ABSTRACT

The presence of a cleistogamous (obligately selfing) plant breeding system may have implications for reproductive isolation between species. The effects of this trait on plant hybridization previously have received little consideration. *Triodanis perfoliata* subsp. *perfoliata* and *Triodanis perfoliata* subsp. *biflora* (Campanulaceae) are two annual sister taxa that co-occur across most of their distribution in North America. Any given plant can produce both open chasmogamous flowers (CH) which can cross-pollinate and closed cleistogamy (CL) which obligately self-pollinate. The sister taxa differ in their allocation to cleistogamy and have been reported to hybridize. To test for hybridization between the subspecies, I used a combination of amplified fragment length polymorphism (AFLP) genetic marker data and morphological data. At four zones of contact. where the sister taxa co-occur, in Pitt County, NC, individuals of *Triodanis* were marked for sampling at 2 m intervals along a transect spanning the two subspecies populations (N = 40- 75). Ten individuals of each parental subspecies, identified by distinguishing diagnostic traits, also were sampled at each site. For each individual sampled, four diagnostic morphological traits (bract base, bract length to width ratio, capsule pore position, and CH/CL ratio) were measured and AFLP genotypes were determined using three primer pairs.

Principal component analysis (PCA) of morphological traits revealed two distinguishable groups representing subspecies-like individuals as well as putative hybrids showing intermediate morphology. Principal component analysis of genetic data gave congruent results. Putative hybrids identified using morphological and genotype data were confirmed using the program STRUCURE 2.3.4, which estimated proportions of ancestry (Q values) for individuals from AFLP genotype data. Of the total 169 plants sampled along the transects, 9.47% were shown to be hybrids. Backcrosses to subsp. *perfoliata* were over four times as frequent as backcrosses to

subsp. *biflora*. Sites with similar abundances of the two subspecies supported populations with a greater number of hybrids.

Components of fitness were estimated in parental taxa and in hybrids identified by genetic markers by measuring biomass, total fruits produced, and seed set. F_1 hybrids produced a greater biomass, more total fruits, and more seeds than parental taxa. Although hybrid vigor is evident, the extent of hybridization appears to be limited by prezygotic reproductive isolation mechanisms such as habitat isolation, divergent chasmogamous flowering phenology, and the presence of cleistogamy.

HYBRIDIZATION IN TWO SUBSPECIES OF *TRIODANIS PERFOLIATA*, A CLEISTOGAMOUS ANNUAL PLANT (CAMPANULACEAE)

A Thesis

Presented to

The Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirement for the Degree

Master of Science in Biology

by

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April 2013

Hybridization in two subspecies of *Triodanis perfoliata*, a cleistogamous annual plant
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AKNOWLEDGEMENTS

I would like to thank Dr. Carol Goodwillie for her knowledge, guidance, and friendship over the past four years. Her warm heart and passion for research has developed a lab that truly enjoys their work. My only regret is not being able to work alongside such a wonderful teacher for a longer period of time. I will never be able to thank Dr. Goodwillie enough for the impact she has made in my life.

I would also like to thank my field assistants Christen Gillis and Joshua Thigpen for their assistance with early morning fieldwork and friendly company; you made collecting fruits and possible Lyme disease much more bearable.

A big thanks to my committee members Dr. Ashley Egan, Dr. Claudia Jolls, and Dr. Kevin O'Brien for their very helpful comments, suggestions, and insights.

I would also like to thank Dr. Cindy Putnam-Evans for allowing me to use land located on her property as one of my field locations.

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INTRODUCTION

Hybridization. Over 25% of all plant species are estimated to participate in hybridization events, the production of viable offspring by two genetically distinct taxa (Mallet 2005). With such a large number of species experiencing this process, it is important to understand factors that may prevent or promote hybridization and its evolutionary consequences.

A number of factors determine the extent of hybridization between taxa. These mechanisms of reproductive isolation are classified according to the timing of their action. Isolation occurring prior to mating, prezygotic isolation, can result from geographical, ecological, temporal, and behavioral differences between parental species (Pascarella 2007, Ito et al. 2008, Lowry et al. 2008). Alternatively, postzygotic isolation occurs after hybrid offspring are formed. Such barriers include reduced hybrid fitness, hybrid sterility, and F_2 breakdown due to segregation of alleles from the two parents (Dobzhansky 1937, Mayr 1942, Ramsey et al. 2003). Reduced fitness in hybrid offspring is thought to be caused by a negative interactions of alleles that have differentially fixed in the parental species (Dobzhansky 1937, Muller 1942). However, studies also have reported hybrid offspring with increased fitness, termed heterosis. Heterosis can cause a breakdown in reproductive isolation between taxa. Hybrids may outperform parental taxa because of genetic factors such as heterozygote advantage (Fenster and Galloway 2000, Rosas et al. 2010) or increased performance in particular environments (Emms and Arnold 1997, Fritsche and Kaltz 2000, Rhode and Cruzan 2005).

There are several evolutionary outcomes to hybridization. When newly diverged species come into secondary contact, gene flow may readily occur between the parental species. This uninterrupted gene flow may allow the convergence of the two species. On the other hand,

hybridization producing offspring with reduced vigor may promote greater divergence between parental taxa. Through a process called reinforcement, traits that promote prezygotic isolation might be selected for to minimize production of hybrids with low fitness (Dobzhansky 1937). This process eliminates the waste of gametes on the production of genetically inferior offspring and causes speciation to occur at a faster rate. Hybridization also can result in the formation of new species. Mechanisms of reproductive isolation can be selected for that block backcrossing between the parental species and the hybrids, causing hybrid speciation (Coyne 1994, Levin 2000, Wu 2001). Hybrid speciation is possible through two different mechanisms; homoploid speciation, production of hybrid of the same ploidy level as parents (Rieseberg 2006), or more commonly through allopolyploid speciation, restoration of fertility to sterile hybrid offspring by means of chromosome doubling while hybridization is blocked between hybrid and parental species (Soltis and Soltis 2009) In either case, hybridization can create new species, increasing diversity within the plant kingdom.

In some cases hybridization can move genes across species boundaries, introducing new genetic variation. Backcrossing, mating between hybrid offspring and parental plants, is a common occurrence in hybrid zones (Rieseberg 1997). This can cause introgression, gene flow from one species to another through repeated hybridization events in which the hybrid form is preferred (Anderson and Hulbricht 1938, Anderson 1953). Gene flow within a hybrid zone is influenced by a combination of genetic and environmental factors. In some hybrid zones gene flow may occur more when one species serves as the maternal parent than in the other direction. Divergent selfing rates may cause asymmetry in gene flow between hybridizing taxa because of differing pollen-ovule ratios (Cruden 1977); this asymmetry in gene flow may result in an increase in frequency of backcrossed individuals with the more highly outcrossing parental taxa.

Other factors that may contribute to gene flow asymmetry in hybrid zones include: the ratio of species abundance (Burgess et al. 2005, Martin and Willis 2007), differences in pollinator preference (Campbell et al. 1997), and pollen competition (Rieseberg et al. 1995, Carney et al. 1996, Howard 1999). With asymmetry in hybridization there will be directionality in the potential for introgression.

Hybridization can lead to taxonomic complications. The biological species concept presented by Mayr (1963) states that a species is a group of interbreeding individuals that are reproductively isolated from other such groups. This concept is widely accepted for most animal species but has been argued to be less readily applied to plant species, in part because of the common occurrence of hybridization (Ehrlich 1961, Raven 1976). Geographic overlap, mechanisms of reproductive isolation, and the potential for hybridization are highly variable in the plant kingdom. This often makes defining a species a difficult task. When hybridization does occur, it also creates questions concerning whether divergent taxa should be considered distinct species, subspecies, or varieties (Hamilton and Reichard 1992).

Mating Systems. Modes of reproduction are highly variable in plants and can play a role in determining the potential for hybridization and speciation. Darwin himself studied plant mating systems extensively and marveled at their immense variation (Darwin 1877). Most plants are hermaphrodites with male (anthers) and female (pistils) organs produced within the same flower. In hermaphrodites, mating systems range from autogamy (self-fertilization within a flower or selfing) to cross-fertilization (outcrossing), which is promoted by a number of mechanisms including temporal or spatial separation of male and female functions (Barrett 2003).

A particularly interesting form of selfing is cleistogamy, in which flowers are formed that never open and are therefore obligately self-fertilized. Chasmogamy refers to the production of typical open flowers that may be cross-fertilized. Most species with cleistogamy also produce some chasmogamous flowers, resulting in a mixed selfing and outcrossing mating system. Cleistogamous breeding systems have been recorded in 50 families, 228 genera, and 693 species of plants and is most common in species in the families Poaceae (326), Violaceae (80), Fabaceae (61), Orchidaceae (24), and Acanthaceae (19) (Culley and Klooster 2007).

If a cleistogamous breeding system is to evolve and persist, there must be greater and distinct fitness advantages associated with cleistogamous (CL) flowers compared to chasmogamous (CH) flowers (Culley and Klooster 2007). As with selfing in general, offspring of cleistogamous fruits might suffer from inbreeding depression, the expression of deleterious recessive alleles (Charlesworth and Charlesworth 1987). Another cost of cleistogamy is complete pollen discounting (Winn and Moriuchi 2009), a reduction in the amount of pollen available for outcrossing caused by selfing (Holsinger 1991). Benefits of cleistogamy are especially pronounced in unfavorable environmental conditions. Since CL flowers never open or produce showy flowers, they conserve a considerable amount of resources and are therefore favored when resources are limited (Schemske 1978, Waller 1979). Cleistogamy is also beneficial in populations where few potential mates or pollinators are available for cross fertilization (Mitchell-Olds and Waller 1985).

Implications of Cleistogamy for Hybridization. The contribution of selfing to reproductive isolation has been previously studied (Levin 1971, Wendt et al. 2002, Husband and Sabara 2004), however the implications of cleistogamy on reproductive isolation rarely have been

considered (Bradley 1975). Cleistogamous flowers cannot participate in hybridization as a pollen or ovule parent. Therefore, the proportion of CL flowers on a plant has major implications for the amount of hybridization possible. Furthermore, if hybridization is occurring between two diverged species and their offspring are genetically inferior or are sterile, selection might act to increase the number of CL flowers produced, limiting future hybridization events. This form of reinforcement selection (Dobzhansky 1937) has not been considered previously. In all, cleistogamy is likely to affect the extent of hybridization and, in turn, hybridization may have implications for the production of CL flowers.

Triodanis. Two sister taxa in the annual genus *Triodanis* (Campanulaceae) provide an opportunity to study hybridization and the role of cleistogamy in reproductive isolation. *Triodanis perfoliata* (L.) Nieuwl. (hereafter referred to as *T. perfoliata* subsp. *perfoliata*) and *Triodanis perfoliata* subsp. *biflora* (Ruiz & Pavon) Lammers are winter annual plants that are widely distributed across North and Central America. Their distribution overlaps throughout much of the southern range of *Triodanis perfoliata* subsp. *perfoliata* (USDA, NRCS 2012). These subspecies produce viable offspring when crossed and putative hybrids have been reported in natural populations (Bradley 1975). Both taxa produce both CH and CL flowers, but the taxa have diverged in their allocation to the two flower types; *Triodanis perfoliata* subsp. *perfoliata* produces several CH flowers, while *Triodanis perfoliata* subsp. *biflora* generally produces only one or two CH flowers, allocating most of its resources to CL flowers. These taxa also differ in a number of diagnostic traits including the shape of floral bracts and the location of the dehiscent pore on fruit capsules (Figure 1). The taxa have been recognized as separate species in the past, but the most recent revision assigns them subspecies status (Lammers 2006).

My study explores the extent of hybridization between the two subspecies and the role of cleistogamy in reproductive isolation. I examined three local zones of co-occurrence using both morphological and AFLP genetic marker data for hybrid identification. The correlation between morphological traits and genetic makeup of individuals is explored. I investigate the potential for the formation of F_1 and backcross hybrids by examining CH flower production in parental subspecies and hybrid classes. I then estimate components of fitness of parental plants, F_1 hybrids, and backcrossed individuals using biomass, fruit set, and seed set to ascertain whether there is evidence of hybrid vigor.

MATERIALS AND METHODS

Study species. *Triodanis perfoliata* subsp. *perfoliata* (hereafter "subsp. *perfoliata*") and *T. perfoliata* subsp. *biflora* (hereafter "subsp. *biflora*") are annual herbs that frequently occur on roadsides and in disturbed habitats, such as previous agricultural lands and mowed areas. The subspecies produce CH flowers that are 1-1.5 cm in diameter, with five blue to violet petals. CH flowers in both subspecies are protandrous, with anthers dehiscing before anthesis and stigmas opening one to two days later. Small bees, wasps, and beetles are common visitors to the CH flowers of both subspecies (Gara and Muenchow 1990; pers. obs.). Cleistogamous flowers of the two subspecies are produced primarily in the lower axils of long inflorescences and mature from base to apex. CH flowers are produced after CL flowers in both subspecies. In subsp. *biflora* one to two CH flowers are produced at the top of the inflorescence, while multiple CH flowers are produced along the length of the inflorescence in subsp. *perfoliata*. In both subspecies, smaller secondary CL fruits are produced later in plant development, within the same bracts as primary

CL fruits, along the inflorescence. Secondary CL fruits mature after CH fruits in subsp. *biflora* and simultaneously to CH fruit maturation in subsp. *perfoliata*. Flowering phenology of the two subspecies is partially overlapping. *Triodanis perfoliata* subsp. *biflora* flowers in early to mid-May and subsp. *perfoliata* flowers mid-May to early June. Fruits mature throughout May and June.

Sites. Four sites containing both subspecies, based upon visual inspection of morphological traits, were selected for sampling; these sites differed in their light climates. A Time for Science (ATFS), a natural area located in Grifton, Pitt Co., NC established for research and science education, was partially shaded throughout the day. Eastside Park (ESP), a natural area located in eastern Greenville, NC, was mostly in full sun throughout the day. A site located in Falkland, NC (FALK), on privately owned land, was in full sun throughout the day. Paramore Park (PARA), a city park located in southern Greenville, NC, was partially shaded (Figure 2). All sites were formerly agricultural land, except for Paramore Park, and appeared to be historically disturbed by mowing. Observation of charred trees and shrubs at Eastside Park suggested a recent fire at this location. For geographical coordinates of sites and distances between sites see Table 1.

Transects were used at each site to sample individual plants. I constructed each transect through areas where *Triodanis* was most dense and both subspecies appeared to be present. Individuals were collected at 2 m intervals without regard to subspecies designation (ESP N = 75, ATFS N = 65, PARA N = 55, FALK N = 40). At each site, ten plants of each parental subspecies were identified, based on morphological traits, and sampled. When possible, the parental plants collected were isolated from the other subspecies by spatial separation; however, plants sampled at ESP and ATFS were more distant from a zone of contact than were parental

plants at FALK and PARA. For each plant sampled, leaf tissue was collected for DNA extraction and two bracts subtending flowers were collected for morphological analysis. Mature, indehiscent fruits were collected throughout the season at ESP including: the first CL fruit (typically located at the bottom of the plant), a second CL fruit halfway up the stem of the plant, a third CL fruit at the top of the plant, a secondary CL fruit, and up to four CH fruits per plant as available. Dates of fruit collection were recorded. After senescence, all sampled plants were collected to quantify total CL and CH fruit production and aboveground biomass.

Morphological Measurements. Two bracts per plant were measured for length, width, and the length from the point of petiole attachment to the end of the proximal bract lobe (leaf auricle, hereafter referred to as "bract base") using digital calipers. Capsule pore position was measured on three cleistogamous fruits and one chasmogamous fruit per plant using a dissecting scope and ocular micrometer. Capsule pore position was calculated as the distance to lower rim of the pore divided by the total length of the fruit. Fruits were also measured for their width.

Fitness Measures. After senescence, plants were cut at ground level, allowed to dry in paper bags using a drying oven at around 80 degrees Celsius, and then weighed to the nearest 0.0001 g to determine biomass. Before bagging, the number of CH and CL flowers was counted for both the primary and lateral stems on each plant. The number of lateral stems was also recorded. Seeds that appeared viable based upon their size and shape were counted in fruits collected before dehiscence from plants at ESP. A relationship was established between fruit volume and seed set from the ESP data using linear regression and used to estimate seed set for fruits at FALK and PARA from fruit measurements. Fruit volume was calculated from length and width

(converted to diameter) measurements using the formula for the volume of an elliptical cone [$V = (\pi d^2 h) / 6$].

Genetic Markers. High rates of herbivory limited the collection of morphological and fitness data at the ATFS site, thus, genetic analysis was not performed for plants sampled there. For all remaining sampled plants, the subspecific or hybrid status was determined using data generated from AFLP (amplified fragment length polymorphism) genetic marker analysis. AFLP is a dominant genetic marker that has been used widely in studies of plant population genetics and hybridization (O'Hanlon et al. 1999, Schulte et al. 2010). Results were scored as presence-absence data for fragments of specified lengths.

Leaf tissue of plants sampled at each site was washed in SDS (sodium dodecyl sulfate) and water solution and then frozen. DNA extraction followed a modified version of the Doyle and Doyle (1987) plant DNA extraction protocol. Frozen leaf tissue was ground in liquid nitrogen using a mortar and pestle and added to a CTAB (cetyltrimethylammonium bromide) solution. A chloroform-isoamyl alcohol solution was later added to extract DNA from plant cells. The DNA was then purified in a series of ethanol precipitations.

To generate AFLP markers, DNA was digested with two restriction enzymes (Mse I and EcoRI) to produce fragments with "sticky" ends, and adaptors were ligated to the fragments to serve as primer attachment sites. Two consecutive rounds of selective PCR (polymerase chain reaction) were implemented: pre-amplification and selective amplification. During pre-amplification, primer pairs were designed to complement the previously ligated adaptors with a single selective nucleotide added to each. PCR amplified only the DNA fragments present that matched the selective nucleotide. The PCR product was then used as a template for further

selective amplification using pairs of primers with three selective nucleotides each. This further reduced the number of DNA fragments amplified. After screening of 14 selective primer pairs, three (M-CAG/ E-AGG, M-CAC/E-AGG, M-CTA/E-AGG) were chosen to yield a sufficient number of easily resolvable polymorphic loci for subspecies differentiation.

The EcoRI primer used during selective amplification was labeled with an infrared fluorescent dye that can be scanned with a laser and analyzed using an automated sequencer. We used the automated sequencer located in the core facility in the ECU Department of Biology to analyze our DNA products. Data from the sequencer were analyzed using Genemapper ® software to determine fragment lengths. A fluorescent DNA ladder with 26 evenly spaced bands ranging from 50- 625 bp was used as a size standard for fragment length. Data were then manually scored from inspection of chromatograms for the presence or absence of DNA fragments of specific lengths.

Data Analyses

Morphological Data Analysis. To test whether morphological traits differed between the two subspecies in natural populations, analysis of variance (ANOVA) was used with subspecies as a fixed effect and site as a random factor nested within subspecies. Data were transformed to meet assumptions of the ANOVA model, using the arcsine square-root for capsule pore position, and using the logarithmic function for bract base, bract ratio, and CH/CL flower ratio. Transformed variables were checked for homogeneity of variance using nonparametric Levene's test.

To further examine morphological differences between subspecies, principle component analysis (PCA) was used with the diagnostic traits (bract base, bract length to width ratio,

CH/CL flower ratio, and capsule pore position) of parental subspecies individuals from all sites combined. To visualize the distribution of morphological variation along the transects and evidence for hybridization, a separate PCA was performed using diagnostic traits of parental subspecies and transect plants from all sites combined.

Genetic Data Analysis. Principle coordinate analysis (PCoA) examined genetic differences between subspecies and transect individuals. Population genetics software STRUCTURE v. 2.3.4 was used to assign membership of individuals to their most probable hybrid or subspecies class based on AFLP data (Pritchard et al. 2000). The program uses a Bayesian approach based on Markov chain Monte Carol (MCMC) to define clusters of individuals based on their genotypes at multiple loci. The number of populations (K) was set at two to account for the two parental subspecies. Each run comprised a 50,000 iteration burn-in, followed by 50,000 sampled iterations. We implemented the admixture model as well as the correlated allele frequencies model. Two Q values were assigned to each individual, corresponding to genetic contributions from the two subspecies.

The population genetic structure of parental subspecies was further analyzed using AFLP genotypes and GenAlEx ver. 6.5 software (Peakall and Smouse 2006, 2012). Six populations, comprising populations of two subspecies at each of three sites, were split into two groups, with three populations nested within each of the two subspecies. A Nei genetic distance (Nei 1972, 1978) matrix was formed to evaluate the genetic differentiation between subspecies and among populations of each subspecies. Analysis of molecular variance (AMOVA) was used to calculate the proportion of genetic variation that was found between subspecies, among populations of each subspecies and within populations.

Analysis of morphological v. genetic data. To determine if there was congruence between morphological and genetic data, Spearman's nonparametric correlation was used to compare the relationship between Q values, the genetic indicator of subspecies or hybrid status generated by STRUCTURE, and morphological traits. The relationship between Q values and the first principal component (PC1) from the overall morphological analysis was also tested.

Fitness data analysis. Kruskal-Wallis nonparametric one-way ANOVA was used to compare differences in biomass, flower production and ratios, and seed production and ratios between the subspecies at ESP. Kruskal-Wallis tests were also used to compare biomass, flower production and ratios, and estimates of seed production and ratios among subspecies, F_1 hybrids, and backcrossed individuals across all sites. SPSS, version 19 (SPSS., Armonk, NY) was used for all morphological and fitness data analyses.

RESULTS

Impact of Herbivory. Some of the morphological traits and fitness data could not be recorded for some individuals at each site because of herbivory. Herbivory was most severe at ATFS, affecting 44.6% of transect plants, but also affected sample sizes at PARA (16.3%), ESP (14.7%), and FALK (2.5%). Deer were observed grazing at each site throughout the study and were likely responsible for removal of the top portion of a large number of study plants. *Triodanis perfoliata* subsp. *perfoliata* individuals suffered a greater amount of herbivory than subsp. *biflora*, which may have been due to the tree line habitat preference of subsp. *perfoliata* plants and the preference of deer to browse near forest edges. Transect plants at ATFS were located along a water-filled ditch. Plants near the ditch also experienced severe herbivory from snails (pers. obs.). Snails consumed the fruits and flowers of transect plants leaving only a bare stem.

Subspecies Morphology. Analysis of variance confirmed that diagnostic morphological traits differed significantly between the two subspecies. There was no significant difference in means of morphological traits among sites within species. The interaction between subspecies designation and site was significant for each morphological trait, except for capsule pore position (Table 2). Compared to subsp. *biflora*, subsp. *perfoliata* plants were found to have pores significantly closer to the proximal end of the capsule (lower capsule pore position value), significantly lower bract length to width ratio, significantly higher CH/ CL flower ratios, and significantly higher bract base length (Table 3). Principle component analysis of morphological traits in parental plants revealed two distinct morphological types (Figure 3). The first

component (PC1) explained 76.79% of the total variance in the data and was strongly correlated with each of the morphological variables (bract ratio: 0.95, capsule pore location: 0.94, bract base: -0.87, CH/CL ratio: -0.73). PC2 explained an additional 15.33% of the total variance and was strongly correlated with only the CH/CL ratio (0.67).

Morphology and Hybridization. Analysis of both parental and transect plants revealed that many transect plants at each site were similar in morphology to subsp. *biflora* and subsp. *perfoliata* while some showed intermediate morphology, suggesting hybrid status (Figure 4). This pattern is also evident in a plotting of the frequency distributions of PC1 values for transect and parental plants (Figure 5). In PCA, the first component (PC1) explained 76.1% of the total variance in the data and PC2 explained an additional 13.6% of the total variance. Plotting of PC1 against plant's location along the transect (Figure 6) showed that individuals with intermediate morphology as well as individuals with subsp. *biflora* and subsp. *perfoliata* characteristics were somewhat spatially mixed at FALK and PARA, but very little spatial mixing was observed at ATFS and ESP.

AFLP and Hybridization. Among 229 individuals, a total of 209 fragments, ranging in size from 50-500 bp, were amplified using three primer pairs. The proportion of polymorphic loci was 64.59% (Table 4). From the total 209 loci, 69 were hand-scored from inspection of chromatograms for presence or absence of a fragment in each individual. The fragments that were not scored were either present in all samples or gave ambiguous results. An average of 24 replicates per primer pair was included in the analysis, and results for these were found to be 100% consistent between replicates. Of the 69 loci that were scored, two were fixed present in one subspecies and fixed absent in the other, 21 were fixed in subsp. *perfoliata* and polymorphic

in subsp. *biflora*, and 15 were fixed in subsp. *biflora* and polymorphic in subsp. *perfoliata*. The remaining 31 loci were polymorphic in both subspecies.

Analysis of molecular variance revealed that, of the total genetic variation present, 65% was between the subspecies, 5% was among populations within subspecies, and 30% was within subspecies within sites. Comparison of pairwise Nei's genetic distance (Nei 1972) between populations revealed that mean differentiation of subsp. *biflora* populations ($\overline{D}$ = 0.079) was over two times greater than mean differentiation in subsp. *perfoliata* ($\overline{D}$ = 0.038) (Table 5).

Principle coordinate analysis of genotype data yielded results comparable to those of the morphological data. Plotting of PC1 frequencies against PC2 frequencies revealed transect plants that were genetically similar to both subspecies as well as individuals with intermediate genetic composition at each site (Figure 6). The first component (PC1) explained 62.1% of the total variance in the data while the second component (PC2) explained an additional 14.9% of the total variance.

A visual inspection of Q plots generated by STRUCTURE analysis revealed that a few plants previously identified by morphological traits as subsp. *biflora* and subsp. *perfoliata* were likely to be hybrids (Figure 8). These individuals were removed from all analyses (morphological and genetic) as subspecies indicators. Subspecies and hybrid classification was performed using midpoint cutoffs between theoretical Q values for F_1 hybrids (Q = 0.5) and backcrosses (Q = 0.75 for either subspecies). Individuals with Q values between 37.5 and 62.5 were assigned F_1 hybrid status. Individuals with Q values between 62.5 and 93.75 were designated as backcrosses to that subspecies. Classification of backcrossed individuals included both backcrossed individuals (expected Q of 75) and second generation backcrosses (expected Q values of 87.5). Individuals with Q values between 93.75 and 100 were identified as pure

subspecies. Of the 255 plants sampled, STRUCTURE identified a total of nine F_1 hybrids (six at PARA, two at ESP, one at FALK), two backcrosses to *biflora* (one at ESP, one at PARA), and 9 backcrosses to *perfoliata* (five at PARA, three at FALK, one at ESP) (Figure 9). There were three individuals (one at ESP and two at PARA) with mixed ancestry that slightly exceeded the estimated proportion of ancestry conditions for backcrosses to subsp. *perfoliata*; these individuals were classified as pure subsp. *perfoliata* although they might have been later generation backcrosses.

Congruence of Genetic and Morphological Data. Spearman's rank correlation nonparametric analyses revealed that morphological traits were highly congruent with molecular data. The Q values (estimated proportions of ancestry) from STRUCTURE analysis were strongly correlated with bract base (ρ (228) = 0.649, $P < 0.001$; bract ratio: ρ (228) = -0.747, $P < 0.001$; CH/CL ratio: ρ (228) = 0.750, $P < 0.001$; capsule pore location: ρ (219) = -0.723, $P < 0.001$ and with PC1 from the PCA of morphological data (ρ (198) = -0.707, $P < 0.001$). Individuals with intermediate Q values had intermediate values for each morphological trait; pore position was chosen to depict this relationship since it was collected for all individuals sampled (Figure 10).

Hybrid Fitness. Kruskal-Wallis nonparametric analysis of fruit production and seed set in subsp. *biflora* and subsp. *perfoliata* individuals from ESP revealed that subspecies differed significantly in their flower and seed production. *Triodanis perfoliata* subsp. *perfoliata* produced a greater number of CH flowers ($P = 0.007$), a higher CH/CL flower ratio ($P < 0.001$), a greater number of seeds from CH fruits ($P = 0.038$), and a greater proportion of seeds from CH fruits (P

< 0.001) when compared to subsp. *biflora*. *Triodanis perfoliata* subsp. *biflora* produced a greater number of CL flowers ($P = 0.004$) and a greater number of seeds from CL fruits ($P = 0.005$) when compared to subsp. *perfoliata*. Subspecies did not significantly differ in their total seed production (Table 6).

Linear regression revealed a significant and strong relationship between fruit volume and seed set (r (484) = 0.79, $P < 0.001$). Regression coefficients were used in conjunction with the linear equation [number of seeds per fruit = 0.009 (fruit volume) + 42.681] to estimate seed set from fruit size data for individuals at FALK and PARA. Although there was a strong correlation between fruit volume and seed set at ESP, this relationship may somewhat differ at other sites.

Nonparametric analyses found evidence for increased fitness in F_1 hybrid individuals. F_1 hybrids had a significantly higher fruit production ($P = 0.017$) and significantly higher biomass ($P = 0.008$) than subspecies and backcrossed individuals. Variation in seed number among subspecies and hybrids was not significant ($P = 0.199$) but the trend was toward higher seed production in hybrids (Table 7, Figure 11). F_1 hybrids also produced a number of CH fruits similar to subsp. *perfoliata* (Figure 12).

DISCUSSION

Hybridization. Hybridization between *Triodanis perfoliata* subsp. *perfoliata* and *T. perfoliata* subsp. *biflora* in local zones of co-occurrence was detected in this study with the use of AFLP molecular markers. PCoA of genetic data identified individuals in each contact zone that were intermediate to the parental taxa (Figure 7). These intermediate individuals were confirmed to be hybrid individuals based on the proportion of ancestry from each parental taxa estimated using STRUCTURE analysis. Hybridization has been reported between subsp. *perfoliata* and subsp. *biflora* based on anecdotal observations of morphology (Radford et al. 1968, Bradley 1975) but the extent of hybridization between the sister subspecies has not been previously quantified. In a greenhouse study of Triodanis, an increased difference in morphological traits between the subspecies in offspring of CL fruits compared to offspring of CH fruits provided further evidence for hybridization (Goodwillie and Stewart 2013). In the current study 9.47% of all transect individuals were estimated to be hybrids based on STRUCTURE analysis and varied among the study sites. This rate of hybridization was lower than expected given the spatial mixing of the subspecies and previous reports of robust hybrids (Bradley 1975). The hybridization rate estimated in the current study is intermediate to those reported in similar studies. Many studies have found evidence for very low rates of hybridization, typically less than 1% (Marshall and Abbot 1980, Vickery 1990, Hodges et al. 1996). There have also been reports of hybridization rates similar to and greater than rates estimated by the current study, 7.5% in *Helianthus* (Rieseberg et al. 1998), 14% in *Eucalyptus* (Field et al. 2011), and 31.7% *Quercus* (Bacilieri et al. 1996).

Sites with more equivalent Triodanis subsp. biflora and subsp. perfoliata abundance were found to have a higher degree of hybridization as has been reported in other studies (Burgess et al. 2005, Martin and Willis 2007) (Figure 9). The site with the most even abundance of parental plants (PARA) also had the highest frequency of hybrids, 16.4%, in transect plants.

Morphological data generally agreed with results from the molecular data. All of the morphological traits were significantly and highly correlated with values indicating proportion of genetic ancestry (Q values). In particular, intermediate capsule pore position values for F_1 hybrid individuals were distinguished from parental taxa with little overlap (Figure 10). This indicates that capsule pore location may be used as a hybrid identifier in future studies. Although bract ratio was more highly correlated with Q values, it is not as useful a hybrid indicator because values for hybrids were somewhat overlapping with those of the parental subspecies. Due to its dominant inheritance (Bradley 1975), CH/CL ratio was least effective when distinguishing F_1 hybrids from subsp. *perfoliata*. CH/CL ratio may also show phenotypic plasticity. Although the presence of cleistogamy is obligate in both subspecies, the ratio of chasmogamy to cleistogamy may be affected by environmental conditions. Other studies have examined the presence of facultative cleistogamy as a phenotypically plastic trait. In *Bromus carinatus* CL flowers are produced only under drought conditions (Harlan 1945). *Lamium amplexicaule* which typically produces CL flowers produces CH flowers during long, warm spring and summer days (Lord 1982).

Components of Reproductive Isolation.

Postzygotic Isolation. Sterility (Mayr 1942, Wagner 1970) and reduced viability in hybrid offspring is common (Stebbins 1958, Dobzhansky 1970). These components of postzygotic

isolation have generally been attributed to the expression of incompatibilities between epistatic genes that have differentiated in parental genomes (Dobzhansky 1936, Muller and Pontecorvo 1940). Likewise, genomes that have not significantly differentiated may produce hybrids with high fitness. Hybrids with fitness that exceeds the parents (heterosis) is often seen in closely related species (Grant 1975) and may be due to dominance, overdominance, or epistasis (Mitchell-Olds 1995).

Results of a previous greenhouse study revealed that subsp. *perfoliata* and subsp. *biflora* could produce viable hybrid offspring (Bradley 1975) when hand-crossed. The current study is the first to measure and compare fitness in Triodanis subspecies and hybrid plants located in their natural habitat. Results of this study suggest that F_1 hybrids have increased fitness, or hybrid vigor, when compared to subsp. *perfoliata* and subsp. *biflora* (Figure 10, Table 6). Hybrid vigor has been recorded in studies of other plant species (Arnold and Hodges 1995, Graham et al. 1995, Emms and Arnold 1997), especially when F_1 hybrids are located in novel environments (Wang et al. 1997). However, there were indications of reduced fitness in backcrossed *Triodanis* subsp. individuals (Figure 11). This reduced fitness may be due to F_2 hybrid breakdown, which occurs when newly formed recombinant genotypes arise with negative interactions between loci (Fenster et al. 1997). Note that variation in fitness traits was generally much greater in backcrosses than in F_1s (Figure 11), also perhaps reflecting segregation of genes. Postzygotic isolation due to abortion of ovules fertilized with heterospecific pollen may have occurred, but was not studied. Several other components of fitness such as pollen production, germination, and seedling survival were not measured in the current study. These components should be examined for a more complete estimate of hybrid fitness. However, preliminary results from a greenhouse study have indicated that F_1 hybrids produce viable pollen (56,080 pollen grains per flower) that

is intermediate in number to that of the subspecies (subsp. *perfoliata* 64,886 pollen grains per flower, subsp. *biflora* 38725 pollen grains per flower).

Prezygotic Isolation. Despite lack of evidence for postzygotic isolation, rates of hybridization are lower than expected and the subspecies remain genetically and morphologically distinct (Figure 7). Habitat isolation between the subspecies is likely limiting the amount of hybridization. *Triodanis perfoliata* subsp. *perfoliata* is typically found in shaded areas while subsp. *biflora* is found in open, sunny locations, although the subspecies are somewhat intermixed. Light availability has been reported to increase up to 50 fold in sunny areas when compared to shaded areas (Galloway 2005). In heterogeneous environments with both shaded and unshaded areas pollinators have been shown to prefer unshaded areas due to higher temperatures in sunny areas (Herrera 1995, Comba 1999, Liow et al. 2001, Totland 2001). Kilkenny & Galloway (2008) reported a seven fold increase in pollinator visitation to *Campanulastrum americanum* located in sunny areas versus plants located in the shade. Primary pollinators of *C. americanum* were reported to be bumblebees (*Bombus*) and halictids (Galloway et al. 2002). These pollinators are similar to pollinators observed in *Triodanis* (personal observation). At ESP where separation between the subspecies was most apparent due to habitat preference, the least amount of hybridization was found. Pollinator visitation may have been reduced between subspecies as subsp. *perfoliata* was located in highly shaded locations. Conversely a larger number of hybrids were found at PARA where shading of the entire transect (including parental individuals) occurred at times during the day (Figure 9). A greater extent of hybridization at PARA may have been due to a more evenly shaded environment, increasing pollinator movement between the subspecies.

Potential for hybridization has been reported to be limited by differing chasmogamous flowering phenology (Waser 1983, Armbruster and Herzig 1984, McGuire and Armbruster 1991, Johnston 1998, Leebens-Mack and Milligan 1998). Chasmogamous flowering time of subsp. *perfoliata* sometimes occurred after that of subsp. *biflora*. This was evident at ESP where CH flowering time of subsp. *perfoliata* occurred a week after CH flowering in subsp. *biflora*. At sites where parental habitats differed less so than ESP, such as PARA and FALK, CH flowering time overlapped somewhat. Chasmogamous flowers that were pollinated with both conspecific and heterospecific pollen may have experienced further prezygotic isolation due to conspecific pollen precedence, the siring advantage of conspecific pollen over heterospecific pollen, especially in more highly chasmogamous subsp. *perfoliata* (Diaz and Macnair 1999).

Cleistogamy. Although both hybridization and self-fertilization in plants have been well studied, the effects of an autogamous mating system on rates of hybridization have received relatively little consideration. Flowers receiving self pollen before the opportunity for receipt of heterospecific reduce the potential for hybridization. Floral traits associated with selfing species, such as smaller floral displays (Goodwillie et al. 2010) and low pollen production (Cruden 1977), may reduce pollinator attraction to selfing species decreasing the potential for hybridization. Although Coyne and Orr (2004) argue that selfing is not a true form of reproductive isolation because the reduction in gene flow is equal both within and among taxa, selfing remains an impeding mechanism to overall gene flow (Martin and Willis 2007). When fertilization of an outcrosser's ovules with pollen from a mostly selfing species is considered, selfing is indeed a substantial barrier to interspecific gene flow. Implications of self-fertilization for reproductive isolation have been considered in hybridizing *Leptosiphon jepsonii* and *L.*

androsaceus (Goodwillie and Ness 2013) and *Mimulus nasutus* and *M. guttatus* (Fishman and Willis 2001, Martin and Willis 2007). In both sets of species, reproductive isolation due to selfing greatly reduced the potential for hybridization.

The effects of obligate selfing, through cleistogamy, on reproductive isolation have not been well studied. As suggested by Bradley (1975), in *Triodanis perfoliata*, the presence of obligately selfing CL flowers is likely to contribute greatly to prezygotic isolation by reducing the potential for hybridization between the subspecies. In subsp. *biflora* only 7% of seeds were produced by CH flowers and 60% of seeds produced by subsp. *perfoliata* were from CH flowers (Table 7).

F_1 Bridge. The observed asymmetry in cleistogamy may have implications for the direction of gene flow between the subspecies. Many studies on prezygotic barriers to hybridization have reported evidence of asymmetry in reproductive isolation (Smith 1968, Carney et al. 1994, 1996, Emms et al. 1996, Rieseberg et al. 1995, Hodges et al. 1996). Asymmetry in hybridization has also been reported due to differences in plant mating systems. Martin and Willis (2007) reported asymmetry in hybridization between the two closely related species, *Mimulus guttatus* (largely outcrossing) and *M. nasutus* (largely selfing), with rates of introgression higher in the outcrossing species. Differing allocation to chasmogamy between the subsp. *perfoliata* and subsp. *biflora* is likely to have important implications for the direction of hybridization and introgression. Hybridization between the subspecies is limited in both directions by the very few CH pollen and ovules available in subsp. *biflora* (Figure 13). Inheritance of CH/CL flower ratio is a dominant trait (Bradley 1975); therefore F_1 hybrids have a high CH/CL flower ratio that similar to subsp. *perfoliata* (Figure 12). Backcross formation with subsp. *biflora* is limited by

few pollen and ovules available in subsp. *biflora*, however backcross formation with subsp. *perfoliata* is likely because of the large number of pollen and ovules available (Figure 13). This hypothesis was supported by the results of estimated proportions of ancestry reported by STRUCTURE. Backcrosses to subsp. *perfoliata* were considerably more common than were backcrosses to as subsp. *biflora*, and also more common than F_1 hybrids. This directional component of hybridization is expected to promote introgression from subsp. *biflora* into *perfoliata*. According to Mayr (1963), introgression should generally weaken isolation between the subspecies. However, if introgression from subsp. *biflora* to subsp. *perfoliata* reduces the amount of chasmogamy in subsp. *perfoliata*, rates of hybridization could be further reduced, causing an increase in divergence between the subspecies.

Life History and Implications for Genetic Structure. Though comparisons between the subspecies have been discussed largely based on CH/CL flower ratio thus far, there are several other important life history differences between the subspecies. In both subspecies, the majority of primary CL flowers are produced prior to CH flower production. Secondary CL flowers, a third flower type, are produced later in plant development. Secondary CL flowers are produced after CH flowers in subsp. *biflora* and simultaneously to CH flower production in subsp. *perfoliata*. Fruits formed by these secondary flowers are generally smaller than primary cleistogamous fruits and are much smaller in subsp. *perfoliata* than in subsp. *biflora*. On average subsp. *biflora* produced 21 secondary fruits on its primary stem and 12 secondary fruits on its lateral stems, while subsp. *perfoliata* produced 4 secondary fruits on its primary stem and less than one secondary fruit on each lateral stem. Secondary fruits contributed 34.45% of total fruit set in subsp. *biflora* and 16.11% of total fruit set in subsp. *perfoliata*. F_1 hybrids produced a

mean of 33 secondary fruits on the main stem and 44 secondary fruits on lateral stems, with 55.14% of total fruit set produced by secondary fruits. Thus, secondary fruits produced just before senescence contribute a substantial amount to fitness, particularly in subsp. *biflora* (Table 7).

Cleistogamy and selfing appear to play a role in the population genetic structure of the subspecies. Selfing reduces effective population sizes, recombination rates, and heterozygosity causing an increase in isolation between both individuals and populations; therefore highly selfing species are expected to diverge more than species with high outcrossing rates (Wright 1951, 1965, Schoen and Brown 1991, Charlesworth 2003). Our results were congruent with this idea; genetic diversity in subsp. *biflora* was over two times greater than genetic diversity in subsp. *perfoliata* (Table 5). Similar results were found in populations of *Arabidopsis lyrata* that vary for their strength of self- incompatibility and realized outcrossing rates; genetic diversity of predominantly self- incompatible populations was over two times greater than that of selfing populations of *A. lyrata* (Mable and Adam 2007).

Broader Implications. The extent of hybridization between *Triodanis perfoliata* subsp. *perfoliata* and subsp. *biflora* measured in this study was considerably high compared to rates recorded in other hybrid studies (Rieseberg and Carney 1998). Since co-occurrence appears to be common, our study was likely to be representative of the broader patterns of gene flow between the subspecies. In an informal survey of local sites, populations of the subspecies were found to occur frequently together; of the total 20 *Triodanis perfoliata* subsp. *perfoliata* populations found in Pitt County, NC, 75% occurred with *biflora*, and of the 19 total *Triodanis perfoliata* subsp. *biflora* populations found in Pitt County, NC, 79% occurred with *perfoliata* (personal

observation). Radford, Ahles, and Bell (1968) also reported in their manual of vascular flora of the Carolinas that the subspecies are found to commonly co-occur in many locations. Although this manual describes flora of the Carolinas it is conceivable that this observation is applicable across much of the overlapping range of the subspecies.

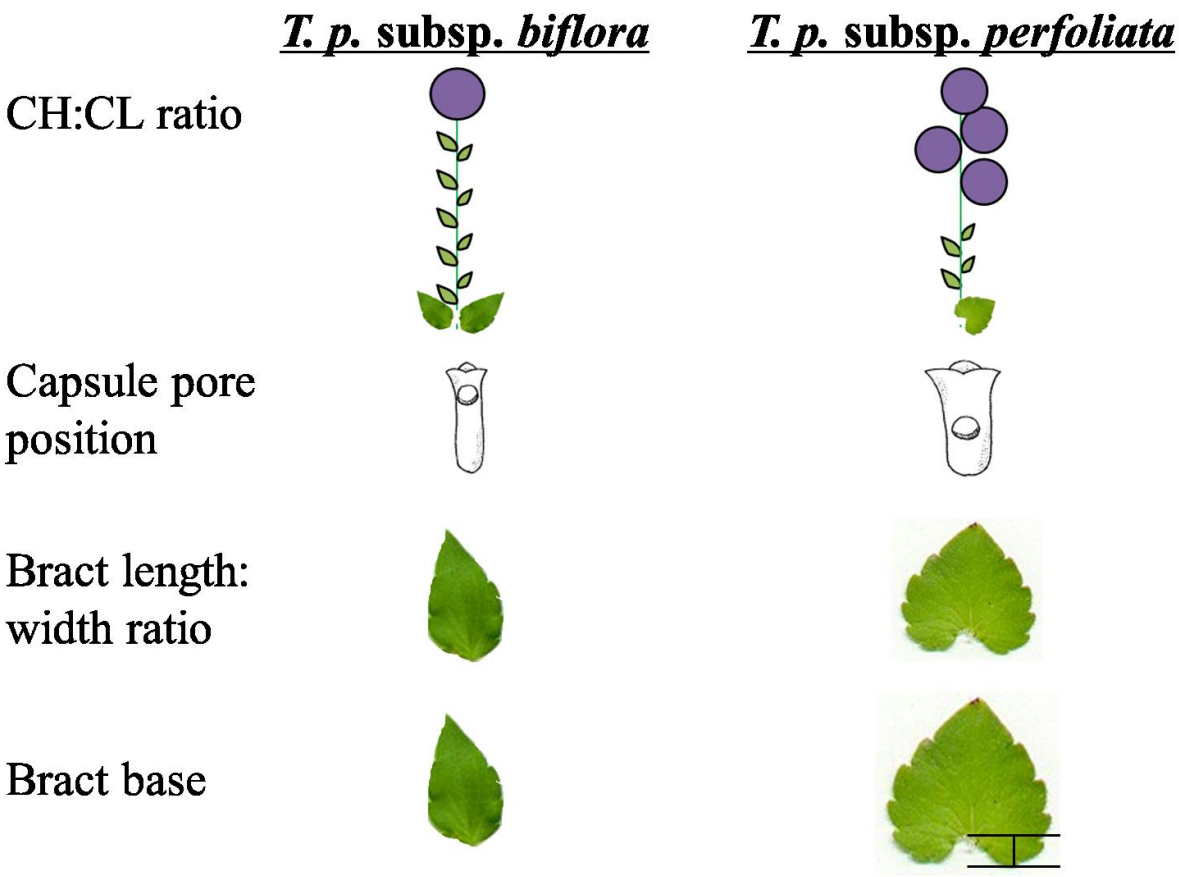

Figure 1. Morphological differences between *Triodanis perfoliata* subsp. *biflora* and subsp. *perfoliata*.

Figure 2. Satellite images (Google Earth 2013) of the four sites samples for *Triodanis*. Orange line represents approximate location of transect. Orange circles represent approximate location of sampled parental individuals. Note: Only one parental population shown for ATFS due to distance from transect.

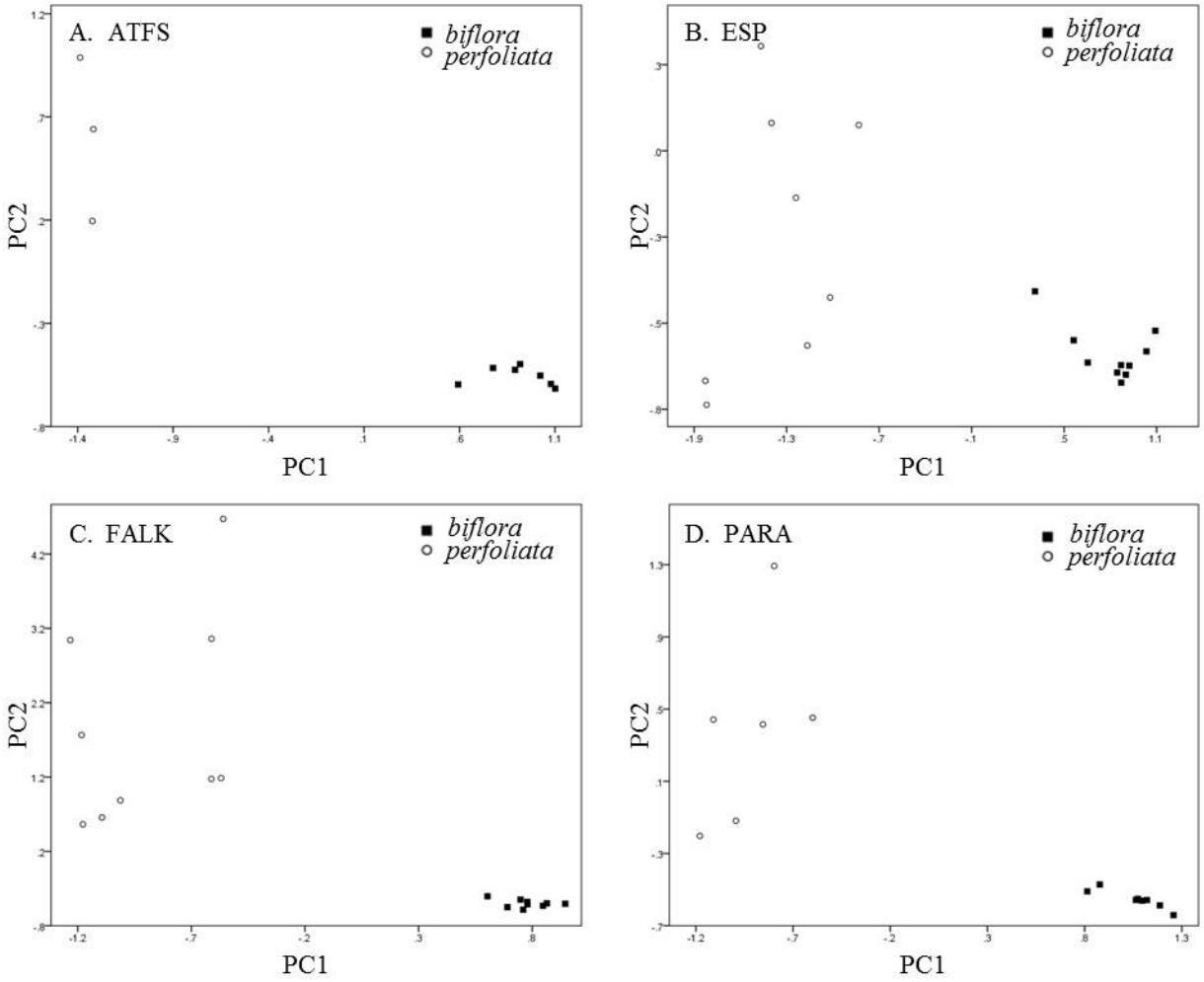

Figure 3. Scatterplot of principle component scores calculated using morphological data for parental *Triodanis* at all sites (transect individuals not used in PCA). Data plotted separately for each site.

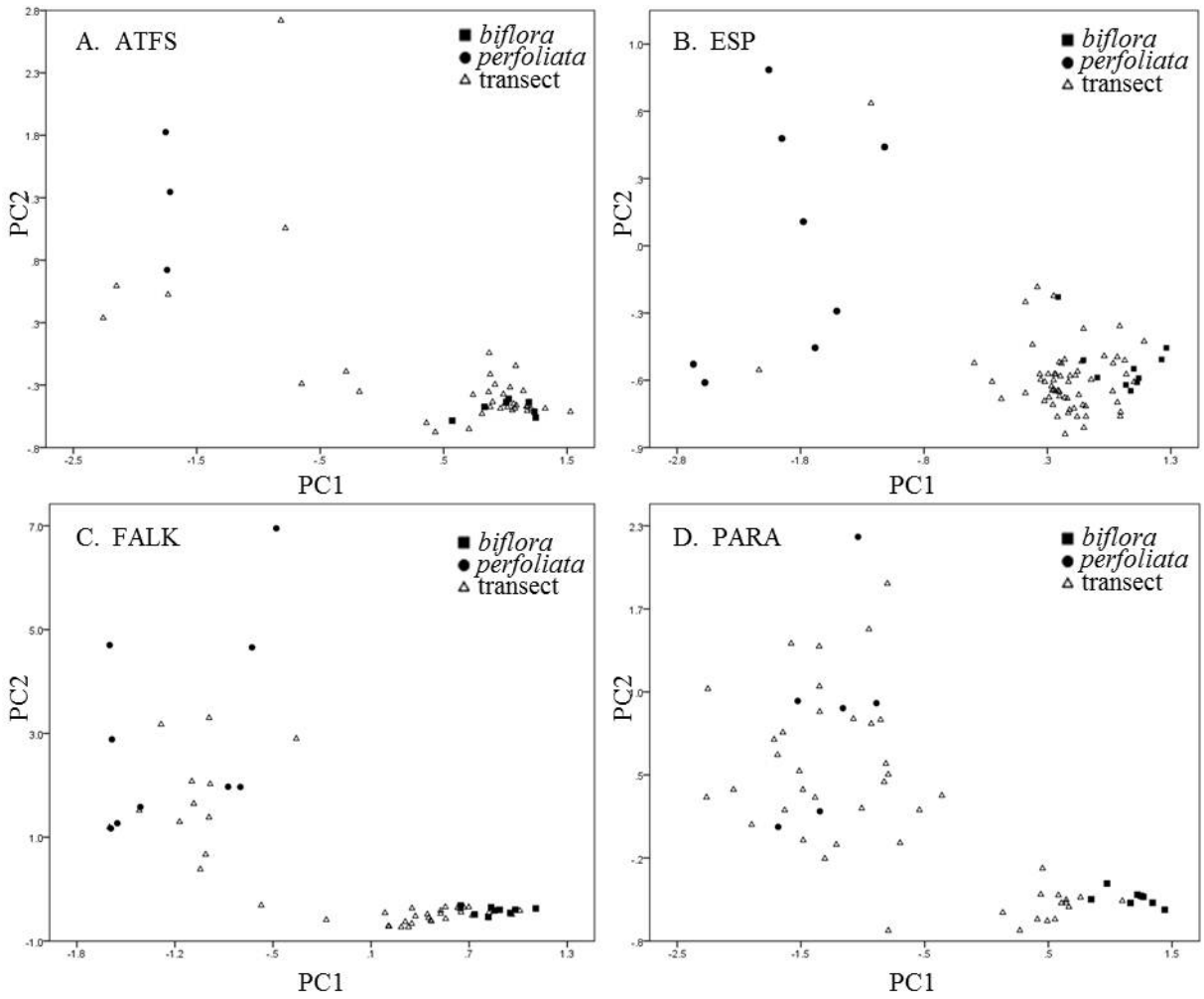

Figure 4. Scatterplot of principle component scores calculated using morphological data for parental and transect individuals. Data plotted separately for each site.

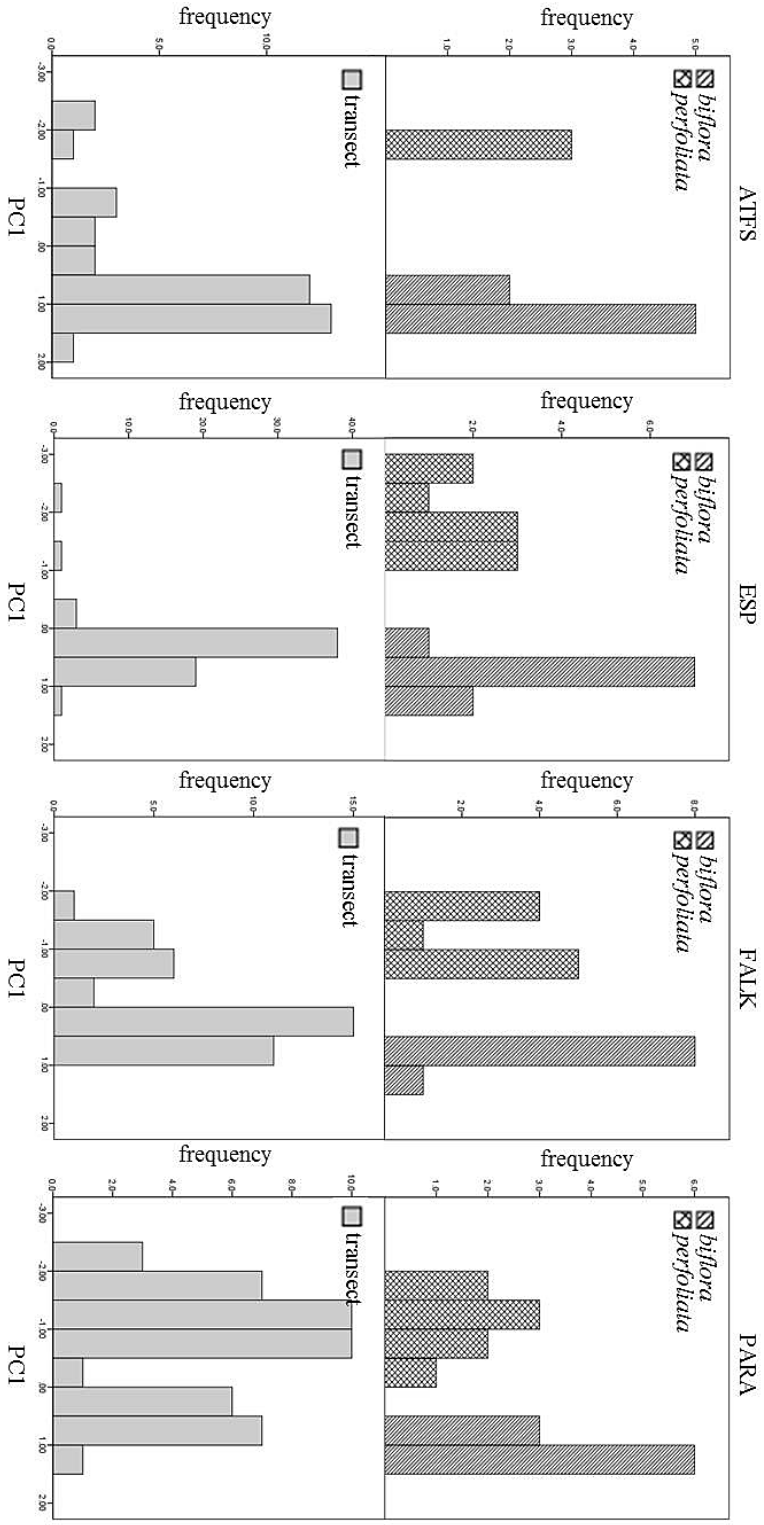

Figure 5. Distribution of parental PC1 scores for each site located on top row. Distribution of PC1 scores for transect individuals at each site located on bottom row.

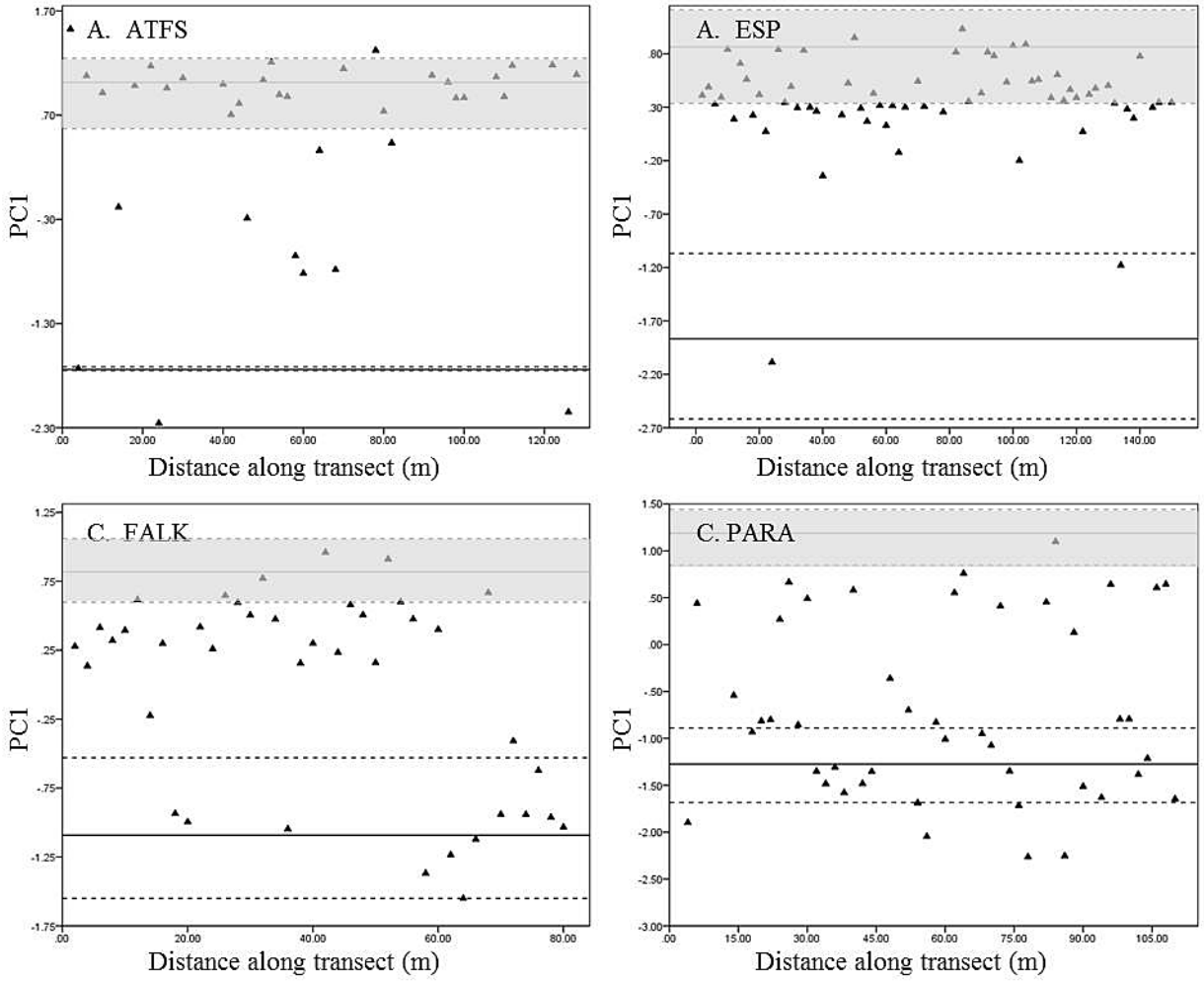

Figure 6. Spatial distribution of individuals collected along transects in hybrid zones. PC1 scores were calculated for morphological traits of parental and transect individuals from each site simultaneously. Gray solid lines indicate PC1 means for *T. perfoliata* subsp. *biflora*. Black solid lines indicate PC1 means for *T. perfoliata* subsp. *perfoliata*. Gray dashed lines indicate minimum and maximum values for PC1 scores in subsp. *biflora*. Black dashed lines indicate minimum and maximum values for PC1 scores in subsp. *perfoliata*. Individuals were collected at 2 m intervals without regard to subspecies identity; ATFS (N = 65), ESP (N = 75), FALK (N = 40), PARA (N = 55).

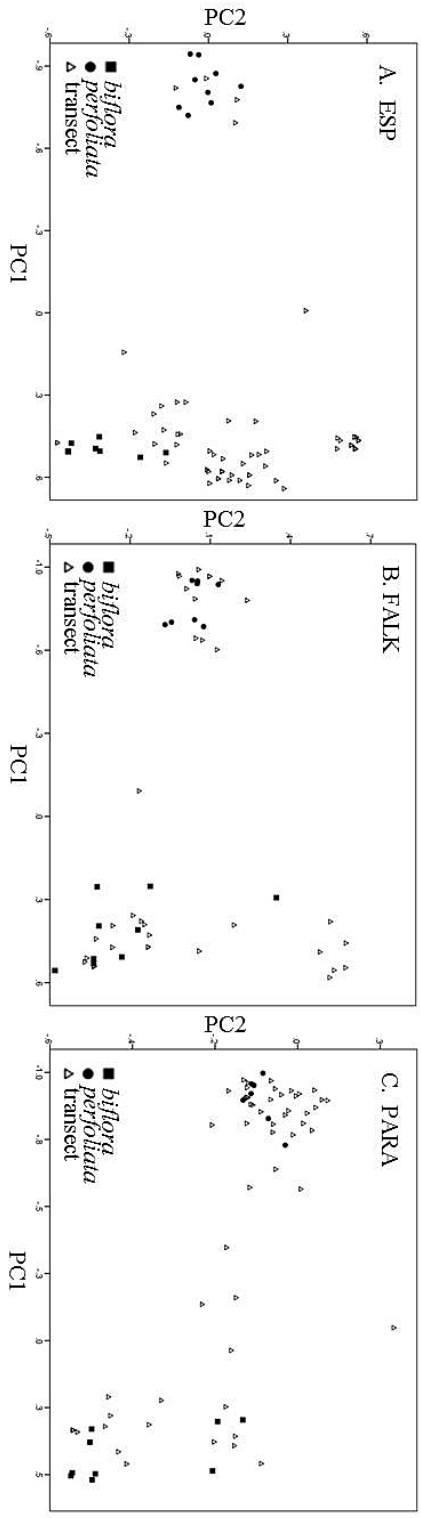

Figure 7. Scatterplot of principle coordinate scores calculated using AFLP genetic marker data for parental and transect individuals. Data plotted separately for each site.

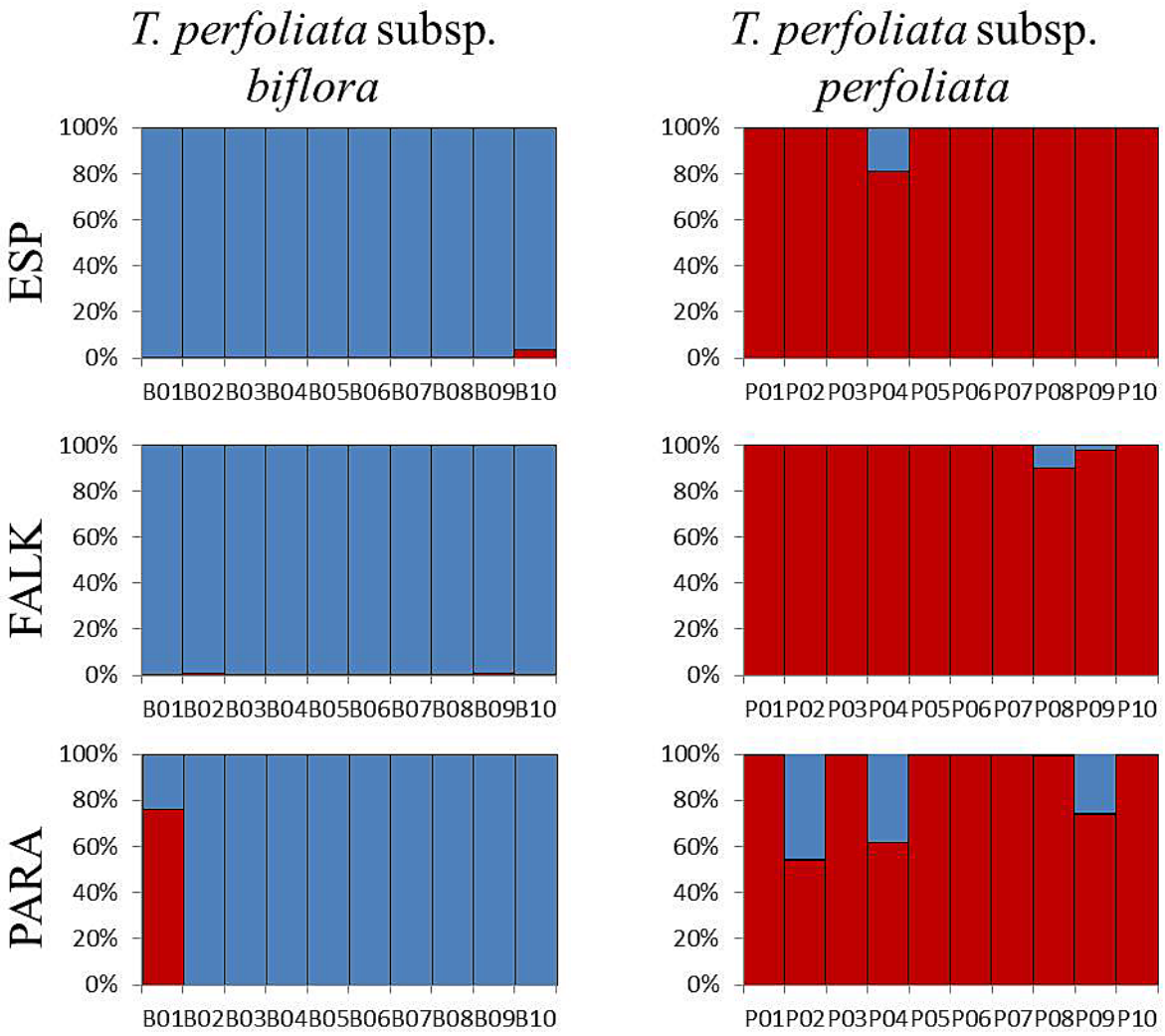

Figure 8. Estimated proportion of ancestry (Q value) generated using population genetics software STRUCTURE. Q values were calculated using AFLP genetic marker data for individuals from each site identified as parental from morphological data. Blue indicates ancestry from *T. perfoliata* subsp. *biflora*, red from subsp. *perfoliata*.

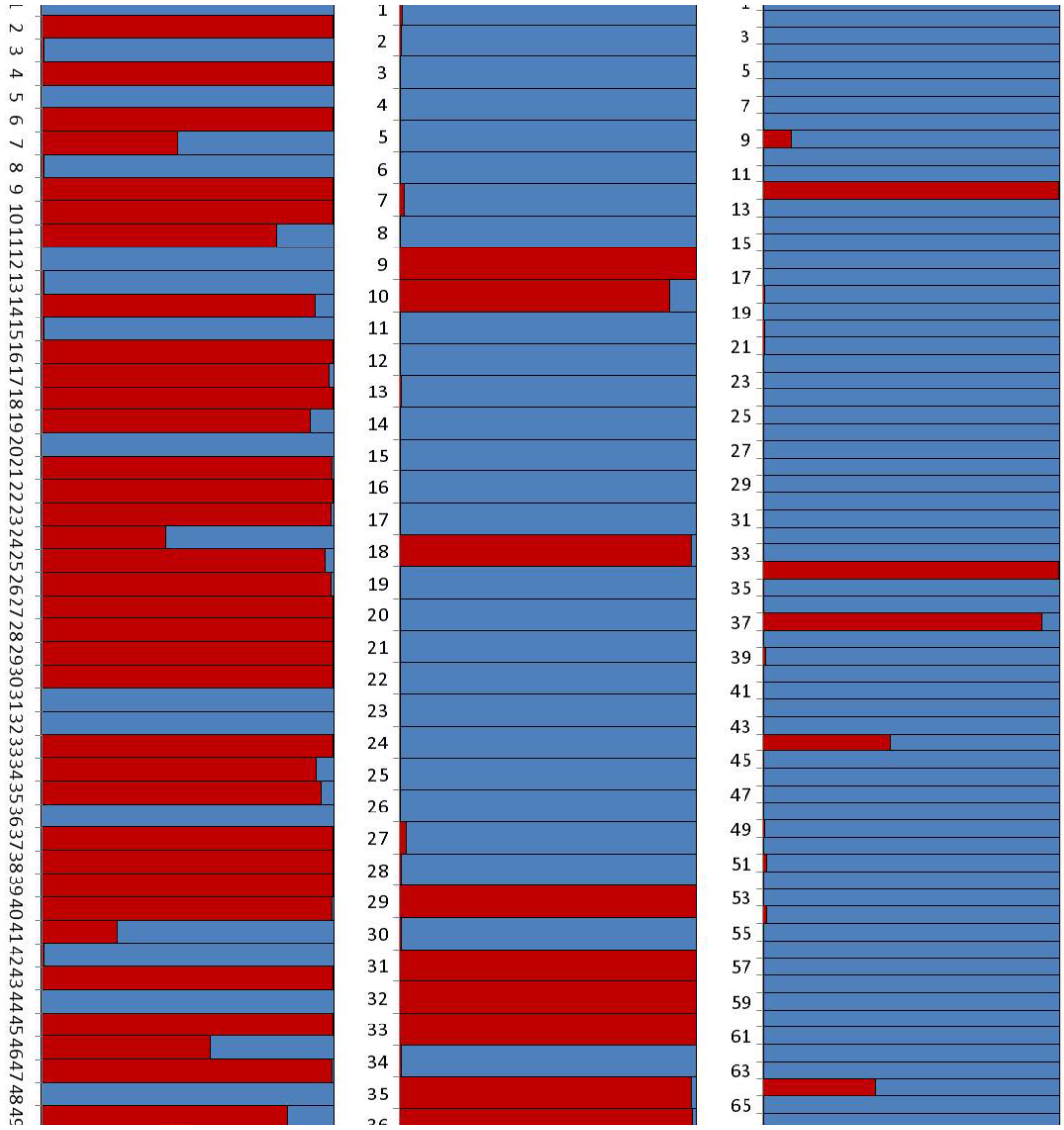

Figure 9. Estimated proportion of ancestry (Q value) generated using population genetics software STRUCTURE. Q values were calculated using AFLP genetic marker data for transect individuals from each site. Blue indicates ancestry from *T. perfoliata* subsp. *biflora*, red from subsp. *perfoliata*.

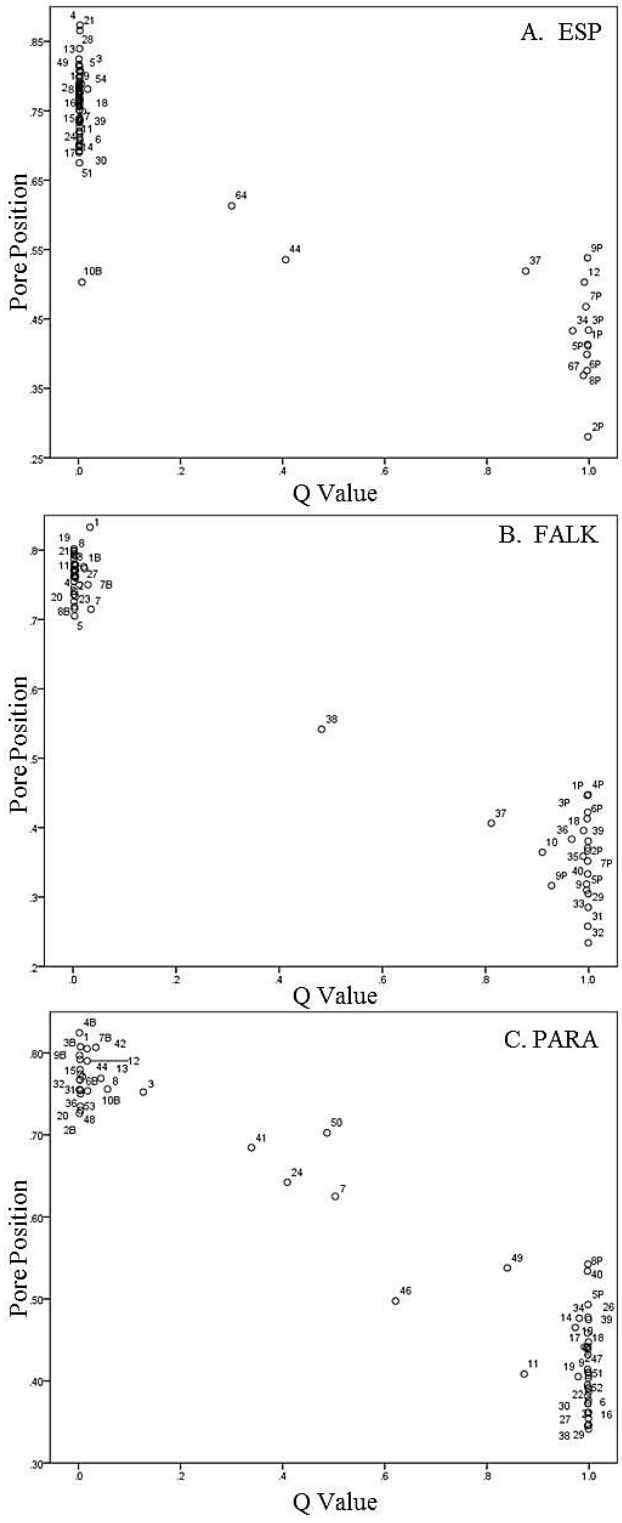

Figure 10. Scatterplot of Q values versus pore position for parental and transect individuals at ESP, FALK, and PARA**.**

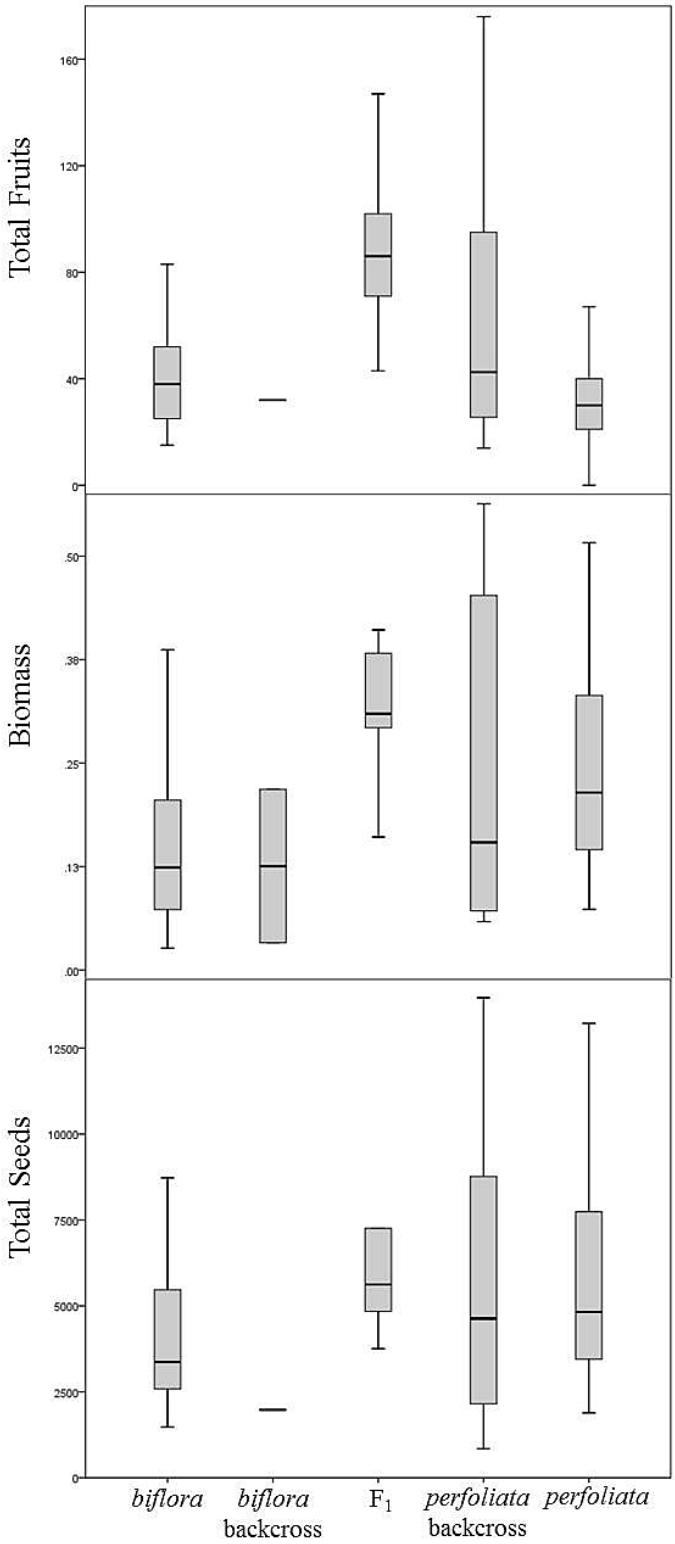

Figure 11. Bars indicate minimum and maximum observations. Boxes indicate the lower quartile, median, and upper quartile.

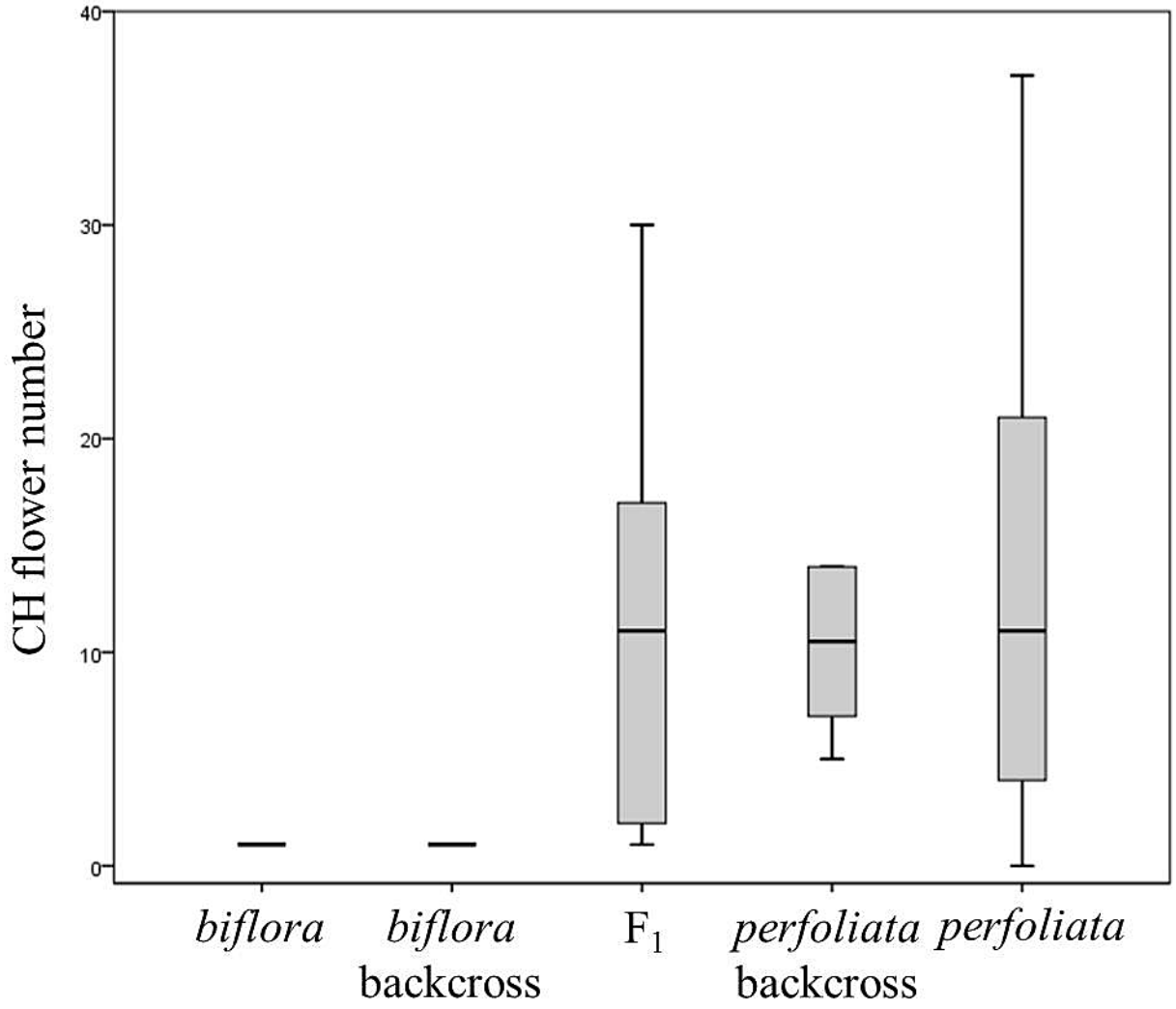

Figure 12. Comparisons between subspecies, hybrid, and backcrossed individuals identified by STRUCTURE. Bars indicate minimum and maximum observations. Boxes indicate the lower quartile, median, and upper quartile.

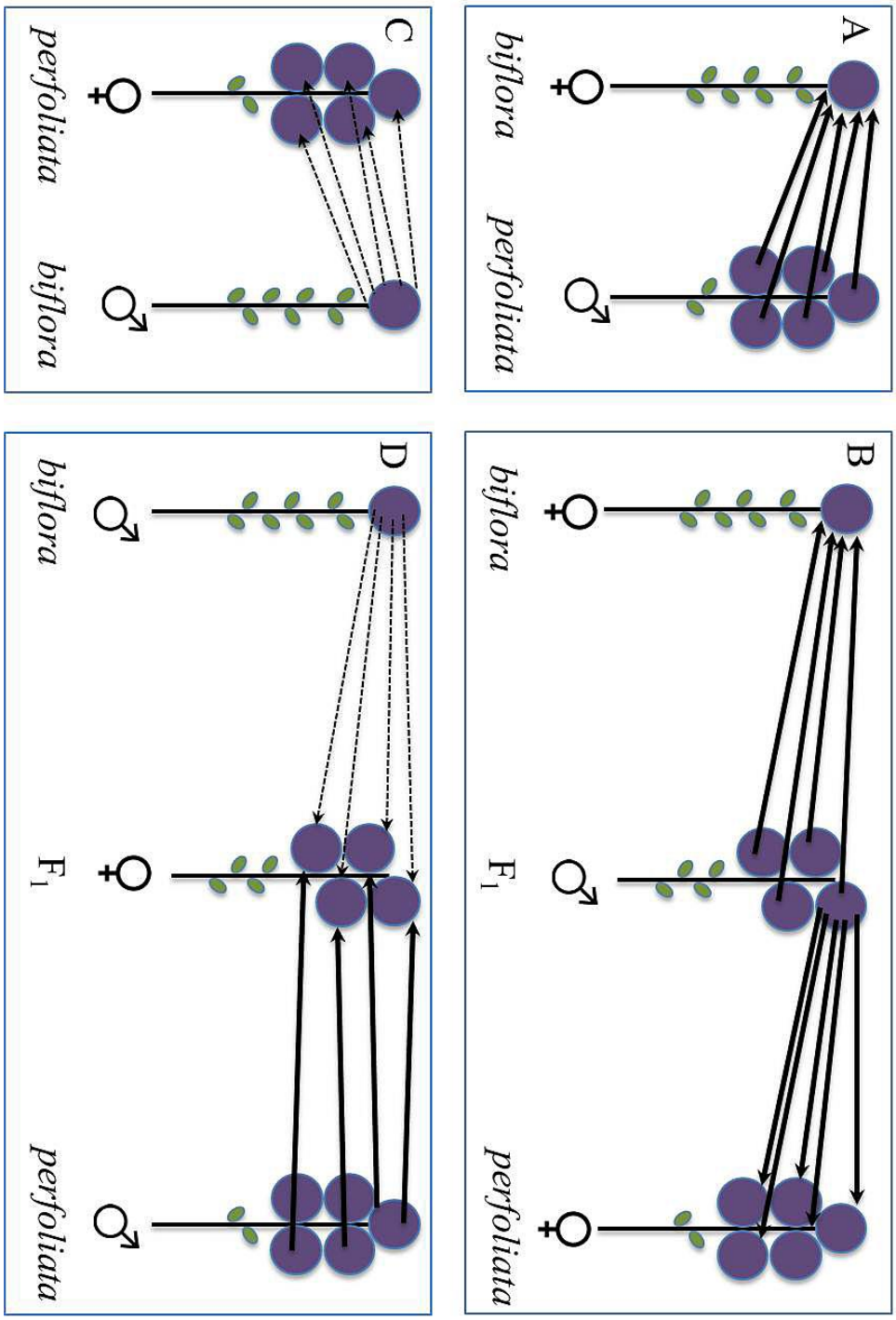

Figure 13. Depiction of hybridization between the subspecies (A and C). Depiction of backcross potential between an F_1 and the two subspecies (B and D). Narrow dashed arrows indicate low potential for gene flow and thick black arrows indicate high potential for gene flow. Purple circles indicate CH flowers. Green ovals represent CL flowers.

Table 1. Distances between sites of *Triodanis*. Geographical coordinate locations for each site were as follows: ATFS 35°26’18”N 77°28’17”W, ESP 35°35’21”N 77°18’28”W, FALK 35°43’21”N 77°31’19”W, and PARA 35°33’24”N 77° 22’40”W.

	ATFS	ESP	FALK
ATFS			
ESP	22.4 km		
FALK	32.0 km	24.4 km	
PARA	15.7 km	7.3 km	22.6 km

Table 2. Analysis of variance for morphological traits in *Triodanis perfoliata* subsp. *perfoliata* and subsp. *biflora* from four naturally occurring locations. Subspecies designation was treated as a fixed factor; site was treated as a random factor.

Source	d.f.	SS	*F*	*P*
Bract Base (mm)				
subspecies designation	1/3.011	3.377	286.023	<0.001
site	3/3	0.136	3.817	0.150
subspecies designation*site	3/66	0.036	2.988	0.037
error		0.262		
Bract Length to Width Ratio				
subspecies designation	1/3.008	3.083	332.902	<0.001
site	3/3	0.037	1.326	0.411
subspecies designation*site	3/66	0.028	4.487	0.006
error		0.137		
CH/CL Ratio				
subspecies designation	1/3.034	1.265	16.350	0.027
site	3/3	0.280	1.137	0.459
subspecies designation*site	3/55	0.246	11.589	<0.001
error		0.389		
Pore Position				
subspecies designation	1/3.039	2.338	239.717	0.001
site	3/3	0.077	2.592	0.227
subspecies designation*site	3/56	0.03	2.553	0.065
error		0.216		

Table 3. Summary statistics for morphological variables in *Triodanis perfoliata* subsp. *biflora* and subsp. *perfoliata*

	T. perfoliata subsp. biflora	***T. perfoliata* subsp. *perfoliata***
ATFS		
Bract Base (mm)	0.033 (0.103) N = 10	2.151 (0.314) N = 10
Bract Length to Width Ratio	2.012 (0.229) N = 10	0.682 (0.052) N = 10
CH/CL Ratio	0.006 (0.018) N = 9	1.073 (0.076) N = 3
Pore Position	0.727 (0.026) N = 8	0.286 (0.072) N = 5
ESP		
Bract Base (mm)	0.242 (0.134) N = 10	2.500 (0.849) N = 9
Bract Length to Width Ratio	1.903 (0.156) N = 10	0.795 (0.109) N = 9
CH/CL Ratio	0.026 (0.037) N = 10	0.540 (0.363) N = 9
Pore Position	0.734 (0.086) N = 10	0.415 (0.074) N = 8
FALK		
Bract Base (mm)	0.049 (0.084) N = 10	1.479 (0.057) N = 9
Bract Length to Width Ratio	1.686 (0.190) N = 10	0.713 (0.058) N = 9
CH/CL Ratio	0.054 (0.019) N = 9	2.454 (1.430) N = 9
Pore Position	0.759 (0.024) N = 9	0.381 (0.054) N = 9
PARA		
Bract Base (mm)	0.00 (0.00) N = 9	1.716 (0.391) N = 7
Bract Length to Width Ratio	2.043 (0.223) N = 9	0.773 (0.076) N = 7
CH/CL Ratio	0.060 (0.018) N = 8	0.963 (0.516) N = 6
Pore Position	0.779 (0.032) N = 8	0.425 (0.066) N = 7

Table 4. Summary of total loci, polymorphic loci, and fixed loci from AFLP scoring analysis. MSE I (M- XXX) and ECORI (E-XXX) adaptors used are shown.

	M-CAG/ E-AGG	**M-CAC/ E-AGG**	**M-CTA/ E-AGG**	**Total**
Number of loci	71	74	64	209
Number of polymorphic loci	51	47	37	135
Number of polymorphic loci used	22	25	22	69
Number of fixed loci	20	27	27	74
Proportion of polymorphic loci	71.83%	63.51%	57.81%	64.59%
Proportion of fixed loci	28.17%	36.49%	42.19%	35.41%

Table 5. Nei's genetic distance calculated using data from AFLP marker data. Within subspecies comparisons are in bold type.

	ESP *biflora*	**FALK *biflora***	**PARA *biflora***	**ESP *perfoliata***	**FALK *perfoliata***	**PARA *perfoliata***
ESP *biflora*	0.000					
FALK *biflora*	**0.134**	0.000				
PARA *biflora*	**0.095**	**0.038**	0.000			
ESP *perfoliata*	0.670	0.418	0.477	0.000		
FALK *perfoliata*	0.695	0.419	0.482	**0.032**	0.000	
PARA *perfoliata*	0.800	0.503	0.570	**0.030**	**0.051**	0.000

Table 6. Means and standard deviations (in parenthesis) for flower and seed counts from parental plants at ESP.

	subsp. *biflora*	subsp. *perfoliata*
Flowers		
N	9	9
Central stem CH	1.56 (1.67)	9.11 (4.91)
Lateral stem CH	1.44 (2.35)	0
Central stem 1° CL	26.33 (7.47)	15.56 (5.36)
Central stem 2° CL	19.67 (15.38)	5.22 (5.49)
Lateral stem 1° CL	23.00 (41.37)	0
Lateral stem 2° CL	6.56 (13.68)	0
Total CH	3.00 (3.20)	9.11 (4.91)
Total 1° CL	49.33 (45.83)	15.56 (5.36)
Total 2° CL	26.22 (25.73)	5.22 (5.49)
Total CL	75.56 (65.64)	20.78 (9.54)
Total flowers	78.56 (67.90)	29.89 (12.24)
CH/CL ratio	0.06 (0.03)	0.68 (0.44)
Seeds		
Seeds per CH	214.56 (98.65)	242.22 (83.98)
Seeds per 1° CL	109.03 (32.71)	139.41 (28.45)
Seeds per 2° CL	73.75 (20.37)	63.00 (46.67)
Total CH seeds	607.22 (684.74)	2181.52 (1470.96)
Total 1° CL seeds	5454.99 (4815.10)	2146.84 (814.21)
Total 2° CL seeds	1285.78 (2415.21)	185.33 (422.01)
Total CL seeds	6740.77 (6149.84)	2332.17 (1022.32)
Total seeds	7347.99 (6736.55)	4513.69 (2171.80)
CH/CL ratio	0.10 (0.04)	1.17 (0.87)

Table 7. Means and standard deviations (in parenthesis) for biomass, flower counts, and seed count estimates for subspecies, F1 hybrid, and backcross groups assigned using STRUCTURE.

	subsp. *biflora*	**subsp. *perfoliata***	**F1 hybrids**	**subsp. *biflora* backcross**	**subsp. *perfoliata* backcross**
N	27	24	8	2	8
Biomass	0.17 (0.17)	0.26 (0.18)	0.43 (0.37)	0.13 (0.13)	0.24 (0.21)
Flowers					
N	26	26	6	1	8
CH	1.46 (1.88)	14.62 (12.61)	12.00 (10.86)	1	11.63 (6.48)
1° CL	31.31 (30.24)	14.15 (12.89)	28.00 (10.26)	12	21.00 (19.68)
2° CL	17.77 (20.05)	5.62 (5.23)	49.17 (28.31)	19	31.88 (30.55)
Total CL	49.08 (46.35)	19.77 (15.82)	77.17 (37.45)	31	52.88 (49.86)
Total (CH+CL)	50.54 (47.62)	34.38 (24.75)	89.17 (34.64)	32	64.50 (55.22)
CH/CL ratio	0.05 (0.03)	1.35 (1.21)	0.47 (0.35)	0.08	0.83 (0.48)
Seeds					
N	26	26	6	1	8
CH	364.95 (459.72)	3243.07 (2569.48)	1657.04 (1387.55)	77.29	2623.02 (1701.37)
1° CL	3669.31 (3359.27)	1811.37 (1238.00)	2892.42 (1539.67)	1289.1	2731.53 (2579.00)
2° CL	1500.95 (1833.11)	465.01 (503.20)	1835.79 (1757.36)	607.11	1127.49 (992.67)
Total CL	4881.61 (4485.29)	2169.07 (1513.95)	4728.20 (3203.17)	1896.21	3436.21 (3289.77)
Total (CH+CL)	5246.56 (4880.42)	5412.14 (3303.04)	6364.39 (2589.25)	1973.5	5736.94 (4589.67)
CH/CL ratio	0.07 (0.04)	2.13 (2.30)	0.55 (0.47)	0.04	1.19 (0.69)
Seeds per 1° CL flower	117.47 (26.62)	137.93 (46.21)	100.53 (18.61)	107.42	121.35 (48.19)
Seeds per 2° CL flower	84.57 (9.23)	83.87 (16.21)	32.51 (14.69)	31.95	26.14 (14.02)
Seeds per CH flower	210.85 (132.95)	269.87 (152.45)	149.10 (39.95)	77.29	226.39 (130.02)

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