Chapter 7

Reproductive Isolation in Angiosperms

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Contents

Introduction

This exercise is intended to illustrate several forms of isolating mechanisms in angiosperms as exhibited by three sympatric species of *Silene* (catchfly or campion). The experimental portion of the exercise consists of pollinating flowers of one species, *S. alba*, with pollen from conspecifics and from the two other species (*S. dioica* and *S. noctiflora*), then observing pollen tube growth in excised styles. *Silene alba* is a particularly suitable subject for several reasons: the plants are dioecious, eliminating the need to emasculate flowers before pollination; the five styles are completely separate, allowing replicate observations from a single flower; and the flowers are large enough (1.5–2.0 cm long) to be easily manipulated and show impressive pollen tube growth. All three species are native to Europe but are now naturalized as weeds in North America. They can also be readily raised from seed in a greenhouse.

The exercise takes up to 90 minutes, including some waiting time for pollen tubes to stain, and so can be readily combined with other activities in a longer lab period. It is appropriate for an evolutionary lab in an introductory course that presents the biological species concept and fits well with a lab exercise on plant-pollinator coevolution. Alternatively, *Silene alba* can be used by itself in a plant biology lab to show pollen tube growth, a distinctive event in angiosperm reproduction. Pollen tubes growing in stylar tissue are much longer than those produced by grains placed in an *in vitro* germinating medium. In more advanced genetics or evolution courses, especially those at summer field stations, students could examine pollen tube growth (and fruit set) in crosses using locally available native North American species to determine whether reproductive barriers have evolved in geographically separated species and if so, what kind.

Materials

Female flowers of *Silene alba*, already pollinated (one for every 1–2 students)
Female flowers of *Silene alba*, unpollinated (one for every 1–2 students, or as needed for lab the next day)
Male flowers of *S. alba* (1–2 for each female flower of *S. alba* to be pollinated)
Male flowers of *S. dioica* (1–2 for each female flower of *S. alba* to be pollinated)
Flowers of *S. noctiflora* (1–2 for each female flower of *S. alba* to be pollinated)
Display plants, one each per lab, of *S. alba* (male and female), *S. dioica* (only male required), and *S. noctiflora*
Labels, paper self-adhesive; red, green, and blue (one sheet of each color per lab)
Flasks, small, from 25 to 125 ml (2 per lab group, or to hold 3–4 flowers each; one flask for already pollinated flowers, one for unpollinated flowers)
Hotplate, marked with setting for 45–55°C (1 per lab group of 4–6 students)
Petri dish, glass, bottom (deeper) half only for use as water bath (1 per lab group of 4–6 students)
Thermometer (1 per lab group of 4–6 students)
Ring stand with clamp to hold thermometer (1 per lab group of 4–6 students)
Beaker, 10 ml, labeled "a," "d," or "n" (1 beaker per student)
Beaker, 100 ml, filled with water and placed with hotplate (1 per lab group of 4–6 students)
Forceps (1 pair per student)
Needles, dissecting (1 per student)
Scissors, fine-pointed (1 pair for each pair of students)
Pencil, wax (1 per lab group of 4–6 students)
Buffer, pH 6 (10 ml per student)
NaOH, 6 N (4–6 ml per student)
Aniline (methylene) blue stain, 0.1 percent in pH 6 buffer (1–2 ml per student)
Bottle, 125 ml glass amber labeled "buffer" (1 per lab group of 4–6 students)
Bottle, 125 ml glass screw top, labeled "NaOH" (1 per lab group of 4–6 students)
Bottle, 500 ml glass screw top, labeled "NaOH waste" (1 per lab group of 4–6 students)
Bottle, 30 ml dropping, labeled "aniline blue" (1 per lab group of 4–6 students)
Bottle, 30 ml dropping, labeled "distilled water" (1 per lab group of 4–6 students)
Bottle, 125 wash labeled "distilled water" (1 per lab group of 4–6 students)
Plate, porcelain with 12 concavities for staining (1 for each pair of students)
Pipettes, Pasteur (4 per lab group of 4–6 students, two by hot plate and two by staining plates)
Slides, glass microscope (3 per student)
Coverslips, glass (6 per student)
Microscope, dissecting (1 per student)
Microscope, compound (1 per student)

Student Outline

Background

According to the biological species concept, a species can be defined as a group of organisms that are capable of interbreeding and that are reproductively isolated from other groups under natural conditions. Reproductive isolation can be achieved in a variety of ways that fall into two broad categories: *pre-zygotic (pre-fertilization) isolating mechanisms,* which act to prevent the formation of hybrid zygotes, and *post-zygotic (post-fertilization) isolating mechanisms*, which lead to reproductive failure after fertilization has occurred. The two categories are hierarchical. Post-zygotic mechanisms come into play if pre-zygotic mechanisms have not worked, and include both the failure of an embryo to develop and the formation of hybrid offspring that are sterile or have greatly reduced fitness.

Pre-zygotic isolating mechanisms can themselves be divided into two categories depending on when they act: *pre-mating isolating mechanisms*, which prevent mating from taking place, and *post-mating isolating mechanisms*, which prevent gametes from fusing once mating has occurred. Post-mating mechanisms are often less noticeable than pre-mating ones, and include failure of the sperm to penetrate the egg, or especially in angiosperms, failure of a pollen grain to germinate on the stigma or failure of the pollen tubes to grow through the style to the ovary. Note that post-zygotic mechanisms are by definition also post-mating.

Pre-mating mechanisms are quite varied. The simplest is *geographical separation;* the potentially interbreeding groups simply do not exist together. However, in the absence of accompanying trait differences to indicate evolutionary divergence, this form of isolation alone is usually not enough to warrant designating a separate species. In areas where potentially interbreeding species do co-occur, pre-mating isolation can be maintained in a variety of ways: ecologically, temporally, mechanically, or behaviorally. In *ecological isolation* the species live in the same geographical area but occupy different habitats and thus do not come into contact with each other. *Temporal isolation* is similar, but with the separation occurring in time rather than space; the species are active or reproduce in different seasons or even times of day. In *mechanical isolation* the species are unable to mate successfully because of morphological incompatibilities. In insects this may be due to the shape of the male genitalia, while in flowers it may due to the location of anthers and the placement of pollen on a pollinator's body. *Behavioral isolation* is achieved by differences in the courtship and mating rituals that animals use to recognize members of their own species. Without the proper actions or signals by a potential partner, mating is inhibited.

Isolating mechanisms arise during the course of speciation as groups that have become reproductively separated diverge evolutionarily. A number of these mechanisms may be operating simultaneously, and effective reproductive isolation may depend on several partial mechanisms acting together. Where groups that have differentiated come into contact once again, some of the mechanisms may be strengthened by natural selection since they prevent the formation of less fit hybrid offspring. Why might you expect selection to be much more likely to act on pre-zygotic mechanisms than on post-zygotic ones?

Compared with mobile animals, plants have the additional complication in mating that they must depend on external agents, wind or animals, to deliver male gametes from one individual to another. Individual plants thus have no direct control over where their pollen goes (i.e. who they actually "mate" with), and as a result a certain amount of pollen may end up on the wrong stigmas. However, because pollination and fertilization are two separate events, a plant can still avoid a potential loss of fitness from producing hybrid seed if the pollen that it receives from another species fails to germinate, or if the pollen tubes fail to reach the ovules. Such post-mating isolation is common in

angiosperms and is a consequence of interactions between the pollen grains and the stigma or style. These interactions give the pollen-receiving plant considerable control over fertilization; in many angiosperm species the interaction between the style and the pollen also prevents self-fertilization in the event a plant is pollinated with its own pollen. (Why might it be advantageous for a plant that usually outbreeds to avoid self-fertilization?)

Pollen Tube-Style Interactions and Post-Mating Isolation in *Silene*

In this exercise you will examine pollen-style interaction in three species of *Silene* ("campion" or "catch-fly"): *S. alba*, *S. noctiflora*, and *S. dioica*. All are European species with overlapping geographical ranges; they are also now established in North America. *Silene alba* and *S. noctiflora* are both weedy species of disturbed habitats—fields and roadsides—and in Europe may be found growing together. *S. dioica* also occurs in disturbed habitats but it prefers moister conditions and is more common in woodlands. *S. noctiflora* and *S. alba* both bloom at night and produce white, tubular flowers that are scented when the flowers are open, distinctly so in *S. noctiflora. Silene dioica*, on the other hand, produces red, mostly unscented flowers that open in the day. As its name suggests, it has separate male and female flowers, like *S. alba* but unlike *S. noctiflora*, which has perfect flowers (stamens and pistils in the seam flower.) See the illustration of flowers of *Silene alba* in Figure 7.1.

Observe the potted examples of these three species on display in the lab, and identify the floral differences and similarities described above. (Note: For the purposes of the lab, the flowers of *S. alba* and *S. noctiflora* used for the pollination experiments have been kept cool and shaded to reduce their tendency to wilt and close during the day.) Despite having similar floral morphologies, these three plants can be readily separated into different species. For example, *S. dioica* and *S. alba* differ in flower color, time of blooming, and appearance of the capsule (fruit). *Silene noctiflora* differs from *S. alba* and *S. dioica* in having flowers with both pistils and anthers. Also, female flowers of *S. alba* and *S. dioica* usually contain five styles while flowers of *S. noctiflora* typically have only three styles. Given that these plants represent morphologically distinct species, the question then is: by what means are they reproductively isolated, and more specifically, can post-mating isolation due to pollen-stigma interaction help prevent gene flow between pairs of species?

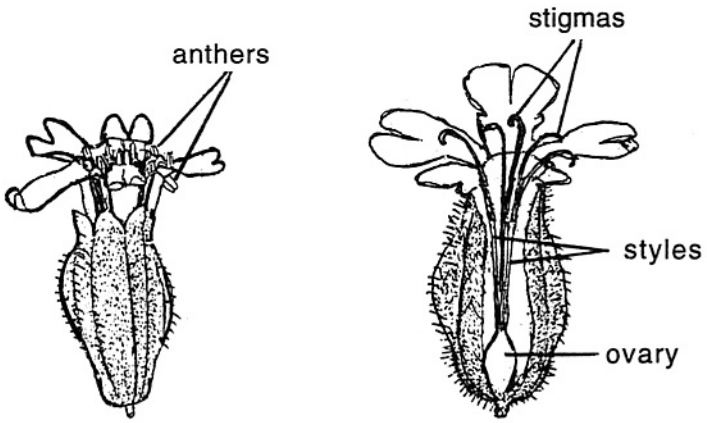


Figure 7.1. Male flower of *Silene alba* (left) and female flower (right, shown in cutaway view). Drawings are about 1.5 ∞ life size.

A full investigation of this latter question would require matings between all pairwise combination of the three species, using each species as both a female parent—pollen recipient—and as a male parent—pollen donor. (How many combinations are involved?) To simplify matters, you will use only *S. alba* as a female parent and will compare the performance of pollen tubes from *S. alba*, *S. dioica,* and *S. noctiflora* in the *S. alba* styles.

Which of the three pollination treatments is the control?

Based on the descriptions in the paragraphs above, propose a hypothesis concerning the relative performance of pollen from the three species in the *S. alba* styles. Do you think the performance will differ among donor species, and if so, how and why?

Preparation of Styles

1. Examine a flowering stem of *S. alba* and identify marked flowers that were pollinated the day before with pollen from either *S. noctiflora*, or a male of *S. dioica*, or a male of *S. alba.*
   Look at flowers on your table and adjacent tables. Do flowers in the three pollination treatments look the same? If not, how do they differ?
   Cut off a flower from *one* of the treatments. If there are fewer flowers than people at your table, ask your instructor about obtaining a flower from another group; otherwise work with a partner. Your instructor will ensure that each table has at least one flower from each pollination treatment.
2. Using forceps, carefully remove the styles and put them in a 10-ml beaker labeled "**a**" for pollination by *S. alba*, "**d**" for pollination by *S. dioica*, or "**n**" for pollination by *S. noctiflora*.
3. Add concentrated (6 N) NaOH to the beakers until they are about one fourth full. *Be careful with the NaOH; it is lye and will burn skin.*
4. With the other pairs of students at your lab table, heat water in a glass Petri plate on a hot plate to 45–55°C, and place the beakers in the water bath. To save time, turn the hot plate on ahead of time and start with hot tap water in the Petri plate. The hot NaOH softens and clears the styles, making them more transparent and allowing the stain to penetrate and reach the pollen tubes.
5. Let the styles soften for 5–15 minutes. To help ensure that they soften evenly, use forceps to "dunk" them until they stay mostly submerged. *Then put the forceps in a large beaker of water by the hotplate to avoid dripping hot NaOH on the lab table!* Initially the styles will look frosted white, but as they clear they will become a pale, translucent green. Keep a close watch on your styles and remove the beaker from the water bath as soon as the styles become translucent over their entire length. If you leave them in too long they will break apart. The time needed to reach the translucent stage varies considerably (5–15 minutes), depending on the age of the style, its thickness, and especially the temperature of the water. Check the water with a thermometer periodically, and try to keep it in the 45–55°C range.
6 Using a Pasteur pipette, remove all of the NaOH from the beaker and put it in the NaOH waste bottle at your table. Try not to suck up the styles, which will now have the limp consistency of well-cooked spaghetti. Fill the beaker with distilled water, and soak the styles for five minutes to leach out the NaOH. Repeat this step, placing the water removed from the beaker in the waste bottle and letting the styles soak in the newly added water for five more minutes. Then again remove the water, and this time fill the beakers halfway with a pH 6 buffer solution to neutralize the NaOH. Let the styles soak in the buffer for five minutes. Pipette out the buffer, fill the beakers halfway with buffer a second time, and allow them to soak for an additional 4–5 minutes.
7. Working in pairs, use the wax pencil to label a row of concavities on a porcelain staining plate "**a**," "**d**," or "**n**." Gently transfer the softened styles to an appropriately labeled depression,

putting 2–3 styles into one depression. Next add several drops of 0.1% aniline blue stain in pH 6 buffer, and if necessary, use forceps to completely submerge the styles. Let the styles stain for 20–30 minutes. Then carefully remove the stain with a Pasteur pipette (again avoiding the styles) and replace it with water. Let the styles rinse for approximately one minute.

8. Label three microscope slides and make *separate* wet mounts of 1–2 styles from each treatment as described below. Each person at a table should obtain all three kinds of styles, and none of the stained styles at a table should be left unexamined. Share styles with students in other groups as directed by your instructor. To mount your styles proceed as follows. Place the styles on the slide, and with a dissecting needle gently arrange them so that they are extended smoothly over their full length. For very long styles you may need to curve one end gently so that the entire style will fit under the coverslip. Because you want to be able to trace the paths of the pollen tubes, it is important not to let the styles become tangled with each other or fold back on themselves. Check that the tip of the style, the stigma, is free and clearly visible. Then add a small drop of water and a coverslip.

Applying Pollination Treatments

While you are waiting for the styles to stain during step 8 above, pollinate flowers for the next day's class. Your instructor will tell you how many flowers of each treatment your lab group should do; *wait for instructions before proceeding.* If necessary, carefully cut off any left over tags from flowers already used, then apply a green tag marked "**a**" for flowers receiving *S. alba* pollen, a blue tag marked "**n**" for flowers receiving *S. noctiflora* pollen, or a red tag marked "**d**" for flowers receiving *S. dioica* pollen. Obtain a pollen-donor flower of the appropriate type, and with forceps remove a mature, open anther with visible pollen. Examine the anther under a dissecting scope to be sure you see pollen grains. Touch the anther to the stigma at the tip of each style of a labeled flower. Verify that you have been an effective pollinator by looking for a dusting of pollen grains adhering to the stigma surface, the distal 1–2 mm of the style. Be sure to pollinate *every* style on the labeled flower; for best results you will need to use several anthers.

Observing Stained Styles

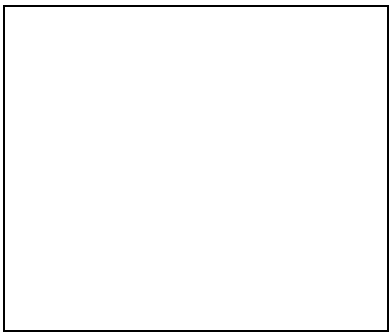
Examine your mounted, stained styles, starting with those pollinated with *S. alba* pollen. For a good overview, use your dissecting microscope. If the flower was successfully pollinated, you should see a swarm of blue-stained pollen tubes. Pollen tubes contain a compound, callose, that is preferentially stained by aniline blue. Depending on how well the stain penetrated the style, the tubes may or may not be stained through their entire length. Sketch a whole style in the box below.

Do the tubes reach the base of the style?

How are pollen tubes arranged in the style? Are they bunched together or does each one follow a separate path? What does this suggest about pollen tube growth through the style?

Are the pollen grains mostly darkly or lightly stained? (Note: more stain is taken up by grains that fail to germinate or that produce only short tubes.)

Style of *Silene alba* pollinated with *S. alba* pollen



Detailed view of stigma showing pollen grains and tubes

Now flatten the style slightly by applying *gentle* pressure on the coverslip with a pencil eraser or wooden end of a dissecting needle. Examine the style under your compound scope. Notice the numerous finger-like projections at the tip and extending part way down the style. These define the region of the stigma.

What do you think is their function?

Trace several pollen tubes back to the grain where they originate, and see how they emerge.

In the box above, sketch a detailed view of part of the stigma showing pollen grains and their tubes.

Under the compound microscope (and possibly the dissecting scope) you will also likely see a blue-stained strand running the length of the style. Do not confuse this with a pollen tube. Rather, this is a strand of *vascular tissue*. Observe that the cells of the xylem, the vessels elements, are characterized by helical thickenings of the cell wall and so look like long springs. The presence of vascular tissue in the style is evidence that the pistil of a flower is an evolutionary modified leaf; essentially what you are seeing is a reduced leaf mid-vein.

Now examine the styles that received pollen from *S. noctiflora* and *S. dioica*, and compare them with the styles receiving *S. alba* pollen. Sketch a style from each treatment in the boxes below.

How many pollen tubes (or approximately what proportion of the tubes) reach the base of the style? How far do most of them get?

Try tracing the tubes from various pollen grains. How do they compare with the tubes of *S. alba* you examined earlier?

How darkly are the pollen grains stained?

Style of *Silene alba* pollinated with *S. noctiflora* pollen

Style of *Silene alba* pollinated with *S. dioica* pollen

Report your observations to your instructor, who will tabulate results for the class as a whole.

Thought Questions

1. What do you conclude about the effectiveness of pollen-style interactions as an isolating mechanism separating *S. alba* from *S. noctiflora* and *S. dioica*?
2. Based on your observations of pollen-style interactions, with which species is *S. alba* most likely to form hybrids? What mechanisms contribute to keeping these two species reproductively isolated? How do these species differ in the pollinators they attract?
3. Why might it not be surprising to find differences in pollen tube growth among different crosses between the same combination of species? What factors can you think of that could cause such variation? (Hint: Compare the selective pressures acting on the female plant and on the pollen grain landing on a stigma.)
4. Flowers of *Silene alba* treated with pollen from two native, eastern North American species, *S. virginica* (fire pink) and *S. cucubulus* (bladder campion) both show good pollen tube growth in the styles yet seeds fail to develop. How might you explain these results?

Notes for the Instructor

Two problems to watch out for in this exercise are overcooking the styles in NaOH and failure to completely remove the NaOH before staining. The styles are ready to be rinsed when they turn from white to a translucent pale green or yellow; the change is very much like that seen when sautéing a chopped onion. Often the styles clear unevenly over their length, with whitish patches persisting in some regions longer than others. Also, the cut end at the base of the style may take longer to clear than the remainder of the tissue. Styles should be removed from the NaOH once all of the regions above the base have turned translucent. The time required for the styles to clear can vary from 5–15 minutes or more, depending on the style's age, thickness, and especially the temperature of the NaOH. To avoid clearing the styles too rapidly and risk having them fall apart, it is best to calibrate the hotplates before lab to determine a setting that yields a temperature in the Petri plate water baths in the range of 45–55°C.

The cleared styles must be thoroughly rinsed because the aniline blue stain turns yellow in basic solutions, providing no contrast between the pollen tubes and surrounding stylar tissue. Although the multiple rinses with distilled water and buffer should be sufficient to remove and neutralize all of the NaOH, if the staining solution starts to turn lighter blue or clear, the style should be removed and soaked again in buffer before continuing. If the styles are very soft from the clearing treatment, it is especially important to pipette out the rinsing solutions carefully to avoid breaking them.

As part of the exercise, students pollinate the flowers to be used by classmates the next day. (Other arrangements are of course required to provide pollinated flowers for the first lab sections.) Because it takes at least 6 hours for maximum pollen tube growth, it is not feasible for a morning section to do the pollinations for the same afternoon. Success with student-applied pollination

treatments requires close supervision by the instructor. To avoid mixing up pollination treatments, flowers should be tagged individually immediately before a treatment is applied, and as much as possible the same treatment should be used on adjacent flowers on the same stalk or cut flowers in the same container (see below). It is also essential for the students to verify that the anthers they are using for pollination are indeed open with ample pollen exposed; this requires examining the anther under a dissecting scope. To ensure full pollination, *each* stigma on a flower should be touched with an anther twice. Assuming that the anthers were touched only to the stigmas, successful pollen transfer can be verified by re-examining the anthers under the dissecting scope and noting whether pollen was removed. It is also often possible to recognize pollinated stigmas with the naked eye under bright illumination because they look slightly more grainy.

It is not necessary to bring potted plants into each lab to provide the female *S. alba* flowers for the pollination treatments. Rather, flowering stems can be cut and placed in small flasks filled with water, provided that the stems are then re-cut underwater. The flowers will remain usable for up to 48 hours, 72 hours or longer if kept in a refrigerator. Long flowering stems can be cut again once students have applied the pollination treatments so that the flowers can be sorted into flasks containing a representative of each treatment for groups in the next lab. Although using cut flowers is more convenient than whole plants, a slight disadvantage of this technique is that buds that may otherwise have opened two or three days later usually do not continue to develop.

Flowers of *S. alba* typically wilt and close up during the day, but as long as they haven't abscised they are still viable and can be pollinated. Pollinating a wilted flower may first require moving the petals out of the way. However, the flowers will usually remain open if they are kept cool and out of bright light. Flowers of *S. noctiflora*, in contrast, almost always look very shriveled during the day, but they perk up remarkably in the evening and emit a strong sweet scent. It is sometimes possible to reverse their day/night cycle so flowers will be will be open during daytime lab hours, but this is not necessary to obtain pollen. Gently peeling back the rolled up petals will expose the anthers inside the corolla tube.

As is true in many species, flowers of *S. alba* shrivel soon after the ovules have been successfully fertilized and the petals are no longer needed to attract pollinators. As a result, flowers receiving pollen from a *S. alba* or *S. dioica* male often look more withered than those receiving *S. noctiflora* pollen. This pattern does not always hold because very young flowers, even when fertilized, may not wither as quickly as older ones, and as described above, all of the flowers may look wilted during the day regardless of pollination treatment. Fortunately, the condition of the petals does not affect the styles, although in thoroughly withered flowers the styles may have to be carefully disentangled from the petals.

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APPENDIX A
Expected Results and Answers to Thought Questions

Results

Under the dissecting microscope the pollen tubes in the control flowers, those receiving pollen from *S. alba*, look like bundles of thin, blue strands of spaghetti extending to the base of the style. (Students may mistake the strand of vascular tissue that runs the entire length of the style as a single large pollen tube.) The tubes occur in an intermingled group and are bunched together rather than distributed uniformly across the style. This suggests that the tubes interact with each other as they grow toward the ovules—with the fastest tubes leading the way—or that the maternal tissue of the style regulates how the pollen tubes grow. The control of pollen tube growth in styles is an area of active research; certainly the pollen tubes must depend on the styles for nutrition because the resources to produce a 150–200 mm long tube cannot be contained in a 27–32 μm pollen grain.

Under the higher magnification of the compound microscope it is possible to see the finger-like projections near the tip of the style that define the stigmatic region. These increase the surface area to which pollen grains can adhere and produce the compounds that stimulate pollen germination. The germinated pollen grains on control styles are largely empty, having emptied their contents into the growing pollen tubes, and thus they take up relatively little stain. In a well-stained style under 400x magnification, pollen tubes can be seen emerging from the pollen grains, one tube per grain. The individual pollen tubes are stained intermittently because callose, the pollen tube component stained preferentially by aniline blue, is not deposited continuously. (Note: callose is a branched glucose polymer found in a variety of plants tissues, notably the sieve plates of phloem cells. In pollen tubes it forms plugs at intervals as the tube elongates, shortening the cytoplasmic stream within the tube. It may also seal small holes in the tube during growth.) At the higher magnification it is also possible to discern the helical thickenings of the vessel members in the xylem tissue of the vascular strand. These are conclusive evidence that this strand of blue is indeed not a pollen tube. See Motten (1992) for black and white photographs of the pollen tubes in styles.

The styles receiving pollen from *S. dioica* look much like the controls, although sometimes a slightly smaller proportion of the tubes may reach the base of the style if the pollinations were performed less than 12 hours before the lab. Crosses between *S. alba* and *S. dioica* are compatible and produce fully fertile hybrids. These two species are thus reproductively isolated by other means—see below. In contrast, styles receiving pollen from *S. noctiflora* are characterized by numerous ungerminated pollen grains that appear dark bluish-brown. Most of the tubes extend only a short distance down the style, and with high magnification it is sometimes possible to find the end of tubes that have burst. In some styles, bands of pollen tubes may grow most of the length of the style with a few tubes reaching the bottom, but not in the high proportions found in the control or *S. dioica*-pollinated styles. These longer tubes apparently do not successfully fertilize the ovules as crosses between *S. alba* and *S. noctiflora* do not yield seeds (Prentice, 1978).

Note that not all styles stain the same way. Those that take up an excess of stain (or were not rinsed sufficiently well before being mounted) may have blue-tinted regions of tissue that could be mistaken for groups of pollen tubes, especially near the base of the style. Alternatively, thick styles, or ones that were not fully cleared, may not stain over their entire length, and the strands of pollen tubes will fade in and out along the style. Sometimes, too, the stigmatic tip of a style stain does not stain as well as the base, making it harder to see pollen tubes emerging from the pollen grains. It is thus a good idea for students to share styles to be sure of seeing a well-stained one for each treatment. They should also examine the control styles first to learn how to recognize the pollen tubes definitively.

Thought Questions

The pollen-style interaction effectively maintains complete reproductive isolation between *S. alba* and *S. noctiflora*, the two species that overlap most in habitat, blooming time, and pollinators. In contrast, pollen from *S. dioica* grows just fine in styles of *S. alba*, and these two species can form viable hybrid progeny. Reproductive isolation between the two species thus depends not on pollen-style interactions but instead on differences in habitat reinforced by differences in both flowering time and pollinators. The floral morphology of *Silene* favors lepidopterans, which can use their long tongues to reach the nectar at the base of the corolla. (Other insects may also visit the flowers for pollen.) However, butterflies are attracted to the pinkish red, day-opening flowers of *S. dioica* while moths are attracted to the night-opening, sweet-scented flowers of *S. alba* and *S. noctiflora*.

Variation in pollen tube growth is particularly evident in the *S. noctiflora*-pollinated styles. This variation reflects differences in the intensity of the interaction between different maternal (stylar) and pollen genotypes. Presuming that there is a fitness advantage to the female to prevent her ovules from being fertilized by pollen of another species, there should be selection to recognize foreign pollen and halt pollen tube growth or even to prevent pollen grain germination. On the other hand, once a pollen grain lands on a stigma, it has zero fitness unless it can successfully deliver the sperm nuclei to fertilize an ovule. There should thus be strong selection favoring germination and rapid pollen tube growth. Selection is all the more potent on pollen grains because they are haploid, and alleles affecting germination or pollen tube growth are constantly exposed and cannot be hidden in the heterozygous condition. The competing interests of the maternal parent and the pollen grains—male gametophytes—are thus likely to be expressed as variation in pollen tube performance if there is genetic variation among male or female parents.

Silene alba presumably evolved independently of *S. virginica* and *S. cucubulus* and had no contact with these species until humans brought *S. alba* to the New World. There has thus been no selection on *S. alba* styles to recognize and reject pollen from the North American species, unlike the situation between the co-occurring *S. alba* and *S. noctiflora*. However, during the time since *S. alba* last shared a common ancestor with the North American plants, the species have apparently diverged sufficiently far genetically that they can no longer form viable hybrid embryos or seeds.

APPENDIX B
Sources of Materials

Obtaining and Growing *Silene*

Silene alba is a widespread weed of fields, roadsides, and waste places across North America. It is common in the northern U.S. and southern Canada and ranges as far south as the mountains of North Carolina. *Silene noctiflora* has a similar distribution but seems to be somewhat less common. *Silene dioica* occurs locally in eastern North America as far south as Virginia. All three species bloom in the summer and set fruit in mid to late summer. If a suitable population can be located, there is little problem in obtaining seeds as hundreds are produced by even a single capsule. Regional floras can be used to locate and identify other, native species of *Silene*, a number of which occur throughout North America. A particularly dramatic one is *Silene virginica*, which has bright crimson flowers that attract hummingbirds. It occurs in woodlands in the eastern U.S. and blooms in early summer.

If seeds cannot be obtained from a natural population it may be possible to find a commercial supplier, although the weedy species of *Silene* are not in much demand horticulturally. One seed company that has listed *S. alba* in its catalog is Far North Gardens (16785 Harrison, Livonia, Minnesota 48154). Other suppliers that feature wildflower species offer several additional, mostly European-derived species. (Note: although *S. alba*, *S. dioica*, and *S. noctiflora* are thought to be closely related (Prentice, 1978), *S. alba* and *S. dioica* are sometimes listed in the genus *Lychnis* because they have five styles whereas *S. noctiflora* and many other members of the genus have three styles. Another genus name that has been used for these species is *Melandrium*. *Silene dioica* has also been described as *S. rubra*, and the most recent nomenclatural revision for *Silene alba* has this species as *S. latifolia*.)

Silene alba is easy to grow in a garden or greenhouse. The seeds need no special conditions to induce germination and soon form rosettes that produce flowering stalks after about 15 to 16 weeks. With continuous long-day illumination in a greenhouse *S. alba* omits the rosette stage and will flower in as few as 6–8 weeks. However, these plants produce few inflorescences so it is better to allow the plants to develop more fully vegetatively. If necessary, this can be encouraged by cutting off the initial inflorescences. Standard six inch or two quart pots work well for growing plants with multiple inflorescences. To stimulate flowering during the "off-season" (winter and spring) it helps to put the plants under high intensity lights that are on in the evening to create a 16-hour day and that also come on for an hour in the middle of the night to interrupt the dark period. Flowering from the rosette stage or in dormant individuals also seems to be encouraged by cold treatment, e.g., 4–6 weeks of temperatures between 0–5°C in a lighted cold room or protected outdoor location. Keeping plants in a lighted cold room is also an effective way to obtain flowers in the fall or early winter. Individuals that are flowering normally in summer can be cut back and stored in the cold for as long as needed. After being returned to warm, well-lighted conditions they will resume flowering in 3–4 weeks. Plants kept in the cold still need to be watered occasionally to prevent their drying out.

The cultivation requirements for *S. dioica* are similar to those of *S. alba*, but this species is more prone to vegetative growth and more recalcitrant about flowering. Long days with a high intensity light and/or a cold treatment are especially helpful. For established individuals, flowering also seems to be stimulated by application of a high phosphorous, low nitrogen fertilizer. Both *S. alba* and *S. dioica* are short-lived perennials, and if they are cut back after the flowers are used they will reflower for several seasons. *S. dioica* persists somewhat longer in cultivation than *S. alba* and can be propagated vegetatively by dividing the large, dormant rosettes, thereby saving the trouble of starting new plants from seed.

Silene noctiflora is an annual that grows from the seedling to the rosette stage in about eight weeks. These can be grown successfully in four inch square pots. Plants started in the fall are much more likely to bolt and flower if given a cold treatment of 4–6 weeks with temperatures below 5°C, then exposed to long-day conditions for 6–8 weeks. This seems to be less necessary for plants started in the spring and used in mid summer. Like *S. alba* and *S. dioica*, plants of *S. noctiflora* that are ready to flower in summer can be maintained in a cold room for use in the fall and winter. Unlike *S. alba* and *S. dioica*, seeds of *S. noctiflora* germinate better if given a cold treatment of 4–6 weeks at 4°C.

The number of plants needed to produce flowers for the exercise depends on their size and vigor. Females of *S. alba* growing well in six inch pots can be expected to bear up to 3–4 flowers during the course of a one week lab period, with individual flowers lasting four days if unpollinated. *Silene noctiflora* in four inch pots typically produce 1–3 flowers during a week. Males of *S. alba* and *S. dioica* are more prolific, producing 5–10 or more flowers a week. In all cases, however, it is desirable to have extra plants on hand because not all individuals may bear flower buds that develop on the same schedule as your lab. Especially for *S. dioica* and *S. noctiflora*, some plants may remain vegetative, or in the case of *S. noctiflora*, may produce some flowers that are pistillate and cannot be used for pollination. When starting *S. alba* and *S. dioica* from seed it is also important to grow extra plants to be sure of obtaining enough individuals of the correct sex as this cannot be determined until the plants begin to flower.

Other Supplies

The buffer used in the exercise is Carolina Biological's standard pH 6 buffer solution. Other commercially prepared buffers would presumably work as well. The aniline blue stain can be purchased as a powder from Carolina Biological; it is also available from Aldrich Chemical Company and Sigma Chemical Company. The stain is prepared as a 0.1% solution in the pH 6 buffer.

The porcelain plates with 12 concavities, used to stain the styles, are sold by Fisher ($15.00 US each) for observing color reactions. Styles could also be stained in deep depression microscope slides, watch glasses, or other similar shallow (1–5 mm deep) chambers. For tagging flowers, the Avery brand "self-adhesive removable labels" work well. The 0.25 inch by 0.75 inch labels can be cut in half lengthwise to fit more easily around the short stems under some flowers.