

CONSPECIFIC POLLEN PRECEDENCE AND ITS  
CONTRIBUTION TO SPECIATION IN *TRIODANIS*  
*PERFOLIATA*

by

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## **Introduction**

### **Reproductive Isolation**

Speciation is the process by which one species becomes two. This can happen for several reasons, and in order for speciation to occur, reproductive isolation must be present. Reproductive isolation refers to the ability of individuals of a species to mate exclusively with individuals from their own species. When two different species are in sympatry, meaning they exist in the same environment at the same time, reproductive isolation becomes more difficult to achieve. Organisms can be reproductively isolated by several factors that can either be prezygotic, or before the formation of an offspring, or postzygotic, after the formation of an offspring zygote. In plants, prezygotic isolation mechanisms include geographic separation, which refers to the separation of two species by a significant distance or geographic barrier like a mountain. Another is adaptation to different microenvironments, for example, adapting to shade or high sun in the same area. As well as different flowering times within the year, so essentially if two plants do not have open flowers at the same times of year, or even the same time of day, they cannot hybridize. Also, if different pollinators visit different species it will isolate each of them because pollen cannot be picked up by the pollinators and transferred to the other individuals. Postzygotically, low hybrid fitness can contribute to speciation and reproductive isolation because if a hybrid is formed, but it is not viable, a hybrid species cannot be formed. Finally, a little studied or understood isolation

mechanism is conspecific pollen precedence, which will be discussed in further detail in the following section.

### **Competitive Gametic Interactions**

Conspecific pollen or sperm precedence is a phenomenon that contributes to speciation and reproductive isolation between two diverging plant subspecies. In plants, conspecific pollen precedence can be defined as the preferential use of pollen from plants of the same species for fertilization when both conspecific and heterospecific pollen is present on the stigma of the individual (Howard, 1999). As a part of this process, individuals then produce fewer hybrid offspring than would be expected based on the frequency of each sperm or pollen type present (Howard, 1999).

The genetic basis of conspecific pollen precedence may fall into two main categories, male-male competition and/or female choice. For example, in pure pollen competition, differences in style length, mating system or ovule number may be the basis of conspecific pollen precedence (Fishman, 2008). This type of precedence would result in one species being dominant over the other regardless of the recipient of the pollen load. This would be an example of male-male competition. In the case of female choice, a style and pollen grain may have coevolved so as to make both tissues specific for only the complementary tissue. Proteins associated with the process of siring may have also coevolved in the same way as the tissues. In this type of precedence, each species would have an advantage in its own species'

tissues, but a disadvantage in the opposite species' tissue. Some plants also have differences in ploidy levels that can restrict the success of siring (Fishman, 2008).

In species of Louisiana irises, it was found that when *I. brevicaulis* was pollinated by mixed pollination with competing pollen from *I. fulva*, it was predicted based on pollen tube growth that the offspring would be mostly *I. fulva*, but it was found that only 39% of offspring were *I. fulva*. However, when *I. fulva* was pollinated by mixed pollination with competing *I. hexagona* pollen it was predicted based on pollen tube growth that each species would have equal success, but the offspring were 95% *I. fulva* (Simon, 1996). In the first instance, this represents conspecific pollen precedence because the species of pollen that was the same as the maternal plant outcompeted the other, even though the other species was predicted to have more success based on pollen tube growth (Simon, 1996). In the second case, it also represents conspecific pollen precedence, but in a different way. The two species were predicted to have equal siring success, but the conspecific pollen dominated over the other. This is a very clearly defined example of how conspecific pollen precedence can be reproductively isolating and restrict gene flow from one species to another. In this case in particular, it prevented hybridization in 95% of the cases, which is very significant.

The same principle applies in other organisms such as mammals. In case studies, a variety of organisms were found to possess the reproductively isolating barrier of conspecific pollen or sperm precedence. Its potency in isolating two

species varied however, and in some cases, it was a secondary barrier in conjunction with other reproductive barriers, either prezygotic or postzygotic. In other organisms, such as a study species of coral, during mass spawning episodes, it was found that conspecific sperm precedence was present, and in 13 out of 14 cases, conspecific sperm sired all larvae. This indicates that conspecific sperm precedence is a strong prezygotic barrier. However, there are also temporal and spatial differences in the release of their sperm that contribute to reproductive isolation (Fogarty, 2012). In still other cases, there are no other known mechanisms for reproductive isolation. Such is the case with two cricket subspecies, *Allonemobius fasciatus* and *Allonemobius socius* found in the Eastern United States. These organisms are present in the same environment at the same time. Post zygotic isolation mechanisms are found to be very weak. The only strong reproductive isolator is conspecific sperm precedence. This precedence is continually present, even when the female is only mated to one conspecific male, and multiple heterospecific males (Howard, 1999).

## **Asymmetry**

In some cases, the dominance of conspecific sperm or pollen precedence is asymmetric, meaning one subspecies exhibits a stronger preference for conspecific sperm or pollen than the other. For example, in a two subspecies of grasshopper, *Chorthippus parallelus* subsp. *erythropus* and *Chorthippus parallelus* subsp. *parallelus*, some conspecific sperm precedence contributes to reproductive

isolation, however it found to be stronger in *parallelus* females than in *erythropus* females (Howard, 1999).

There is a case of different species of *Mimulus*, which is a weedy perennial herb. In mixed pollinations of *M. lewisii* the offspring generation produced the number of hybrids expected if there was no pollen competition occurring (Ramsey, 2003). In contrast, mixed pollinations of *M. cardinalis* produced very few hybrids, even when loaded with a majority of heterospecific pollen. Pollen competition was estimated to have higher impact on reproductive isolation in *M. cardinalis* than in *M. lewisii* (Ramsey, 2003). These results suggest that pollen competition plays an asymmetric role in reproductive isolation. This means that pollen precedence was isolating in *M. cardinalis*, but it did not seem to be isolating in *M. lewisii*. This indicates that pollen precedence is restricting gene flow to *M. cardinalis* but not to *M. lewisii*.

## **Cleistogamy**

In hermaphroditic plant species, a phenomenon known as a mixed mating system can occur. In this system, one individual can produce flowers that are open and can be fertilized by any pollen that is applied, including pollen from other individuals, and can also produce flowers that are closed and obligately self-fertilizing. In unfavorable conditions, this can be very advantageous because it gives the plant the ability to still reproduce without the necessity of pollen from other individuals. This is unique to plants because due to their sedentary nature, they cannot quickly adapt or move away from unfavorable conditions. The ability to self

fertilize ensures that the plant can still produce and release viable seeds regardless of conditions. These aforementioned closed flowers are known as cleistogamous flowers. This breeding system is known as cleistogamy (Culley, 2007). Under environmentally poor conditions, some plants may produce more cleistogamous flowers in efforts to conserve energy, since presumably these flowers are less energetically costly to produce. Cleistogamous systems are relatively uncommon and can have interesting repercussions in reproductive isolation. If a plant has a high volume of cleistogamous flowers, it makes it more unlikely that the plant will hybridize, or produce offspring that are a product of two different species of plant (Martin, 2007).

### ***Triodanis perfoliata***

*Triodanis perfoliata* is one such species that exhibits this mixed mating system that includes cleistogamy. Two subspecies of an annual weedy herb, *Triodanis perfoliata* subsp. *perfoliata* and *Triodanis perfoliata* subsp. *biflora*, are widely distributed throughout the United States and Canada, with overlapping distributions (USDA, 2015). Yet, in the wild, viable putative hybrids have been seen but are not prevalent. This raises the question why the two subspecies are remaining separate. Each subspecies as well the two hybrid varieties exhibit distinct trait sets with regards to the ratio of open, chasmogamous flowers and closed, cleistogamous flowers produced along the main stem and the position of the pore on each fruit produced. This study will explore the possibility that conspecific pollen precedence plays a role in reproductive isolation of the two subspecies. In

order to investigate the causes of the differences between two subspecies and also their hybrids, this study will focus on the morphological indicators of hybridization. Trends of these indicators will also be supplemented by AFLP marker data that will be generated by extracting DNA from each plant and performing AFLP primer testing on it and drawing comparisons between individuals based on this data.

## Materials and Methods

### Study species

*Triodanis perfoliata*, clasping Venus' looking- glass, is a dicot in the family Campanulaceae. It is an annual herb that is native to most of the continental United States and Canada. *Triodanis perfoliata* is a weedy plant and is able to grow in many different conditions and climates.

*Triodanis perfoliata* plants produce two types of flowers, chasmogamous, (open) flowers and cleistogamous (closed) flowers. Chasmogamous flowers of *Triodanis perfoliata* are protandrous, meaning that the male function precedes the female function. Protandry is a characteristic state for hemophroditic systems. It is described as the development of male organs, in this case the anthers, and/or production of male products before the development of the female organs. In the case of *Triodanis perfoliata* the anthers dehisce before the flowers open, placing pollen on the outside of the stigma. Later in development, the stigma lobes open to reveal the receptive surface. Chasmogamous flowers of *Triodanis perfoliata* have purple to lavender five-lobed corolla, five stamens and a three-lobed stigma on a thick style. Cleistogamy is a unique feature to this species and it represents the



ability of the plant to produce flowers that are obligately self-fertilizing. No visible corolla, stamen or stigma is present in a cleistogamous flower. All organs are inside of the capsule.

Leafy bracts subtend both kinds of flowers. *Triodanis perfoliata* produces capsules that are ellipsoid in shape and have persistent sepals at the top. They are approximately 80-100 cm in length, initially green in color, and then change to brown as they mature. During maturation, a pore appears in the medial portion of the capsule, and when fully mature, this pore will open and drop mature seeds. The seeds are ellipsoid and brown in shape and color and can be up to 3/8 of an inch long.

*Triodanis perfoliata* is classified into two subspecies, *Triodanis perfoliata* subsp. *perfoliata* and *Triodanis perfoliata* subsp. *biflora*. The two subspecies are distinguished by differences in several morphological traits. The most obvious distinction is a difference in the ratio and placement of closed flowers and open flowers, on each individual plant. Plants of subsp. *biflora* typically have only cleistogamous flowers distributed along the main stem length and a few chasmogamous flowers produced at the top most portion of the stem. In contrast, plants of subsp. *perfoliata* typically have more chasmogamous flowers distributed along the entire stem length. The two subspecies also differ in pore position on the capsules. In subsp. *biflora*, this capsule is more distally located on the upper part of the fruit. Subsp. *perfoliata* individuals tend to have larger, medially located pores on the fruits. Each subspecies also has a unique shape to their bract, which is the leafy

portion that subtends each fruit. The bract shape in subsp. *biflora* tends to be longer and more rectangular in shape. In subsp. *perfoliata*, the bract shape tends to be shorter and more heart shaped.

In order to determine whether conspecific pollen precedence is a contributing factor in reproductive isolation between the two subspecies, an experiment was designed in which the parental plants were grown from seeds collected in the field. These seeds were collected at the A Time for Science facility in Grifton, North Carolina. Pollinations were performed on eight individuals of each subspecies. Flowers on these individuals were emasculated by removing the anthers before they dehisced during the floral bud stage; this prevents self-fertilization from occurring. When stigmas opened, pollen was then applied by hand. One lobe was fully coated with pollen from subsp. *perfoliata* and one lobe was coated in pollen from subsp. *biflora* for each individual plant. One lobe was not coated in any pollen.

The morphological traits noted above have been characterized as diagnostic for both subspecies and hybrids by the results found in a previous experiment. In this experiment, breeding two individuals, one from each subspecies, together, generated hybrids. This crossing was done in both directions, meaning individuals of subsp. *perfoliata* were fertilized with pollen from individuals of subsp. *biflora*, as well as an individuals of subsp. *biflora* were fertilized with pollen from subsp. *perfoliata*. The results of the previous experiment indicated that the differences between the 4 groups were the most pronounced in the measurements of pore size,

and the number and type of flowers generated. Thus, these traits can be considered indicative of the identity of each plant and will indicate for the current experiment what type of individual was generated by the mixed pollination.

In the offspring generation, individuals from subsp. *perfoliata* and subsp. *biflora* were predicted using visual morphological indicators described above. The flower ratio along each main stem was quantified by counting the number of each type of flower on each individual.

The size and placement of the capsule pore was measured using a dissecting microscope with an ocular micrometer. The length from the base of the capsule to the top of the fruit, the base of the fruit to the base of the pore, the base of the fruit to the top of the pore, and the width of the pore were measured on a sample fruit of each type (cleistogamous and chasmogamous) from each individual plant. Based on previous data, we expected pure subsp. *biflora* to have more distal pores and fewer open flowers than their hybrids. In pure subsp. *perfoliata*, we expected more medial pores and more open flowers than their hybrids. Leaf bract shape data were not collected in this study.

## **AFLP**

To confirm the identification of hybrids and pure individuals based on morphological data, molecular genetic analyses were carried out for a subset of individuals. Individuals were selected that showed a strong morphological

resemblance to one subspecies type or the other. The confirmation was done using Amplified Fragment Length Polymorphism (AFLP) analysis, using a protocol modified from Vos et al. (19xx). To begin AFLP analysis, DNA from selected plants was extracted. The extraction was done using a CTAB buffer protocol modified from Doyle and Doyle, 1987. The tissue was frozen with liquid nitrogen and then ground using a pestle and mortar method to break the cell walls and release DNA. The ground plant tissue was then placed into microfuge tubes that contained a CTAB buffer. The DNA was isolated from other molecules and cellular debris using a chloroform and isoamyl alcohol extraction technique. Several purification steps were then performed through precipitation and resuspension.

The purified DNA was then cut with restriction enzymes EcoRI and MseI. These enzymes are designed to cut DNA at specific recognition sequences. DNA adaptors were then attached to the template DNA fragments using T4 ligase. Following this, amplification of the DNA was performed using primers that were complementary to the adaptor sequences. Initially, pre-selective amplification was done using one selective nucleotide that was added to each adaptor region. The final amplification was performed using three selective nucleotides. This was done to reduce the number of DNA fragments amplified to a level that can be resolved and scored. One of the final primers had a fluorescent label attached so as to be detected by the automated sequencer. Three different primer combinations with different selective nucleotides were used.

To analyze the fragments, the DNA was run through a capillary sequencer model ABI 373. The size markers were Rox 625 (ChimerX, Madison, WI). These size markers served as a ladder sequence for comparison when looking at the experimental DNA. The capillary sequencer separated fragments based on size by flowing a charge through a capillary tube filled with polyacrylamide gel. As the DNA fragments move through the tube, they are traced by using the fluorescent label. By using a GeneMapper software program, these traces are translated into peaks, displayed on a chromatogram. The chromatographs that were generated by each individual were then scored by visual inspection of the peaks. When analyzing the read out, any peaks that were polymorphic for different individuals were recorded. 33 loci were identified across 3 different primer pairs. If a peak was present at a loci, the individual was assigned a value of 1 at that loci, if a peak was absent it was assigned a value of 0. This data was then entered into a program that used non-metric dimensional scaling to visualize the data. This analysis generated a plot that showed the relationship between individuals so as to see if the morphological relationships that were observed could be reinforced by genetic data. If the morphological identifications were correct, one would expect to see strong correlation between the pattern of peaks of individuals in related groups. For instance, we would expect to see similar peaks in all individuals that were hypothesized to be subsp. *biflora* pure offspring, and one would expect to see them grouped together on the statistical plot generated by this data.

## Results

## Morphological Analysis

In subsp. *biflora*, one would expect a cluster of pure individuals with high pore position numbers and low CH flower numbers and a cluster of hybrids with lower pore position and higher flower number. If there is no conspecific pollen precedence, these groups would be similar in number of individuals. If conspecific pollen precedence were acting on subsp. *biflora*, a large cluster of pure individuals and a small cluster of hybrids would be seen. With the results obtained, a hybrid cluster and pure cluster are distinguishable and there are very few pure individuals and many hybrid individuals (Figure 1).

When observing results from subsp. *perfoliata*, one would expect a cluster of pure individuals with low pore position numbers and high CH flower numbers and a cluster of hybrids with higher pore position and lower flower number. If there were no conspecific pollen precedence, then these groups would be similar in number of individuals. If conspecific pollen precedence were acting on subsp. *perfoliata*, a large cluster of pure individuals and a small cluster of hybrids would be seen. A hint of two clusters is seen in this data set; however, the second smaller set does not correspond to what is expected for a hybrid variety. Based on the data, it can be concluded that most individuals produced are pure subsp. *perfoliata* (Figure 2).

## AFLP Analysis

From three different primer pairs, 33 polymorphic resolvable loci were identified. Three individuals were removed due to data that was unreadable for

certain primer pairs. This data was then entered into a program that generated a non-metric multidimensional scaling plot that compressed the data from the 33 loci to visualize the relationships between individuals. The closer individuals are on this plot, the more closely related they are genetically. Individuals were chosen for this analysis based on the morphological traits they exhibited. If individuals exhibited similar morphological trends then one would expect them to be closely related on the plot. In the results, this was confirmed and the morphological data and AFLP peaks aligned (Figure 3). Interestingly, two individuals of subsp. *biflora* scored identically at every loci and are shown as the same point on the graph since they scored genetically identical.

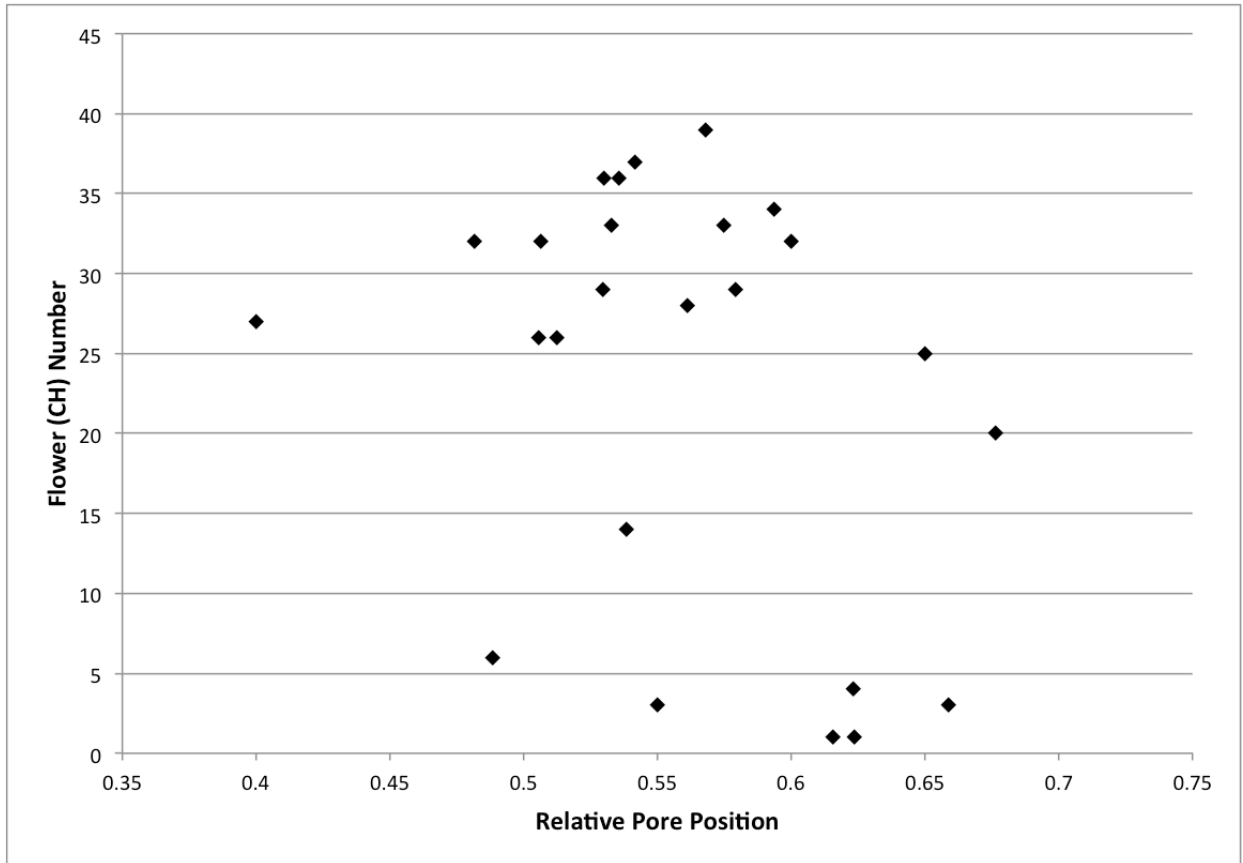


Figure 1: The morphological data for individuals that were produced when maternal *Triodanis perfoliata* subsp. *biflora* was pollinated in a mixed pollination, with a 50:50 mix of pollen from *Triodanis perfoliata* subsp. *biflora* and *Triodanis perfoliata* subsp. *perfoliata* is shown here. The relative pore position is the distance from the base of the fruit to the pore divided by the total distance of the fruit from bottom to top.



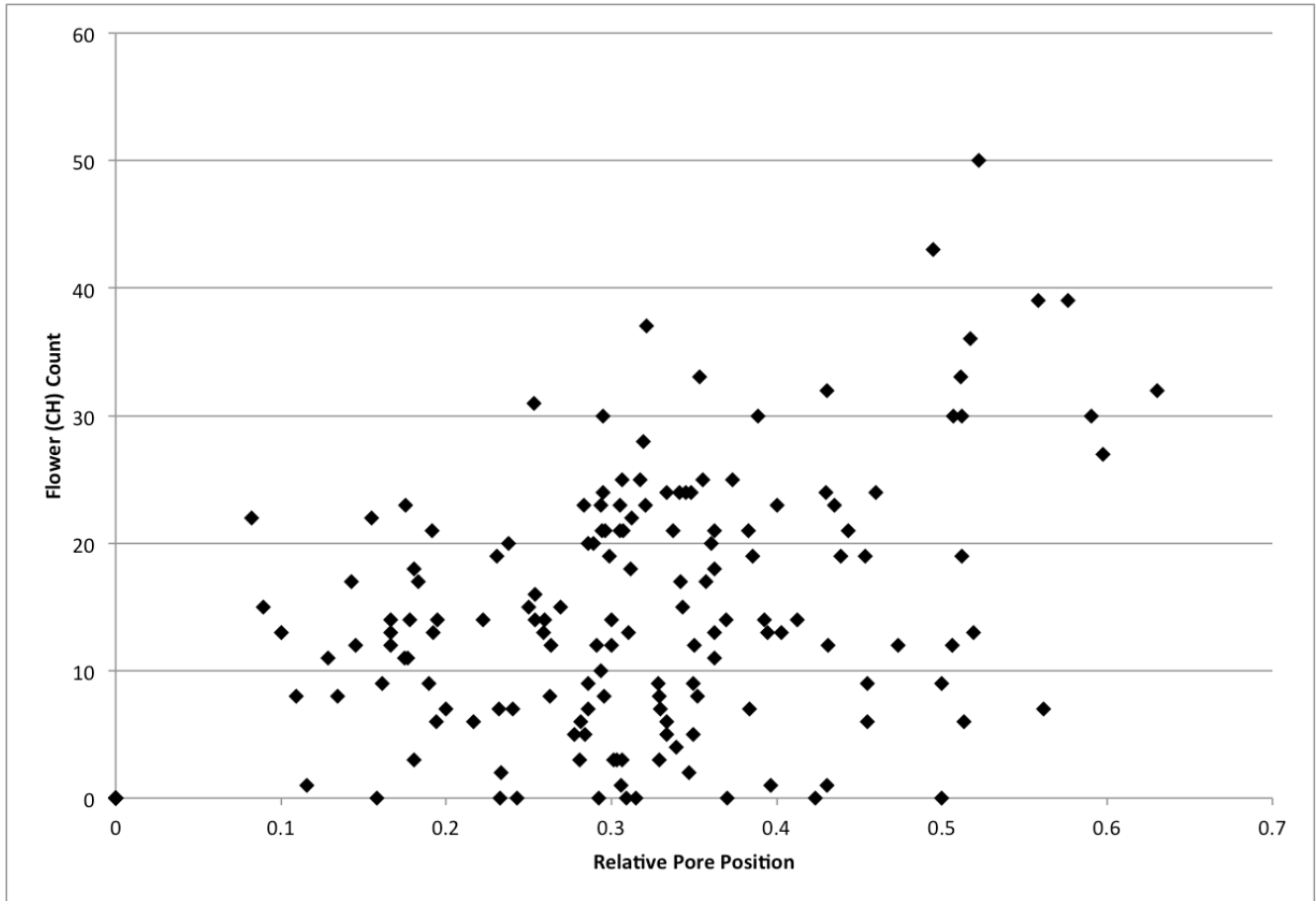


Figure 2: The morphological data for individuals that were produced when maternal *Triodanis perfoliata* subsp. *perfoliata* was pollinated in a mixed pollination, with a 50:50 mix of pollen from *Triodanis perfoliata* subsp. *biflora* and *Triodanis perfoliata* subsp. *perfoliata* is shown here. The relative pore position is the distance from the base of the fruit to the pore divided by the total distance of the fruit from bottom to top.

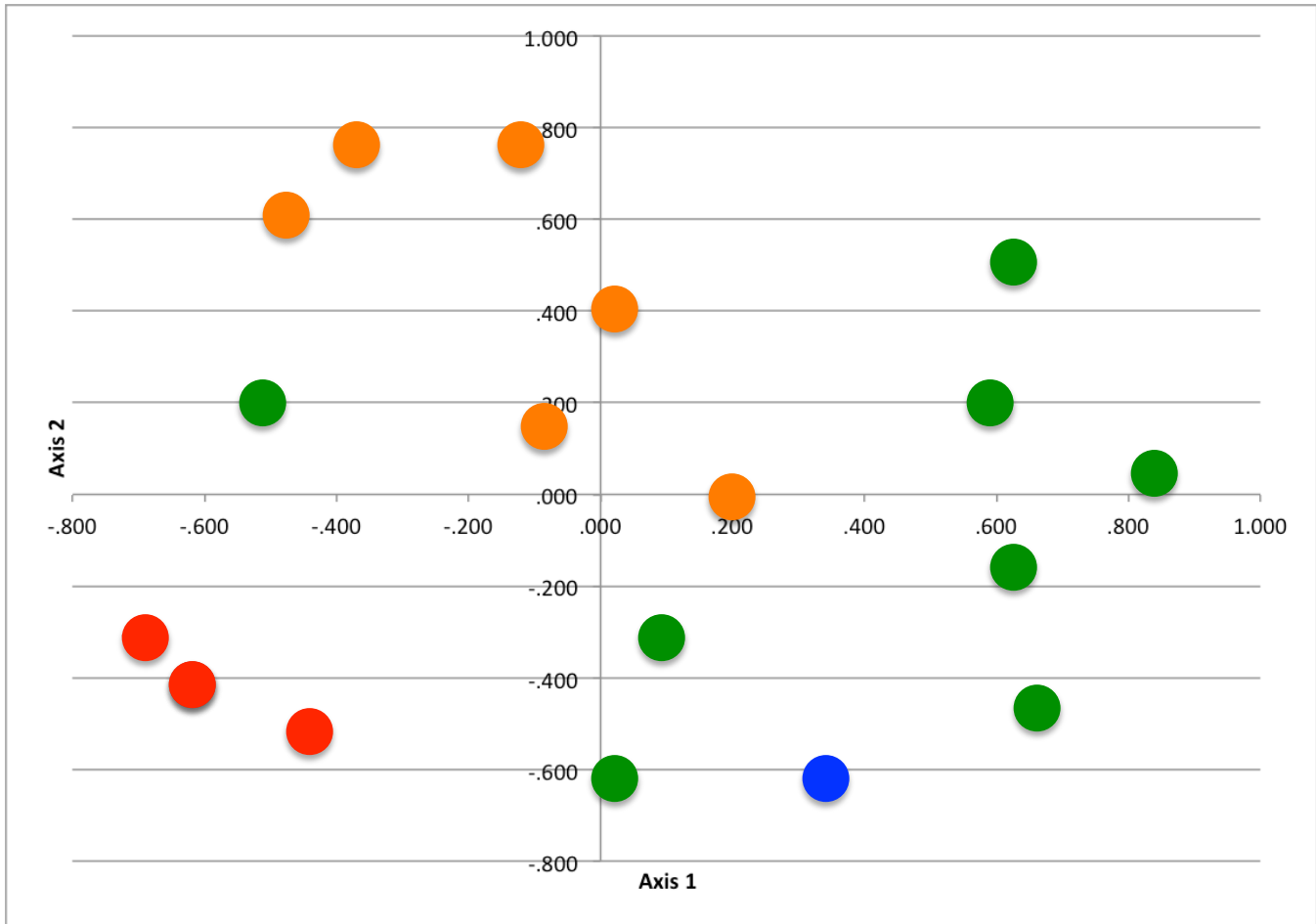


Figure 3: The plot shown is a non-metric multidimensional plot. The AFLP analysis was performed on offspring individuals of a mixed pollination. The colors on the graph represent putative identities of the individuals based on the morphological traits that were observed.

Color Key:

Red: Pure *Triodanis perfoliata* subsp. *biflora*

Orange: Hybrid *Triodanis perfoliata* subsp. *biflora*

Green: Pure *Triodanis perfoliata* subsp. *perfoliata*

Blue: Hybrid *Triodanis perfoliata* subsp. *perfoliata*

## Discussion

In *Triodanis perfoliata*, reproductive isolation seems to be occurring between the two subspecies. When mixed pollen was applied to subsp. *perfoliata* stigmas, fewer hybrids were produced than expected. In contrast, it was found that subsp. *biflora* readily accepted subsp. *perfoliata* pollen, meaning there were more hybrids produced in the offspring generation than expected. When extracting DNA from the individuals for comparison, AFLP scoring was done based on 33 polymorphic peaks that were generated through Gene Mapper software. This data seemed to support the visual trends observed. From this it can be concluded that subsp. *biflora* exhibited heterospecific pollen precedence and that subsp. *perfoliata* seemed to exhibit conspecific pollen precedence. After conducting the experiment to see if conspecific pollen precedence could be a contributing factor to isolation, it was found that pollen precedence was occurring asymmetrically between the two subspecies. However, it is important to note that the results lacked resolution in subsp. *perfoliata*. When plotting the relationships between individuals based on AFLP markers, subsp. *perfoliata* did not have any distinct sets of clusters that would suggest a separate hybridized group.

One possible reason for the lack of resolution in the results for subsp. *perfoliata* is plasticity in response to light. Plasticity refers to the ability of an individual to adapt its physiology in response to a change in the environmental conditions (Bradshaw, 1964). This phenomenon is very important for plants since

they are sessile and cannot move locations to escape unfavorable conditions. Light can be a stressing factor for plants if they are adapted to one type of light and receive a different treatment of light. It has been shown that in some cases, plants that receive low light will reallocate resources to their stems such as in a study on *P. peregrina* (Navas, 2002). In other cases, such as a study on a cleistogamous weed *Rueilia nudiflora*, in shaded treatments, plants exhibited an earlier end to the production of chasmogamous flowers than plants in direct light treatments (Munguia-Rosas, 2013). In the *Triodanis perfoliata* lab, each plant rack was placed in a different place that would theoretically get the same amount of light. However, due to the placement of the windows in relation to the rest of the room, it is possible that some racks of plants received more sunlight than others. It has been shown through the experimentation of a colleague that this increase in sunlight may lead to decreased chasmogamous flower production in *Triodanis perfoliata*. This may account for some of the subsp. *perfoliata* plants producing fewer chasmogamous flowers than expected and appearing more like hybrids. Placing the plants on a rotating cycle that ensures each rack received equal amounts of time in higher light levels could eliminate this variable in future studies.

Results suggest that pollen from subsp. *biflora* was outcompeted by subsp. *perfoliata* pollen. Several factors could explain this pattern. In the lab, pollen grain samples were collected from both subspecies and counted using a hemacytometer to view the number of grains. Through analysis, it was found that subsp. *biflora* produces fewer pollen grains per flower when compared to subsp. *perfoliata*. There

is no indication that the pollen produced by subsp. *biflora* was less viable than subsp. *perfoliata* however. But when applying the pollen to the stigma lobes, it is possible that, despite attempts to equalize the number of pollen grains deposited from each subspecies, subsp. *perfoliata* pollen was nevertheless more abundant and therefore achieved the dominant status that was seen in the results. Pollen tube growth rate differences may also be a cause of the differences seen in preferences of pollen. In some species of plants, differences in flower size encourage faster growth rate of pollen tubes in order to fertilize the plant. When this faster growing pollen is placed on a smaller flower, the pollen will fertilize the plant faster due to the shorter distance but the same growth rate. It is important to note however, in *Triodanis perfoliata*, the two subspecies have very similar flower size. Also, as previously mentioned, incompatibility of the tissue types between pollen and stigma may play a role in the growth rate. However, based on the results seen in subsp. *biflora*, it can be concluded that there are no compatibility issues between the pollen of one species and the stigma of the other.

*Triodanis perfoliata* is divided into two subspecies with distinct trait sets and they are reproductively isolated from each other to a degree. These two subspecies grow sympatrically in much of the United States and Canada. However, the subspecies can also hybridize and create viable offspring. The results of this study suggest that conspecific pollen precedence is not an important mechanism of reproductive isolation between the two subspecies. Pollen precedence appears to be asymmetric between both subspecies. In subsp. *biflora*, it appears that pollen

precedence is promoting hybridization, which is the opposite of reproductive isolation. In subsp. *perfoliata*, it appears that there is some conspecific pollen precedence, which could serve to restrict gene flow into subsp. *perfoliata*. Other experiments on *Triodanis perfoliata* have shown that the same pollinators visit both subspecies, and both subspecies also have the same flowering phenologies. Therefore, neither of these factors contributes to reproductive isolation either. Instead, it is thought that reproductive isolation may be attributed to the cleistogamous or selfing activity of the plant. A high volume of cleistogamous flowers means that there are fewer flowers that are available for hybridization. It has also been seen that each subspecies prefers different microenvironments, with subsp. *biflora* preferring low sand content in its soil and high light conditions, and subsp. *perfoliata* preferring high sand content in its soil and lower light conditions. These different preferences may also prove to be reproductively isolating.

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