. Within each sampled population, individuals were chosen randomly with replacement. Once the sample had been reconstituted in this way, loci were randomly sampled with replacement. Average difference in allele frequency was then calculated as follows: first the population gene frequencies were calculated for each locus. These were then averaged to obtain an average frequency of each locus in each species. The absolute values of the differences in frequency between species were then calculated for each locus, and these were averaged over loci to obtain a final value.

Bootstrap samples for allele frequencies at the flower-color locus were calculated in similar fashion. I started with a data set that contained either 100 or 200 individuals in each population, depending on the number of individuals sampled. Genotypes for these

individuals were assigned based on the censused frequency of white alleles using either Method 1 or Method 2 (see above). For each bootstrap sample, populations were randomly sampled with replacement within species, and individuals were then randomly sampled with replacement for each sampled population. Population gene frequencies were averaged for each species and the difference in gene frequency between species was calculated as the difference population averages. One thousand bootstrap samples were used in the analysis.

Test for selective sweep- To test for a selective sweep, I compared the genetic diversity of the *R2R3-Myb* gene in the two species. I performed a similar analysis on the anthocyanin gene *DFR-B* as a control to determine whether any reduction in diversity in *I. lacunosa* could be the result of a genetic bottleneck. I sampled a single copy of these genes from 20 white flowered *I. lacunosa* and 15 purple flowered *I. cordatotriloba*, each from a different population in NC and SC. I sequenced 974 b.p. of each copy of the *R2R3-Myb* gene. The sequenced region spanned from the second exon to 345 bp downstream of the stop codon. For *DFR-B* I sequenced a 655 bp region that included parts of exons 1 and 2 and the entire first intron (Table 20). Both genes were direct sequenced in the forward and reverse direction. For quality control, individuals with singleton variants were sequenced at least twice. Chromatograms of each sequence were visual inspected and consensus sequences were generated. The number of haplotypes was determined by visual inspection of the data.

To determine whether there was a reduction in diversity in *I. lacunosa*, I compared the Shannon-Wiener (S-W) diversity index of the haplotypes between species. Historically, the S-W index has been used to test diversity within and between species (Hutchinson, 1970; Shannon, 1948). The S-W diversity index is useful because it reflects both the number of haplotypes as well as their relative frequencies. I used a jackknife procedure (Cohen, 1969; Gray & Schuncary, 1972) with 10,000 iterations to test the hypothesis that the amount of genetic variation of the *R2R3-Myb* as well as *DFR-B* is statistically different for individuals of *I. cordatotriloba* and *I. lacunosa*. The means calculated by the jackknife procedure are asymptotically normal, allowing me to perform a standard t-test to determine if the parameter estimates are significantly different from each other (Gray & Schuncary, 1972).

3.3 Results

HPLC- While the anthocyanins cyanidin and peonidin are abundant and trace amounts of pelargonidin can be found in purple-flowered *I. lacunosa* and *I. cordatotriloba* individuals, they were not detectable in white flowered individuals of *I. lacunosa* confirming that white flowers result from a lack of anthocyanin production (Table 21). Interestingly, anthocyanins were produced in the stem of white flowered *I. lacunosa*, indicating that the anthocyanin genes are functional in the plant.

Flower color genetics- Crosses between white and purple flowered *I. lacunosa* from a population in NC indicate that flower color is controlled at two loci with major effects that interact epistatically. Three phenotypes were evident in the S₂ progeny

scored: (i) individuals with purple pigment in both the throat and corolla limb; (ii) individuals with white corolla limbs with rays of purple and purple throats; and (iii) individuals with white throats and little or no pigment in the corollas. Out of 732 S₂ progeny created from ten purple and white parental crosses, 530 individuals had a purple throat, while 202 had a white throat. These numbers are consistent with the hypothesis that pigmentation in the throat of *I. lacunosa* is controlled by a single dominant Mendelian locus, as indicated by lack of deviation from the expected 3:1 ratio of purple to white (pooled G-value=2.57, df=1, p=0.11). I tentatively designate this locus *Anl1* (*Anthocyaninless 1*).

The gene controlling the corolla limb color acts epistatically with the gene controlling the throat of the flower. If the throat is white, then there is little pigment in the limb, and the effect of the locus on limb pigmentation is too small to be quantified. On the other hand, if the throat is purple, then anthocyanins controlled by the gene in the outer petal can be assessed. Results from the purple *I. lacunosa* individuals scored revealed 22 with light-, 54 with medium-, and 27 with dark-purple limbs, indicating that anthocyanins in the outer petal are likely controlled by a co-dominant Mendelian locus with a 1:2:1 ratio of light-, medium-, and dark-purple flower colors, respectively (pooled G-value=0.74, d.f.=2, p=0.69). An additional 30 S₃ individuals were scored for other purposes, and their flower color was consistent with expectations of the single-locus model (Table 22). I designate this locus *Anl2* (*Anthocyaninless 2*). The remainder of this

study focuses on the gene controlling throat color (*An11*), since the white genotype at this locus lacks pigment throughout the flower.

Gene expression level differences- I first examined expression levels of anthocyanin genes in the flowers of field-collected *I. lacunosa* individuals. Q-PCR results revealed that *CHS-D* is almost 100-fold down-regulated (ANOVA, $F_{1,6}=93.80$, $p=0.0002$) and *DFR-B* is more than 100- fold down-regulated (ANOVA $F_{1,6}=39.63$, $p=0.0015$) in the throats of white- compared to purple-flowered individuals (Figure 10). This coordinate down regulation suggests that the white phenotype is due to a genetic change in one of the transcription factors that regulates these anthocyanin genes. Additionally, Q-PCR showed that the *R2R3-Myb* is also down-regulated 100- fold (ANOVA, $F_{1,6}=533.95$, $p<0.0001$) in white compared to purple field collected plants (Figure 10). White S_2 individuals, created from an original purple X white cross, show significant down regulation in all three genes compared to purple individuals (*CHS-D* ANOVA, $F_{1,6}=52.14$, $p=0.0007$; *DFR-B* ANOVA, $F_{1,6}=554.28$, $p<0.0001$; *R2R3-Myb* ANOVA, $F_{1,6}=31.44$, $p=0.0025$) (Figure 10). Since the white phenotype appears to be controlled by a single locus, these co-segregation results suggest that down regulation of the *R2R3-Myb* is the cause of white flower color *I. lacunosa*, although it is possible that a substitution in an upstream regulator of *R2R3-Myb* could be responsible.

Co-segregation analyses- Co-segregation analysis supports the conclusion that the *R2R3-Myb* corresponds to *An11*. I scored 41 B_2 offspring generated from backcrossing F_1 (*I. X leucantha* X *I. lacunosa*) individuals to white *I. lacunosa*. Among

these offspring, variation at the *Myb* gene exhibited perfect association with variation in flower color (Families 1-3) (Table 23). Furthermore, the same association was found in 12 F₃ offspring generated from a purple F₂ backcrossed to white *I. lacunosa* (Family 4). By contrast, variation in *DFR-B* did not co-segregate with flower color (Table 23). Family 4 was not included in the *DFR-B* analysis because it did not contain an informative marker in the gene when backcrossed to white *I. lacunosa*. These results are consistent with the hypothesis that a *cis*-regulatory change in the *Myb* gene results in its own down-regulation, as well as that of the two enzyme-coding genes.

Allele-specific expression- To further test this hypothesis, I used allele-specific expression. If down regulation of the *Myb* gene is due to a *cis*-regulatory change, then in heterozygotes the “white” allele should be expressed at much lower levels than the “purple” allele. By contrast, if a *trans*-acting regulatory is responsible, then the two alleles should be expressed at approximately the same level (Wittkopp et al., 2004). Allele-specific expression results obtained by pyrosequencing indicate that the down regulation of the *R2R3-Myb* in white flowers is due to a *cis* change (Figure 11). In F₁ individuals created from a cross between white-flowered *I. lacunosa* and purple-flowered *I. X leucantha*, the “purple” allele is expressed at a significantly higher level in cDNA than the “white” allele. In all three replicates, the “purple” allele constituted more than 90% of the transcripts. By contrast, the two alleles are expressed approximately equally in the genomic DNA control. This difference between cDNA and gDNA is highly significant for each replicate: (Family 1 (ANOVA, $F_{1,6}=443.36$, $p<0.0001$); Family 2

($F_{1,6}=39.60$, $p=0.0007$); Family 3(ANOVA, $F_{1,6}=219.02$, $p<0.0001$). This increased expression of the purple allele in F_1 individuals indicates that the expression difference in *Myb* between white- and purple-flowered individuals due to a *cis*-regulatory change.

Test for neutral divergence in flower color- As expected, the average frequency of the white allele at *An11*, as reflected by the frequency of white-flowered individuals, differs between *I. lacunosa* and *I. cordatotriloba*. The average frequency for *I. lacunosa* was 0.98 ± 0.004 (number of populations = 43), while that for *I. cordatotriloba* was of 0.21 ± 0.02 (number of populations = 50). This difference of 0.77 is highly significant (ANOVA $F_{1,91}=164.29$, $p<0.0001$). I demonstrated in chapter 1 that *I. lacunosa* and *I. cordatotriloba* are not significantly differentiated at four microsatellite loci. Averaged over populations and loci, the average difference in allele frequency between species was 0.14, much smaller than the difference for *An11*. To determine whether this difference was statistically significant, I conducted a bootstrap analysis to generate a distribution of likely values for allele frequency differences. This analysis used a subset of the populations from the census because I had microsatellite data from only 8 *I. lacunosa* and 7 *I. cordatotriloba* populations (Table 24, Figure 12) Nevertheless, these populations are representative of the large sample of census populations for frequency of white flowers: The mean frequencies of white flowers for *I. lacunosa* and *I. cordatotriloba*, respectively were 0.981 and 0.135, which do not differ significantly from the proportions for samples not used (ANOVA on arcsin(square-root)-transformed data, $F_{1,48}=0.33$, $P=0.57$ and $F_{1,41}=0.19$, $P=0.67$) for *I. cordatotriloba* and *I. lacunosa*, respectively).

Regardless of whether I used Method 1 or Method 2 to estimate *AnII* allele frequencies, the bootstrap analysis gave non-overlapping distributions for microsatellite frequencies and *AnII* frequency differences between the two species (Figure 13). With Method 1, there was an overlap of one out of 1000 values, indicating that the two distributions differ at a significance level of $P=0.001$. With Method 2, there was no overlap, indicating that the two distributions differ at a significance level of $P<0.001$. It thus seems that the magnitude of divergence at *AnII* is not consistent with neutral divergence.

Molecular signature of selection- To test whether the *R2R3-Myb* in *I. lacunosa* exhibits a signature of a selective sweep, I compared the haplotype diversity of this gene in *I. lacunosa* and *I. cordatotriloba*. In a sample of 20 alleles from *I. lacunosa*, there was only one haplotype. By contrast, *I. cordatotriloba* exhibited substantially greater diversity: in a sample of 15 alleles, there were four haplotypes, three of which occurred at similar frequencies, with the fourth represented by a single copy (Table 25). The Shannon-Wiener haplotype diversities are 0 and 1.27 for *I. lacunosa* and *I. cordatotriloba*, respectively. This difference in haplotype diversity is highly significant ($P < 0.001$), as judged by a t-test based on jackknifing the data. This pattern is consistent with the recent occurrence of a selective sweep of the white allele in *I. lacunosa*.

An alternative explanation of this pattern is that *I. lacunosa* has recently undergone a population bottleneck that greatly reduced genetic diversity at all loci. To assess this possibility, I performed a similar analysis on haplotype diversity of the *DFR-B*

gene. The S-W index was slightly lower for *I. lacunosa* (1.03 vs. 1.44 for *I. cordatotriloba*), but this difference was not significant ($P = .07$). It thus appears that a severe bottleneck is unlikely and that the *Myb* gene likely underwent a recent selective sweep.

3.4 Discussion

The objective of this investigation was to determine whether evolutionary loss of pigmentation in *Ipomoea lacunosa* was a result of natural selection or genetic drift.

There are cogent reasons for believing either possibility might have occurred. Selection for reduced selfing may promote the evolution of white flowers to reduce pollinator visitation and thereby increase selfing rate. Alternatively, the evolution of high rates by other means (e.g. decreased anther-stigma separation) would likely have freed it plants from dependence on pollinator visitation. With no need to maintain floral pigmentation to attract pollinators, there may have been no purifying selection to eliminate mutations that cause loss of pigmentation. In the absence of such purifying selection, if there is little cost to pigment production, such a mutation could fix by drift alone. On the other hand, it is easy to imagine that floral pigment production could be costly. If so, once freed of purifying selection, the costs could have led to selective elimination of pigmentation.

Two lines of evidence indicate that selection drove the near fixation of pigment loss in *I. lacunosa*. First, genetic differentiation between *I. lacunosa* and *I. cordatotriloba* at the *An11* locus is substantially and significantly greater than genetic

differentiation at presumably neutral microsatellite loci. Fixation of white flowers in *I. lacunosa* thus appears inconsistent with neutral divergence. Second, the *R2R2-Myb* locus corresponding to *An11* exhibits a signature of a recent selective sweep. In a sample of 20 individuals from throughout the range of *I. lacunosa* I detected no sequence variation in this gene, whereas I found substantial diversity in *I. cordatotriloba*. While this reduction in diversity could be due to a recent severe bottleneck, this possibility seems unlikely because diversity is not significantly reduced at a second locus, *Dfr-B*.

Selective fixation of the “white” allele at *An11* suggests that there may be some cost associated with producing floral pigments. A recent survey of the fitness of different genotypes in populations or species that are polymorphic for floral pigmentation indicates that in most cases, any pleiotropic effects favor the pigmented genotypes, suggesting absence of costs (Strauss & Whittall, 2006). However, there are two examples in which the loss-of-pigment genotype is advantageous. In *Linanthus parryae*, under wet conditions, absence of floral pigments is favored by selection (Schemske & Bierzychudek, 2001). And in *Claytonia virginica* pigmented genotypes experience substantially more leaf herbivory than genotypes without floral pigments (Frey, 2004). Although I have no information on the source of costs in *I. lacunosa*, they are unlikely to arise because of pleiotropic effects that occur in non-floral tissues. In other species of *Ipomoea* the expression of the gene orthologous to the *R2R3-Myb* gene identified in this investigation is limited to floral tissue (Morita et al., 2006). This appears to be true in *I. lacunosa* as well, since vegetative tissues produce anthocyanins. Because down

regulation is confined to floral tissue, pleiotropic effects that would give rise to costs are likely confined to floral tissue as well. Without further research, however, the exact nature of these effects remains unknown. Should such research fail to detect costs of pigment production, a likely alternative interpretation would be that white flowers were favored because they directly increased selfing rate by reducing pollinator visitation, an hypothesis that is open to experimental testing.

An additional possible source of selection for white flowers is reinforcement, in which flower color change reduces gene exchange between two species that produce unfit hybrids (Hopkins & Rausher, 2012). While I cannot rule out this explanation, I believe it unlikely for two reasons. One is that the high selfing rate of *I. lacunosa* already limits gene exchange from other *Ipomoea* species. The second is that its geographic distribution overlaps only minimally with other related species of *Ipomoea* in the *Batatus* section with which hybrid offspring can be produced. While reinforcing selection might operate where *I. lacunosa* comes into contact with these species, it would presumably not cause the allele for white flowers to spread throughout the majority of this species range where it does not contact these species.

Parallel genetic evolution- A question of current interest in evolutionary biology is the extent to which parallel phenotypic evolution is caused by parallel genetic or developmental evolution. Previous investigations of the evolutionary loss of floral pigmentation have revealed a remarkable degree of genetic parallelism: in all cases that have been examined, pigment loss has been caused by substitutions in floral *R2R3-Myb*

genes (Streisfeld & Rausher, 2010). This genetic parallelism presumably has occurred because, compared to other genes at which loss-of-function mutations can produce white flowers, inactivation of the floral *Mybs* incurs relatively little deleterious pleiotropy because their normal expression domain is confined to flowers (Streisfeld & Rausher, 2010). My results reinforce this pattern. In addition, they provide the first demonstration that evolutionary loss of floral pigments occurred by a regulatory mutation to the *Myb* gene. By contrast, in *Petunia axillaris*, lack of pigmentation is due to a loss-of-function mutation to the coding region (Quattrocchio et al., 1999). In *Antirrhinum majus* reduction in anthocyanin pigmentation is associated with both a coding-region knockout and down regulation, but it is not possible to determine which was the causal change and which followed after white flowers had evolved (Schwinn et al., 2006) In the case of *I. lacunosa*, I could detect no coding sequence differences between *I. lacunosa* and *I. cordatotriloba* copies of the *Myb*, ruling out coding-sequence change as a possibility. Instead, the *cis*-regulatory change causing down regulation in *I. lacunosa* is clearly causal. These results thus indicate both *cis*-regulatory and coding-region changes can cause parallel evolutionary change because they have equivalent effects: both eliminate activation of the anthocyanin biosynthetic pathway in flowers but not in other tissues. There is thus not strict parallelism at the genetic level (i.e. the same mutation, nor even the same type of mutation, does not cause a similar phenotypic change), but at the developmental level: inactivation of the pigment pathway. A similar pattern has been found for evolutionary shifts from blue to red flowers, where inactivation of pathway

branching enzymes causes a change in the type of anthocyanin produced, but this inactivation can be achieved by loss-of-function coding-region mutations, substitutions in *cis*-regulatory regions of the genes coding for these enzymes, or substitutions in transcription factors that activate them (Des Marais & Rausher, 2010; Smith & Rausher, 2011). Thus, for different types of change in floral color, constraints giving rise to parallel phenotypic evolution appear to operate largely at the developmental level rather than at the genetic level.



A) *I. lacunosa*



B) *I. cordatotriloba*

Figure 8: A) *I. lacunosa* B) *I. cordatotriloba*

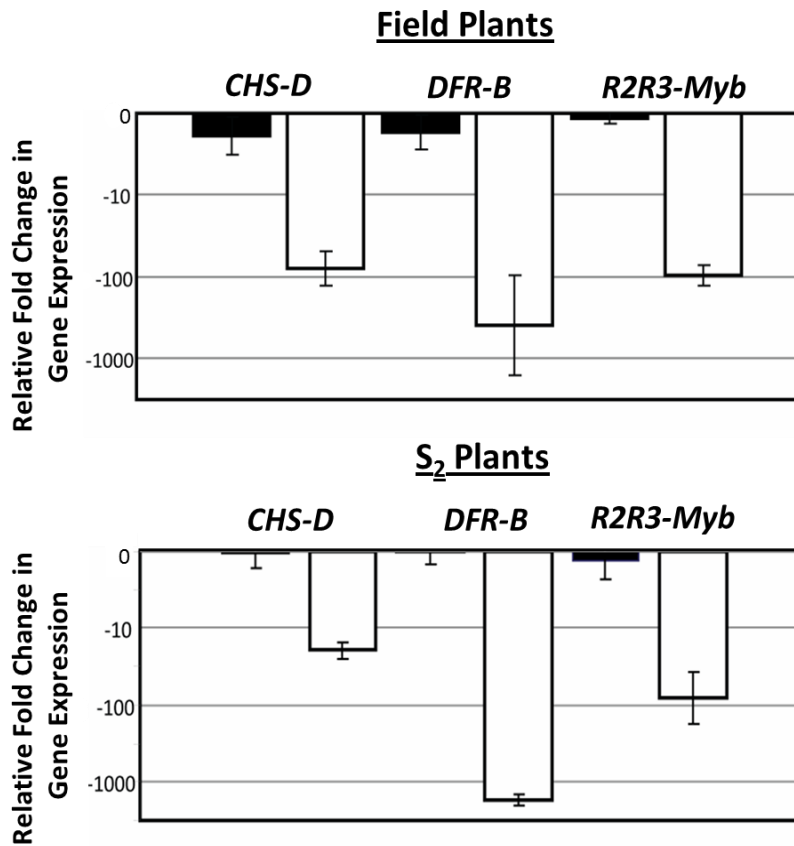


Figure 10: Q-PCR results.

Field individuals (*CHS-D* ANOVA, $F_{1,6}=93.80$, $p=0.0002$; *DFR-B* ANOVA $F_{1,6}=39.63$, $p=0.0015$; *R2R3-Myb* ANOVA, $F_{1,6}=533.95$, $p<0.0001$); *S₂* individuals (*CHS-D* ANOVA, $F_{1,6}=52.14$, $p=0.0007$; *DFR-B* ANOVA, $F_{1,6}=554.28$, $p<0.0001$; *R2R3-Myb* ANOVA, $F_{1,6}=31.44$, $p=0.0025$)

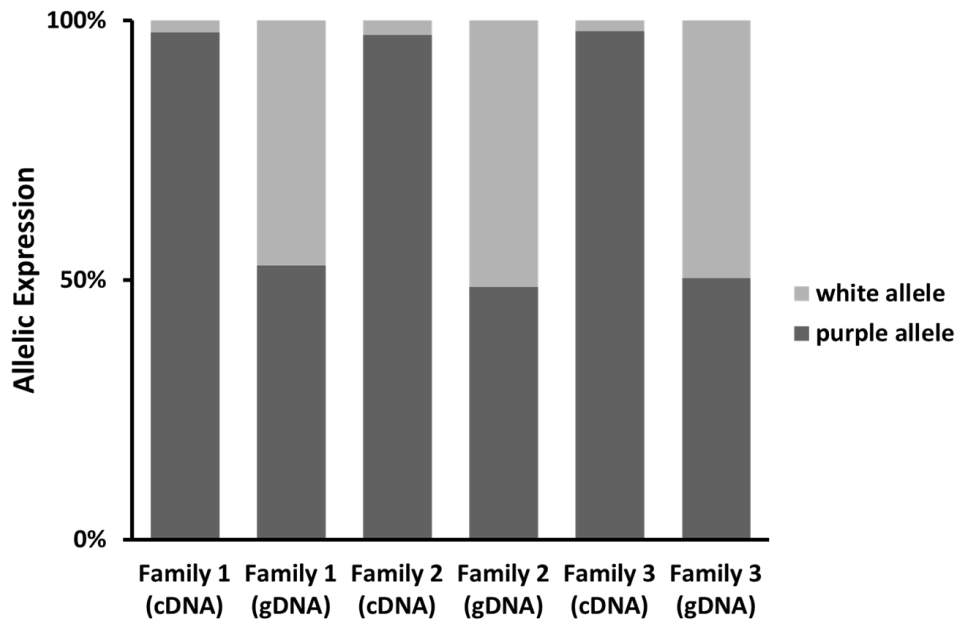


Figure 11: *R2R3-Myb* pyrosequencing results.

Family 1 (ANOVA, $F_{1,6}=443.36$, $p<0.0001$); Family 2 ($F_{1,6}=39.60$, $p=0.0007$); Family 3 (ANOVA, $F_{1,6}=219.02$, $p<0.0001$).

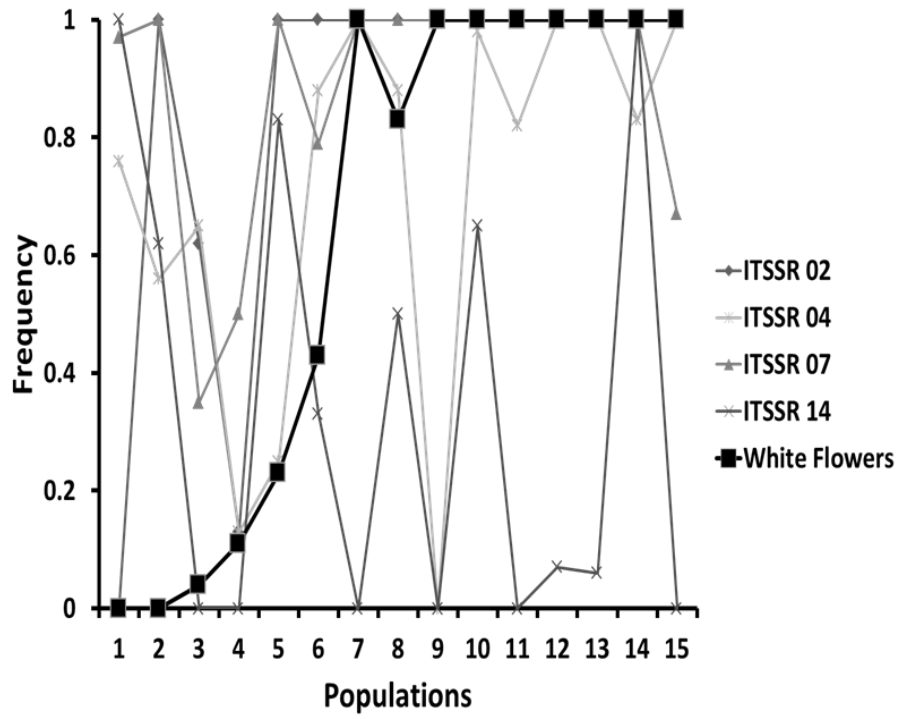


Figure 12: Variation in allele frequencies across 15 populations.

Populations 1-7 contain *I. cordatotriloba* and 8-15 contain *I. lacunosa* individuals.

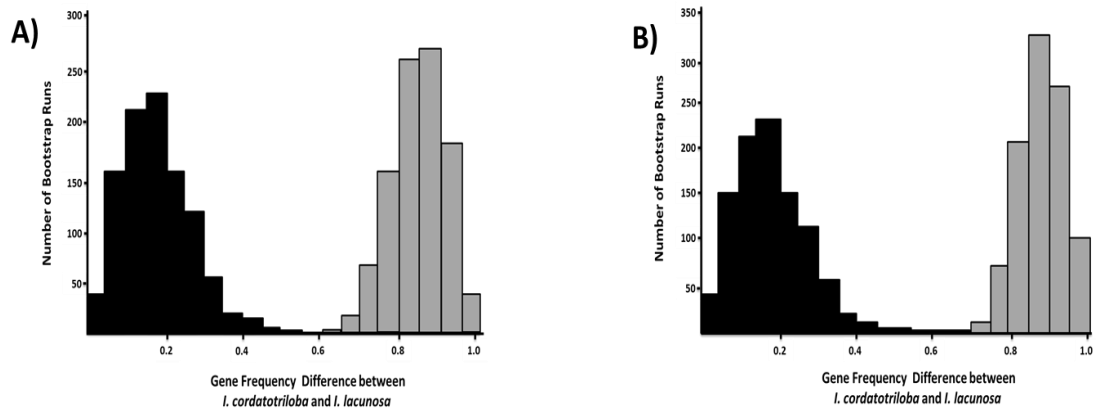


Figure 13: Gene and flower color frequency difference between *I. lacunosa* and *I. cordatotriloba*.

A) Analysis method 1 B) Analysis method 2. Average gene frequency difference across microsatellite loci $0.14 \pm X$ (black). Average gene frequency difference of flower color loci using method 1) $0.85 \pm X$ and method 2) $0.88 \pm X$ (gray).

Table 17: Primers used to amplify partials regions of anthocyanin genes *DFR-B* and *CHS-D* as wells as the anthocyanin transcription factor *R2R3-Myb*.

	Forward Primer (5'→3')	Reverse Primer (5'→3')
<i>DFR-B</i> (cDNA)	ATCGGCTCCTGGTTGGTCATGACA	GTGGCCTCTGCTTAGTAGGTTCTT
<i>CHS-D</i> (cDNA)	CGTCGAGGAGGTCAGAAAGG	TGGAGGACCACGGTTTCGATGGTAA
<i>R2R3-Myb</i> (cDNA)	TCGTCTCCGCGAGTGAGAA	TGTTTCATTGTCGTCGTAGAGCAAAT
<i>R2R3-Myb</i> (3' UTR genomic region)	TCGTCTCCGCGAGTGAGAA	CAATTTGCAGTGATGCCAAG

Table 18: Primers for Q-PCR expression assay.

	Forward Primer (5'→3')	Reverse Primer (5'→3')
<i>CHS-D</i>	GCACCTACCCCGACTACTATTTTCGT	GAAGGCGCCATGTATTCACAAAAGC
<i>DFR-B</i>	ATCGGCTCCTGGTTGGTCAAGACAC	GCAATGGCTTCATCAAAGCTTCCTT
<i>R2R3-Myb</i>	TCGACATTAACCGGAAAAGC	AAATCTTCCCACCACTGCAC
<i>EF-1*</i>	CTGTAACAAGATGGATGCCA	AGACGGAGTGGCTTGTCTG

* primers developed in Streisfeld and Rausher, 2009 (Streisfeld & Rausher, 2009)

Table 19: Primers and restriction enzymes used in co-segregation assay.

Gene	Genetic Variation (cut site bold)	Rest. Enz.	Forward Primer (5'→3')	Reverse Primer (5'→3')	PCR & Restrict. Frag. size (b.p)
DFR-B					
<i>I. X leucantha</i>	TTTCAGCTAGC	NheI	GTCGCTTATTGCTGGC AGAA	GTCTAGCCATGTCCGT AGTATAAACCAA	120, 330
<i>I. lacunosa</i>	TTTCAGGTAGC				450
R2R3-Myb					
<i>I. X leucantha</i>	-----	AseI	GTCTAGCCATGTCCGT AGTATAAACCAA	CAATTTGCAGTGATGC CAAGA	890
<i>I. lacunosa</i>	TATTATTAATTC				630, 260

Table 20: Primers used for genetic diversity analyses.

Primer	Forward Primer (5'→3')	Reverse Primer (5'→3')
<i>R2R3-Myb</i>	GCTGATCTCATTCTGCGCT	CAATTTGCAGTGATGCCAAGA
<i>DFR-B</i>	ATCGGCTCCTGGTTGGTCATGACA	GCAATGGCTTCATCAAAGCTTCCT

Table 21: HPLC results.

Species	N	Flower Color	Corolla	Stem
<i>I. cordatotriloba</i>	2	purple	Yes	Yes
<i>I. lacunosa</i>	3	purple	Yes	Yes
<i>I. lacunosa</i>	3	white	No	Yes

Table 22: S₃ individuals scored.

IC= inner corolla; OC= outer corolla; W= white; P=purple; LP=light purple

Parental Genotype (phenotype)	S ₃ Offspring			
	aa_BB (IC:W, OC: N/A)	A_bb (IC:P, OC:W)	A_Bb (IC: P, OC: LP)	A_BB (IC:P, OC:DP)
AaBb (IC= P, OC= LP)	7	2	5	1
AaBB (IC= P, OC= DP)	0	0	0	7
aa_BB (IC= W, OC=N/A)	8	0	0	0

Table 23: Co-segregation analysis of *R2R3-Myb* and *DFR-B*.

(T=purple allele, t= white allele)

Gene	Family	Genotype	Flower Color		χ^2	P
			purple	white		
<i>R2R3-Myb</i>	1	T_	13	0	24	<0.001
		tt	0	11		
	2	T_	2	0	5	0.03
		tt	0	3		
	3	T_	5	0	12	<0.001
		tt	0	7		
	4	T_	3	0	12	<0.001
		tt	0	9		
<i>DFR-B</i>	1	T_	5	6	0.74	0.39
		tt	8	5		
	2	T_	1	1	0.33	0.57
		tt	1	2		
	3	T_	3	5	0.5	0.48
		tt	2	2		

Table 24: Flower color frequency for 15 populations with concordant microsatellite data.

	population	latitude (N)	longitude (W)	Species	White Allele Frequency
1	c13	34.00996	78.30176	<i>I. cordatotriloba</i>	0
2	clela3	33.95991	78.99147	<i>I. cordatotriloba</i>	0
3	c7	33.89953	80.89953	<i>I. cordatotriloba</i>	0.04
4	c22	34.37833	77.89637	<i>I. cordatotriloba</i>	0.11
5	cle1	33.85097	78.81234	<i>I. cordatotriloba</i>	0.23
6	cl4	34.81235	76.87686	<i>I. cordatotriloba</i>	0.43
7	c19	34.6909	77.97886	<i>I. cordatotriloba</i>	1
8	la43	35.98721	79.26268	<i>I. lacunosa</i>	0.83
9	la3	34.22187	80.40211	<i>I. lacunosa</i>	1
10	lela8	33.98603	80.17159	<i>I. lacunosa</i>	1
11	la15	34.9598	78.17761	<i>I. lacunosa</i>	1
12	la30	35.51501	79.34398	<i>I. lacunosa</i>	1
13	la35	35.86958	80.03384	<i>I. lacunosa</i>	1
14	la7	34.47367	79.8702	<i>I. lacunosa</i>	1
15	ula7	34.87798	77.97215	<i>I. lacunosa</i>	1

Table 25: Haplotype frequency for *I. cordatotriloba* and *I. lacunosa*.

Haplotype	<i>R2R3-Myb</i>		<i>DFR-B</i>	
	<i>I. cordatotriloba</i>	<i>I. lacunosa</i>	<i>I. cordatotriloba</i>	<i>I. lacunosa</i>
A	5	20	2	4
B	5	0	4	6
C	1	0	3	10
D	4	0	6	0

Appendix A: Species present in population surveyed.

Population name and GPS coordinates are given for each location surveyed. Grey boxes indicate populations in which leaf tissue was collected and used in the microsatellite analysis

	Pop Name	latitude (N)	longitude (W)	<i>I. cordatotriloba</i>	<i>I. austinii</i>	<i>I. X leucantha</i>	<i>I. lacunosa</i>
1	c1	33.71054	78.88154	X			
2	c2	33.73008	78.90894	X			
3	c3	33.74665	78.83166	X			
4	c4	33.75048	78.97870	X			
5	u1	33.75352	78.86810		X		
6	cle1	33.85097	78.81234	X		X	
7	u2	33.85156	78.65240		X		
8	cula1	33.85819	80.00819	X	X		X
9	c5	33.86581	78.63960	X			
10	c6	33.87177	78.62401	X			
11	c7	33.89953	80.89953	X			
12	cla1	33.90135	79.13961	X			X
13	c8	33.90598	78.58437	X			
14	lela1	33.92589	78.98779			X	X
15	c9	33.92639	80.15923	X			
16	lela2	33.92680	80.15444			X	X
17	lela3	33.92937	78.98542			X	X
18	c10	33.93110	78.55347	X			
19	clula1	33.93341	79.10781	X		X	X
20	le1	33.93880	78.98620			X	
21	lela4	33.93910	79.10781			X	X
22	lela5	33.94329	78.98803			X	X
23	le3	33.94857	80.08791			X	
24	le5	33.94907	80.09646			X	
25	c11	33.94935	79.02579	X			
26	clela2	33.95099	79.05856	X		X	X
27	lela6	33.95268	80.16554			X	X
28	clela3	33.95991	78.99147	X		X	X
29	c12	33.96198	78.42105	X			
30	lela7	33.96504	80.05355			X	X

31	le7	33.96824	80.16987				X	
32	le9	33.96897	80.04815				X	
33	u3	33.98432	81.03335			X		
34	lela8	33.98603	80.17159				X	X
35	cula2	34.00710	79.22410	X		X		X
36	la1	34.01166	79.22197					X
37	lela9	34.01675	80.02195				X	X
38	c13	34.01905	78.30013	X				
39	lela10	34.03076	80.01170				X	X
40	le11	34.04208	80.03619				X	
41	la2	34.05346	79.24291					X
42	le13	34.05409	80.06216				X	
43	le15	34.05710	80.16683				X	
44	le17	34.09191	79.95067				X	
45	lela11	34.10136	79.92306				X	X
46	c14	34.10871	78.10811	X				
47	le19	34.18089	80.15563				X	
48	culela1	34.18493	80.14191	X		X	X	
49	c15	34.18635	78.07789	X				
50	lela12	34.18890	79.93886				X	X
51	c16	34.19313	78.06443	X				
52	la3	34.22187	80.40211					X
53	lela13	34.22298	80.00309				X	X
54	c17	34.22374	78.01118	X				
55	la4	34.23576	79.69021					X
56	la5	34.24047	79.68680					X
57	la6	34.25922	79.69435					X
58	le21	34.26204	79.44064				X	
59	c18	34.26725	78.09312	X				
60	le23	34.27343	79.70387				X	
61	le25	34.28426	79.42495				X	
62	le27	34.28880	79.44165				X	
63	c19	34.30962	77.97134	X				
64	le29	34.32545	79.89128				X	
65	lela14	34.32771	79.49670				X	X
66	lela15	34.32882	79.49974				X	X
67	c20	34.34259	78.00364	X				
68	le31	34.34700	79.89752				X	

69	c21	34.35167	77.72792	X			
70	c22	34.37833	-77.89637	X			
71	c23	34.39078	77.67836	X			
72	clela4	34.41868	77.88434	X		X	X
73	lela16	34.42209	79.40007			X	X
74	cla2	34.42570	77.87385	X			X
75	la7	34.47367	79.87020				X
76	c24	34.48462	77.56372	X			
77	ula1	34.50415	79.30717		X		X
78	c25	34.51701	77.91437	X			
79	ula2	34.51756	79.10712		X		X
80	ula3	34.52426	79.27620		X		X
81	ula4	34.52973	79.11169		X		X
82	cu1	34.57249	77.94509	X	X		
83	c26	34.60050	77.94480	X			
84	c27	34.63702	77.92169	X			
85	u4	34.64191	79.03985		X		
86	c28	34.64724	77.96371	X			
87	lela17	34.67929	79.88819			X	X
88	la8	34.68127	77.96570				X
89	c29	34.69087	77.97886	X			
90	la9	34.69638	77.97226				X
91	c30	34.69795	76.78481	X			
92	u5	34.73284	77.95270		X		
93	la10	34.74071	77.97177				X
94	c31	34.74557	77.97576	X			
95	cla3	34.75478	76.84532	X			X
96	ula5	34.76182	77.97152		X		X
97	la11	34.79800	79.88852				X
98	u6	34.80929	78.00094		X		
99	cl4	34.81235	76.87686	X			X
100	c32	34.83395	76.87960	X			
101	ula6	34.83951	78.95704		X		X
102	c33	34.85135	76.88751	X			
103	u7	34.85196	77.96004		X		
104	c34	34.85374	76.89292	X			
105	c35	34.86991	76.90060	X			
106	ula7	34.87798	77.97215		X		X

107	u8	34.89089	77.96341		X	
108	la12	34.89755	79.80206			X
109	la13	34.90111	78.22715			X
110	ula8	34.90256	77.99157		X	X
111	u9	34.90592	77.91953		X	
112	u10	34.91049	78.02251		X	
113	u11	34.91860	78.02782		X	
114	ula9	34.92800	78.03465		X	X
115	c36	34.95646	76.95004	X		
116	la15	34.95980	78.17761			X
117	la14	34.96051	78.92429			X
118	c37	34.96248	76.95459	X		
119	la16	34.96613	78.91940			X
120	u12	34.96713	78.92649		X	
121	u13	34.97198	78.18830		X	
122	u14	34.98564	78.15189		X	
123	c38	35.02278	76.99767	X		
124	c39	35.08108	77.03062	X		
125	c40	35.09979	77.05437	X		
126	la17	35.16163	78.72521			X
127	la18	35.23980	77.57603			X
128	la19	35.24480	78.33661			X
129	la20	35.27125	78.61682			X
130	la21	35.28423	77.79586			X
131	la22	35.32614	79.28025			X
132	la23	35.33331	79.36028			X
133	la24	35.33467	79.36006			X
134	la25	35.33599	79.35348			X
135	la26	35.33956	78.55302			X
136	la27	35.36075	77.92425			X
137	la28	35.46023	78.54914			X
138	la29	35.47045	78.19380			X
139	la30	35.51501	79.34398			X
140	la31	35.56867	78.38925			X
141	la32	35.63225	79.01803			X
142	la33	35.68255	78.91405			X
143	la34	35.86155	80.18288			X
144	la35	35.86958	80.03384			X

145	la36	35.93086	79.23156	X
146	la37	35.93593	79.19526	X
147	la38	35.95078	79.92552	X
148	la39	35.95091	79.25802	X
149	la40	35.97611	79.25593	X
150	la42	35.98721	79.26268	X
151	la41	35.99383	78.94802	X
152	la43	36.00923	78.95233	X
153	la44	36.04138	79.55877	X
154	la45	36.07438	79.46184	X

Appendix B: Frequency of white allele.

100 flowers were counted when populations were fixed for flower color. 200 flowers were counted when variation in flower color was found.

# pop	Population	latitude (N)	longitude (W)	Species	frequency of white allele
1	c14	34.10871	78.10811	<i>I. cordatotriloba</i>	0
2	c9	33.92639	80.15923	<i>I. cordatotriloba</i>	0
3	c6	33.87177	78.62401	<i>I. cordatotriloba</i>	0
4	c11	33.94935	79.02579	<i>I. cordatotriloba</i>	0
5	c2	33.73008	78.90894	<i>I. cordatotriloba</i>	0
6	c8	33.90598	78.58437	<i>I. cordatotriloba</i>	0
7	c10	33.93110	78.55347	<i>I. cordatotriloba</i>	0
8	c13	34.00996	78.30176	<i>I. cordatotriloba</i>	0
9	c16	34.19313	78.06443	<i>I. cordatotriloba</i>	0
10	c17	34.22374	78.01118	<i>I. cordatotriloba</i>	0
11	c18	34.26725	78.09312	<i>I. cordatotriloba</i>	0
12	c19	34.30962	77.97134	<i>I. cordatotriloba</i>	0
13	c20	34.34259	78.00364	<i>I. cordatotriloba</i>	0
14	c21	34.35167	77.72792	<i>I. cordatotriloba</i>	0
15	c23	34.39078	77.67836	<i>I. cordatotriloba</i>	0
16	c24	34.48462	77.56372	<i>I. cordatotriloba</i>	0
17	c26	34.60050	77.94480	<i>I. cordatotriloba</i>	0
18	c28	34.64724	77.96371	<i>I. cordatotriloba</i>	0
19	cla2	34.42570	77.87385	<i>I. cordatotriloba</i>	0
20	cu1	34.57249	77.94509	<i>I. cordatotriloba</i>	0
21	c40	35.09979	77.05437	<i>I. cordatotriloba</i>	0
22	c39	35.08108	77.03062	<i>I. cordatotriloba</i>	0
23	c38	35.02278	76.99767	<i>I. cordatotriloba</i>	0
24	c37	34.96248	76.95459	<i>I. cordatotriloba</i>	0
25	c35	34.86991	76.90060	<i>I. cordatotriloba</i>	0
26	c34	34.85374	76.89292	<i>I. cordatotriloba</i>	0
27	c32	34.83395	76.87960	<i>I. cordatotriloba</i>	0
28	c25	34.51701	77.91437	<i>I. cordatotriloba</i>	0
29	clela4	34.41868	77.88434	<i>I. cordatotriloba</i>	0
30	cla1	33.90135	79.13961	<i>I. cordatotriloba</i>	0

31	clela2	33.95099	79.05856	<i>I. cordatotriloba</i>	0
32	clela3	33.95991	78.99147	<i>I. cordatotriloba</i>	0
33	cula2	34.00710	79.22410	<i>I. cordatotriloba</i>	0
34	c7	33.89953	80.89953	<i>I. cordatotriloba</i>	0.04
35	c3	33.74665	78.83166	<i>I. cordatotriloba</i>	0.10
36	c22	34.37833	77.89637	<i>I. cordatotriloba</i>	0.11
37	c15	34.18635	78.07789	<i>I. cordatotriloba</i>	0.05
38	c27	34.63702	77.92169	<i>I. cordatotriloba</i>	0.18
39	clula1	33.93341	79.10781	<i>I. cordatotriloba</i>	0.47
40	cle1	33.85097	78.81234	<i>I. cordatotriloba</i>	0.23
41	cl4	34.81235	76.87686	<i>I. cordatotriloba</i>	0.43
42	c1	33.71054	78.88154	<i>I. cordatotriloba</i>	1
43	c4	33.75048	78.97870	<i>I. cordatotriloba</i>	1
44	c5	33.86581	78.63960	<i>I. cordatotriloba</i>	1
45	c12	33.96198	78.42105	<i>I. cordatotriloba</i>	1
46	c36	34.95646	76.95004	<i>I. cordatotriloba</i>	1
47	c33	34.85135	76.88751	<i>I. cordatotriloba</i>	1
48	c29	34.69087	77.97886	<i>I. cordatotriloba</i>	1
49	c30	34.69795	76.78481	<i>I. cordatotriloba</i>	1
50	c31	34.74557	77.97576	<i>I. cordatotriloba</i>	1
51	ula3	34.52426	79.27620	<i>I. lacunosa</i>	1
52	ula4	34.52973	79.11169	<i>I. lacunosa</i>	1
53	la9	34.69638	77.97226	<i>I. lacunosa</i>	1
54	la10	34.74071	77.97177	<i>I. lacunosa</i>	1
55	ula3	34.75478	76.84532	<i>I. lacunosa</i>	1
56	ula5	34.76182	77.97152	<i>I. lacunosa</i>	0.44
57	la11	34.79800	79.88852	<i>I. lacunosa</i>	1
58	ul4	34.81235	76.87686	<i>I. lacunosa</i>	1
59	ula6	34.83951	78.95704	<i>I. lacunosa</i>	1
60	ula7	34.87798	77.97215	<i>I. lacunosa</i>	1
61	la12	34.89755	79.80206	<i>I. lacunosa</i>	1
62	la13	34.90111	78.22715	<i>I. lacunosa</i>	1
63	ula8	34.90256	77.99157	<i>I. lacunosa</i>	1
64	ula9	34.92800	78.03465	<i>I. lacunosa</i>	1
65	la14	34.96051	78.92429	<i>I. lacunosa</i>	1
66	la15	34.95980	78.17761	<i>I. lacunosa</i>	1
67	la17	35.16163	78.72521	<i>I. lacunosa</i>	1
68	la18	35.23980	77.57603	<i>I. lacunosa</i>	1

69	la19	35.24480	78.33661	<i>I. lacunosa</i>	1
70	la20	35.27125	78.61682	<i>I. lacunosa</i>	1
71	la21	35.28423	77.79586	<i>I. lacunosa</i>	1
72	la22	35.32614	79.28025	<i>I. lacunosa</i>	0.98
73	la23	35.33331	79.36028	<i>I. lacunosa</i>	1
74	la24	35.33467	79.36006	<i>I. lacunosa</i>	1
75	la25	35.33599	79.35348	<i>I. lacunosa</i>	1
76	la26	35.33956	78.55302	<i>I. lacunosa</i>	1
77	la27	35.36075	77.92425	<i>I. lacunosa</i>	1
78	la28	35.46023	78.54914	<i>I. lacunosa</i>	0.99
79	la29	35.47045	78.19380	<i>I. lacunosa</i>	1
80	la30	35.51501	79.34398	<i>I. lacunosa</i>	1
81	la32	35.63225	79.01803	<i>I. lacunosa</i>	1
82	la33	35.68255	78.91405	<i>I. lacunosa</i>	1
83	la34	35.86155	80.18288	<i>I. lacunosa</i>	1
84	la35	35.86958	80.03384	<i>I. lacunosa</i>	1
85	la36	35.93086	79.23156	<i>I. lacunosa</i>	1
86	la37	35.93593	79.19526	<i>I. lacunosa</i>	1
87	la38	35.95078	79.92552	<i>I. lacunosa</i>	1
88	la39	35.95091	79.25802	<i>I. lacunosa</i>	1
89	la40	35.97611	79.25593	<i>I. lacunosa</i>	1
90	la41	35.99383	78.94802	<i>I. lacunosa</i>	1
91	la43	35.98721	79.26268	<i>I. lacunosa</i>	0.83
92	la44	36.04138	79.55877	<i>I. lacunosa</i>	1
93	la45	36.07438	79.46184	<i>I. lacunosa</i>	1

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