COMPONENTS OF REPRODUCTIVE ISOLATION

BETWEEN SUBSPECIES OF AN ANNUAL PLANT

by

Evan Arthur

A Senior Honors Project Presented to the

Honors College

East Carolina University

In Partial Fulfillment of the

Requirements for

Graduation with Honors

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Greenville, NC

May 2014

Approved by:

Faculty Mentor (signature required):

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Dr. Carol Goodwillie

I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree.

Signed:_____   Date:_____

                    Evan B. Arthur

Components of Reproductive Isolation between Subspecies of an Annual Plant

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ABSTRACT - Reproductive isolation is required in the divergence of species. The components of reproductive isolation are separated into either ecological or genetic components. In plants these include: habitat, flowering phenology, pollinator fidelity, gametic incompatibility, zygotic mortality, hybrid viability, male and female hybrid sterility and hybrid breakdown. Cleistogamy, the production of closed obligate self-fertilizing flowers, creates a barrier to gene flow by preventing hybridization. However, there have been few studies that have investigated its impact in reproductive isolation. A unique opportunity arose to determine the degree of isolation that cleistogamy provides. In multiple sites within Pitt County two subspecies of an annual, cleistogamous plant *Triodanis* co-occur. Each subspecies exhibit a mixture of closed (cleistogamous) and open (chasmogamous) flowers. Soil moisture and content were quantified for several sites where they co-occur to calculate the extent that each contributes to the isolation of the two subspecies. Calculations show that there are significant differences between the subspecies in the moisture and composition of soils. Observations in the field were also made to calculate the overlap of flowering and it was found that the species do in fact overlap, therefore, unlikely to impact prezygotic isolation. To quantify the extent of isolation due to cleistogamy, the number of seeds produced was quantified between open and closed flowers. By

using hand pollination, an F1 generation was made and raised in the greenhouse to calculate viability (survivorship and biomass). Results show that F1's are in fact viable and in some cases appear to be as large or larger than parentals. Quantifying a variety of reproductive barriers allowed us to determine the total amount of isolation for each subspecies as well as the relative amount of isolation due to cleistogamy. This study is essential to the understanding of cleistogamy as a component of total reproductive isolation

Acknowledgments

I would like to especially thank all of the friends, family and organizations that made this study possible: Dr. Susan B. McRae, Mr. Kevin Baxter, Emily Stewart, Daniel Harder, Adrian Modzik, Jessica Moore, Dr. Cindy Putnam-Evans,Joshua Thigpen, my parents, brother, grandparents, East Carolina University, the Department of Biology and the Honors College. I would also like to give a special thanks to my mentor and advisor, Dr. Carol Goodwillie, for her investment, direction and enthusiasm. Funding was received from an East Carolina University Undergraduate Research and Creative Award.

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Introduction

Reproductive isolation is required in the divergence of species because of its role in determining gene flow (Coyne and Orr 1998). Reproductive isolation has several components, which fall into two categories, ecological or genetic, either of which can act before or after the formation of a zygote (Widmer 2009). Although there is great interest in understanding reproductive isolation, few studies have looked into the contribution of different isolating barriers in reproductive isolation or have tried to estimate their relative importance. (Ramsey *et al*. 2003).

In plants, isolation can occur in a variety of ways. The most common and well known of these mechanisms in plants include: habitat, flowering phenology, pollinator fidelity, gametic incompatibility, zygotic mortality, hybrid viability, and male and female hybrid sterility. Plant species are rarely isolated solely by a single mechanism but rather a combination (Widmer 2009). More specifically, components work in a sequential combination to each other; as a consequence, each component acts only on the potential gene flow remaining after previous barriers have acted. For example, the first barrier, divergence in habitat preference, isolates species based on the relative location of the plants. For populations that co-occur and are not isolated by location, the second mechanism, flowering phenology, can isolate them dependent upon the blooming time. However, since the components act sequentially, the relative isolation that flowering phenology provides can only act on the amount of gene flow left over from the previous barrier. If flowers of both species are open simultaneously, then pollen transfer can be limited if flowers are visited by different pollinators. Once the pollen transfer has taken place, the gametes can be incompatible. If the gametes are compatible then a zygote can

form. Once the zygote forms and a hybrid has grown, that hybrid may have decreased size and biomass which causes it to be less competitive. If the hybrid is large enough to compete, then in the next mechanism, species can be isolated by the inability of the hybrids to reproduce. Not only do these components act in sequential combination to each other but they also work asymmetrically. The amount of isolation that species A experiences from species B may be significantly different than the amount of isolation species B experiences from species A.

One factor that has received little to no consideration for its effect in reproductive isolation is self-fertilization. Self-fertilization, also known as selfing, is the merging of male and female gametes that come from the same individual. Selfing facilitates speciation by preventing gene flow between individuals (Martin and Willis 2006).

In plants, an extreme version of selfing is cleistogamy. Cleistogamy is the production of closed, obligate self-fertilizing flowers which creates a barrier to gene flow by preventing hybridization. This unique characteristic has been studied since the work of Charles Darwin in his book, The Different Forms of Flowers on Plants of the Same Species (1887). However no research has been done on the role it plays in reproductive isolation.

Study Species

Studies that have looked at isolating mechanisms typically target one or a few barriers to gene flow without consideration of the other components of isolation (Ramsey *el al* 2003). A unique opportunity arose to determine the degree of isolation that cleistogamy provides with respect to other components of isolation in a local plant

species. Two subspecies of an annual, cleistogamous plant, *Triodanis* co-occur in Pitt County, NC: *Triodanis perfoliata* subsp. *perfoliata* (hereafter referred to as *perfoliata)* and *Triodanis perfoliata* subsp. *biflora* (hereafter referred to as *biflora)*. Each subspecies exhibits a mixture of closed (cleistogamous) and open (chasmogamous) flowers but their divergence has created a difference in the ratio each produce. They occur in sympatry throughout most of the southern region of the United States (USDA, NRCS 2012) and some hybridization has been reported between the two subspecies but they continue to stay morphologically distinct (Goodwillie and Stewart 2013). Another unique characteristic of these plants is in the sexual phase observed in their chasmogamous flowers. When chasmogamous flowers first open, the stamens, have deposited pollen on the outside of the stigma. This is considered the male phase because the receptive surface of the stigma is covered. After a few days, the stigma lobes open and expose the receptive surface of the stigma making it readily available to accept foreign pollen, the female phase. My study quantifies the components that contribute to the reproductive isolation of these two subspecies, including cleistogamy, by studying them in both their natural habitat and in a laboratory setting. By calculating the extent that each component contributes to isolation we will calculate the total isolation for each subspecies as well as the relative contribution that cleistogamy provides.

Materials and Methods

To quantify the relative contribution of cleistogamy to reproductive isolation, we first had to quantify the known components of isolation. In this study we quantified habitat, flower phenology, seed germination, hybrid viability, female fertility and male fertility with the addition of cleistogamy. Since these components act sequentially, the first step was to determine where cleistogamy would be placed in the sequential time line of isolation. We propose that if that the first barrier states the flowers must occur in the same place and the second barrier states they have to bloom at the same time, then we assumed that prior to flowering phenology, the plant must produce chasmogamous flowers that can open.

Prezygotic isolation

Habitat Preference

The two subspecies of *Triodanis perfoliata* occur in sympatry on a continental scale, especially in the south eastern region. The same, although, cannot be said on a local scale. The first component, habitat preference, was calculated with the aid of Dr. Carol Goodwillie and her lab whom over the past five years have done an extensive search throughout the Greenville/Pitt County area and identified 23 different sites where one or both subspecies of *Triodanis perfoliata* occur. Each site was identified as either a mixed site, where both subspecies occur, or pure site, where only one subspecies is present.

The anecdotal observation of differentiation in habitat led us to look at the microhabitat, specifically soil moisture and composition, to quantify the divergence in

habitat. To determine soil moisture, a Decagon EC 5 moisture sensor was used in the previously identified sites that are known to contain on or both subspecies. Each site was designated as either a pure *biflora*, pure *perfoliata*, or mixed site. Three readings were taken at each site at the same time for a more accurate result and averaged together for comparison.

To compare composition we also dug out a shovel length (approximately 6" x 4") soil sample. Each soil sample was filtered through a large screen and placed in a test tube. After filling the test tube 1/3 with soil and 2/3 with water and detergent, the test tube was shaken for five minutes and allowed to settle for 24 hours. This allowed the soil to be disturbed so that the heaviest particles in soil settle first, sand, followed by silt then clay. Once all the soil is settled, measurements of each component were taken as vertical height along the test tube and recorded. The relative amount of each component was then calculated to determine the classification of soil.

Cleistogamy

The extent of cleistogamy was calculated using the greenhouse grow out. By carefully counting the cleistogamous vs. chasmogamous flowers of 20 plants for *perfoliata* (P), and *biflora* (B), we were able to find the ratio of flowers for cleistogamous flowers vs. total flowers. Using data generated by Emily Stewart in a previous study of seeds produced by each type of flower, we were able to extrapolate seed counts for each plant and average them. This number was used to more accurately weigh our calculations of reproductive isolation due to cleistogamy.

Flowering Phenology

If plants of the subspecies are not blooming at the same time then gene flow between them will be shut off, known as asynchrony in flowering phenology. We quantified this component of reproductive isolation by studying sites in three areas where the subspecies co-occur: a site in Greenville, Falkland, and Winterville. In each site, three distinct transects were mapped to inspect each sampling day for open flowers. The phenology data were intricate because with *Triodanis perfoliata,* both subspecies have a male and female phase in their chasmogamous (open) flowers. So instead of only counting the chasmogamous flowers that were open, we had to take into consideration the sexual phase of the flower.

The first task was to identify each subspecies. Identification was based on known identifiable traits: pore position, chasmogamous flower vs. cleistogamous flower position and ratio, leaf shape and pollen color (Stewart 2013). *Biflora* plants typically have narrow leaves and cleistogamous fruits a pore position found close to the top. Chasmogamous flowers in *biflora* are found in much fewer number per stalk and exhibit a determinate inflorescence with the apical meristem becoming a chasmogamous flower. These typically have whitish pollen.

Perfoliata plants, in contrast, have heart shaped leaves. The cleistogamous fruits are shorter and broader with pore position found close to the bottom. Open, chasmogamous, flowers are found in high number per stalk with dark purple pollen.

Once the subspecies of an open flower was determined, each flower was closely examined to determine whether it was exhibiting a male or female phase. Approximately

every other day at each site, the number of open flowers of each subspecies was flower counted and sexual phase was noted. Data were recorded throughout the flowering period from early May to early June.

Postzygotic isolation

In the greenhouse, plants of each subspecies were raised and crosses were made in both directions to create an F1 generation. Pure *biflora* and *perfoliata* were grown, as well as hybrids in both directions--- *perfoliata* ovules by *biflora* pollen (P x B) and the reverse (B x P)--- to see if maternal or paternal effects play a role in growth and survivorship.

Seed Germination

The seed germination trials were done within a petri dish. A small piece of filter paper was placed at the bottom of a petri dish. Just enough water was added to saturate the paper. Three sets of 20 seeds were germinated for each cross type then placed evenly throughout the dish. Once placed, the dishes were wrapped and sealed to prevent bacteria/ other organisms from getting in. Seeds were given two weeks to germinate until seeds they were considered inviable.

Sixty plants from each cross type --- P, PxB, BxP, B --- were individually planted in containers. Plants were then randomized and placed on a grid in the greenhouse. All plants were sub-irrigated and rotated twice a week to control for variation in the light environment.

Hybrid Viability

During the hybrid grow out, 20 plants of each cross type were counted with respect to number of branches, chasmogamous/cleistogamous flowers on the main stem, chasmogamous/cleistogamous flowers on the side branches and main stem height. After the counts were done, all of the plants were placed in the drying over and weighed for biomass. These two measurements were used together to portray hybrid viability.

Ovule Fertility

To calculate ovule fertility in F1 plants, seeds were counted in one cleistogamous fruit of 12 plants of each cross type from the lab grow out.

Male Fertility

To quantify male fertility in F1 plants, pollen counts were conducted. One flower was collected from approximately nine plants of each of the four cross types. One recently opened chasmogamous flower was taken and the anther and stamen were plucked as to ensure all pollen was obtained. Pollen was stained with 30 µL of methylene blue and centrifuged. Pollen was then viewed with a hemocytometer. Pollen grains were counted under the microscope and then multiplied to find total pollen grains per flower.

Calculations

The model for calculating reproductive isolation (RI) values for each component was modeled after Ramsey *et al.* (2003). This value indicates the extent to which a given pre

or postzygotic component reproductively isolates and typically varies between 0 and 1 (Ramsey 2003). It also takes into account negative values for situations such as hybrid vigor, where hybrid performance is greater than intraspecific performance. Total values between *perfoliata* and *biflora* were calculated based on a multiplicative function of the individual components of reproductive isolation (Ramsey *et al* 2003). In order to ensure the sequential aspect of isolation, absolute contribution is calculated as shown in equations 1-4.

(1) $AC_1 = RI_1$

(2) $AC_2 = RI_2(1-AC_1)$

(3) $AC_3 = RI_3[1-(AC_1+AC_2)]$

Generally summarized:

(4) $AC_i = RI_n(1-\sum_{i=1}^{n-1} AC_i)$

This formula ensures that for any given component of reproductive isolation can only act to limit the gene flow that has not previously been isolated.

Once these numbers have been calculated, we simply sum the absolute contribution to find total reproductive isolation (T) which also varies from 0 to 1, so for *m* components of isolation:

(5) $T = \sum_{i=1}^{m} AC_i$

The relative contribution (RC) of a reproductive barrier at stage *n*, is then calculated as a ratio of absolute contribution to total contribution:

(6) $RC_n = \dfrac{AC_n}{T}$

We used the Excel spreadsheet made available at

http://www.plantbiology.msu.edu/schemske.shtml to calculate total isolation and absolute

contributions to the total.

<div align="center">**Results**</div>

Prezygotic isolation

Habitat preference

To investigate habitat isolation, populations of *biflora* and *perfoliata* were found in 23 sites throughout the Greenville, Pitt County area. It was found that 12 sites were mixed, 6 sites were pure *perfoliata* and 5 sites were purely *biflora.* The next step was to derive an equation so that RI was 0 the subspecies always occurred together in the same area and 1 if they never overlapped. In order for these conditions to be met, we calculated the RI value as 1 minus the ratio of mixed sites to total sites where the subspecies were found (mixed sites plus pure sites).

(P) RI_p = 1 – (mixed(12)/total perf(18)) = 0.333

(B) RI_b = 1 – (mixed(12)/total bif(17)) = 0.294

When characterizing differences in soil habitats of the two subspecies percent sand was chosen as the factor to compare. In the test tube assays, each layer of soiled was measure with respect to height as an indication of volume. By comparing the height of each layer to the total height of the soil (T), percent composition was calculated:

(7) % Sand $= \dfrac{S}{T}$ x100

Another trait of soil we used to compare was average moisture based upon the readings taken at each site. By using a t-test we found that both characteristics of the soil have significant differences. While *biflora* prefers a moister environment with less sand, *perfoliata* thrives in environments with significantly less moisture and more sand shown in Table 1.

	Average Moisture (m^3/m^3)	Average % Sand
biflora	0.125	53.9%
perfoliata	0.0398	73.9%
t-test P-value	<0.001	<0.001

Table 1. – Soil moisture and sand content in soils of two subspecies of *Triodanis perfoliata*

Cleistogamy

Unlike the other prezygotic isolating barriers, cleistogamy was studied in the lab. Using data generated by Emily Stewart (2013), average seed count for each *perfoliata* and *biflora* were found. Reproductive isolation caused by cleistogamy was calculated using the following formula, which yields a value of 0 when all seeds are chasmogamous (CH) and 1 when all seeds are cleistogamous and therefore cannot be hybridized.

(P) R.I. = 1 – (CH seeds (15,400)/total seeds(46,347.9)) = 0.668

(B) R.I. = 1 – (CH seeds(5547.41)/total seeds(59,564.68)) = 0.9069

Flowering Phenology

To quantify reproductive isolation caused by asynchrony in flowering phenology we modeled the system used by Martin and Willis (2006) in their paper on ecological divergence in mating systems. Figure 1 and 2 shows the overlap of flowers open on each day throughout the blooming period (note that for hybridization to occur a male *biflora*

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flower must be open simultaneously with a female *perfoliata* or vice versa). These numbers were used in our calculation of RI. Because the frequency of hybridization depends on the relative frequency of the species' flowers; let q_{0p} represent the proportion of all hybrids formed from *perfoliata* ovules and similairly q_{0b} represent the proportion of all hybridized flowers that were formed by *biflora* throughout the season. The subscripts will direct us on the stage of isolation (time 0) and which subspecies fertilized the ovules.

On any given, i^{th} day, the frequency of hybrid pollen that is deposited on *perfoliata* $q_{1,p,,I}$ or *biflora* $q_{1,b,I}$ is the fraction of flowers open on that day that are *biflora* (b_i) or *perfoliata* (p_i) respectively ($p_i + b_i = 1$). Allow the proportion of all *perfoliata* flowers across the season on the ith day be s_i and for *biflora* be t_i. The expected hybridization of *perfoliata* ovules then is $q_{1,p,,i} = \sum s_i * b_i$ and the expected frequency for *biflora* ovules would be $q_{1,b,i} = \sum t_i * p_i$. Once these numbers have been derived, we can then calculate the strength of the barrier on each subspecies as

$$w_{1p} = \frac{q1,p,/q0p}{(1-q1,p,)/(1-q0p)}$$

$$w_{1b} = \frac{q1,b,/q0b}{(1-q1,b,)/(1-q0b)}$$

These values were used to calculate RI values due to flowering phenology as

(P) R.I. $= 1 - w_{1p} (1.01) = -0.01$

(B) R.I. $= 1 - w_{1b} (0.095) = 0.095$
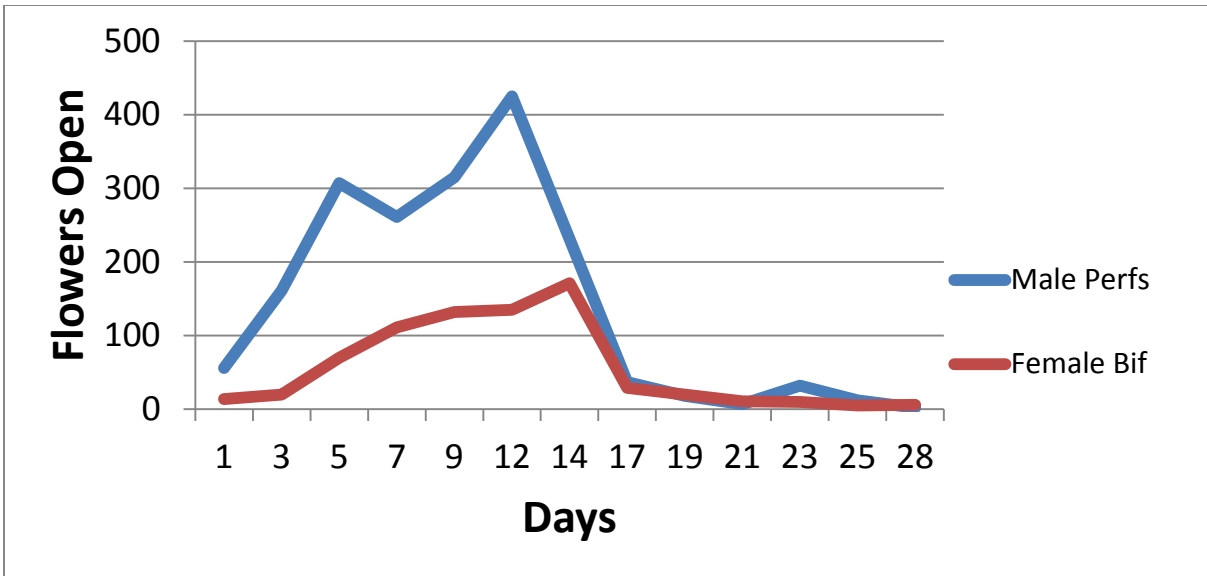
(Ramsey *et al* 2003)

Figure 1 – Overlap of open male *perfoliata* and female *biflora* chasmogamous flowers
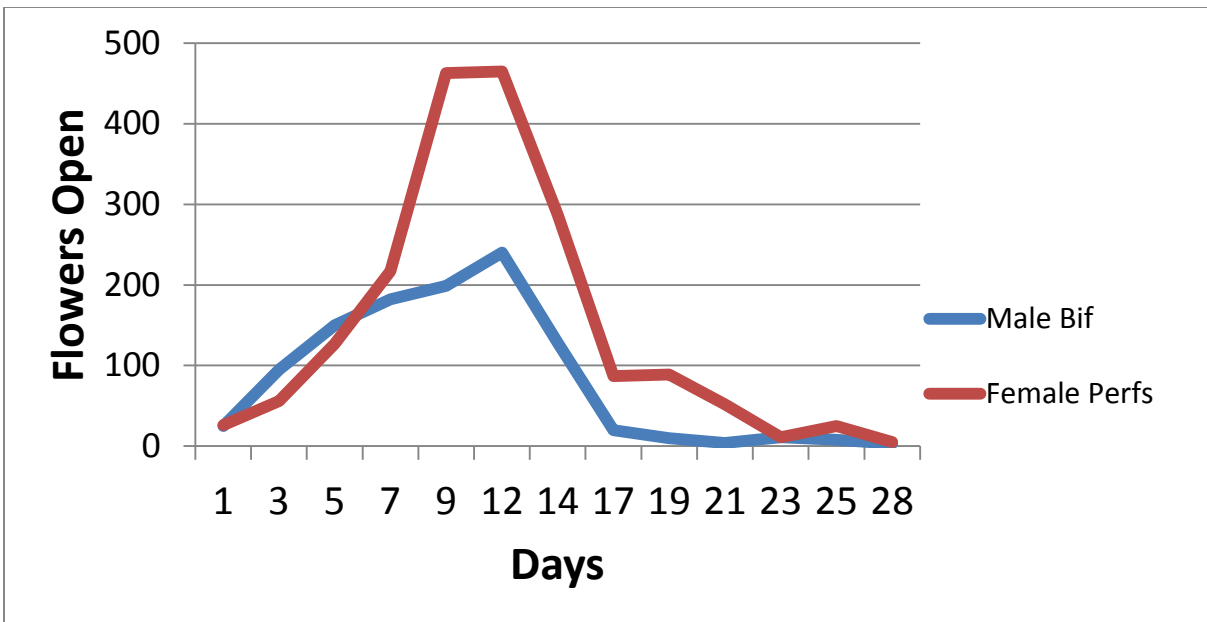

Figure 2 – Overlap of open Male *biflora* vs. Female *perfoliata* chasmogamous flowers

Postzygotic isolation

For all components of postzygotic isolation the general formula can be applied:

(8) RI = 1 – (hybrid performance/parental performance)

Seed germination

The first component of postzygotic isolation was seed germination. By counting the mean number of seeds out of 20 that had germinated, that number was recognized as "performance" and plugged in to our generalized equation (8) for this component. Figure 3 shows the number/performance for each cohort.
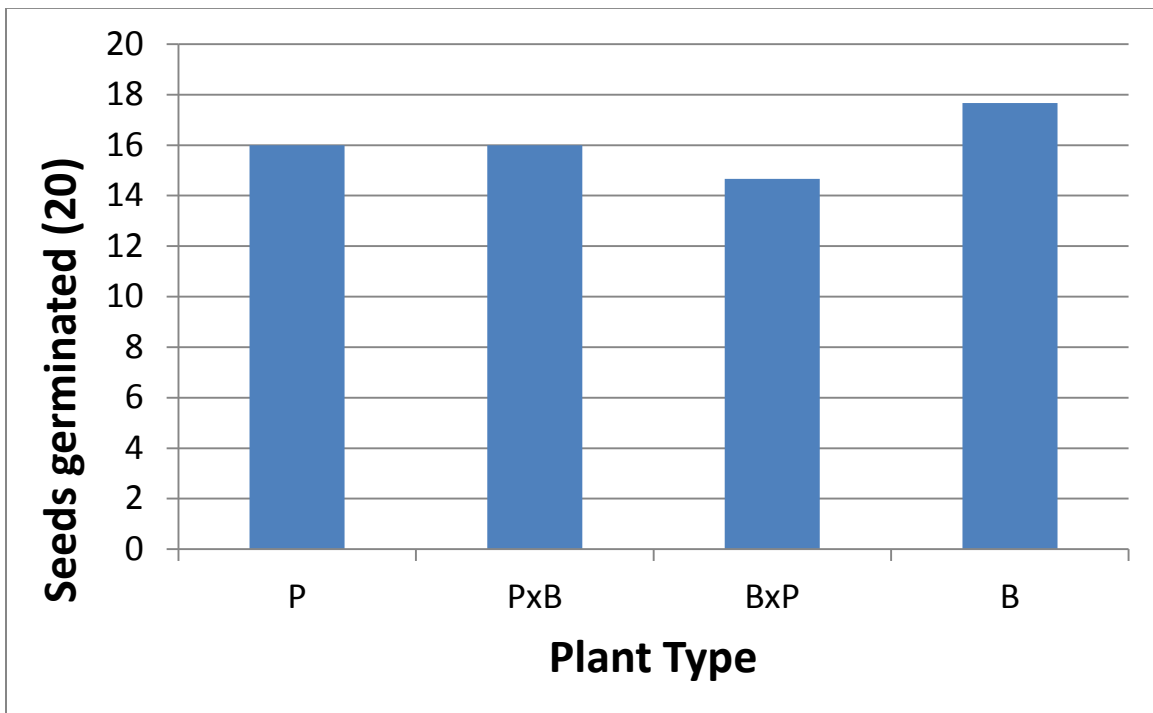

Figure 3– Results of seed germination trials. Values shown are means of three replicate dishes of 20 seeds

RI calculations were as followed for germination rates based on equation 8:

(P) RI = 1 − (16/16) = 0.00

(B) RI = 1 − (15/17) = 0.17

Because of to the positive linear relationship between flower count and dry biomass (Figure 4), we compared the means for dry biomass in Figure 5 as the most relevant source of information to quantify hybrid viability. PxB far outperformed *perfoliata* which was quantified as a negative RI value for *perfoliata*. This negative number shows the strength of hybrid vigor, the more negative the RI value, the stronger the hybrid vigor. This trend, however, was not seen in *biflora* as BxP did not perform as well as *biflora.*
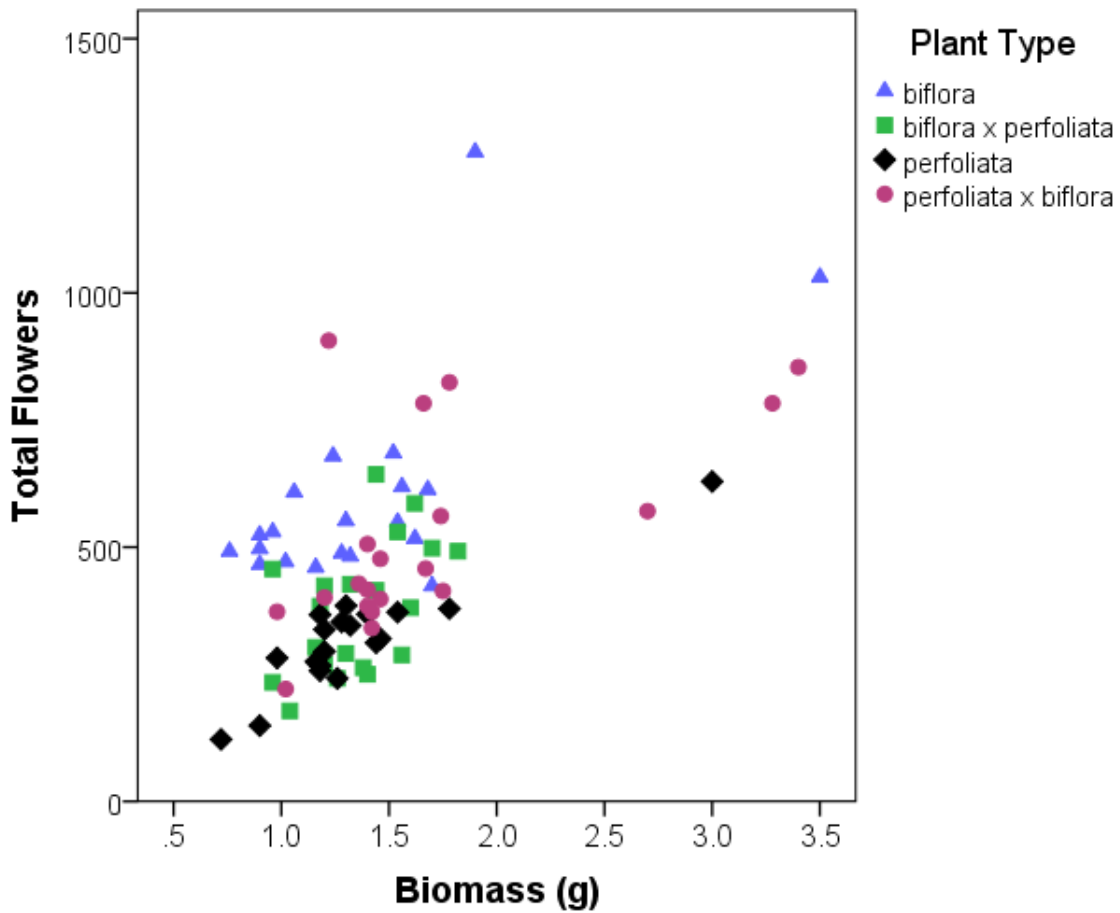
Figure 4 – A positive linear relationship was found between flower number and biomass. General trends also show that pure *biflora* seems to produce more flowers on average per gram of biomass than the other cohorts
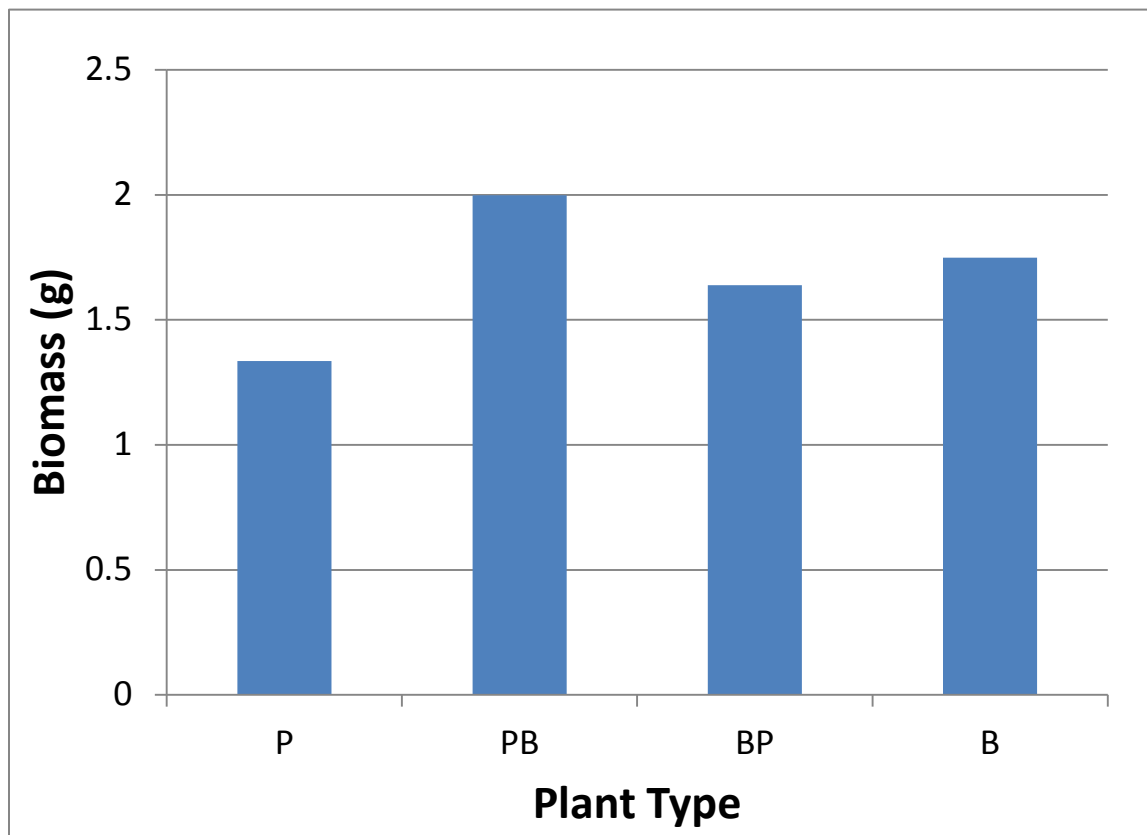
Figure 5 - Average biomass of each cohort shows a substantial amount of hybrid vigor in *perfoliata* with the PB plants out performing its parental

Applying equation 8 to results found for biomass:

(P) RI = 1 – (2.14/1.34) = -0.597

(B) RI = 1 – (1.63/1.75) = 0.0686

Ovule fertility

Average number of seeds produced per flower was chosen as our performance factor for ovule fertility as shown in Figure 6. In this experiment, *biflora* continued to just lightly outperform BxP, but there was a large difference in counts for *perfoliata* and PxB. PxB fell off in its production of seeds dramatically compared to *perfoliata*.
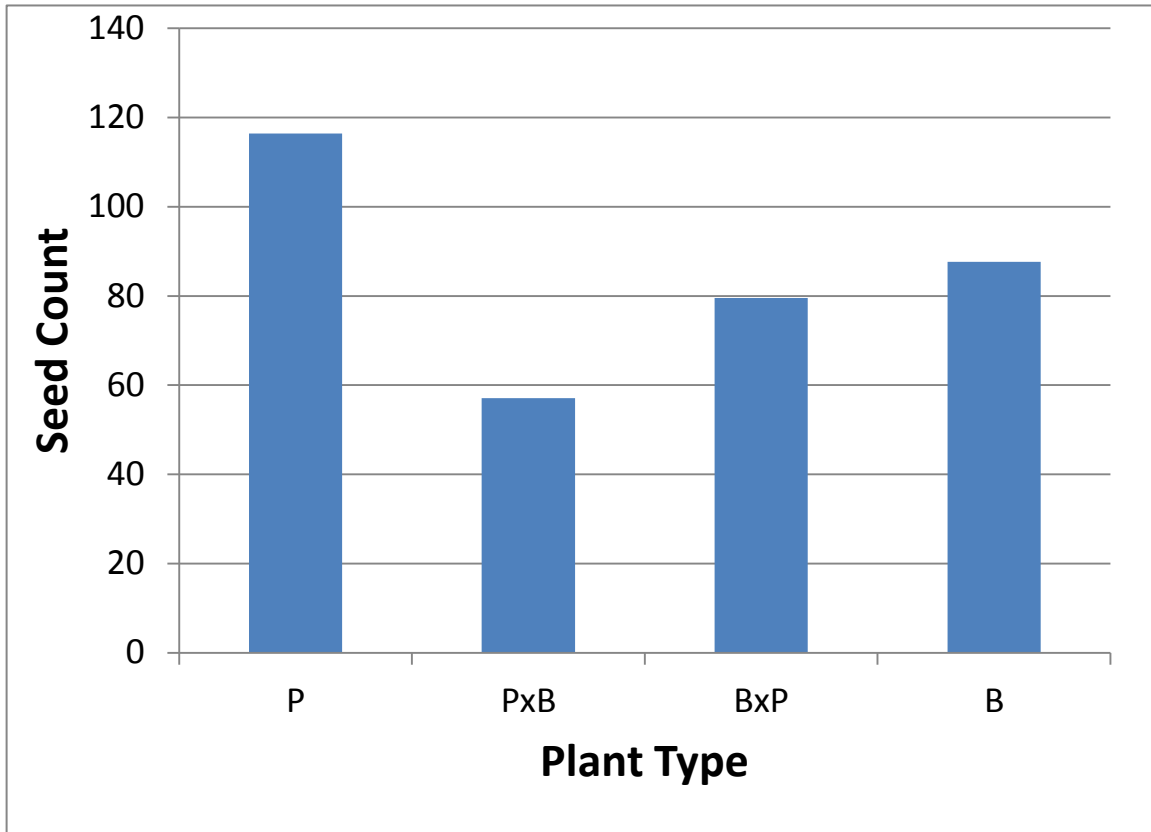
Figure 6 – Ovule fertility parental and F1 offspring.

Applying equation 8 to results found for seed count:

(P) RI = 1 – (57.08/116.42) = 0.5098

(B) RI = 1 – (79.54/87.62) = 0.0923

Male fertility was calculated based on pollen counts taken in the lab using the hemocytometer. Pollen counts were multiplied by 300 (based on the volume of solution in the hemocytometer and the total volume of the solution). Intermediate values for PxB and BxP were exhibited, yet, BxP exhibited a closer resemblance to *perfoliata* while PxB resembled *biflora* more. Total pollen counts per flower are shown in Figure 7.
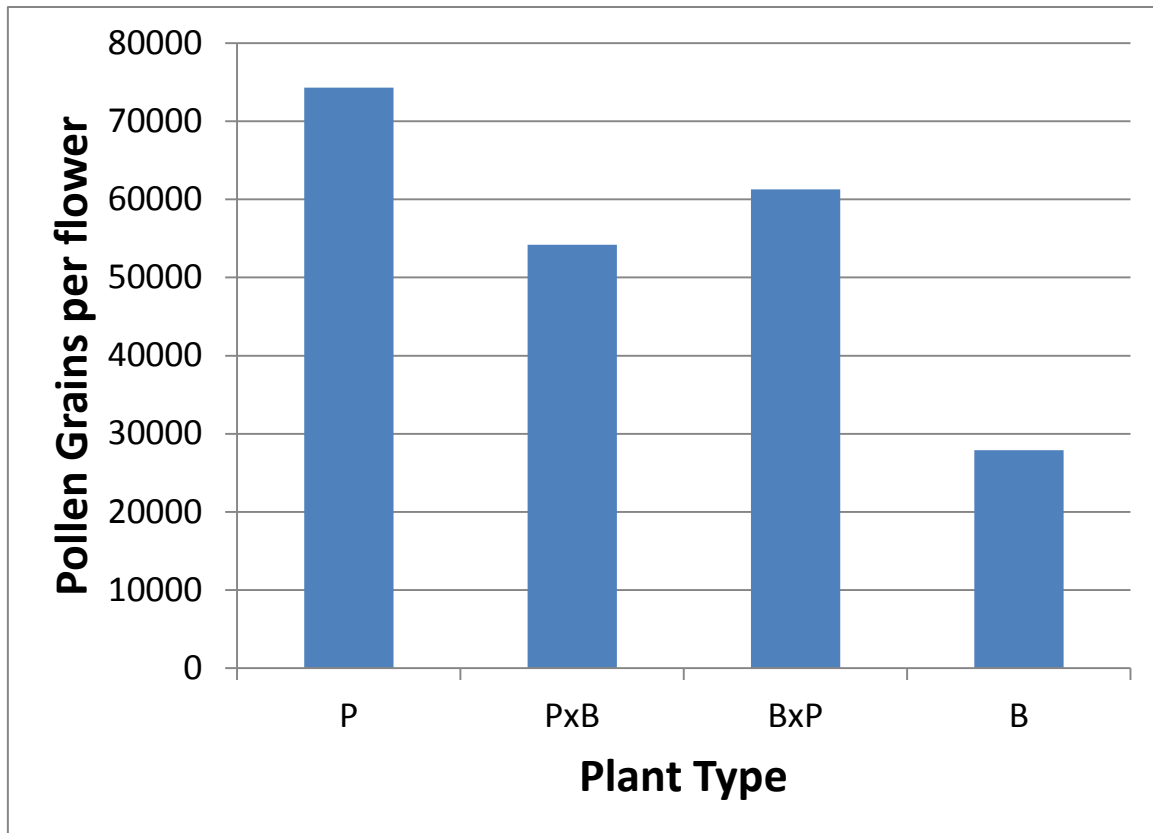
Figure 7 – Average pollen grains per flower, extrapolated
from counts using a hemocytometer

After plugging in the counted values into equation 8, the quantified amount of isolation

were as follows:

(P) RI = 1 – (204.2/93) = 0.271

(B) RI = 1 – (180.67/247.7) = -1.196

Summarized Results

Once RI values were calculated we then calculated absolute contribution (AC_n).

Results for absolute contribution (AC_n), relative contribution (RC_n), and total

reproductive isolation (T) are shown in Table 2 for *perfoliata* and Table 3 for *biflora.* A

graphical analysis for relative contribution to isolation is shown in Figure 8 for *perfoliata* and Figure 9 for *biflora*.

Barrier	*perfoliata* Absolute Contribution	*perfoliata* Relative Contribution
(1) Habitat	0.3300000000	0.3785338487
(2) Cleistogamy	0.4475600000	0.5133836646
(3) Flowering Phenology	-0.0022244000	-0.0025515476
(4) Seed Germination	0.0000000000	0.0000000000
(5) Hybrid Viability	-0.1341246468	-0.1538506629
(6) Female Fertility	0.1829106561	0.2098117412
(7) Male Fertility	0.0476630439	0.0546729559
Total Reproductive Isolation: 0.8718		

Table 2 ▲

Table 3 ▼

Barrier	*biflora* Absolute Contribution	*biflora* Relative Contribution
(1) Habitat	0.2940000000	0.3236682596
(2) Cleistogamy	0.6402714000	0.7048827542
(3) Flowering Phenology	0.0062442170	0.0068743362
(4) Seed Germination	0.0101123451	0.0111328066
(5) Hybrid Viability	0.0033869218	0.0037287044

(6) Female Fertility		
	0.0042444262	0.0046727417
(7) Male Fertility		
	-0.0499218651	-0.0549596027
Total Reproductive Isolation: 0.9083		

Table 2 and Table 3 – Relative contribution of each component shows that cleistogamy is the most important isolating mechanism for both *perfoliata* and *biflora*
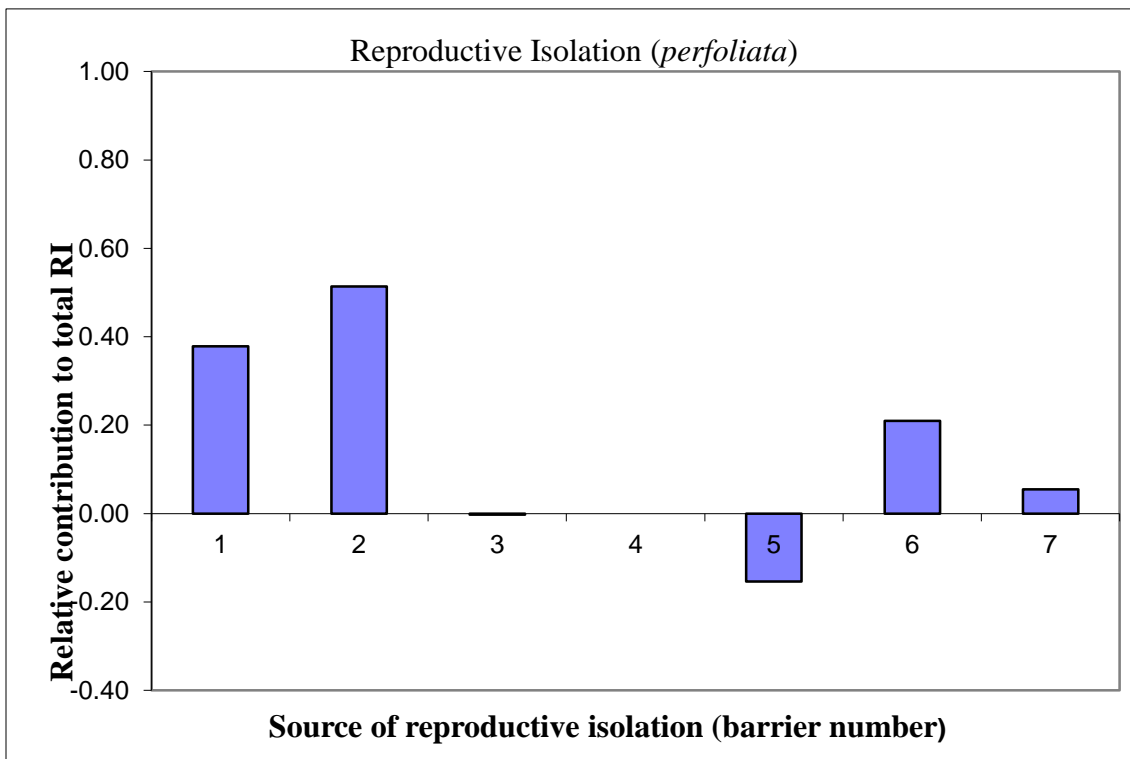

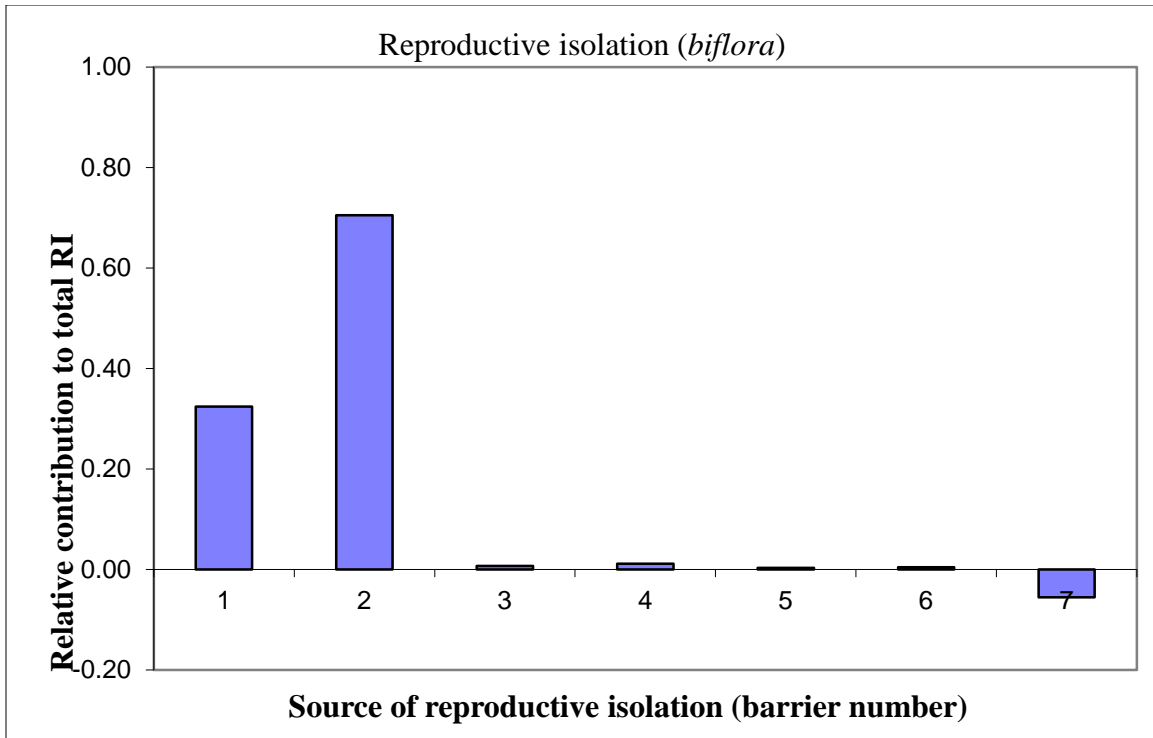Figure 8 ▲ shows the relative contribution that each barrier provides to the isolation of *perfoliata*

Figure 9 ▲ shows the relative contribution that each barrier provides to the isolation of *biflora*

Discussion

This study contributes to our understanding of reproductive isolation and specifically the effect that cleistogamy has as a component of reproductive isolation between divergent plant taxa. In both subspecies, cleistogamy was the strongest barrier in the reproductive isolation. *Biflora* was almost exclusively isolated by the first two barriers: cleistogamy and habitat preference, yet cleistogamy, despite being the second barrier, isolated more than twice the amount then did habitat preference. The relative amount cleistogamy provided to the total isolation was over two thirds for *biflora*. A similar result occurred with *perfoliata* as well. The two most strongly isolating barriers were still habitat preference and cleistogamy, however cleistogamy contributed just over one half of the total isolation while habitat preference contributed just over a third. It was expected that *biflora* would be isolated by cleistogamy more than *perfoliata* since *biflora* produces almost exclusively cleistogamous flowers with relation to chasmogamous flowers, much more than we see in *perfoliata*.

"Detailed knowledge of the strength and nature of these barriers provides insight into ecological and genetic factors that directly or indirectly influenced their origin, and may help predict whether they will be maintained in the face of sympatric hybridization and introgression" (Martin and Willis 2006).

As we look at each component of isolation and its relation to total reproductive isolation there are some limitations to keep in mind. Our study only focused on seven potential barriers to isolation and did not look at every possible component such as pollinator fidelity, pollen competition, F2 or F3 viability. It is unlikely, however, that adding these factors would change the overall findings of the study. While it might

produce a slight change in the total reproductive isolation, and as a result, relative contribution (RC_n), it is unlikely to change the absolute contribution cleistogamy provides. Because cleistogamy is only the second barrier to isolation, and all the barriers act sequentially, the potential that cleistogamy has does not change with the addition of additional barriers (since these added factors act later in the timeline of isolation). Thus stating that with such a large amount of total isolation due to habitat and cleistogamy alone, then even if other components were added, their potential for isolation would not make a significant difference in the findings of this study.

In the habitats of these subspecies, the significant differences we found in soil moisture and composition are likely to provide explanation for spatial separation in the occurrence of the subspecies. Another factor that seemed to differ in their spatial separation was different type of light environments. *Perfoliata* is typically found near the edge of woods while *biflora* is typically found farther in the open, yet,we did not measure them in this study.

When looking at the relative amount of isolation that each component provides for *biflora* and *perfoliata*, we see that although cleistogamy provides a substantial amount of isolation for *perfoliata*, there is still a significant contribution from other barriers. Since the F1s resulting from hybridization of *perfoliata* by *biflora* pollen showed a negative RI value for biomass (hybrids were larger than parental offspring), barriers acting after that contribute a larger amount, relatively. More specifically, after *perfoliata* F1s performed so well in viability, we see that the opposite trend occurs in female fertility, with F1s having lower pollen production than parental offspring. The RI values for hybrid viability and female fertility almost exactly counteracted each other. Perhaps

the lack of performance in female fertility was due to the lack of energy the plant was able to devote to reproduction because its use of so much energy in creating a large viable hybrid. We saw hybrid vigor contribute to the negative RI value in biomass for *perfoliata,* while pollen production was lower in hybrids because the trait seemed to be intermediate.

It is also known, thanks to Emily Stewarts study on these plants, that it takes substantially less energy for the plants to produce cleistogamous flowers than chasmogamous flowers. Since *biflora* is producing almost all of its seeds through self fertilization in cleistogamous flowers, it in turn does not have to devote so much energy to reproduction. Due to the less amount of energy that is required for reproduction it is able to perform better or devote more energy, to its biomass/hybrid viability.

A potentially interesting trend in the hybridization of these subspecies is seen in male fertility. In most plant species mitochondrial DNA is known to be passed on by the maternal plant, therefore we would expect to see intermediate values for F1s produced by *perfoliata* (PxB) and F1s of *biflora* (BxP) that more closely resembled the maternal subspecies. If this were the case we should have seen a steady decline in pollen grains per flower as we go from P to PxB to BxP to B. Instead we see quite the opposite. We do see intermediate values for pollen grains, as expected, however the values for each hybrid seem to more closely resemble the paternal than the maternal subspecies. What then is the cause for this observation? More study to explain why this occurs is being done. A remote possibility could be that the mitochondrial DNA is paternally inherited in this subspecies, as is true in some plants.

The total reproductive isolation values were consistent with what was observed in the field as well as with the results of previous genetic marker studies of *Triodanis perfoliata* hybrid zones (Stewart 2013). With such high amount of isolation, it is rare to observe hybrids, yet the possibility still exists. The substantial amount of reproductive isolation between the subspecies, though, does explain why gene flow is limited between the two and why the subspecies are remaining divergent phenotypically. Our results also tell us that for both subspecies, the isolation of these subspecies occurs almost entirely in the prezygotic stage. If pollen transfer does take place, there is almost nothing due to postzygotic isolation that prevents the formation of a strong viable hybrid, able to reproduce. The dominant force of isolation, nonetheless, is cleistogamy and should be considered further as an important component of reproductive isolation.

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