DISSERTATION

INVESTIGATION OF MULTIPLE INTERSPECIFIC REPRODUCTIVE BARRIERS IN *SOLANUM* SECTION *LYCOPERSICON*

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2016

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ABSTRACT

INVESTIGATION OF MULTIPLE INTERSPECIFIC REPRODUCTIVE BARRIERS IN *SOLANUM* SECTION *LYCOPERSICON*

Interspecific reproductive barriers (IRBs) act to prevent hybridization between close relatives and provide insight on how species maintain their integrity in nature. Wild tomato species (*Solanum* Section. *Lycopersicon*) are useful for studying IRBs. The monophyletic tomato clade includes 13 closely related species that possess a variety of mating systems and complex IRBs. IRBs can be classified according their operation during reproduction in plants; IRBs occurring before mating (premating prezygotic barriers), those operating after mating but before fertilization (postmating prezygotic barriers), and those acting after fertilization (postzygotic barriers). In the tomato clade, postmating prezygotic barriers regulating pollen tube growth in pistils are known to be important for preventing hybridization. Interspecific pollen rejection frequently displays the SI x SC rule, in which crosses between self-incompatible (SI) species and self-compatible species (SC) are successful in one direction but the reciprocal crosses fail, resulting in unilateral incompatibility (UI). This implies that mechanisms involved in SI and IRB systems overlap.

I tested multiple aspects of IRBs in the tomato clade at different points in reproduction. First, I assessed pollen grain size and style length among nine species in the tomato clade to test the hypothesis that larger pollen is required to traverse longer styles. I found no correlation between pollen grain size and style length, and thus it is unlikely that either of these factors act as a reproductive isolating mechanism among the wild tomato species. Second, I assessed pollen-pistil interactions in interspecific crosses among 13 species of tomato species in order to test the SI x SC rule in the tomato clade (*Solanum* sect.

Lycopersicon). I found that the SI x SC rule was generally followed at the species level, but exceptions to the SI x SC rule were observed with more recently evolved SC populations. My results further revealed differences in strength of both pistil and pollen IRBs in the tomato clade. Third, I assessed a series of IRBs between geographically co-occurring species of the tomato clade from 12 sympatric sites. My previous study assessed the relationship between interspecific populations that do not share range overlap, so this study was performed to understand IRBs in an ecologically relevant context. I did not find consistent reductions in stigma exsertion (which would contribute to lower outcrossing rates) of the SC species *Solanum pimpinellifolium* from sympatric sites, suggesting that this floral trait is unlikely to act as a reproductive barrier in this species. In six instances, I detected strong post-mating prezygotic IRBs, in which pollen tubes of SC *S. pimpinellifolium* were consistently rejected by pistils of their SI sympatric partner. I also identified a possible case of conspecific pollen preference (relatively slower interspecific pollen tube growth) in one sympatric species pair. In cases where prezygotic IRBs were not observed, I mostly found strong post-zygotic IRBs in the form of abnormal seed development in which embryos only progressed to the globular stage. Although I identified multiple IRBs between sympatric pairs, normal seed was formed in three crosses resulting in F1 hybrid plants. These studies suggest that most sympatric populations in the tomato clade exhibit a combination of prezygotic and postzygotic IRBs that prevent hybridization between species, although there may be exceptions. Finally, I investigated whether a low activity S-RNase protein (SI pistil factor) is involved in IRBs in the wild SC species *Solanum neorickii*. Populations of *S. neorickii* located at northern and southern margins of the distribution reject interspecific pollen and express a low activity S-RNase protein, whereas those in the center of the species range do not reject interspecific pollen and lack expression of the S-RNase. To determine whether this low activity S-RNase is sufficient for the observed IRB (or if another factor is involved), I crossed individuals from populations which show difference in S-RNase expression and interspecific pollen tube rejection and generated F1 hybrids and F2 lines. In the F2, I observed individuals that express S-RNase and reject interspecific pollen tubes, and those that lack S-RNase and are not capable of rejecting interspecific pollen tubes, as expected. However, I also observed individuals that express S-RNase but do not reject interspecific

pollen tubes. These findings suggest that a low activity S-RNase is necessary but not sufficient to reject interspecific pollen tubes in *S. neorickii*. The findings presented in my dissertation research are major advances that aid in our understanding of reproductive barriers in wild populations. Further, studies of reproductive barriers in tomato, a major food crop, have important implications for agronomic improvement. Many QTL conferring disease resistance, fruit quality and other important traits have been introgressed into cultivated tomato from wild species, but the success of introgression is often inhibited by reproductive barriers.

ACKNOWLEDGEMENTS

I here acknowledge many people who support contribute to the making this PhD. First of all, I would like to thank my advisor, Dr. Bedinger, for her patience support, many insights, and time spent mentoring. I was very fortune to earn my PhD with her and to be a part of her lab. I also want to thank my committee members Daniel Bush, Cris Argueso, and Mark Brick for their support and guidance.

I also thank people who are and were in Dr. Bedinger's lab, Amy Ashford, Amanda Broz, Paul Covey, Ashley Denny, Margaret Fleming, Laura Hantzis, Chris Miller, April Randle, Suzanne Royer, Shelley Sianta, and all of undergraduate assistances, Brie Genuchi, Nicole Irace, Lauren Nalezny, and Olivia Todd. In particular, I would like to thank Amanda Broz again for all of her efforts on mentoring me to accomplish my work including sharing ideas, answering all of my scientific or non-scientific questions and editing on thesis. I thank Ashley Denny for providing a great friendship and helping me to have a grammatical correct thesis. I thank to all of our collaborators, especially Alejandro Tovar-Mendez who not only participated in my research, but also gives a good friendship and advice.

Lastly, I really appreciated my family members who are really good supporters even though they are far away in Korea. I always appreciated for being loved.

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This chapter is a part of the published paper (Bedinger et al., 2011)

"Interspecific reproductive barriers in the tomato clade: Opportunities to decipher mechanisms of reproductive isolation."

Bedinger PA, Chetelat RT, McClure B, Moyle LC, Rose JK, Stack SM, van der Knaap E, Baek YS, Lopez-Casado G, Covey PA, Kumar A, Li W, Nunez R, Cruz-Garcia F, Royer SM.

Sexual Plant Reproduction 24:171-187

Author's contributions

I participated in prezygotic barriers section as measuring pollen grain size and style lengths.

Acknowledgments

This work was supported by the National Science Foundation, grant number DBI-0605200. We thank Ms. Ashley Denney and Ms. Margaret Fleming for helpful editing

Chapter 3: Testing the SI x SC rule: pollen-pistil interactions in interspecific crosses between members of the tomato clade (*Solanum* Section *Lycopersicon,* Solanaceae)

This chapter has been published (Baek et al., 2015).

"Testing the SI x SC rule: Pollen-pistil interactions in interspecific crosses between members of the tomato clade (*Solanum* section *Lycopersicon,* Solanaceae)."

Baek YS, Covey PA, Peterson JJ, Chetelat RT, McClure B, Bedinger PA.

American Journal of Botany 102(2):302-11

Acknowledgements

The authors thank the Charles M. Rick Tomato Genetics Resource Center for seeds, A. Ashford for plant care, A. Martin, O. Todd, B. Genuchi and N. Allen for help with compositing microscopic images, Dr. S. Royer for help preparing figures, and Drs. A. Tovar-Mendez, A. Broz, and A. Randle for critical reading of the manuscript. This work was supported by grant number DBI-0605200 and MCB-1127059 from the Plant Genome Research Program of the National Science Foundation.

Author's contributions

I obtained and analyzed most of the data with some contribution from other authors.

Chapter 4: Interspecific reproductive barriers between sympatric populations of wild tomato species

This chapter is being submitted for publication.

Baek YS, Royer SM, Broz AK, McClure B, Covey PA, López-Casado G, Nuñez R, Kear P, Bonierbale P, Orillo M, Stack SM, Chetelat CT, and Bedinger P.A. Interspecific reproductive barriers between sympatric populations of wild tomato species.

Author's contributions

Yousoon Baek and Suzann Royer contribute equally in this publication.

I measured stigma exertions of members from 10 sympatric sites at Colorado State University. I assessed pollen pistil interaction in 10 sympatric sites to test interspecific pollen tube rejection. In case where pollen tube rejection was not observed, I performed crosses and collected interspecific fruits and seeds. Also, I analyzed fruit characters, measuring size and weight.

Acknowledgements

This article is dedicated to the memory of Dr. Matilde Orillo. The authors thank the Charles M. Rick Tomato Genetics Resource Center for most seeds used in this study and thank Universidad Técnica

Particular de Loja for seeds imported from Ecuador under a Materials Transfer Agreement between Colorado State University and Universidad Técnica Particular de Loja, export authorization 009-2014-IC-FAU-DPL-MA. We thank A. Ashford for plant care, and A. Martin, O. Todd, T. Randall, B. Genuchi and L. Kassenbrock for help with compositing microscopic images. This work was supported by grant number DBI-0605200 and MCB-1127059 from the Plant Genome Research Program of the National Science Foundation

Chapter 5: Testing whether a low activity S-RNase is involved in interspecific pollen tube rejection in the wild tomato species *Solanum neorickii*

This work will be published in the future.

Baek YS, Tovar-Mendez A, Bedinger PA.

Author's contributions

I performed all crosses, performed genomic PCR and RT-PCR on samples I collected. I obatined stylar extracts and Dr. Tovar-Mendez performed immnuoblots on those samples.

Acknowledgements

This work was supported by grant number DBI-0605200 and MCB-1127059 from the Plant Genome Research Program of the National Science Foundation

CHAPTER 1

INTRODUCTION

The Biological Species Concept (BSC) defines a species as "a group of populations that are potentially able to interbreed in nature" (Mayr 1942). Interspecific Reproductive Barriers (IRBs) preserve species integrity by preventing interspecific gene flow for hybridization, a crucial aspect of the biological species concept. Understanding and identifying which reproductive isolating mechanisms occur between closely related taxa will demonstrate how species maintain their integrity when they co-occur sympatrically (co-occur) in the wild. Numerous reproductive isolating barriers have been identified that preclude or reduce gene flow, preventing hybridization between species (Dobzhansky 1937; Mayr 1942; Ramsey et al., 2003; Coyne and Orr 2004; Rieseberg and Willis 2007; Lowry et al., 2008; Baack et al., 2015). These reproductive barriers have been classified depending on the timing of their occurrence during reproduction (Levin 1971; Grant 1981).

In plants, premating prezygotic barriers act before pollination and represent an initial barrier to gene flow between species. This type of barrier can involve geographic isolation, changes in floral morphology, or differences in floral traits that lead to pollinator shifts (pollinator preference). Geographic isolation between different taxa in plants greatly reduces or prevents contact of two lineages and thus reduces the opportunity for gene flow (Mayr, 1963; Schemske, 2000; Sweigart et al., 2007). Even when different lineages are in contact, different species/taxa may effectively be isolated from each other due to local adaptation to defined environments or microhabitats that exhibit distinct soils, temperatures, or herbivores (Stebbins, 1950; Anderson et al., 2014; Anacker and Strauss, 2014; Oakley et al., 2014; Baack et al., 2015). Despite overlapping of ranges between different taxa, plants cannot co-occur at fine spatial scale because they are restricted to specific environmental niches.

Ecological divergence of species that occur in sympatry is also often related to flowering timing, i.e. phenology. For example, the flowering time of *Clarkia parviflora*, which produces flowers during the

dry season, shows very little overlap with the flowering time of *C. xantiana*, a species which produces flowers much later in the season (Briscoe Runquist et al., 2014). Fishman et al. (2014) identified two major QTLs (LG7 and LG8) responsible for photoperiod of flowering using an introgression line between summer-flowering *M. guttatus* and spring-flowering *M. nasututs*. The different phenologies of *M. guttatus* and *M. nasututs* can be fully explained by these two QTLs (Fishman et al., 2014).

Morphological traits can also reduce the probability of mating even when lineages co-occur (Grant, 1981; Levin, 1971). Flower morphologies including floral size (i.e., style length), shape, or location of floral organs can prevent hybridization between species. For example, in some cases pollen from short-styled species cannot traverse long-styled species, leading to a failure of sperm delivery (Darwin, 1884; Williams and Rouse, 1990; Lee et al. 2008). Evolutionarily, reduction in flower size and a more inserted stigma are often closely associated with the transition from outcrossing to self-compatibility (SC). Changes to both of these traits make plants more likely to be self-pollinated (autogamy) than cross-pollinated (allogamy), since smaller flowers attract fewer pollinators and inserted stigmas receive more self-pollen (Rick et al., 1978; Peralta and Spooner, 2005; Georgiady, 2002; Chen et al., 2007; Goodwillie et al., 2010; Sicard and Lenhard 2011). It was found in the tomato clade that stigma exsertion is controlled by a few genes. Chen et al. (2004) identified a single QTL on chromosome 2, *stigma exsertion 2.1,* and found that one locus controlling style length (*Style 2.1*) had the greatest impact on stigma exsertion. It is likely that mutations at this locus have contributed to the evolutionary trend from allogamy to autogamy in the cultivated tomato and closely related SC red-fruited species.

In plants, premating barriers can involve complex interactions between traits of both flowers and pollinators, since many flowering plants rely on external insect pollinators. Differences in floral traits like floral color or scent have been found to influence pollinator preference (Grant and Grant 1965; Grant 1994; Bradshaw et al., 1995; Bradshaw and Ramsey 2003; Ramsey et al., 2003; Hoballah et al., 2007; Cooley et al., 2008; Whitehead & Peakall, 2009; Hopkins and Rausher, 2012; Xu et al., 2012). In orchids, increased nectar rewards are generally found in larger flowers that are more visible than their smaller counterparts (Blarer et al., 2002).

Floral color is one of the best-studied premating barriers in plants. For instance, differences in gene expression and/or loci for pigment synthesis pathways are known to be directly responsible for flower color differences leading to changes in preference by pollinators such as *YUP* (*YELLOW UPPER* - yellow pigment concentration locus), *AN2* (*ANTHOCYANIN2,* transcription factor involved in purple pigmentation synthesis) (Bradshaw and Schemske, 2003; Hoballah et al., 2007). Pink flowers of *M. lewiskii* and red flowers of *M. cardinalis* have different pollinators, bumblebees and hummingbirds, respectively. The *YUP* allele in *M. lewiskii* prevents the deposition of carotenoid resulting in pink color flowers, whereas *M. cardinalis* contains the *yup* allele that allow the deposition. Near isogenic lines (NILs) were produced by repeated backcrossing to the two species to obtain substitution of one species' *YUP* allele for the other and show that pollinator shifts among NIL plants depend on alleles of *YUP* (Bradshaw et al., 1995; Bradshaw and Schemske, 2003). In another example, *Petunia integrifolia* and *P. axillaris* are also reproductively isolated due to pollinator specificity which has been linked to differences in floral color. *P. intagrifolia* has bee-pollinated purple flowers while *P. axillaris* has mothhawk-pollinated white flowers. An alteration on a single locus, *AN2,* was sufficient to lead to a shift in pollinator preference (Hoballah et al., 2007). Hopkins and Rausher (2012) have also shown that the concentration of anthocyanin pigmentation controls floral color intensity and reduces shared pollinators between *Phlox drummondii* and *P. cuspidata*.

Although premating prezygotic barriers play an important role in preventing hybridization between species, pollination between species does still occur. Once pollen grains land on the stigmatic region of pistils in pollination, postmating prezygotic barriers can act to prevent hybridization for gametic isolation. In compatible crosses, pollen grains first land on the stigma followed by adhesion of pollen grains, "foot" (pollen coat-stigma) formation, hydration and germination of pollen grains. Next, germinated pollen tubes grow through the style and into the ovary (Cheung, 1996; Dresselhaus and Franklin-Tong, 2013). However, postmating prezygotic barriers can prevent interspecific pollen tube delivery of sperm cells to ovules (egg cells) any time during pollen-pistil interactions, thus leading to failure of fertilization.

Postmating prezygotic barriers can occur in plants that have dry stigmas when pollen grain adhesion or hydration is prevented. This barrier is controlled by interactions between the extracellular pollen coat

and stigmatic papillae. In *Brassicas*, two major determinants of this stigmatic pollen rejection are the stigma specific S receptor Kinases (SRK) and small pollen coat proteins, *S*-locus cysteine rich or *S*-locus protein 11 (SCR/SP11). Genes encoding these proteins are tightly linked and polymorphic. Although the interaction between stigma and pollen coat is important in self-incompatibility, it can also prevent interspecific gene flow between species. For example, much lower adhesion of pollen from non- Brassicaceae species donors was observed on the stigmas of *Brassica oleracea* and *A. thaliana* (Hiscock and Diskinson, 1993; Luu et al., 1999; Zinkl et al., 1999). The failure of pollen grain adhesion shows unilateral incompatibility (UI) that often follows the SI x SC rule, in which crosses between pistils of SI species and pollen of SC species fail, but the reciprocal crosses are successful. For instance, pollen tube penetration failed on SI *B. oleraceae* pistils when pollinated with different Brassiceae species pollen, while pollen grains from wide range of species were able to grow in pistils of SC *A. thaliana* (Hiscock and Dickinson, 1993). This suggests that the pollen-stigma mechanisms involved in the self-incompatibility response are related to interspecific interactions.

Other families of plants that have wet stigma surfaces will allow almost any kind of pollen to germinate but can inhibit pollen tube growth in styles. In Solanaceae, pollen tube rejection occurs in the self-incompatibility response to prevent self-fertilization, but pollen tube rejection also occurs after pollination in interspecific crosses and has a critical role in preventing hybridization. Self-incompatible pollen tube rejection is controlled by *S*-locus factors, with pistil-side determinant S-RNase, and pollen side determinant *S*-locus F-box proteins. Non *S*-locus factors such as pistil HT proteins and pollen ubiquitin ligase complex components Cullin1 and Skip1 have also been implicated in pollen tube rejection (Kao and Tsukamoto, 2004; McClure, 2004; McClure and Franklin-Tong, 2006; Kubo et al., 2010; Li and Chetelat, 2014). Interspecific pollen tube rejection often displays UI according to the SI x SC rule, as described above. For example, pistils of SI *S. pennellii* reject pollen from domesticated tomato, SC *S. lycopersicum*, while pistils of *S. lycopersicum* do not reject SI *S. pennellii* pollen tubes (Murfett et al., 1996). While there is overlap of mechanisms between SI and IRB systems in pollen tube rejection in the Solanaceae, SI system-independent interspecific reproductive barriers also exist. In the tomato clade, SC *S. habrochaites* that does

not express S-RNase and is unable to reject self-pollen can reject pollen from *S. lycopersicum* (Covey et al., 2010). Further details and complexities of UI in the tomato clade will be discussed more detail below.

Another postmating prezygotic barrier is conspecific pollen preference. Conspecific pollen preference refers to slow relative growth of interspecific pollen tubes compared to faster-growing conspecific pollen tubes, which leads to lower rate of delivery of interspecific sperm to egg cells. In these cases, mixed-pollen (inter- and conspecific pollen) is loaded on the stigma, conspecific pollen dominates in fertilizing ovules and producing progeny (Ramsey et al., 2003; Fishman et al., 2008).

Upon pollen tube arrival in the ovary, a positive interaction is required between pollen tubes and ovules by species-specific chemical attraction for successful fertilization. Once pollen tubes enter the ovary, pollen tubes can be directionally guided to target ovules by female derived chemoattractants (Marton et al., 2005; Higashiyama et al., 2006; Escobar-Restrapo et al., 2007; Takeuchi and Higashiyama, 2012). The mechanistic basis of female chemoattraction has been well-studied in *Torenia* and *Arabidopsis*, and involves cysteine rich polypeptides (CRP) called LURE proteins which are expressed in synergid cells of ovules (Higashiyama et al., 2006; Okuda et al., 2009; Takeuchi and Higashiyama, 2012). *In vitro* experiments to test species-specific pollen-ovule interactions have been done in both *Torenia* and *Arabidopsis* systems. For example, higher attraction rates were found in intraspecific versus interspecific pollen-ovule signaling in *T. fournieri* and *T. concolor* (Kanaoka et al., 2011). In addition, there was significantly less attraction of *A. thaliana* pollen tubes to *A. arenosa* ovules, suggesting ovule targeting acts as an important barrier to interspecific hybridization in some species (Takeuchi and Higashiyama, 2012).

Although prezygotic barriers contribute largely to reproductive isolation, postzygotic barriers are also significant in some plant species (Lowry et al., 2008; Baack et al., 2015). The range of postzygotic barriers is very wide and includes seed development, hybrid seed germination, hybrid necrosis, hybrid sterility, and hybrid breakdown. These interspecific hybrid incompatibility can result from genetic conflicts between parents or epigenetic incompatibility.

Although pollen tubes can successfully deliver sperm to egg cells in ovules, hybrid seed formation can still fail due to abnormal embryo development. Hybrid seeds generated between diploid *A. thaliana* and

A. arenosa display abnormalities of seed development such as arrest of embryo and delayed development (Josefsson et al., 2006; Burkart-Waco et al., 2012), resulting in seed death. This may be associated with genome dosage of parents, since hybrid seeds between tetraploid *A. thaliana* and diploid *A. arenosa* were able to produce almost normal seeds whereas crosses between equal ploidy parents produce less than 10% viable seeds (Josefsson et al., 2006). A recent transcriptome study comparing developing seeds of hybrid and compatible crosses found that genes involved in seed coat regulation and endosperm were mis-expressed in hybrids. In addition, several genes involved in immune response were hyper-activated in hybrid seeds, and could possibly be responsible for the observed seed death (Burkart-Waco et al., 2012 and 2013).

In cases where hybrid seeds undergo full development and germinate, F_1 hybrid plants can display hybrid necrosis or lethality (Sawant, 1956; Ramsey et al., 2003; Bomblies et al., 2007; Yamamoto et al., 2010). For example, hybrid necrosis occurs when a disease resistance allele from one species interacts incorrectly with another allele from the other species, resulting in an autoimmune response and damage to hybrids (Bomblies, 2010): *Cladosporium fulvum* resistance locus (*Cf*-2) in tomato, nucleotide-binding leucine-rich-repeat (NB-LRR) in *Arabidopsis,* and defense-related gene RIN4 (*RPM1 interacting protein4*) in lettuce (Langford, 1948; Kruger et al., 2002; Bomblies et al., 2007; Jeuken et al., 2009).

F_1 hybrids are often sterile due to genetic incompatibility, and this type of postzygotic hybrid incompatibility is common in plants (Moyle and Graham, 2005; Kubo et al., 2008; Bomblies, 2010; Yamamoto et al., 2010). Sterility in hybrid plants is often related to chromosomal rearrangements such as inversions (modification of the order of genes within a chromosome) or translocations (transfer of genes onto a different chromosome). However, other factors can also influence this phenotype. For instance, cytoplasmic male sterility can occur due to variations in mitochondrial genes that are tightly coupled to nuclear genes. In the rice hybrid between *indica* and *japonica,* three linked loci are involved in female hybrid sterility, in which abortion of ovules results from two loci for killer phenotypes and loss of protector allele at a third locus (Li et al., 1997; Ouyang and Zhang, 2013).

Even when hybrids are fertile, other defects are often detected. Some fertile hybrids express an

intermediate phenotype that results in low fitness in parental environments. This type of hybrid breakdown, due to low fitness, can be observed in both the F1 and in following generations (Stebbins, 1958; Rick et al., 1976; Rundle and Whitelock, 2001; Rhode and Cruzan, 2005; Baack et al., 2015).

The tomato clade

The wild tomato species (*Solanum* sec. *Lycopersicum*) provide an excellent model system for studies of Interspecific Reproductive Barriers (IRBs) (Bedinger et al., 2011). The tomato clade comprises 12 wild species related to the single domesticated tomato according to recent taxonomic studies (Fig. 1.1, Peralta et al., 2008; Rodriguez et al., 2009). The distribution range of these wild species is from central Ecuador through Peru to northern Chile on the western Andean Slopes, and includes two species endemic to the Galápagos Islands (Rick, 1979; Darwin et al., 2003; Peralta et al., 2008; Moyle, 2008; Rodriguez et al., 2009). Diverse mating systems, morphological characteristics and habitat preferences are evident among different wild tomato species (Moyle, 2008; Peralta et al., 2008).

There are three major types of mating systems exhibited in the tomato clade: autogamous self-compatibility (SC), facultative SC, and self-incompatibility (SI). *S. lycopersicum, S. galapagense, S. cheesmaniae, S. pimpinellifolium*, and *S. neorickii* are autogamous SC species, and, as such, they accept their own pollen tubes and make fruits without a requirement for pollinators. Facultative self-compatible species such as *S. chmielewskii* are self-fertile but possess floral morphology traits that promote outcrossing and genetic diversity. Allogamous self-incompatible species reject self-pollen, which forces outcrossing and maintains genetic diversity. Additionally, there are species that are mostly SI but contain some SC populations such as *S. arcanum, S. habrochaites,* and *S. pennellii* (Rick et al., 1978; Peralta and Spooner, 2005; Moyle, 2008; Bedinger, 2010).

Within the tomato clade, the *Lycopersicon* group is comprised of four species that are self-compatible (SC; autogamous) with red to orange fruits and are hereafter referred to as "red-fruited" species. These include *S. lycopersicum* (the domesticated species), *S. pimpinellifolium*, and the two species endemic to the Galápagos Islands, *S. galapagense* and *S. cheesmaniae*. The *Arcanum* group consists of 3 species, two of which are SC – *S. chmielewskii* and *S. neorickii* – and one SI species with one SC population, *S.*

arcanum. The *Eriopersicon* group contains five SI species: *S. habrochaites*, *S. peruvianum*, *S. corneliomulleri*, *S. chilense*, and *S. huaylasense*. *Neolycopersicon* has a single SI member, *S. pennellii*, which is considered to be the most distantly related member of the tomato clade (Peralta and Spooner, 2001; Peralta et al., 2008).

All tomato species are diploid (2n = 24) with a high degree of synteny (Chetelat and Ji, 2007). Many tomato genetic resources are available including complete domesticated genome sequencing and genomic resources for wild species, extensive collections of wild species, collections of expressed sequenced tags, and mutants (Moyle, 2008; Bedinger et al., 2011; Tomato Genome Consortium, 2012, 2013; Lin et al., 2014).

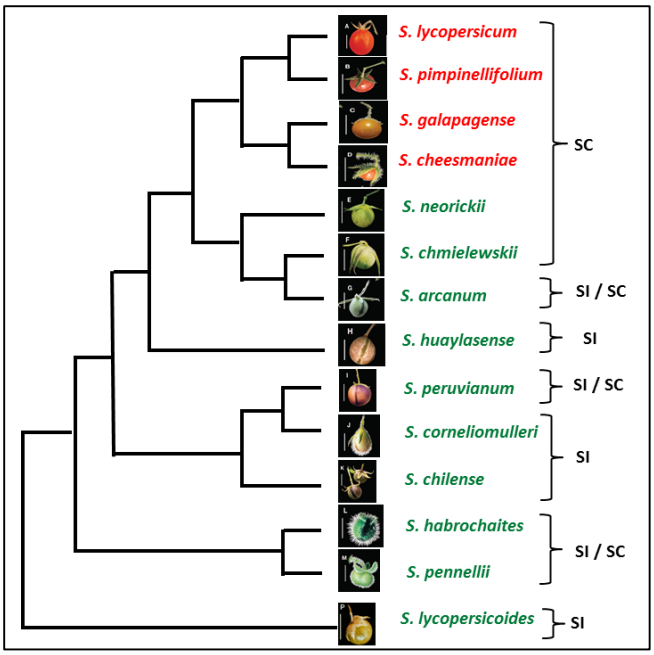

Figure 1.1 a) Phylogenetic tree of the tomato clade. SC= self-compatible, SI=self-incompatible, SI/SC= SI populations and SC populations wihtin a species. Red colored species: species produce red-fruited species, green colored species: species produce green-fruited species. (Modified from Peralta et al., 2008; Bedinger et al. 2010).

Pollen-pistil interactions: pollen tube rejection

Self-Incompatibility in the tomato clade

Self-incompatibility (SI) is a widely distributed prezygotic intraspecific reproductive barrier in angiosperms. The SI system is controlled by the highly polymorphic *S*-locus containing female and male

determinants. In the SI system, female tissue recognizes and inhibits self-pollen tubes to prevent self-fertilization, which enforces outcrossing with genetically different individuals of the same species (de Nettancourt, 1997). The SI system is well understood at the molecular level. There are two systems of SI, sporophytic and gametophytic, which evolved independently. Both types of SI are controlled by male and female recognition proteins that are encoded at the complex *S*-locus. The sporophytic SI system (SSI) is found in at least 7 plant families including Brassicaceae (Igic et al., 2008). In SSI the interaction between pollen coat proteins from the tapetum and receptors in the stigmatic papillae determines self-pollen rejection (Takayama and Isogai, 2005; Chapman and Goring, 2010). Gametophytic SI systems (GSI) are found in more than 36 plant families including Solanaceae, and represents one of the best understood pollen rejection mechanisms (McClure, 1989; Kao and Tsukamoto, 2004; McClure, 2004; McClure and Franklin-Tong, 2006; Igic et al., 2008; Kubo et al., 2010; Li and Chetelat, 2014). In GSI pollen rejection occurs when the single *S*- haplotype of the haploid pollen matches with either of the two *S*-haplotypes in the diploid style.

In the most common form of GSI, the S-locus-encoded stylar secreted ribonuclease, S-RNase, is the known female determinant of self-incompatibility (SI) (Anderson et al., 1986; McClure et al., 1989). Gain-of-function and loss-of-function experiments in transgenic systems have demonstrated that S-RNase is required for the recognition and rejection of self-pollen tubes. For example, expressing an *S-RNase* gene from SI *Nicotiana alata* in SC *Nicotiana* allowed for the recognition and rejection of pollen tubes expressing *N. alata* genotypes (Murfett et al., 1994). In addition, transformation of the S_3*-RNase* gene from SI *Petunia inflata* into plants with the S_1S_2 genotype allowed rejection of S_3 pollen tubes (Lee et al., 1994). When *Petunia inflata* S_3-RNase was suppressed by introducing an antisense *S-RNase* gene construct, S_3 pollen tubes were not rejected (Lee et al., 1994), demonstrating that S-RNase is required for the recognition of the corresponding pollen S genotype.

Known S-RNases have been characterized as containing five conserved regions (C1 to C5) and two hyper-variable regions (Ioerger et al., 1991). The C2 and C3 conserved regions contain a histidine residue associated with enzymatic catalysis (Kawata et al., 1988; Ioerger et al., 1991). Ribonuclease activity of S-RNase is required to reject self-pollen tubes (Huang et al., 1994). Pistils of SC *S. arcanum* (LA2157)

express S-RNase protein, however, this S-RNase has low enzymatic activity due to loss of the histidine residue at the active site of the enzyme (Kowyama et al., 1994; Royo et al., 1994). The two hyper-variable regions are responsible for haplotype recognition. For example, substitution of the hypervariable regions between two S-RNases of different alleles (S_1 and S_3) led to loss of specificity for the rejection of self-pollen in transgenic *Petunia inflata* (Kao and McCubbin, 1996). In addition, alteration of four amino acids in the hypervariable region led to loss of self-incompatibility in transgenic *Solanum chacoense* (Matton et al., 1997).

In pollen, the determinants of male specificity encoded by the S-locus are F-box proteins (SLFs), which are part of a ubiquitin ligase E-3 complex (known as the SCF complex, Skp1, Cullin, F-box) that is involved in ubiquitin-mediated protein degradation (Lai et al, 2002; Qiao et al., 2004; Hua and Kao, 2006; Kubo et al., 2010). Sijacic et al. (2004) performed transformation experiments in *Petunia* to confirm SLF as a candidate gene in the SI system, in which the *PiSLF2* gene was transformed into SI S_1S_1 plants. In the normal response, pistils of S_1S_1 plants reject pollen carrying the S_1 haplotype and do not reject pollen carrying the S_2 haplotype. Only those pollen expressing *PiSLF2* were able to grow through the styles of transformed S_1S_1 plants (Sijacic et al., 2004).

Other non-*S*-locus pistil factors are also required for the SI system. McClure *et al.* (1991) identified the first pistil-specific 'modifier genes' of the SI response in *Nicotiana* species. The identified HT proteins are small, around 100 amino acids, and asparagine rich. HT proteins are secreted into the transmitting tract of the style and are expressed late in style development (McClure *et al*., 1999). Suppressing HT-B expression in the pistils of hybrids between SI and SC *Nicotiana* using an antisense construct attenuated the ability for *S*-specific pollen rejection (McClure et al., 1999). Two paralogous genes encoding HT proteins, *HT-A* and *HT-B*, were subsequently discovered in *Solanum chacoense* (O'Brien, 2002). *HT-A* and *HT-B* are tandemly repeated and tightly linked on chromosome 12, located 1.57 kb apart in *S. lycopersicum* and 4.5 kb apart in *S. habrochaites* (Covey et al., 2010). Kondo et al. (2002) examined both *HT* genes in cultivated tomato *S. lycopersicum* and other wild tomato species. They detected point mutations in the coding regions of *HT-A* and *HT-B*, as well as reduced *HT-B* transcript expression in styles of all SC species.

However, Covey et al. (2010) found that *HT-B* genes in all SI and SC *S. habrochaites* contain premature stop codons, resulting in truncated HT-B proteins. This suggests that the SI system does not require both *HT-A* and *HT-B* genes, at least in SI *S. habrochaites*.

Pistil factors other than HT proteins are involved in the SI system including S-RNase binding (Cruz-Garcia et al., 2003, 2005). For example, in *Nicotiana*, a style-specific 120-kD arabinogalactan protein (120K) formed complexes with S-RNase and is involved in *S*-specific pollen tube rejection, since suppressing 120K in transgenic plants resulted in a failure to reject *S*-specific pollen tubes (Hancock et al., 2005).

Although the GSI system in the Solanaceae is one of the best characterized, it is still unclear how exactly S-RNase inhibits pollen tube growth. Two different pollen tube rejection models have been suggested, a degradation model and a compartmentalization model (Kao and Tsukamoto, 2004; Goldraij et al., 2006; Hua et al., 2008; Zhang et al., 2009; Kubo et al., 2010; McClure et al., 2011). Both models suggest that incompatible and compatible pollen tube growth is determined by the interaction between S-RNase (pistil determinant) and SLF (pollen determinant), and that pollen tube rejection results from degradation of pollen RNA. The degradation model proposes that S-RNases act as S-allele-specific cytotoxins that degrade RNA from self-pollen tubes after being taken up into the pollen tube cytosol. According to this model, non-self SLFs provide resistance to the cytotoxic effects of S-RNase by recognizing and degrading non-self S-RNases through ubiquitin-mediated protein degradation (Kao and Tsukamoto, 2004; Hua et al., 2008; Zhang et al., 2009; Kubo et al., 2010). The compartmentalization model was proposed based on immunolocalization experiments showing that both compatible and incompatible pollen tubes take up S-RNases (self or non-self) and sequester them in membrane-bound organelles (i.e., vacuoles). In the case of incompatible pollen tubes, vacuoles containing S-RNases are disrupted and the S-RNases are released into pollen tube cytoplasm, resulting in pollen tube rejection. In compatible interactions, S-RNase proteins are unable to exit the vacuole and cannot exert their cytotoxic activity in growing pollen tubes. Since it was observed in the immunolocalization experiment that HT-B is stable in incompatible pollen tubes but degraded in compatible pollen tubes, HT-B might be involved in the breakdown of compartmentalization

leading to cytotoxic effects in incompatible pollen tubes (Goldraij et al., 2006; McClure et al., 2011); although this has not yet been proven.

Interspecific pollen tube rejection

While the SI system prevents inbreeding and helps to maintain genetic diversity within a species, interspecific reproductive barriers (IRBs) prevent gene flow between species so that a species' genetic integrity can be maintained. These IRBs often show unidirectional pollen tube rejection, called unilateral interspecific incompatibility (UI).

The UI system often follows the SI by SC rule (Lewis and Crowe, 1958; Martin, 1967; Hogenboom, 1973), in which pistils of SI species reject pollen tubes of SC species, while pollen tubes of SI species are not rejected by pistils of SC species in the reciprocal cross (Lewis and Crowe, 1958; Liedl et al., 1996; Covey et al., 2010; Tovar-Mendez et al., 2013). The SI x SC rule suggests that there is a relationship between the SI system, which dictates the ability to reject self-pollen tubes, and interspecific pollen tube rejection (i.e. UI). Previous studies strongly support an overlap of SI and UI mechanisms. For example, one UI QTL maps to the *S*-locus in *S. habrochaites* while another UI QTL maps to a region on chromosome 12 encoding HT proteins (Bernacchi and Tanksley, 1997; Covey et al., 2010). Direct evidence that SI factors are involved in rejection of other species' pollen tubes has been provided more recently. On the pistil side, Tovar-Mendez et al. (2014) showed that transgenic introduction of two SI factors, *S-RNase* and *HT-A/B*, into the SC cultivated tomato (*S. lycopersicum*), which lacks both SI factors, recapitulated the rejection of pollen tubes from red-fruited SC species.

A pollen side UI factor *ui6.1* was identified by QTL mapping and encodes the Cullin 1 protein, an essential component of the SCF complex. The pollen UI factor *ui6.1* is only functional in conjunction with *ui1.1*, another pollen UI QTL located at the *S*-locus (Li et al., 2010; Li and Chetelat, 2010). A recent study found that *ui1.1* encodes an *S*-locus F-box protein (SLF-23) in pollen of *Solanum* (Li and Chetelat, 2015). Suppressing the pollen UI factor *Cullin 1* in *Solanum arcanum* caused self-pollen tube rejection, which suggests that *Cullin1* is also involved in SI (Li and Chetelat, 2014). It is thought that other components of

the SCF complex such as Skp1 may also be involved in interspecific pollen rejection (Hua and Kao, 2006; Li and Chetelat, 2010).

Although there is significant mechanistic overlap between SI and UI systems, UI can nonetheless occur in an *S-RNase*-dependent or -independent fashion. For example, SC *S. pennellii* (LA0716; note that *S. pennellii* is mostly SI, but there are SC populations) is able to reject pollen from red-fruited species, despite the fact that SC *S. pennellii* LA0716 does not express S-RNase (Covey et al., 2010; Chalivendra et al., 2013). This suggests that pollen tube rejection of red-fruited species is due to an S-RNase independent system in this population. In *Nicotiana*, the class III pistil-specific extensin-like protein, PELPIII, has also been shown to be involved in (S-RNase independent) species-specific pollen tube inhibition. Antisense experiments demonstrated that suppression of PELPIII in pistils of SC *N. tabacum* led to loss of the ability to inhibit pollen tubes of *Nicotiana obtusifolia* and *N. repanda* (Eberle et al., 2013).

Overview of experiments

My thesis includes four chapters that demonstrate how interspecific reproductive barriers in wild tomato species prevent hybridization.

Chapter 2: Testing for a correlation between pollen grain size and style length that potentially contributes to reproductive isolation

In chapter 2 I examined the correlation of pollen grain size and style length in members of the tomato clade to see whether the combination of these factors could act as a reproductive barrier. In previous studies, a correlation between pollen grain size and style length led to the hypothesis of pollen grain provisioning, in which pollen grains contain only enough nutrients to grow through styles of certain lengths (Delpino, 1867; Torres, 2000; Aguilar et al., 2002). However, in other plants, these traits were not correlated as single sized pollen grains were able to traverse variable style lengths (Darwin, 1884). The failure of sufficient pollen provisioning could act as premating reproductive barrier if pollen from short-styled species failed to traverse to the ovaries of long-styled species.

Chapter 3: Testing the SI x SC rule: pollen-pistil interactions in interspecific crosses between members of the tomato clade (*Solanum* Section *Lycopersicon,* Solanaceae)

In Chapter 3 I examined post-mating prezygotic barriers in wild tomatoes by analyzing pollen tube growth in interspecific crosses. Most studies of prezygotic UI barriers (interspecific barriers) use the cultivated tomato. However, this species is not found in natural populations and thus, the relevance of these experiments to the presence of IRBs in the wild is unknown. For this reason, I examined pollen tube growth using crosses of all members of the tomato clade in both directions to find actual IRBs acting in wild tomato species. Results of this chapter supported the mechanistic relationship between SI and UI and additionally revealed some SI-independent UI cases, indicating additional genetic factors are involved in the UI response.

Chapter 4: Interspecific reproductive barriers between sympatric populations of wild tomato species

In Chapter 4 I examined interspecific barriers between wild tomato species from twelve sympatric sites to investigate how IRBs function in the wild to maintain species integrity. Four types of reproductive barriers were examined: 1) stigma exsertion (premating barrier), 2) interspecific pollen tube rejection (postmating prezygotic barrier), 3) conspecific pollen preference (postmating prezygotic barrier), and 4) fruit and seed set (postzygotic barrier). I compared stigma exsertion between allopatric and sympatric SC *S. pimpinellifolium* populations to test a pattern of less exserted stigma in sympatry as a reproductive barrier. Also, I examined whether UI barriers act between sympatric pairs by assessing pollen tube growth in interspecific crosses. In cases where pollen tube rejection was absent, fruit development and seed set were assessed to see whether postzygotic barriers act to prevent hybridization.

Chapter 5: Testing whether a low-activity S-RNase is involved in interspecific pollen tube rejection in the wild tomato species *Solanum neorickii*

In Chapter 5 I tested the hypothesis that differences in expression of a low-activity S-RNase protein correlate with the observed variability in rejection of pollen tubes of red-fruited SC species in pistils of

different groups of SC *S. neorickii*. Using four distinct geographic groups of *S. neorickii*, I carried out cross-pollinations to examine pollen-pistil interactions with red-fruited species, assessed allelic variation in S-RNase in expression and examined expression of S-RNase proteins. Inter-group hybrids were created to further examine the correlation between *S-RNase* variability and interspecific pollen tube rejection. Results from these experiments will clarify the relationship between this SI factor (*S-RNase*) and IRBs in *S. neorickii.* Additional experiments are being performed by our collaborators.

CHAPTER 2

TESTING FOR A CORRELATION BETWEEN POLLEN GRAIN SIZE AND STYLE LENGTH THAT POTENTIALLY CONTRIBUTES TO REPRODUCTIVE ISOLATION[1]

Pollen size, style length, and stigma architecture

It has been proposed that pollen size (or more directly, ‘‘pollen provisioning’’) can limit growth in pistils (Torres 2000; Aguilar et al. 2002). Indeed, in some plant species a positive correlation of pollen grain size and style length has been demonstrated, supporting the idea that larger pollen carry more provisions and can therefore traverse longer styles to reach the ovary (Delphino 1867; Cruden and Lyon 1985; Aguilar et al. 2002). Some dramatic examples of this include heterostyled species where the shorter styled morphotype has larger pollen grains than the longer styled morphotype (Delphino 1867; Cruden and Lyon 1985; Williams and Rouse 1990). However, as Darwin concluded (1884), there are many exceptions to this tenet, both within heterostyled species where pollen grain size can be identical in the two morphotypes, and between species wherein pollen grains are similarly sized but must grow greatly varying distances in styles. While style length does not vary as much in the tomato clade as in some other taxa (Lee et al. 2008), there is more than twofold variation in style length, from 5.35 to 11.76 mm, and threefold variation in pollen size (volume), from 4,419 to 13,388 μm^3 (Fig. 2.1). It should be noted that pollen size variation within the tomato clade shown here is in general agreement with that reported by Garcia (2007) and Chetelat et al. (2009).The results shown in Fig. 2.1 indicate that *S. habrochaites* pollen grains are among the smallest in the tomato clade—only *S. arcanum* has smaller pollen. However, *S. habrochaites* styles are the longest found in this clade. *S. habrochaites* style length is very similar to that

[1] BEDINGER, P. A., R. T. CHETELAT, B. MCCLURE, L. C. MOYLE, J. K. ROSE, S. M. STACK, E. VAN DER KNAAP, et al. 2011. Interspecific reproductive barriers in the tomato clade: opportunities to decipher mechanisms of reproductive isolation. *Sexual Plant Reproduction* 24: 171-187.

of *S. pennellii*, the species with the largest pollen grains. Therefore, style length and pollen grain size do not correlate within the tomato clade.

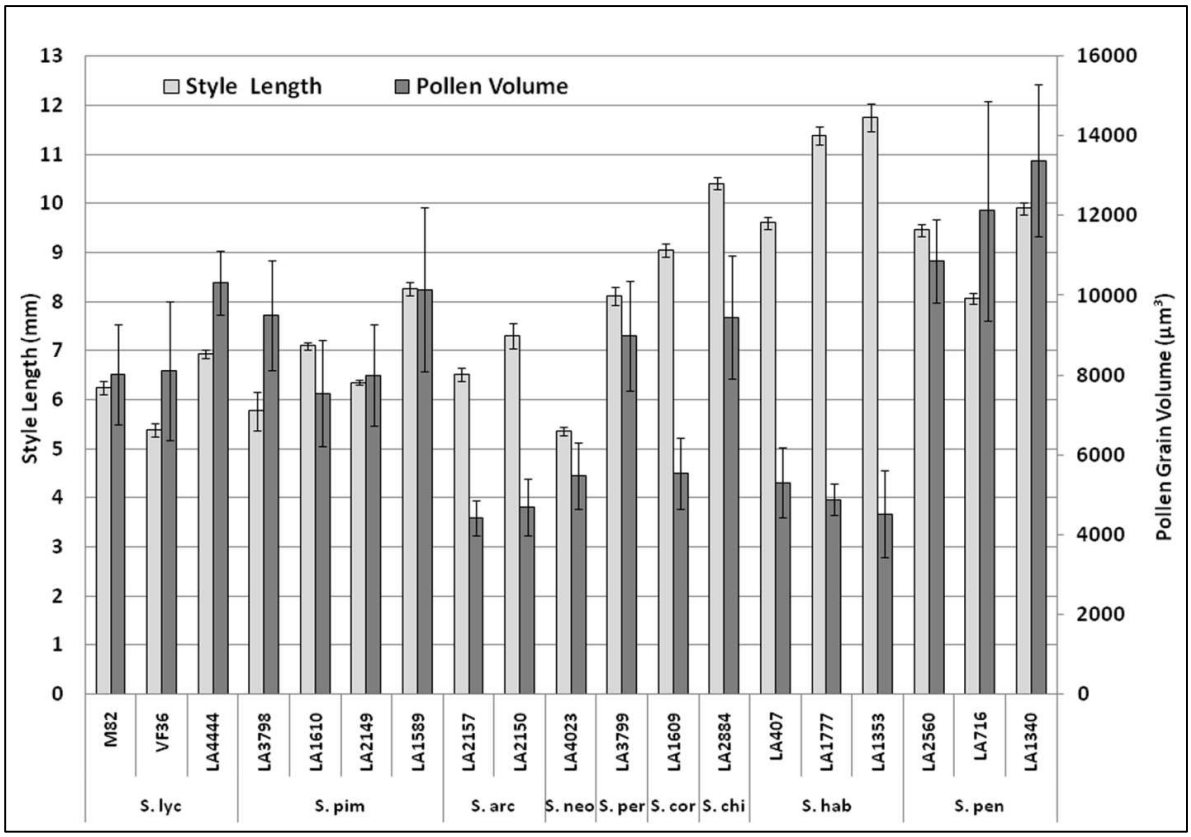

Figure. 2.1 Pollen grain size and style length for selected accessions and species in the tomato clade. Species abbreviations are the same as those in Figure 1.1a. Pollen was hydrated on a microscope slide with pollen germination medium and imaged with a Leica DM5500 B microscope using IPLab software. At least 15 hydrated pollen grains from each accession were measured using Image J 1.33u (http://rsb.info.nih.gov/ij/). Style lengths of emasculated flowers were measured on the day of bud break using images taken with a dissecting microscope. Measurements are from the top of the stigma to the top of the ovary. Fifteen styles were measured for each accession.

CHAPTER 3

TESTING THE SI X SC RULE: POLLEN-PISTIL INTERACTIONS IN INTERSPECIFIC CROSSES BETWEEN MEMBERS OF THE TOMATO CLADE (*SOLANUM* SECTION *LYCOPERSICON*, SOLANACEAE)[2]

Summary

Premise of study: Interspecific reproductive barriers (IRBs) act to ensure species integrity by preventing hybridization. Previous studies on interspecific crosses in the tomato clade have focused on the success of fruit and seed set. The SI × SC rule (SI species × SC species crosses are incompatible, but the reciprocal crosses are compatible) often applies to interspecific crosses. Because SI systems in the Solanaceae affect pollen tube growth, we focused on this process in a comprehensive study of interspecific crosses in the tomato clade to test whether the SI × SC rule was always followed.

Methods: Pollen tube growth was assessed in reciprocal crosses between all 13 species of the tomato clade using fluorescence microscopy.

Key results: In crosses between SC and SI species, pollen tube growth follows the SI × SC rule: interspecific pollen tube rejection occurs when SI species are pollinated by SC species, but in the reciprocal crosses (SC × SI), pollen tubes reach ovaries. However, pollen tube rejection occurred in some crosses between pairs of SC species. This demonstrates that a fully functional SI system is not necessary for pollen tube rejection in interspecific crosses. Further, gradations in the strength of both pistil and pollen IRBs were revealed in interspecific crosses using SC populations of generally SI species.

Conclusion: The SI × SC rule explains many of the compatibility relations in the tomato clade, but exceptions occur with more recently evolved SC species and accessions, revealing differences in strength of both pistil and pollen IRBs.

[2] BAEK, Y. S., P. A. COVEY, J. J. PETERSEN, R. T. CHETELAT, B. MCCLURE, AND P. A. BEDINGER. 2015. Testing the SI × SC rule: Pollen–pistil interactions in interspecific crosses between members of the tomato clade (*Solanum* section *Lycopersicon*, Solanaceae). *American Journal of Botany* 102: 302-311

Introduction

One premise of the biological species concept (BSC) is that reproductive barriers act to prevent interbreeding between species. While the BSC is not universally applicable, interspecific reproductive barriers (IRBs) between species can be detected in many cases. For example, in the tomato clade (*Solanum* sect. *Lycopersicon*) prezygotic IRBs can prevent hybridization between certain species (Rick, 1956, 1979; Martin, 1961a, b, 1964; Hardon, 1967; Rick et al., 1976; Liedl et al., 1996; Bedinger et al., 2011). In compatible crosses, a pollen grain on a stigma germinates, forming a pollen tube that grows through the style and into the ovary to fertilize the ovum (Cheung, 1996; Dresselhaus and Franklin-Tong, 2013). However, in incompatible crosses, pollen tubes can be prevented from reaching the ovary by active rejection processes. In a number of wild tomato species, there are two types of pollen–pistil incompatibility systems involving rejection of pollen tubes in pistils. First, self-incompatibility (SI) can prevent inbreeding through the rejection of self-pollen tubes. In the Solanaceae, SI depends on the interaction of *S*-locus pistil-expressed S-RNases and pollen-expressed F-box proteins, as well as non-*S*-locus factors, such as pistil HT proteins and pollen SCF ubiquitin ligase components including Cullin1 (CUL1) (Kao and Tsukamoto, 2004; McClure, 2004; McClure and Franklin-Tong, 2006; Kubo et al., 2010; Li and Chetelat, 2014). Second, in some crosses between species, unilateral incompatibility (UI) occurs, such that pollinations are compatible in one direction and incompatible in the other direction (Levin, 1971; Grant, 1981; Hogenboom, 1984; McClure et al., 2000, 2011; Hancock et al., 2003). UI barriers, thus, contribute to the reproductive isolation of species. The directionality of UI often follows the SI × SC rule (Lewis and Crowe, 1958; Martin, 1967; Hogenboom, 1973): SI species reject pollen tubes from SC species, while the reciprocal SC × SI species cross is compatible. The generality of the SI × SC rule suggests that UI and SI are related, and genetic studies provide further support for this relationship. For example, both pollen and pistil UI QTL map to known SI loci in wild tomato species (Chetelat and Deverna, 1991; Bernacchi and Tanksley, 1997; Covey et al., 2010). Recent experiments provide direct evidence that SI and UI use at least three different common factors. Expression of two pistil SI factors, S-RNase and HT, introduced a UI barrier in *S. lycopersicum* (Tovar-Méndez et al., 2014). Further, when the pollen UI factor CUL1 was downregulated in *S. arcanum*,

SI was suppressed (Li and Chetelat, 2014). It is important to note, however, that there are redundant UI pollen rejection mechanisms, some of which are independent of S-RNase (Murfett et al., 1996; Tovar-Méndez et al., 2014).

The tomato clade, *Solanum* section *Lycopersicon*, comprises 13 closely related species possessing diverse mating systems, making it an excellent system in which to investigate the relationship of IRBs to mating systems (Rick, 1979; Mutschler and Liedl, 1994; Peralta et al., 2008; Rodriguez et al., 2009; Bedinger et al., 2011). Four species are self-compatible (SC; autogamous) and produce red, orange, or greenish-yellow fruits and are hereafter referred to as "red-fruited" species: *S. lycopersicum* (the domesticated species), *S. pimpinellifolium*, and two species endemic to the Galápagos Islands, *S. galapagense* and *S. cheesmaniae*. The remaining nine species, with green to purple fruits, include two entirely SC taxa, *S. chmielewskii* and *S. neorickii*, and seven mostly SI species, *S. arcanum*, *S. huaylasense*, *S. peruvianum*, *S. corneliomulleri*, *S. chilense*, *S. habrochaites*, and *S. pennellii*. Previous studies of cross compatibility between species in the tomato clade have measured the success of fruit set and seed production (Mutschler and Liedl, 1994). Since the SI × SC rule is robust in this group and SI systems act during pollen tube growth, it is of interest to examine pollen tube growth more directly in interspecific crosses. The relatively few studies that have analyzed pollen tube growth in interspecific crosses are limited in scope because generally only the domesticated species, *S. lycopersicum*, was used in reciprocal crosses with wild species (Martin, 1961a; Hardon, 1967; Liedl et al., 1996; Covey et al., 2010). These previous studies reveal a UI relationship: pistils of cultivated tomato accept pollen tubes from the wild species, but in the reciprocal crosses, pollen tubes of cultivated tomato were rejected by pistils of wild species. Lewis and Crowe (1958) found a similar result in interspecific crosses with SC *S. pimpinellifolium* as male with two SI species. Covey et al. (2010) discovered two modes of pollen tube rejection in UI crosses with *S. lycopersicum*; in most cases, rapid rejection was manifested after 1–2 mm of pollen tube growth, but pistils of *S. chmielewskii* and an SC population (LA0407) of *S. habrochaites* showed slower rejection, manifested after 6–7 mm of pollen tube growth.

In this paper, we assess whether the SI × SC rule applies consistently in the tomato clade by

examining pollen tube growth in reciprocal crosses between all of the species in this group. We found that in general the SI × SC rule is followed in crosses between pairs of SI and SC species. However, we found that some SC species, and SC populations of otherwise SI species, exhibited an array of pollen–pistil behaviors in interspecific crosses, suggesting that incomplete loss of SI or other IRB factors can modulate interspecific compatibility.

Materials and Methods

Plant material

Seeds of *S. lycopersicum* cultivars VF36, M82, and VFNT Cherry (LA1221), and accessions of the wild tomato species (Appendix S3.1) were obtained from the Charles M. Rick Tomato Genetics Resource Center at the University of California Davis (http://tgrc.ucdavis.edu/) and grown in greenhouses in Pro-Mix-BX soil with 16 h of light at 26°C and 8 h dark at 18°C, or in fields at Colorado State University or UC Davis.

Pollinations and pollen tube analysis

Flower buds were emasculated 1 d before anthesis (Brukhin et al., 2003) and pollinated. At least three different female plants of each species were tested in each interspecific cross. Self-pollinations were performed to confirm mating system in each species. Pollinated pistils were collected after 48 h, unless otherwise noted, fixed, cleared, and stained with aniline blue fluorochrome as previously described (Covey et al., 2010). Images were composited in either Adobe Photoshop (http://www.photoshop. com/) or Image Composite Editor (ICE; http://research.microsoft.com/en-us/ um/redmond/groups/ivm/ice/) after capture at 5× magnification using a DAPI emission filter. For all figures, fluorescence images of pistils were inverted, contrast adjusted to optimize appearance of pollen tubes, and placed on a white background. Lengths of pollen tubes and styles (from top of stigmas to the bottom of styles) were measured using the program ImageJ 1.33u (National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/). Images from crosses with at least seven pollen tubes in pistils were used to measure the length of the majority of pollen tubes (the point at which no more than three pollen tubes passed) and the longest pollen tube.

Averages and standard deviations were calculated using Microsoft Excel software 2011 (Redmond, Washington, USA).

Results

To assess the presence and strength of IRBs acting during pollen–pistil interactions, we performed reciprocal crosses between the 13 species of the tomato clade (*Solanum* section *Lycopersicon*). We examined pollen tube growth in each cross by staining pollen tube cell walls in pistils, as shown in Fig. 3.1. In crosses in which interspecific pollen tube rejection occurred, the points where pollen tubes ceased growth were measured from the stigma surface to the point where the majority of pollen tubes tips were observed (Fig. 3.1 insert, dashed line). Usually, only 1–3 pollen tubes could be observed beyond this point, and these rarely grew more than 1 mm past the majority of pollen tubes (Fig. 3.1 insert, solid line, Appendix S3.2). Table 3.1 summarizes the results of reciprocal interspecific crosses across the entire clade. Shown are SI and SC status in self-crosses and, where appropriate, seed set results, or the points where the majority of pollen tubes were observed (i.e., ovary or the distance from the stigma surface).

SC × SC crosses among the SC red-fruited species

Crosses among the four SC red-fruited tomato species (the domesticated species *S. lycopersicum* and three wild species, *S. pimpinellifolium*, *S. galapagense*, and *S. cheesmaniae*) did not result in pollen tube rejection, and fruit and seeds were produced, consistent with previously reported results (Rick, 1956, 1967, 1979; Rick et al., 1976). Pollen tube growth to ovaries in reciprocal crosses between *S. pimpinellifolium* and the other SC red-fruited species is shown in Appendix S3.3 (A–C) as representative of these types of crosses.

SC × SC crosses between SC green-fruited species

Two green-fruited SC species, *S. chmielewskii* and *S. neorickii*, are reported to be interfertile. Appendix S3.3 (D and E) shows that no pollen–pistil prezygotic IRBs exist between these species; pollen tubes grew to the ovaries in reciprocal crosses. However, a postzygotic barrier reducing the fertility of these crosses has been reported. Hybrid breakdown results in F_2 generation seeds that germinate poorly relative to seeds from self-pollination of the two parent species (Rick et al., 1976).

SC × SC crosses between SC red- and green-fruited species

Crosses between red- and green-fruited SC species can display UI and, therefore, deviate from the SI × SC rule in the strictest sense. Pistils of all four of the SC red-fruited species accept pollen tubes of the two SC green-fruited species (Rick et al., 1976; Rick, 1979). Appendix S3.3 (F and G) shows *S. pimpinellifolium* × *S. neorickii* and *S. pimpinellifolium* × *S. chmielewskii* as representative examples. In the reciprocal crosses, when the two SC green-fruited species *S. chmielewskii* and *S. neorickii* are used as females in crosses with SC red-fruited species, interspecific pollen tubes can fail to reach the ovaries (Figs. 3.2, 3.3).

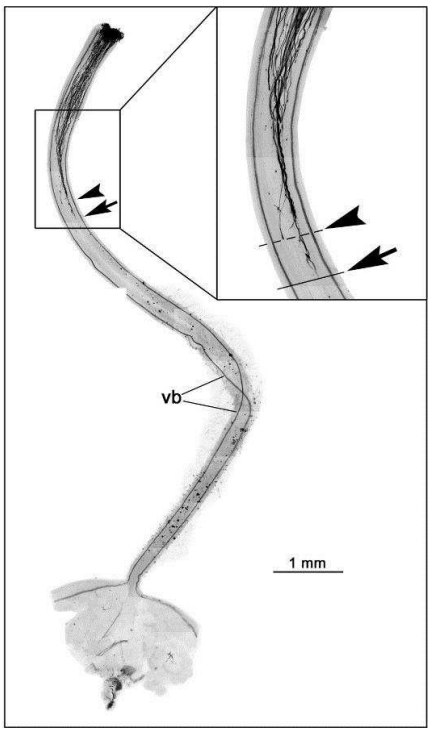

Figure. 3.1. Visualization and measurement of pollen tube growth in interspecific crosses. After pollinated pistils were fixed and stained as described in Materials and Methods, pollen tubes were measured from the top of the stigma to the point at which the majority (arrowhead, dashed line in insert) and the longest pollen tubes (arrow, solid line in insert) stopped growing. The image shown is from a cross between SI *Solanum corneliomulleri* (female) and SC *S. neorickii* (male), and demonstrates rejection of pollen tubes in the upper third of the style. vb = vascular bundles.

When *S. chmielewskii* is used as female in crosses with red-fruited species, pollen tubes grew to 7.3 mm in styles in 48 h; whether interspecific pollen tubes reached the ovaries may depend on the style

length (Fig. 3.2). For example, pollen tubes from the four red-fruited species grew 6.5–7.3 mm but did not reach the ovaries even after 72 h in accessions with long styles (8–9 mm), such as *S. chmielewskii* accessions LA1316 or LA1317. In contrast, pollen tubes from the red-fruited species reached the ovaries in 24 h in crosses with accessions of *S. chmielewskii* with shorter (i.e., 6–7 mm) styles, such as LA1325, LA3656, and LA3653.

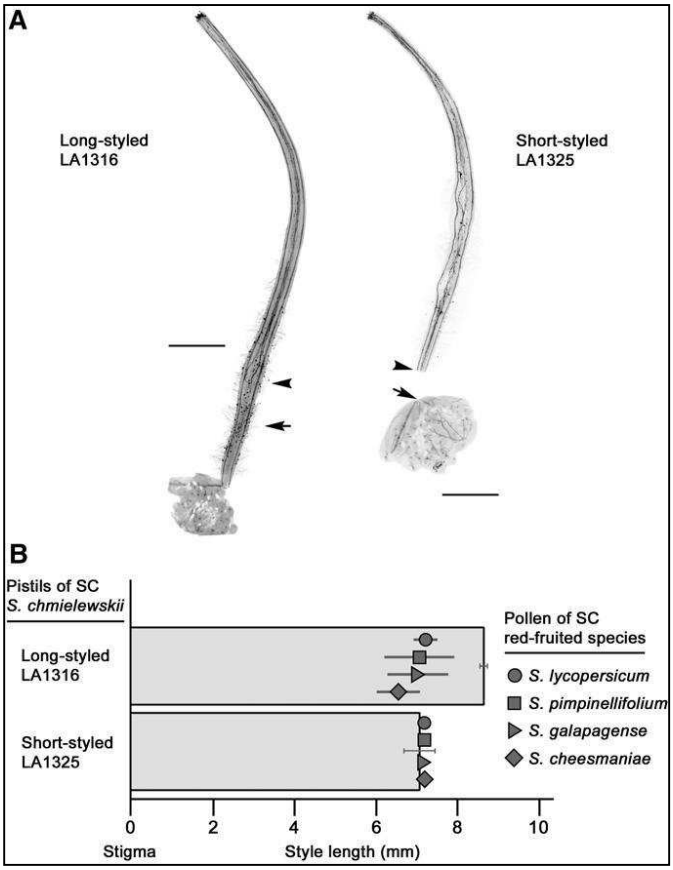

Figure. 3.2. Growth of pollen tubes of red-fruited SC species in pistils of different accessions of SC *Solanum chmielewskii*. (A) Representative images of crosses with SC *S. chmielewskii* LA1316 (left) and SC *S. chmielewskii* LA1325 (right) as female, pollinated with SC red-fruited *S. galapagense* LA1408. Arrowhead indicates where the majority of pollen tubes stop; arrow indicates the end of the longest pollen tube. Bars = 1 mm. (B) Lengths of pollen tubes from red-fruited species after 48 h growth in pistils of SC *S. chmielewskii* LA1316 with long styles (upper shaded rectangle) and after 24 h in LA1325 with shorter styles (lower shaded rectangle). Pollen tube lengths are shown in millimeters, with the averages of the majority of pollen tube lengths (symbols) and standard deviations (bars).

Solanum neorickii is an SC species with a large geographical range that extends from near Paute in central Ecuador to the Cusco area in southern Peru. When *S. neorickii* accession LA4023 from the northern limit of the species range in Paute, Ecuador was used as the female in crosses with red-fruited species as

the male, pollen tubes of all four red-fruited species were rejected after about 2.5 mm of growth into styles (at about the midpoint of the style). However, pollen tubes of the red-fruited species grew into the ovaries of some accessions of *S. neorickii*, including LA2403 and LA0247, which were collected near Huanuco, Peru in the center of the species range. This parallels the results of Chmielewski (1962) and Rick et al. (1976), who reported compatibility of *S. lycopersicum* with another accession of *S. neorickii* (LA0735) from the Huanuco region. Thus, it is likely that genetic variability in *S. neorickii* influences the strength or timing of interspecific pollen tube rejection on the female side. Figure 3.3 shows the variation in pollen tube growth in crosses with *S. neorickii* as the female and red-fruited species as the male, with pollen tube rejection at 2.0–2.6 mm in styles of the Paute accession and pollen tubes reaching the ovaries in pistils of the Huanuco accession.

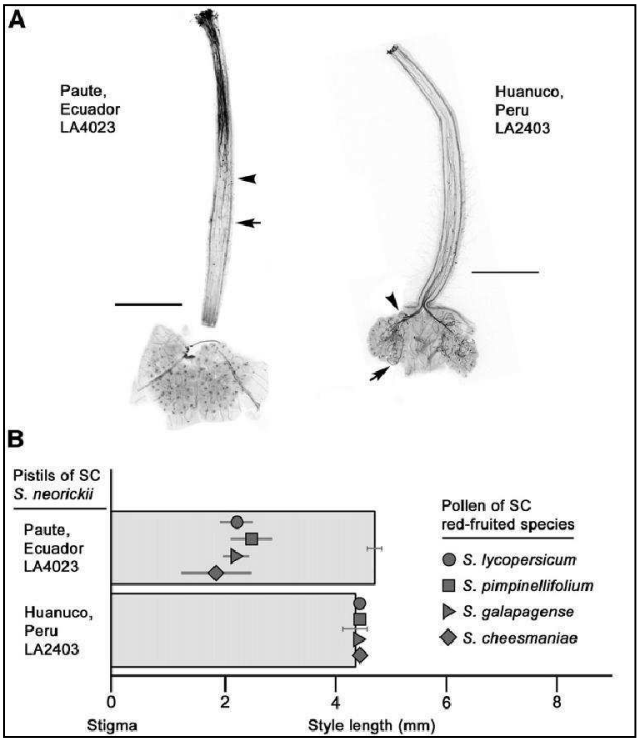

Figure. 3.3. Growth of pollen tubes of red-fruited SC species in pistils of different accessions of SC *Solanum neorickii*. (A) Representative images of crosses with SC *S. neorickii* accession LA4023 from Paute, Ecuador (left) and SC *S. neorickii* accession LA2403 from Huanuco, Peru (right) as female, pollinated with red-fruited SC *S. cheesmaniae* LA0522. Arrowhead indicates where the majority of pollen tubes stop; arrow indicates the end of the longest pollen tube. Bars = 1 mm. (B) Lengths of pollen tubes from red-fruited species in pistils of SC *S. neorickii* LA4023 from Paute, Ecuador (upper shaded rectangle) and LA2403 from Huanuco, Peru (lower shaded rectangle). Pollen tube lengths are shown in millimeters, with the averages of the majority of pollen tube lengths (symbols) and standard deviations (bars).

SI × SI crosses among SI species

The seven SI species in the tomato clade (*S. arcanum, S. huaylasense, S. corneliomulleri, S. peruvianum, S. chilense, S. habrochaites,* and *S. pennellii*) were intercrossed, and pollen tube growth was assessed. *Solanum corneliomulleri* and *S. peruvianum* are not well resolved taxonomically, and since no differences were observed in crosses (data not shown), data was combined for these two species. Appendix S3.4 (see online Supplemental Data) shows images of pollen tubes in representative SI × SI crosses. In general, pollen tubes of all SI species reach the ovaries of other SI species within 48 h. However, Fig. 3.4. shows that when pollen from either SI and SC *S. arcanum* is used in crosses with *S. habrochaites* and *S. pennellii*, *S. arcanum* pollen tubes grow more slowly than conspecific pollen tubes.

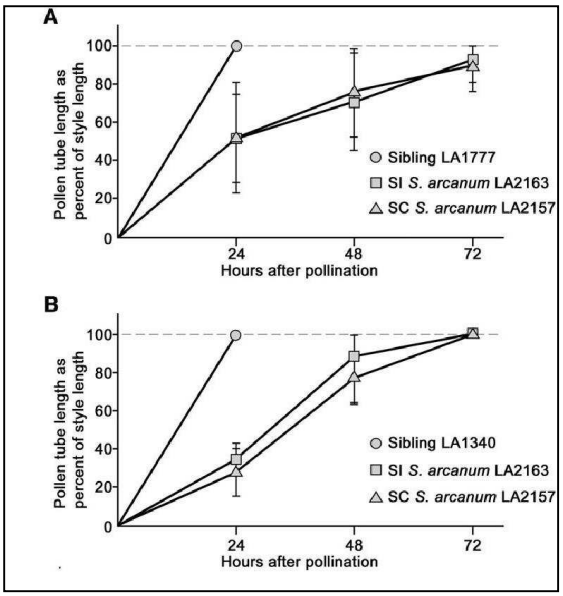

Figure. 3.4. Time course of *S. arcanum* pollen tube growth in pistils of (A) SI *Solanum habrochaites* LA1777 and (B) SI *S. pennellii* LA1340. Pollen tubes and styles were measured at 24, 48, and 72 h after pollination. The majority of pollen tubes reach ovaries within 24 h in intraspecific sibling crosses (circles), but the majority of pollen tubes of both SI (squares) and SC (triangles) *S. arcanum* do not consistently reach ovaries until 72 h postpollination. Pollen tube growth is shown as a percentage of style length, with the averages of the majority of pollen tube growth (symbols) and standard deviations (bars).

The majority of *S. habrochaites* or *S. pennellii* pollen tubes reach the ovaries in 24 h in sibling crosses (as do sibling pollen tubes in *S. arcanum* pistils). In contrast, at 24 h post-pollination, *S. arcanum* pollen tubes rarely reach ovaries in pistils of *S. habrochaites* and *S. pennellii*; in most crosses the majority

of *S. arcanum* pollen tubes are about mid-way between the stigma and the ovary. At 48 h, *S. arcanum* pollen tubes reach the ovary in approximately a third of crosses, but in most crosses the majority of pollen tubes have only traversed about 80% of the style. The majority of *S. arcanum* pollen tubes reach the ovaries in crosses with both *S. habrochaites* and *S. pennellii* by 72 h post-pollination.

SI × SC and SC × SI interspecific crosses

Reciprocal crosses were performed using the six SC and seven SI species in the tomato clade described above. Consistently, rejection of pollen tubes from all SC species is observed in pistils of all SI species, while in the reciprocal crosses SI species' pollen tubes reach the ovaries (Table 3.1). Therefore, the SI × SC rule holds across this set of interspecific crosses. SI species reject pollen tubes from red-fruited SC species after 0.8 - 1.9 mm of growth into the styles, while pollen tubes of green-fruited SC species are rejected somewhat later, after 1.5- 2.8 mm of growth (Table 3.1, Fig. 3.5 white rectangles, Appendix S3.5). An exception is seen in pistils of SI *S. pennellii*, wherein pollen tubes of all SC species, including green-fruited ones, are very rapidly rejected, after 0.9-1.3 mm of growth. In all cases, rejection of interspecific pollen tubes of SC species occurs in the upper third of the styles of SI species.

SC × SC and SI × SC crosses involving SC populations of SI species

In the tomato clade, there are several examples of SC populations of predominantly SI species, including populations of *S. pennellii*, *S. arcanum*, and *S. habrochaites*. Because some SI system components may persist in more recently evolved SC populations that could function in interspecific pollen tube rejection, we tested pollen tube growth in interspecific crosses using several SC populations of normally SI species. Pistil-side differences between the SC populations and SI populations of *S. pennellii*, *S. arcanum*, and *S. habrochaites* in interspecific crosses are shown in Fig. 3.5. Styles of SC populations were shorter on average than those from SI populations as expected: the "selfing syndrome" found in selfing populations of numerous plant species includes smaller flowers compared with those in outcrossing populations (Ornduff, 1969; Goodwillie et al., 2010; Sicard and Lenhard, 2011). In addition, pistils of all SC populations exhibited weakened IRBs compared with SI populations of the same species. In the case of *S. pennellii* LA0716, a well-known SC accession from southern Peru, pollen tubes of all SC species were

rejected, but they generally grew longer than in SI *S. pennellii* accessions—on average, 2.1 mm longer for SC red-fruited species and 4.0 mm longer for SC green-fruited species. A more dramatic difference was seen when SC *S. habrochaites* accession LA0407 was used as the male in crosses; pollen tubes reach ovaries of LA0716, but they penetrated only an average of 2 mm into styles of SI accessions of *S. pennellii* (Fig. 3.5).

There are distinct SC populations of *S. habrochaites* at the northern and southern margins of the species range (Rick et al., 1979). In this study, the northern SC accession LA0407 of *S. habrochaites* was used in interspecific crosses. A number of differences in interspecific pollen tube rejection were observed in pistils of SI *S. habrochaites* accessions compared with LA0407 (Fig. 3.5). Pistils of SI *S. habrochaites* rejected pollen tubes of the all of red-fruited species rapidly, after 1.3 mm of growth, while pistils of LA0407 rejected *S. lycopersicum* pollen tubes at 6.7 mm on average, a finding consistent with that of Covey et al. (2010), and rejected pollen tubes of *S. pimpinellifolium* at 4.4 mm on average. Pollen tubes of the two Galápagos Island species grew even longer. Pollen tubes of *S. galapagense* reached the ovary in 6 of 15 crosses, and those of *S. cheesmaniae* consistently reached the ovaries of SC accession LA0407 of *S. habrochaites* within 48 h. Pollen tubes of the green-fruited SC species *S. chmielewskii* and *S. neorickii* also always reached the ovaries of LA0407, but were rejected in the pistils of SI accessions of *S. habrochaites*. Therefore, our results showed that pistil IRBs in SC *S. habrochaites* LA0407 were significantly weaker than those in SI *S. habrochaites*. It should be noted that other, more northerly, SC accessions of *S. habrochaites* have been reported to produce fruit in crosses with SC red-fruited species as male and thus have even weaker pistil IRBs (Chmielewski, 1966).

Solanum arcanum LA2157 is the only known SC accession of this species. As in the other SI species, we find that IRBs in pistils of LA2157 were substantially weaker than those in pistils of SI *S. arcanum*. Pollen tubes from *S. lycopersicum* were rejected after 3.9 mm of growth in the styles of LA2157, compared with only 1 mm in styles of SI accessions of *S. arcanum*. Pollen tubes from the other red-fruited species grew even longer (on average 5.4 m) in styles of LA2157; pollen tubes of *S. pimpinellifolium* and pollen tubes of the two Galápagos Island species reached the ovaries in about half of the crosses. Pollen

tubes of green-fruited SC species *S. neorickii* and *S. chmielewskii*, which were rejected on SI *S. arcanum* (Table 1.1, Fig. 3.5), consistently reached ovaries of LA2157. Finally, when SC accession LA0407 of *S. habrochaites* was used as male in crosses with *S. arcanum* LA2157, pollen tubes reached the ovaries but were rejected at 2.5 mm in styles of SI accessions of *S. arcanum* in some crosses (pollen-side variation in LA0407 is discussed below).

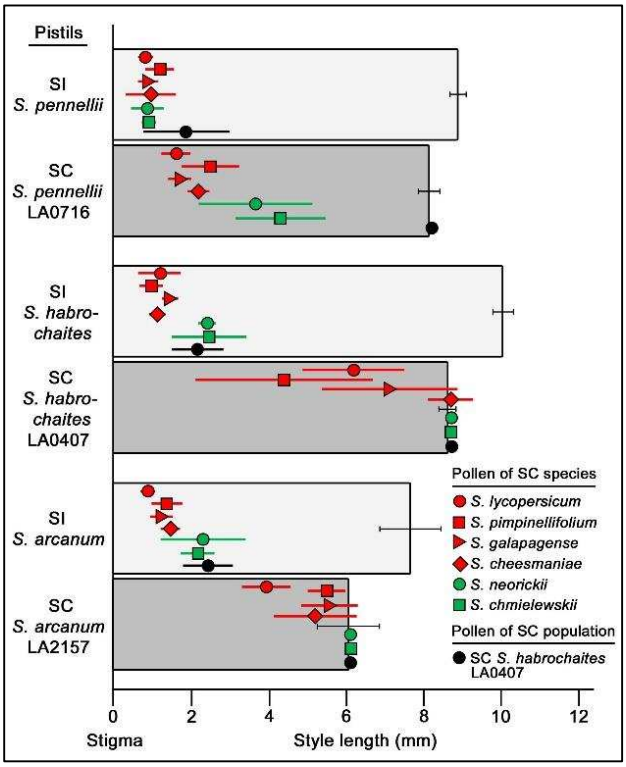

Figure. 3.5. Comparison of pollen tube growth in pistils of SI and SC accessions of *S. pennellii*, *S. arcanum* and *S. habrochaites*. Pollen tube lengths in pistils pollinated with pollen from SC red-fruited (red symbols) and SC green-fruited (green symbols) species and SC *S. habrochaites* accession LA0407 (black circles). Pistils of SI accessions are shown with light gray rectangles and pistils of SC accessions are shown with darker gray rectangles. Pollen tube lengths are shown in millimeters, with the averages of the majority of pollen tube lengths (symbols) and the standard deviations (bars).

We also tested the pollen side behavior of SC accessions of otherwise SI species in interspecific crosses. No pollen-side differences were observed for two of the three SC populations compared with SI populations. For example, when pollen from the SC accession LA0716 of *S. pennellii* was used in interspecific crosses, no differences in pollen tube growth were detected compared with crosses using pollen of SI accessions of *S. pennellii* (LA0751, LA1340, or LA2560); pistils of all SI species in the tomato clade

accepted pollen tubes of both SI and SC *S. pennellii* (Table 3.1; online Appendix S3.6). Similarly, pollen tubes of SC accession LA2157 of *S. arcanum* reached the ovaries in pistils of SI species, although they grew more slowly in pistils of SI *S. habrochaites* and *S. pennellii* (Fig. 3.4, Appendix S3.6).

Since Martin (1961a, 1964) previously showed that pollen from northern SC populations of *S. habrochaites* was rejected by central SI populations of *S. habrochaites*, we hypothesized that pollen from SC accession LA0407 of *S. habrochaites* also may not behave like SI *S. habrochaites* pollen in interspecific crosses. We found that pollen tubes of SC *S. habrochaites* LA0407 reached ovaries in pistils of all SC species and SC populations of SI species (Fig. 3.5). However, pistils of SI *S. corneliomulleri/peruvianum*, *S. habrochaites*, and *S. pennellii* rejected pollen tubes from LA0407, while pollen tubes of SI accessions of *S. habrochaites* always reached the ovaries of the same species in 48 h (Fig. 3.6). There is some variability in the behavior of pollen tubes of SC accession LA0407 of *S. habrochaites*; pollen tubes of 9 of 14 LA0407 individuals tested were rejected in pistils of SI *S. arcanum*, and pollen tubes of 10 of 14 individuals tested were rejected in pistils of SI *S. chilense*. These results show that SC *S. habrochaites* LA0407 is polymorphic in this regard and suggest that pollen-side factors are segregating in this accession.

Discussion

It has long been thought that SI and UI may be related, because the success of interspecific crosses often follows the SI × SC rule across many plant families, including Solanaceae (Harrison and Darby, 1955; Lewis and Crowe, 1958; Mutschler and Liedl, 1994; Liedl et al., 1996; Murfett et al., 1996; Onus and Pickersgill, 2004), Brassicaceae (Hiscock and Dickinson, 1993), Liliaceae (Harder, 1993), and Plantaginaceae (Harrison and Darby, 1955). Studies in the tomato clade provide direct support for an SI–UI relationship because some quantitative trait loci (QTLs) for pollen and pistil UI map to the *S*-locus in *S. habrochaites* and *S. pennellii* (Chetelat and Deverna, 1991; Bernacchi and Tanksley, 1997). An additional QTL for pistil UI maps to the location of the gene encoding the pistil SI factor HT (Covey et al., 2010). Moreover, specific SI and UI genes have recently been directly tested for function in both SI and UI. Expression of two known pistil SI factors (S-RNase and HT) in transgenic cultivated tomato creates IRBs, leading to the UI rejection of pollen tubes of red-fruited tomato species (Tovar-Méndez et al., 2014). On

the pollen side, the pollen UI factor CUL1 has been shown to be required for pollen tube growth in intraspecific pistils expressing a functional S-RNase (Li and Chetelat, 2010, 2014). These results clearly show that SI and UI use common pollen- and pistil-side factors, and, thus, mechanistic overlap is also expected.

While SI and UI mechanisms overlap, there are also significant differences between these two incompatibility systems. In SI, the recognition and destruction of self-pollen tubes by S-RNases is exquisitely allele-specific: a single S-RNase causes rejection of only one specific pollen S-haplotype. However, UI does not show this level of specificity. For example, we find that pistils of all individuals tested in all SI species, which presumably express a wide array of S-RNases, reject pollen tubes from all SC species. This result, as well as the finding that the S_6 S-RNase from *S. arcanum* LA2163 can recapitulate an IRB in transgenic cultivated tomato (Tovar-Méndez et al., 2014), is consistent with the lack of allele specificity in UI. There can be some degree of specificity in pistil-side UI, however, since occasional S-RNases show different behavior. For example, unlike all other S-RNases tested, S_{9811} S-RNase fails to cause rejection of pollen of *Nicotiana plumbaginifolia* in interspecific crosses (Beecher et al., 2001). UI is similarly allele-nonspecific on the pollen side, since pollen tubes of all individuals of SC species are rejected in crosses with all SI species. The allele specificity of pollen-side SI resides in combinations of pollen *S*-locus F-Box proteins (Kubo et al., 2010), which are also components of SCF ubiquitin ligases. In the case of red-fruited SC species, our results make sense because these species exhibit a loss-of-function mutation in the *CUL1* gene (Li and Chetelat, 2010), an essential component of SCF ubiquitin ligases (Hua and Kao, 2006; Sims et al., 2010). Therefore, all SCF ubiquitin ligase complexes without CUL1 would be rendered nonfunctional, eliminating the possibility of *S*-allele-specific pollen rejection.

Redundancy is another major difference between SI and UI. Table 3.2 provides clear evidence for redundant UI mechanisms in crosses involving SC populations of SI species. For example, it is known that pollen from the SC red-fruited species can be rejected by an S-RNase-dependent mechanism (Tovar-Méndez et al., 2014), yet SC *S. pennellii* LA0716, *S. habrochaites* LA0407 and *S. arcanum* LA2157, which all lack functional S-RNase (Kowyama et al., 1994; Royo et al., 1994; Covey et al., 2010; Chalivendra et

al., 2013), consistently reject pollen from these species. Transgenic plant studies also provide clear evidence for S-RNase-independent IRBs (Murfett et al., 1996; Tovar-Méndez et al., 2014).

These results clarify the conditions under which the SI × SC rule applies and what conditions allow exceptions to the rule. In our study, reciprocal crosses between pairs of SC and SI species follow the SI × SC rule (Table 3.1). However, interspecific crosses with SC populations of otherwise SI species show revealing deviations from the SI × SC rule. Lewis and Crowe (1958) made a distinction between interspecific crossing behavior of longstanding and recently evolved SC species and populations. Our finding that pollen tubes of SC accessions of *S. pennellii* and *S. arcanum* behave like those of functional SI accessions in interspecific crosses (Appendix S3.6), along with previous findings that pollen of these SC biotypes is fully compatible on pistils of SI accessions of the corresponding species (Hardon, 1967; Rick, 1986), is consistent with the notion that functional pollen SI/IRB factors have been retained in these SC populations even after the loss of SI due to pistil-side mutations. We also observed exceptions to a corollary of the SI × SC rule that posits that SC × SC crosses should be compatible because SC pistils should lack the capacity to reject SC pollen. Our results do not always meet this expectation, at either the species or population level. For example, pistils of SC *S. neorickii* and SC populations of *S. pennellii*, *S. arcanum*, and *S. habrochaites* can actively reject pollen tubes of the SC red-fruited species. These observations agree with prior reports of pollen rejection or lack of seed set in some SC × SC crosses (Martin, 1961a, 1967; Hardon, 1967; Rick, 1986). These results are best understood as reflecting redundant interspecific pollen rejection systems or persistence of partial interspecific pollen rejection systems, even after the loss of SI (Murfett et al., 1996; Tovar-Méndez et al., 2014). We propose that the variation in whether pollen tubes reach ovaries in interspecific crosses is due to different constellations of pollen and pistil IRB components.

Pistil IRB rejection systems range from very strong in the SI species (especially in *S. pennellii*) to virtually absent in the SC red-fruited species (Tables 3.1, 3.2). Pistils of SC species and populations, which lack functional S-RNase, display a range of weaker pollen tube rejection strength. The SC populations of SI species (Fig. 3.5) range from rejecting pollen tubes of all SC species (SC *S. pennellii* LA0716) to consistently rejecting only pollen tubes of *S. lycopersicum* (SC *S. arcanum* LA2157). Active rejection of

pollen tubes from red-fruited species occurs only in some accessions of SC *S. neorickii*, indicating that there is genetic variation in pistil IRBs in this species (Fig. 3.3). Pollen tubes of the red-fruited species grow quite long in styles of SC green-fruited *S. chmielewskii*, reaching ovaries in some cases; a possible mechanical reproductive barrier (style length) may impede pollen tubes from reaching the ovaries (Fig. 3.2). It should be noted, however, that self-pollen tubes can reach ovaries in accessions of SC *S. pimpinellifolium* with styles that are longer than 8 mm (Bedinger et al., 2011); thus, there may be additional systems that limit interspecific pollen tube growth in *S. chmielewskii*. Pistils of the red-fruited species, which express neither S-RNase nor HT protein (Kondo et al., 2002b; Covey et al., 2010), do not reject pollen tubes of any tomato clade species.

There is a gradation in strength of pollen resistance systems in interspecific crosses as well (Table 3.3). Pollen tubes of all SI species possess IRB resistance systems that allow growth to the ovaries in all the other species (Table 1; Appendix S3.4), with the caveat that pollen tubes of *S. arcanum* grow somewhat more slowly in pistils of some SI species (Fig. 3.4). Two SC populations of generally SI species, *S. pennellii* LA0716 and *S. arcanum* LA2157, retain robust pollen-side IRB resistance as well, even after the loss of SI (Appendix S3.6), explaining their deviation from the SI × SC rule. In the case of SC *S. habrochaites* LA0407, pollen IRB resistance is attenuated, as its pollen tubes are rejected by pistils of all SI species (Fig. 3.6). Resistance has not been completely lost, however; SC *S. habrochaites* LA0407 pollen tubes reach ovaries in pistils of all SC species and SC *S. pennellii* and SC *S. arcanum* accessions (Fig. 3.5). Pollen tubes of the SC green-fruited species *S. chmielewskii* and *S. neorickii* are rejected in pistils of SI species and in pistils of SC *S. pennellii*, yet grow longer than pollen tubes of the SC red-fruited species in most SI pistils (Table 3.1, Fig. 3.5; Appendix S3.5) and reach ovaries in pistils of SC accessions of *S. arcanum* and *S. habrochaites*. Pollen tubes of the red-fruited SC species are rejected by all SI species and by SC *S. pennellii* but vary in their ability to reach ovaries in SC populations of *S. arcanum* and *S. habrochaites* (Fig. 3.5). The weakest degree of pollen IRB resistance is seen in *S. lycopersicum*, since pollen tubes reach ovaries only in crosses with other SC red-fruited species. The red-fruited species are missing at least one important pollen factor: CUL1, a component of SCF ubiquitin ligase that is required for resistance to S-RNases in

both SI and UI (Li and Chetelat, 2010, 2014).

The IRBs observed in this study are relevant to natural populations of wild tomato species, given the numerous sites in South America with two or more sympatric wild tomato species (http://tgrc.ucdavis.edu). For example, sympatric populations of *S. pimpinellifolium* have been independently documented growing in association with six of the seven wild tomato SI species. Active rejection of interspecific pollen tubes would be expected to prevent hybridization at these sites if pollen from *S. pimpinellifolium* was transferred by pollinators to stigmas of SI species. There is also at least one example of sympatric SI *S. arcanum* and SI *S. habrochaites* (http://tgrc.ucdavis.edu, accessions LA1351 and LA1352, respectively) in northern Peru, and comparatively slow growth of *S. arcanum* pollen tubes in pistils of *S. habrochaites* could contribute to reproductive isolation at this site. Our results are also relevant to understanding early stages of speciation in natural populations: since pollen tubes of SC accession LA0407 of *S. habrochaites* are rejected by ancestral SI populations of *S. habrochaites* (Martin, 1961a, 1964), the partial loss of pollen IRBs in LA0407 could represent a step in the establishment of reproductive isolation, as a population differentiates into a separate lineage.

The spectrum of pistil and pollen IRB phenotypes revealed in this study represents a rich genetic resource that illustrates the value of preserving and utilizing the natural genetic diversity in wild crop relatives. Our results will inform the design of further studies aimed at uncovering mechanisms controlling pollen– pistil interactions in interspecific crosses. For example, comparative transcriptomic analysis of genotypes that vary in pistil rejection or pollen resistance behaviors, in conjunction with newly available genomic sequences (Tomato Genome Consortium, 2012, 2014; Lin et al., 2014), should identify candidate IRB genes. The function of candidate genes can then be verified by generating transgenic plants for use in test crosses with the species and populations of varying IRB strength that we have characterized. In addition to aiding in mechanistic studies, the results of this study advance our understanding of pollen–pistil interactions in crosses between species, such that we are better positioned to transfer valuable agronomic traits from wild germplasm into crop species, either directly or through the development of bridging lines for wide crosses.

Table 3.1. Pollen tube growth in interspecific crosses in the tomato clade.

		Male											
		SC						**SI**					
	Female	*S. lyc*	*S. pim*	*S. gal*	*S. che*	*S. chm*	*S. neo*	*S. arc*	*S. hua*	*S.cor / S. per*	*S. chi*	*S. hab*	*S. pen*
SC	*S. lyc*	**SC**	Seed	Seed	Seed	Seed	Seed	Ovary	Ovary	Ovary	Ovary	Seed	Seed
	S. pim	Seed	**SC**	Seed	Seed	Seed	Seed	Ovary	Ovary	Ovary	Ovary	Seed	Seed
	S. gal	Seed	Seed	**SC**	Seed	Seed	Seed	Ovary	Ovary	Ovary	Ovary	Seed	Seed
	S. che	Seed	Seed	Seed	**SC**	Seed	Seed	Ovary	Ovary	Ovary	Ovary	Seed	Seed
	S. chm	7.3 ± 0.1 [a]	7.0 ± 0.59 [a]	7.0 ± 0.8 [a]	6.5 ± 1.2 [a]	**SC**	Seed	Ovary	Ovary	Ovary	Ovary	Ovary	Ovary
	S. neo	2.3 ± 0.26 [b]	2.6 ± 0.2 [b]	2.3 ± 0.15 [b]	2.0 ± 0.31 [b]	Seed	**SC**	Ovary	Ovary	Ovary	Ovary	Seed	Seed
SI	*S. arc*	1.0 ± 0.13	1.4 ± 0.1	1.3 ± 0.12	1.5 ± 0.1	2.2 ± 0.19	2.4 ± 0.57	**SI**	Ovary	Ovary	Ovary	Ovary	Ovary
	S. hua	0.8 ± 0.24	1.1 ± 0.05	1.2 ± 0.16	0.9 ± 0.13	1.9 ± 0.3	1.5 ± 0.6	Ovary	**SI**	Ovary	Ovary	Ovary	Ovary
	S. cor / S. per	1.7 ± 0.18	1.6 ± 0.07	1.5 ± 0.15	1.3 ± 0.04	2.0 ±0.22	2.5 ±0.3	Ovary	Ovary	**SI**	Ovary	Ovary	Ovary
	S. chi	1.5 ± 0.37	1.7 ± 0.11	1.9 ± 0.32	1.6 ± 0.32	2.8 ± 0.35	2.2 ± 0.58	Ovary	Ovary	Ovary	**SI**	Ovary	Ovary
	S. hab	1.3 ± 0.47	1.1 ± 0.13	1.5 ± 0.02	1.2 ± 0.1	2.5 ± 0.93	2.5 ± 0.15	Ovary [c]	Ovary	Ovary	Ovary	**SI**	Ovary
	S. pen	0.9 ± 0.07	1.3 ± 0.13	1.0 ± 0.23	1.2 ± 0.72	1.1 ± 0.12	1.0 ± 0.16	Ovary [c]	Ovary	Ovary	Ovary	Ovary	**SI**

Notes: SC and SI refer to self-compatible and self-incompatible mating systems respectively. "Seed" means that seed was produced in interspecific crosses as previously reported (Rick et al., 1976; Rick, 1979, 1986). "Ovary" means that pollen tubes reached ovaries in pistils within 48h unless otherwise noted; fertilization (fruit/seed set) tests were not attempted in this study. Numbers refer to the lengths in mm of the majority of pollen tubes ± standard deviation in cases of pollen tube rejection. *S. lyc= S. lycopersicum*, *S. pim= S. pimpinellifolium*, *S. gal= S. galapagense*, *S. che= S. cheesmaniae*, *S. chm= S. chmielewskii*, *S. neo= S. neorickii*, *S. arc= S. arcanum*, *S. hua= S. huaylasense*, *S. cor= S. corneliomulleri*, *S. per= S. peruvianum*, *S. chi=S. chilense*, *S. hab= S. habrochaites*, *S. pen= S. pennellii*. [a] Pollen tubes do not reach ovaries in some, accessions of *S. chmielewskii*. [b] Pollen tubes rejection occurs in some, accessions of *S. neorickii*. [c] The majority of pollen tubes reach ovaries in 72h.

Table 3.2. Relative strength of pistil rejection systems in interspecific crosses in the tomato clade.

Rejection	Pistils of :		pollen tubes from:	S-RNase
Strongest	SI *S. pennellii*	**reject**	all SC species and SC *S. habrochaites* LA0407	Functional [a]
	other SI species	**reject**	all SC species but reject those of SC green-fruited species more slowly than pistils of SI *S. pennellii*	Functional [a, b]
	SC *S. pennellii* LA0716	**reject**	all SC species but more slowly than pistils of SI species	Absent [a]
	SC *S. habrochaites* LA0407	**reject**	SC *S. lycopersicum* and *S. pimpinellifolium* and variably those of *S. galapagense*	Absent [a]
	SC *S. arcanum* LA2157	**reject**	SC *S. lycopersicum* and variably those of *S. pimpinellifolium*, *S. galapagense* and *S. cheesmaniae*	Present but non-functional [c]
	SC *S. neorickii* (some accessions)	**reject**	SC red-fruited species	Varies; absent or low activity [b]
	SC *S. chmielewskii* (some accessions)	**impede**	SC red-fruited species from reaching ovaries in long styles	Absent [a, b]
Weakest	SC red-fruited species	**do not reject**	any species	Absent [b]

[a] Covey et al. (2010)
[b] Kondo et al. (2002a; 2002b)
[c] Kowyama et al. (1994) and Royo et al. (1994)

Table 3.3 Relative strength of pollen resistance systems in interspecific crosses in the tomato clade.

Resistance	Pollen tubes of :		ovaries of pistils in:
Strongest	SI species (other than *S. arcanum*) and SC *S. pennellii* LA0716	**reach**	all species
	SI *S. arcanum* SC *S. arcanum* LA2157	**reach**	all species but more slowly than those of other SI species in pistils of SI *S. habrochaites* and SI *S. pennellii*
	SC *S. habrochaites* LA0407	**reach**	all SC species and populations; grow longer than those of red-fruited species in styles of SI species
	SC *S. neorickii* SC *S. chmielewskii*	**reach**	all SC species and SC *S. habrochaites* LA0407 and SC *S. arcanum* LA2157; grow longer than those of red-fruited species in styles of SI species
	SC *S. cheesmaniae*	**reach**	SC red-fruited species and SC *S. habrochaites* LA0407; variably reach ovaries in SC *S. arcanum* LA2157
	SC *S. galapagense*	**reach**	SC red-fruited species; variably reach ovaries in SC *S. habrochaites* LA0407 and SC *S. arcanum* LA2157
	SC *S. pimpinellifolium*	**reach**	SC red-fruited species; variably in SC *S. arcanum* LA2157
Weakest	SC *S. lycopersicum*	**reach**	only SC red-fruited species

CHAPTER 4

INTERSPECIFIC REPRODUCTIVE BARRIERS BETWEEN SYMAPTRIC POPULATIONS OF WILD TOMATO SPECIES[3]

Summary

Premise of study: Interspecific Reproductive Barriers (IRBs) act to prevent hybridization between closely related species when they co-occur at sympatric sites. In the tomato clade, interspecific crosses can be successful under greenhouse conditions, but interspecific interactions between natural sympatric populations have not been evaluated. In this study, we assessed IRBs between co-flowering members of the tomato clade at 12 sympatric sites in Ecuador and Perú.

Methods: Using accessions collected from sympatric sites, we first measured stigma exsertion in a frequent sympatric partner species, *Solanum pimpinellifolium*. We then conducted 26 interspecific crosses and assessed pollen tube growth and examined seed development using microscopy.

Key results: We found that reduced stigma exsertion, a trait associated with reduced outcrossing in the self-compatible (SC) wild tomato species *Solanum pimpinellifolium*, is not consistently found in populations at sympatric sites. However, pollen tubes of this SC species were consistently rejected by pistils of the species partner at sympatric sites, comprising a strong post-mating prezygotic IRB. We found a possible case of conspecific pollen preference at one sympatric site. In most interspecific crosses that lacked prezygotic IRBs, we found strong post-zygotic IRBs that prevented normal seed development, generally resulting in seed-like structures (SLS) containing globular embryos and aborted endosperm. In four interspecific crosses, normal seed was formed that resulted in F1 hybrid plants.

[3] From a thesis submitted to the Academic Faculty of Colorado State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy."

Conclusions: Our findings indicate that in most cases a combination of prezygotic and post-zygotic IRBs would prevent hybridization between species in naturally occurring sympatric populations in the tomato clade.

Introduction

In order to maintain boundaries between closely related species growing in sympatry, gene flow between species is prevented by Interspecific Reproductive Barriers (IRBs) that act to block hybridization. Since a variety of mechanisms contribute to species isolation, understanding different types of reproductive barriers is fundamental to understanding how the genetic integrity of species is maintained (Dobzhansky 1937; Mayr 1942; Ramsey et al., 2003; Coyne and Orr 2004; Rieseberg and Willis 2007; Lowry et al., 2008; Baack et al., 2015).

Reproductive barriers involved in the isolation of species can be classified according to the order of their action in reproduction (Mayr 1963; Levin 1971; Grant 1981). In plants, premating prezygotic IRBs act prior to pollination, through geographical isolation (Mayr 1963; Schemske, 2000; Sweigart et al., 2007), differences in flowering phenology (Kaing and Hamrick, 1978; Martin and Willis 2007; Fishman et al., 2014; Briscoe Runquist et al., 2014s), or floral morphology (Darwin, 1884; Blarer et al., 2002; Hodges et al., 2002; Fenster et al., 2004; Silva-Pereira et al., 2007; Schiestl and Schluter, 2009; Yost and Kay, 2009; Grossenbacher and Whittall, 2011). Pollination between species can be also prevented by other floral characters such as floral color/pigmentation or scent related to pollinator activity or preference (Grant and Grant 1965; Grant 1994; Bradshaw et al., 1995; Bradshaw and Ramsey 2003; Ramsey et al., 2003; Hoballah et al., 2 007; Cooley et al., 2008; Whitehead & Peakall, 2009; Hopkins and Rausher, 2012; Xu et al., 2012).

Post-pollination prezygotic barriers can disrupt pollen adhesion and germination on stigmas (Rougier et al., 1988; Zinkl et al., 1999; Fiebig et al., 2004; Dickinson et al., 2012) or inhibit pollen tube growth through styles to ovaries (Martin, 1961; Hardon, 1967; Murfett et al., 1996; Kay, 2006; Covey et al., 2010; Takeuchi and Higashiyama, 2012; Eberle et al., 2013; Tovar-Mendez et al., 2014; Moyle et al.,

2014; Baek et al., 2015). Post-mating prezygotic barriers that act during pollen-pistil interactions have been shown to play a major role in restricting gene flow between SI and SC species, when an SI species is used as female and SC species is used as male. This general pattern is called the SI x SC rule, which posits that SI species x SC species crosses fail, but the reciprocal cross can be successful (Lewis and Crowe, 1958; Murfett et al., 1996; Onus and Pickergill, 2004; Baek et al., 2015). Pollen competition can also act as a post-mating prezygotic reproductive barrier when interspecific pollen tube growth is slow relative to conspecific pollen tube growth (Darwin 1898; Arnold et al., 1993; Reiseberg et al., 1995; Carney et al., 1996; Howard 1999). Within the ovary, species-specific factors produced by the embryo sac can be required for pollen tube targeting to ovules for fertilization (Marton et al., 2005; Higashiyama et al., 2006; Escobar-Restrapo et al., 2007; Takeuchi and Higashiyama 2012).

Postzygotic barriers can also restrict hybridization in plants (Baack et al., 2015). These barriers can interfere with seed development or seed germination (Cooper and Brink 1945; Scopece et al., 2008; Burkart-Waco et al., 2012; Ng et al., 2012; Lafon-Placette and Kohler, 2015). If hybrid seeds germinate, hybrid lethality or necrosis is sometimes observed in F_1 plants (Sawant 1956; Ramsey et al., 2003; Bomblies and Weigel, 2007; Yamamoto et al., 2010) or F1 plants can be sterile due to pollen inviability (Henderson et al., 1958; Grant, 1971; Reiseberg et al., 1999; Fishman and Willis, 2001; Moyle and Graham, 2005; Sweigart et al, 2006; Kubo et al., 2008; Bomblies 2010). Even if F1 plants are fertile, they may have reduced fitness in specific environments, and subsequent generations can experience hybrid breakdown due to low fitness (Stebbins, 1958; Rick et al., 1976; Rundle and Whitelock, 2001; Rhode and Cruzan, 2005; Baack et al., 2015).

The wild tomato species provide an excellent system for the study of IRBs in sympatric species. The monophyletic tomato clade consists of one domesticated species (*S. lycopersicum*) and 12 wild species found in Ecuador, Peru and Chile (Rick, 1979; Peralta et al., 2008; Moyle, 2008; Rodriguez et al., 2009), all of which are n=12 (diploid) with a high degree of synteny (Peralta and Spooner, 2001; Chetelat and Ji, 2007). The wild species of the tomato clade exhibit a variety of mating systems, from autogamous self-

compatibility (SC) to facultative SC to self-incompatible (SI) species (Rick, 1979; Mutschler and Liedl, 1994; Peralta et al., 2008; Bedinger et al., 2011). The SI x SC rule is followed at the species level in the tomato clade in that pollen tubes of SC species are rejected by pistils of SI species (Rick, 1979; Martin, 1961a, b, 1964; Hardon, 1967; Rick et al., 1976; Liedl et al., 1996; Bedinger et al., 2011; Baek et al., 2015). In reciprocal crosses, prezygotic pollen-pistil barriers are generally not observed; however, significant postzygotic barriers such as failure of fruit and/or seed formation have been reported in some cases (Rick 1979; Mutschler and Liedl, 1994). It should be noted that hybrids can form when pistils of cultivated *S. lycopersicum* (which lack IRBs) are pollinated by wild species, and this has allowed traits of agronomic importance to be introduced into the crop species (McGuire and Rick 1954; Martin 1961 and 1964; Hardon 1967; Hogenboom, 1973, Rick et al., 1976; Rick 1979 and 1986).

Although wild tomato species can have significant range overlap (Moyle 2008; Peralta et al., 2008), previous studies on IRBs in the tomato clade have focused on crosses between species regardless of geographic location (Muschtler and Liedl, 1996; Covey et al., 2010). There are numerous reports of two or more tomato clade species in sympatry, and interestingly, hybrids have not been detected in natural populations. Therefore there is an opportunity to test for interspecies barriers that have relevance for natural populations.

In this study, we assessed IRBs at different stages of reproduction between wild tomato species found in sympatry in natural populations at 12 different sites in Ecuador and Perú. We examined floral morphology, pollen-pistil interactions, and hybrid fruit and seed formation. We found strong prezygotic pollen-pistil IRBs in six cases where pollen tubes of an SC species, *S. pimpinellifolium*, were rejected in styles of the sympatric partner. We also found strong postzygotic seed development IRBs when prezygotic barriers were not detected. Both of these types of barriers would likely act to prevent hybrid formation in the natural populations under study. We recovered healthy hybrid plants in four out of 26 interspecific sympatric crosses, which suggests that, in some cases, hybridization between sympatric wild tomato species could occur.

Materials and Methods

Sympatric sites

Sympatry has previously been documented for collections of wild tomato species through the Tomato Genetics Resource Center (TGRC) at the University of California, Davis (http://tgrc.ucdavis.edu/, Appendix S1). Several of these previously reported sites were visited in 2009, and the continued presence of sympatric species was verified at five sites (Table 4.1).

Some crosses between sympatric species were performed on site in Perú, but since it was not possible to export seed from Perú, most crosses for this study were performed in greenhouses and research fields at Colorado State University and University of California, Davis, using material available through the TGRC. In one case (site 1 in Ecuador), an exporting permit was obtained and seeds from the field site were used in experiments. Accessions not known to be sympatric with other wild tomato species are referred to as "allopatric" here, but it is acknowledged that information on other species at these sites could be incomplete.

Plant material

Seeds of accessions of the wild tomato species were obtained from the Charles M. Rick Tomato Genetics Resource Center at the University of California Davis (http://tgrc.ucdavis.edu) and grown in greenhouses in ProMix-BX soil with 16 h of light at 26 °C and 8 h dark at 18 °C, or in fields at Colorado State University or University of California Davis.

Stigma exsertion measurements

Stigma exsertion was measured using at least three flowers per plant at anthesis from three individuals of each accession. In the field and at University of California Davis, stigma exsertion was measured using a digital caliper. At Colorado State University, mature flowers were collected and sepals and petals were removed. To measure stigma exsertion, flowers were either imaged at 2400dpi on an EPSON Perfection V700 photo scanner or using a Nikon SMZ1500 (http://www.nikon.com) dissecting microscope with Image ProPlus software (http://www.mediacy.com/index/aspx/aspx?page=IPP) connected with a Nikon DMX1200 digital camera (http://www.microscopyu.com/). Stigma exsertion was subsequently measured for each floral image using Image J 1.33 (http://rsb.info.nih.gov/ij/). Measurements

of three or more flowers were averaged for each individual plant, and then those values were averaged to determine the stigma exsertion value for each accession.

Crosses

Floral buds of the female parent were emasculated one day before bud break and allowed to mature an additional 24 hours before the application of pollen. Pollen was obtained from mature flowers of male parents by vibrating anther cones into gelatin capsules using tooth polishers. Stigmas were dipped in pollen, and pistils were collected after 48 hours unless otherwise noted. In crosses not performed in greenhouses, inflorescences were covered with fine-mesh nylon net bags after emasculation to prevent pollination by insects.

For crosses performed in Peru, branches of tomato species containing inflorescences were collected at sympatric sites, and stems were submerged immediately in water. Flower buds were emasculated and pollinated within 6 hours, and pollinated pistils were harvested after 24 hours.

Pollen tube growth analysis

Pollinated pistils were collected and placed in fixative (1:3 acetic acid:ethanol) for at least 24h. Pollen tubes were stained and imaged as previously described (Covey et al., 2010; Baek et al., 2015). In cases where pollen tube rejection occurred in the crosses, pollen tube lengths were determined for where the majority of pollen tubes were arrested (the point at which no more three pollen tubes passed) and where the longest pollen tube stopped. All measurements and analysis of pollen tubes were performed as described in Baek et al. (2015).

Fruit analysis

Pollinated flowers were left on the plants for at least 50 days, until fruits were soft and ripe. After collection, fruits were weighed, and fruit height (longitudinal section) and diameter (longest transverse section) were measured using an electronic caliper. Comparisons of fruit weight and height between interspecific hybrid fruits and control fruit (self or sibling crosses) were converted to the percent of hybrid compared with control.

Seed measurements

All seeds and seed-like structures (SLS) were removed from each fruit and counted. Included in the counts were all SLS showing appreciable enlargement relative to unpollinated ovules. For control fruits, mature seeds and any significantly smaller SLS were counted separately, but both were included in the total number of seeds per fruit. Prior to embedding for microscopy, all seeds and SLS from each fruit were imaged by scanning at 2400 dpi with an Epson Perfection V700 Photo scanner. The gelatinous placental tissue was dissected away from a representative sample of the seeds/SLS before scanning to reveal details of their external appearance. Seed/SLS measurements were obtained using MicroMeasure software (www.biology.colostate.edu/MicroMeasure). Total length and maximum width across the seed body were measured from scanned images. Seed thickness was measured from micrographs of seeds sectioned at right angles to their long axis at the thickest part of the seed.

Seed Fixation and Microscopy

Halved fruits or seed-containing pulp were fixed with 2.5% glutaraldehyde and 3.7% formaldehyde in 0.1 M sodium cacodylate buffer (pH7.3) and stored at 4^{o}C. After fixation for at least 24 h, seeds/SLS were extracted from the pulp. The seed coats of mature seeds (and some more developed SLS) were opened on one or both lateral surfaces. Where possible, part of the seed coat and underlying endosperm were removed to permit penetration of fixative and other reagents. Fixed seeds/SLS were washed with 0.1 M sodium cacodylate buffer, dehydrated through a graded ethanol series, transferred to propylene oxide, and infiltrated with medium-hard Eponate 12 resin (Ted Pella, Inc.). During both fixation and infiltration, exposure to a mild vacuum was used to facilitate penetration. Following polymerization of the embedding resin, seeds and SLS were sectioned using a diamond knife and Reichert-Jung Ultracut E Ultramicrotome. Sagittal or cross sections 1 to 5 micrometers in thickness were mounted on glass microscope slides, stained with toluidine blue, and coverslipped using Cytoseal 60 mountant (Electron Microscopy Sciences). Sections were photographed using a Leica DM5500 B microscope, Leica DFC450 color camera, and Leica Application Suite Version 4.1 image capture software. Figures were prepared using Adobe Photoshop.

Table 4.1. Sympatric sites in this study, north to south

#	Site	Mating system, Species	TGRC accessions	Latitude/Longitude	Co-flowering
1	Manchagrandi, Manabí Ecuador	SC, *S. pimpinellifolium* SC, *S. habrochaites*	Not available Not available	S 01 04 17/W 80 11 06	Yes[+]
2	Timbaruca, Cajamarca, Perú	SC, *S. pimpinellifolium* SC, *S. habrochaites*	LA2176 LA2175	S 05 08 30/W 79 0 30	Yes[#]
3	Puente Muyuna Rio Jequetepeque, Cajamarca, Perú	SC, *S. pimpinellifolium* SI, *S. arcanum*	LA2149 LA2150	S 07 13/W 078 47 13	Yes*
4	Chilete-Rupe Cajamarca, Perú	SI, *S. arcanum* SI, *S. habrochaites*	LA1351 LA1352	S 07 17 14/W 078 49 15	Yes*
5	Rio Pativilca Ancash, Perú	SC, *S. pimpinellifolium* SI, *S. huaylasense*	LA3798 LA3799	S 10 39 17/W 77 26 34	Yes[#]
6	Above Yaso Rio Chillón, Lima, Perú	SC, *S. pimpinellifolium* SI, *S. corneliomulleri* SI, *S. habrochaites*	Not available LA1646 LA1648	S 11 34 18/W 076 43 38	Yes*
7	Surco Rio Rimac, Lima, Perú	SI, *S. corneliomulleri* SC, *S. habrochaites*	LA1294 LA1295	S 11 52 32/W 076 25 42	Yes[#]^
8	Sisacaya Rio Lurin, Lima, Perú	SI, *S. corneliomulleri* SI, *S. pennellii*	LA0752, LA1281 LA0751, LA1282	S 12 01 16/W 076 38 05	Yes[#]^
9	Cacra Rio Cañete, Lima, Perú	SC, *S. pimpinellifolium* SI, *S. corneliomulleri* SI, *S. pennellii*	Not available LA1694 LA1340	S 12 49 07 /W 075 51 40	Yes*
10	Asia-El Piñon Lima, Perú	SC, *S. pimpinellifolium* SI, *S. corneliomulleri*	LA1610 LA1609	S 12 46 56/W 076 33 27	Yes[#]^
11	Ticrapo Rio Pisco, Huancavelica, Perú	SI, *S. corneliomulleri* SC, *S. habrochaites*	LA1722 LA1721	S 13 22 56/W 075 25 55	Yes*
12	Rio Apurimac, Puente Cunyac, Apurimac, Perú	SC, *S. chmielewskii* SC *S. neorickii*	LA2639B LA2639A	S 13 33 30/W 72 35 30	Yes[#]

[+]New site in 2014, UC Davis collections 8121 and 8122 *Confirmed in 2009 ^Single species found at site in 2009
[#]TGRC field notes; photos and/or flowering noted, fruits collected on same date

Molecular marker tests for hybridization

To confirm hybridization between species collected from sympatric sites PCR assays were performed. Genomic DNA was prepared using the 'shorty prep' method: briefly, a small piece of leaf was placed in a 1.5 mL tube tube containing 500 uL of 0.2 M Tris, pH 9, 0.4 M LiCl, 25 mM EDTA and 1% SDS, and ground with a disposable pestle. Insoluble plant material was spun to the bottom of the tube and 300uL of supernatant was mixed with 400uL isopropanol to precipitate DNA. After centrifugation, the supernatant was removed, and the DNA pellet was washed in 1 mL of 70% EtOH and was resuspended in 50 uL of 10 mM Tris, pH 8.

For sympatric site 2 (*S. habrochaites* and *S. pimpinellifolium*), primers were designed to detect a 25 bp deletion in the 17th intron of the *CULLIN 1* gene (GenBank sequence KP210075.1) that is specific to *S. habrochaites* (Gao et al. 2015). Primer sequences were as follows; CUL1 F: 5'- *TGATCATTTTGA GTTCAACTCCCA* -3' and CUL1R: 5'- *TCAACACACTCCAAAATTAGCTGT* -3', and amplified a product of 277bp from *S. habrochaites* and 302 bp from *S. pimpinellifolium*. For sympatric site 12 (*S. neorickii* and *S. chmielewskii),* we used previously identified species-specific *S-RNase* alleles in *S. neorickii* and *S. chmielewskii* (Kondo et al., 2002) as genetic markers to test for hybridization. Primers were designed to amplify the *S. neorickii S-RNase Lpfsn-1* (Genbank sequence AB072475); 5'neosrn-1-FP7: 5'- *ATGGTTAAACCACAACTCACAGCA*-3' and 3'neosrn-1-RP7: 5'-*TGTTGTTCAGCGAAAAAATATTTTT CCGG*-3', and the *S. chmielewskii S-RNase* (Genbank sequence AB072477) 5'chmsrn-FP: 5'- *CAAGTC CGTAATACTGAATAACTGC*-3' and 3'chmsrn-2-RP-1: 5'- *GGAAATGTGGAACTTAATGAGATTGG* -3'.

For all primer sets, PCR was performed using Econotaq Plus Green Mastermix (Lucigen; http://www.lucigen.com/).), 0.5 µM of each primer and approximately 80ng of genomic DNA per 20 µL reaction (95^0 C 90sec; 35 cycles of 95^0 C 30s, 55^0 C 30s, 72^0 C 30s; 72^0 C for 3min). For site 2, PCR products were electrophoresed in a 2% agarose gel and for sites 12, PCR products were electrophoresed in a 1% agarose gel. All PCR products were visualized by ethidium bromide staining.

Results

Incidence of sympatric populations

Sympatric sites with two or more wild tomato species have been documented over the past several decades at 42 sites (http://tgrc.ucdavis.edu/, Darwin et al., 2003, Appendix S1; see Supplemental Data with the online version of this article), but natural hybrids are rarely if ever observed in natural populations. At the 12 sites represented in this study (Table 4.1, Fig. 4.1), eight different species were found in different pairings in sympatry.

Figure 4.1. Sites of sympatry used in this study (circles). White indicates an SC species, black indicates an SI species and hatched indicates an SC population of a generally SI species. Sites are numbered 1-12 from north to south.

Species with varied mating systems were found in sympatry (Fig. 4.1). For example, three different pairs of self-incompatible (SI) species (*S. arcanum* and *S. habrochaites*; *S. pennellii* and *S. corneliomulleri*; *S. corneliomulleri* and *S. habrochaites*) were found at three different sites (Sites 4, 8 and 9). SC populations of *S. habrochaites*, a generally SI species, were found in sympatry with SI *S. corneliomulleri* at two sites (Sites 7 and 11). *S. pimpinellifolium*, a red-fruited SC species, was found in sympatry with five different SI species (*S. arcanum*, *S. corneliomulleri*, *S. huaylasense*, *S. habrochaites* and *S. pennellii*) at five sites (Sites 3, 5, 6, 9 and 10) and with SC populations of *S. habrochaites* at two sites (Sites 1 and 2). Finally, two SC species, *S. chmielewskii* and *S. neorickii*, were found in sympatry at Puente Cunyac in Perú (Rick et al., 1976).

Pre-mating prezygotic barriers

At all 12 sympatric sites, co-flowering of species (Table 4.1) was either confirmed by direct or recorded observation or inferred from concurrent seed collection (http://tgrc.ucdavis.edu/). Members of the tomato clade require bees that collect pollen using buzz pollination for outcrossing success. At some sympatric sites we were able to capture and identify the same bee species on both resident species of wild tomato (data not shown). However, since a pollinator activity study was not conducted, we are not able to evaluate the importance of pollinator visitation as an IRB at these sites.

Since stigma exsertion is known to affect the degree of outcrossing in *S. pimpinellifolium* (Rick 1977), we measured stigma exsertion in known sympatric *S. pimpinellifolium* populations, and compared the measurements to those from populations not known to be sympatric with other species (termed allopatric here). When possible, we also noted whether these were classified as autogamous (selfing) or facultative (outcrossing) SC accessions (Rick et al., 1977). As shown in Table 4.2, on average there is more stigma exsertion in allopatric populations (1.05 mm) than in sympatric populations (0.46 mm) of *S. pimpinellifolium*. However, the range of stigma exsertion was wide for both allopatric and sympatric populations studied. For example, we observed lower than average stigma exsertion in allopatric

populations at Chanchape and Virú – Galunga, Perú and greater than average stigma exsertion at sympatric sites Tembladera, Perú and Manchagrandi, Ecuador.

Table 4.2. Stigma exsertion (mm) in *S. pimpinellifolium* at sympatric and allopatric sites

	Location	**Accession # (field site #)**	**mm exserted**
Allopatric	E of Arenillas, Ecuador	LA1719	0.50 [C]
	Chachapoyas – Balsas, Peru	LA1382	0.99 [D]
	Miramar, Peru	LA1683	1.93[D] (1.5 outcrossing)[A]
	Chanchape, Peru	LA1380	0.03 [D]
	Malpaso, Peru	LA2538	1.06 [D]
	Patapo-La Cria, Peru	LA2536	1.51 [D]
	Patapo, Peru	LA2535	2.21 [D]
	Virú – Galunga, Peru	LA1589	0.19[C] (0.3 selfing)[A]
	Average 1.05 (range 0.03-2.21)		
Sympatric	1. Manchagrandi, Ecuador	(8121)	1.04 [C] 1.59 [F]
	2. Timbaruca, Peru	LA2176	0.39 [C]
	3. Puente Muyuna, Peru	LA2149	0.59 [C]
	5. Rio-Pativilca, Peru	LA3798	0 [C]
	6. Yaso, Peru	(8030)	0.05[F]
	9. Cacra, Peru	(8035)	0 [F]
	10. Asia-El Piñon, Peru	LA1610	0.20 [C] (0.3 selfing)[A]
	Tembladera, Peru	LA2389 (8041)	1.40 [C] 1.44[F]
	Average 0.46 (range 0-1.40) [G]		

[A] Measurements by Rick *et al.* 1977
[C] Measurements performed at Colorado State University
[D] Measurements performed at University of California, Davis
[F] Measurements performed at field site
[G] When measurements were obtained from both the field and common gardens, those from common gardens were used to calculate average and range

Post-mating prezygotic barriers (pollen-pistil interactions)

In order to assess post-mating prezygotic barriers, reciprocal crosses were performed between sympatric species and pollen tube growth was evaluated. In total, pollen tube growth was examined in 26 different crosses between sympatric species pairs.

We found that pollen tubes of SC *S. pimpinellifolium* were always rejected in pistils of their partner sympatric species at six different sympatric sites; SC *S. habrochaites* (Sites 1 and 2), SI *S. arcanum* (Site 3), SI *S. huaylasense* (Site 5), and SI *S. corneliomulleri* (Sites 6 and 10), as shown in Fig. 4.2A.

Pollen tube rejection of SC *S. pimpinellifolium* occurs on average 1.4 mm from the stigma in the styles of SI species, while styles of SC *S. habrochaites* populations at Sites 1 and 2 inhibit pollen tubes at 5 mm and 2.6 mm on average, respectively (Fig 4.2B; black circles). When SC *S. pimpinellifolium* was used as female in crosses with sympatric species, pollen tubes of the partner species consistently reached ovaries (data not shown). Therefore, a strong post-mating prezygotic IRB acts when SC *S. pimpinellifolium* is used as pollen donor, but not in reciprocal crosses.

To determine whether sympatric pollen tubes are rejected more rapidly than allopatric pollen tubes, pollen tube growth of an allopatric *S. pimpinellifolium* (SC) population (LA1589) was measured in pistils of *S. arcanum* (SI), *S. huaylasense* (SI), *S. corneliomulleri* (SI), and *S. habrochaites* (two SC populations) that have *S. pimpinellifolium* as a sympatric partner (Fig. 4.2B, gray squares). There was no significant difference between sympatric and allopatric pollen tube growth of *S. pimpinellifolium* ($p>0.05$) except at site 1 ($p=0.0002$). In this case, pollen tubes from sympatric SC *S. pimpinellifolium* were rejected at 4.8 mm on average in the styles of SC *S. habrochaites* whereas pollen tubes of allopatric *S. pimpinellifolium* accession (LA1589) were rejected at 6.8 mm on average (Fig 4.2b, Site 1), and in several cases (8/20 crosses) pollen tubes even reached the ovaries.

Pollen tube growth was also assessed in crosses between SI species pairs found in sympatry at four sites (Sites 4, 6, 8 and 9), between SI *S. corneliomulleri* and SC populations of SI *S. habrochaites* at two sites (Sites 7 and 11), and between SC *S. neorickii* and SC *S. chmielewskii* (Site 12). Pollen tube rejection was not observed in these crosses between sympatric pairs in either direction.

Interestingly, slower relative pollen tube growth of one sympatric partner was observed in crosses of the sympatric pair at the Chilete-Rupe site (Site 4, Fig. 4.3). SI *S. arcanum* pollen tubes grew only 79% of the way from the stigma to the ovary in styles of sympatric partner SI *S. habrochaites* in 48 h, while sibling pollen tubes of SI *S. habrochaites* reached the ovaries by 48 h. This pollen-pistil interaction does not complete prezygotic barriers such as those described above, because by 72 hours, *S. arcanum* pollen tubes had reached ovaries in the *S. habrochaites* partner.

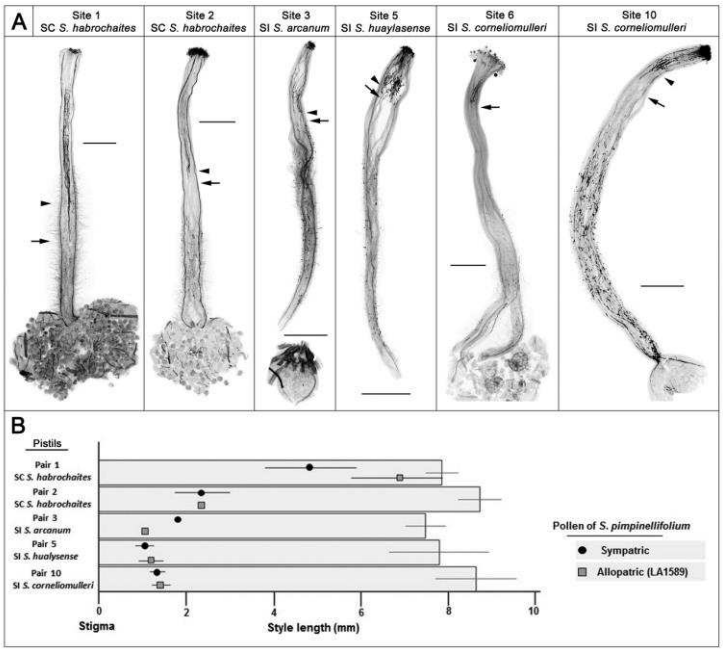

Figure 4.2. Pollen tube rejection of SC *S. pimpinellifolium* by pistils of sympatric species. A. Representative images of SC *S. pimpinellifolium* pollen tube growth in pistils of sympatric species: (left to right) SC *S. habrochaites* at Sites 1 and 2, SI *S. arcanum* at Site 3, SI *S. huaylasense* at Site 5, and SI *S. corneliomulleri* at Sites 6 and 10. Arrows indicate the growth of the longest pollen tube and arrowheads indicate where the majority of pollen tubes stop. B. Lengths of pollen tubes of sympatric (black circle) and allopatric (grey square) accessions of SC *S. pimpinellifolium* after 48h in the pistils (shaded rectangles) of sympatric species as (A). Pollen tube lengths are shown in millimeters with standard deviation (bars).

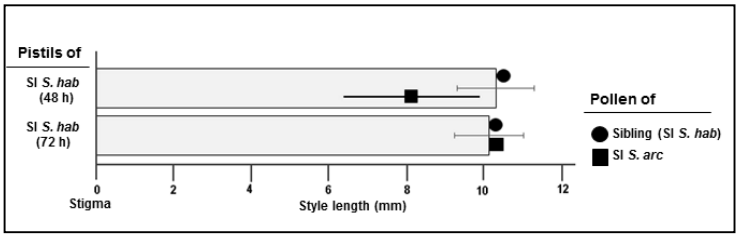

Figure 4.3. Pollen tube growth of intraspecific sibling crosses (squares) and of *S. arcanum* (circles) 48 h and 72 h after pollination in styles of SI *S. habrochaites* (rectangles) at Site 4, Chilete-Rupe, Peru.

Postzygotic barriers

a. Fruit development

From the studies described above, we detected seven cases of post-mating prezygotic IRBs acting between sympatric species. Fruit and seed formation was assessed in 19 interspecific crosses (those for which seed from sympatric sites was available through the TGRC) in which prezygotic barriers were not detected.

In two crosses, SI *S. corneliomulleri* x SC *S. habrochaites* from Site 11 and SC *S. neorickii* and SC *S. chmielewskii* from Site 12, fruit production failed after multiple attempts (>35 and 26 attempts, respectively). Interestingly, even though pollen tubes reached ovaries in these crosses, in most cases they did not appear to target ovules within ovaries (Appendix S4.3).

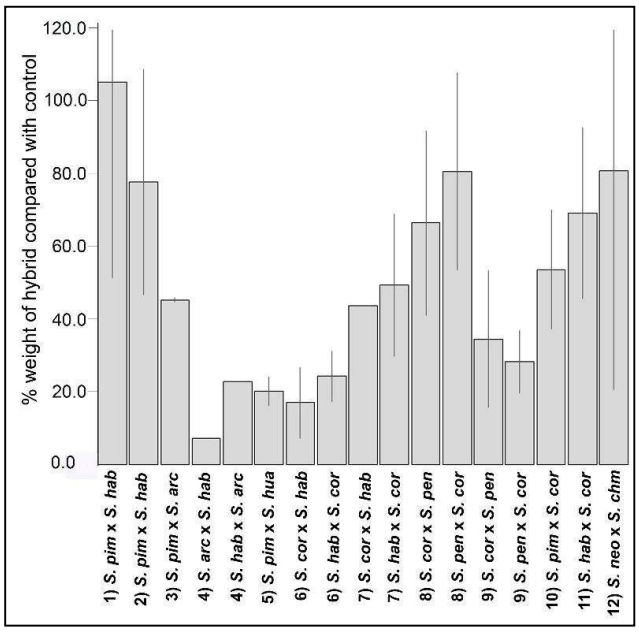

Figure 4.4. Fraction weight of hybrid fruit compared with control sib or self fruit. Grey rectangles represents % of average weight of hybrid fruit compared with controls with error bars showing standard deviation. (Note: only a single fruit from Site 4 was obtained for each reciprocal cross).

b. Seed development

In the majority of crosses (17/19), hybrid fruits were produced from crosses between sympatric species pairs, and fruits contained seeds or seed-like structures (SLS). Sizes (height and diameter) and weights of hybrid fruits were compared to those of control fruits from conspecific crosses (Appendix S4.2).

The majority of hybrid fruits showed a substantial reduction in weight compared to controls (Fig. 4.4). For example, the weight of hybrid fruits from Sites 4, 5, 6 and 9 were reduced to ~25% of control fruits Other sites showed less extreme reductions in hybrid fruit weight, with a ~50% reduction at Sites 3, 7 and 10 compared to control fruits. In four cases (Sites 1, 2, 8 and 12), hybrid fruits were very similar in size and weight to their conspecific controls.

Fruits formed from interspecific crosses contained seeds or SLS of varying sizes and degrees of maturity. In order to analyze the anatomy of the interspecific seeds and SLS, we examined developing seeds of self-pollinated *S. pimpinellifolium* at several developmental stages, Normal structure of *S. pimpinellifolium* seeds at 10 days after self-pollination (the stage most relevant to understanding the development of SLS in the majority of interspecific crosses) and at maturity is illustrated in Fig. 4.5.

At 10 days post-pollination (Fig. 4.5A), the embryo sac is surrounded by a single integument (int), consisting of one layer of endothelial cells (et), numerous parenchymal layers, and an epidermis (ep).The embryo (em) has reached the globular stage and is attached to the embryo sac wall by the suspensor (s) at the micropylar (mp) end. At the opposite end of the embryo sac, a vascular bundle (vb) approaches the chalazal pocket (cp) through the funiculus (f). The cellularized endosperm (es) surrounds the embryo and fills most of the embryo sac. In the mature seed (Fig. 4.5B), the fully developed embryo has assumed a spiral form, with the two cotyledons (cot) curled within the hypocotyl (hyp) and radicle (rad). The embryo and the endosperm (es) surrounding it are contained within a seed coat (sc) consisting of the pigmented inner cell layer of the integument, the endothelium (et) and a tough outer layer of collapsed cells that form the seed coat (testae) with surface hairs (h). A small amount of non-collapsed integumentary parenchyma is present in the remnant of the funiculus.

For each interspecific cross, we examined the structure of normal mature control seeds formed from self or sibling crosses in the maternal species (Figs. 4.6, 4.7, Appendix S4.6 and S4.7). The seed coats of control seeds in different species ranged in color from yellow through brown as a result of pigment in the endothelium. Hairs derived from the cell walls of the outer layer of the seed coat covered the surface to a greater (*S. pimpinellifolium*) or lesser (*S. pennellii*) extent. Longer hairs sometimes formed a tuft at the

distal end of the seed body (*S. pennellii*) or completely surrounded the margins (*S. pimpinellifolium*). Seeds varied in size from approximately 0.9 mm x 1.7 mm (width x length) in *S. pennellii* to around 1.7 mm x 2.9 mm in *S. pimpinellifolium*, with sizes for the other species studied ranging between those extremes. There was also some variation in average seed size among accessions within species. Thickness of seeds ranged from approximately 0.5 mm to 1.0 mm in controls. The internal seed structure of control seeds was very similar to that of *S. pimpinellifolium*, with a spiral mature embryo, as shown in Fig. 4.5B.

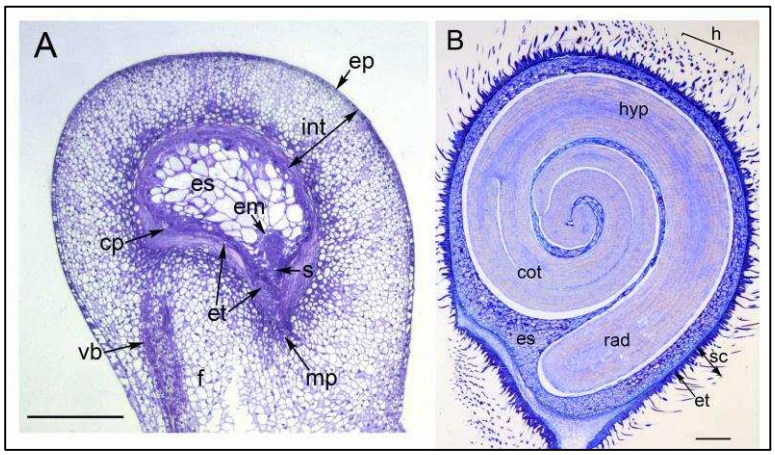

Figure 5. Normal seed development in self-pollinated *S. pimpinellifolium*. A. 10 days post-pollination.B. Mature seed. Abbreviations: cot, cotyledon; cp, chalazal pocket; em, embryo; es, endosperm; ep, epidermis; et, endothelium; f , funiculus; h, hairs; hyp, hypocotyl; int, integument; mp, micropyle; rad, radicle; s, suspensor; sc, seed coat, vb, vascular bundle. Scale bars, 200 µm.

When interspecific fruit developed, seed development was abnormal in the majority of crosses (13/17). In these cases, interspecific hybrid SLS were much smaller than control seeds (average size 0.5-0.75 mm width x 1.0-1.5 mm length, or ~40-70% of the control seed size), and were usually pale and translucent. In many SLS, the outline of the embryos sac was visible through the integument, with a darker dot in the center indicating the position of the embryo (e.g., Fig. 4.6). Fig. 4.6 illustrates the three different abnormal hybrid seed phenotypes that we observed. Type 1, the least developed interspecific seeds (7/13 crosses, Fig. 4.6A), contained a globular embryo and a small amount of endosperm, surrounded by the integument. The cells of the endosperm, which have thicker cell walls than those of embryonic cells, most often appeared to be collapsed. Seed coats were absent or rudimentary, consisting at most of patches of compressed integument cells, sometimes with elaboration of the outer layer into hairs. A second phenotype,

Type 2 (5/13 crosses), was observed when *S. habrochaites* or *S. pennellii* was used as female in interspecific crosses. Type 2 SLS generally resembled those of Type 1 in having a globular embryo, variable amounts of collapsed endosperm, integument, and a patchy or absent seed coat. However, a conspicuously multi-layered endothelium, rather than the single cell layer of endothelium found in normal seeds, was a distinguishing feature of this phenotype (e.g., Fig. 4.6B). The presumption that the extra layers of tissue are derived from the endothelium is based on previous studies in wild tomatoes and potatoes (Cooper and Brink, 1945; Lee and Cooper, 1958). When *S. habrochaites* was the female parent, the overgrown endothelium appeared densely stained in sections, completely surrounded the embryo sac, and often occupied a large part of the seed interior. In cases where *S. pennellii* was the female parent, the thickened endothelial layer tended to stain more lightly and may be discontinuous, with some endosperm cells occasionally appearing to lie outside it (data not shown).

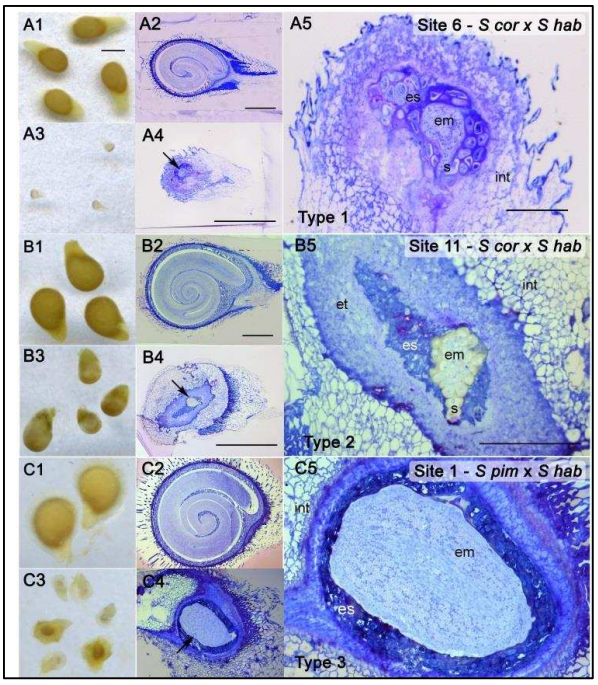

Figure 4.6. Three phenotypes of hybrid SLS, illustrated by examples from interspecific crosses between sympatric species at Sites 6, 11 and 1. Type 1, Type 2 and Type 3 SLS are shown in A5, B5 and C5, respectively. A. Site 6, *S. corneliomulleri* x *S. habrochaites*; B. Site 11, *S. corneliomulleri* x *S. habrochaites*; C. Site 1, *S. pimpinellifolium* x *S. habrochaites*. A1-C1, control seeds of the pistil parent for each cross; A2-C2, sagittal sections of control seeds; A3-C3, seeds and SLS in fruit resulting from the interspecific crosses; A4-C4, sagittal sections of seeds resulting from the interspecific crosses. A5-C5, enlargements of sections of interspecific seeds. Abbreviations: em, embryo; es, endosperm; et, endothelium; int, integument; s, suspensor. Arrowheads in A4-C4 indicate embryos.

A third abnormal phenotype (Type 3) was seen in *S. pimpinellifolium* x *S. habrochaites* crosses made using accessions from sympatric site 1 (Fig. 4.6C). In this case most SLS were similar to Type 1, but there were a small number of SLS (<5%) that are slightly larger, approximately 1.7 mm in length by 1.1 mm in width, with pigment around the embryo sac. Upon sectioning, these larger forms were seen to contain embryos at a "pre-heart" stage of development *i.e.*, epidermal, ground and vascular tissue are apparent, but no cotyledon buds are seen. These embryos are surrounded by collapsed endosperm tissue and have only a rudimentary seed coat.

Formation of normal or nearly normal hybrid seeds and hybrid plants

In hybrid fruits resulting from interspecific crosses, 4/17 crosses using accessions from three sympatric sites (Fig. 4.7) produced normal or nearly normal seeds, which were either slightly smaller or the same size as control seeds. These seeds outwardly resembled control seeds and had well-developed seeds coats with normal pigmentation. Elaboration of seed coat hairs generally resembled seeds of the female parent. Upon sectioning, these seeds were found to contain embryos that were fully developed (or nearly so), with normal endosperm, a single endothelial layer and a distinct seed coat. Interestingly, the embryos resulting from *S. pennellii* x *S corneliomulleri* crosses were often erupting from the seed coat (Fig. 4.7, C4), probably because of the maternally-regulated small *S. pennellii* seed size (Fig. 4.7, C1). It should be noted that fruits of these interspecific crosses generally also contained SLS that were less developed, from to those containing globular embryos to those with post-globular embryos of torpedo, walking stick, or early spiral stages. The ratio of normal seed to abnormal SLS within these fruits was often highly variable, ranging from no normal seeds to over 90% normal. Even in fruits resulting from intraspecific pollinations there is often a significant number of ovules that fail to develop normally, and this number is sometimes as high as 25-50%.

Seed from these four crosses germinated and produced F1 plants. Both leaves and flowers of these putatively hybrid plants were intermediate in phenotype (Appendix S4.8). Molecular markers for species at sites 2 and 12 were used to confirm hybrid production (Appendix S4.9).

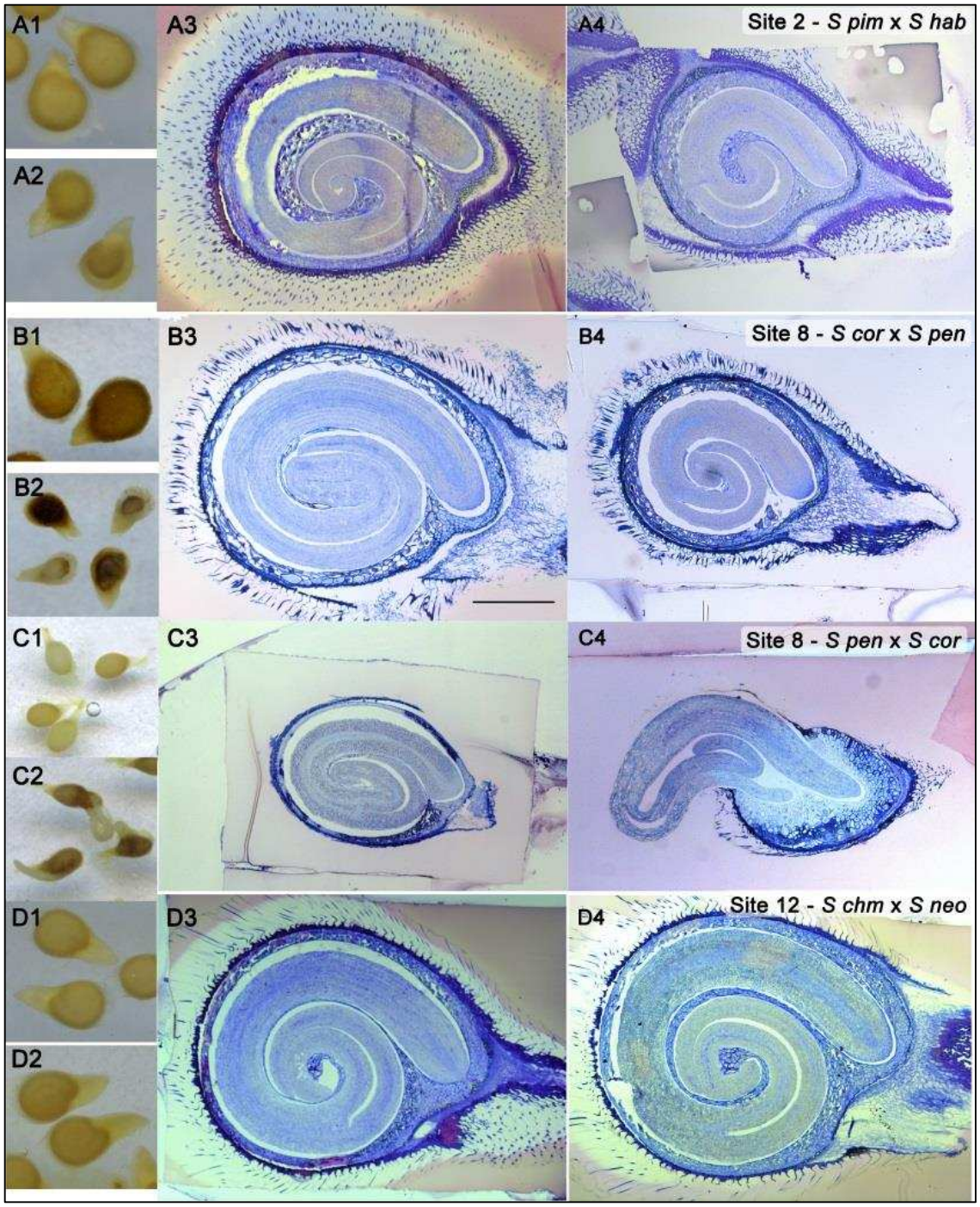

Figure 4.7. Normal or nearly normal hybrid seeds produced by four interspecific crosses at three sympatric sites. A. Site 2, *S. pimpinellifolium* x *S. habrochaites*; B. Site 8, *S. corneliomulleri* x *S. pennellii*; C. Site 8, *S. pennellii* x *S. corneliomulleri*; D. Site 12, *S. chmielewskii* x *S. neorickii*. A1-D1, control seeds of the pistil parent for each cross; A2-D2, seeds and SLS in fruit resulting from the interspecific crosses; A3-D3, sagittal sections of seeds from intraspecific crosses. A4-D4, sagittal sections of seeds resulting from the interspecific crosses.

Discussion

There have been a number of attempts to evaluate the relative strength of different kinds of reproductive barriers between species (Ramsey et al., 2003; Rieseberg and Willis, 2007; Lowry et al., 2008; Schemske, 2010; Baack et al., 2015). While it has been argued that prezygotic barriers contribute more to reproductive isolation than postzygotic ones, numerous post-zygotic barriers including hybrid lethality, sterility and F2 hybrid breakdown are also common (Baack et al., 2015).

We have evaluated both prezygotic and postzygotic reproductive barriers in 26 different interspecific crosses, using accessions from 12 known sympatric natural populations where co-flowering occurs (Table 4.1). We did not conduct pollinator preference studies, which could constitute a significant pre-mating reproductive barrier. Stigma exsertion, a morphological feature that has been associated with outcrossing frequency in wild tomato *S. pimpinellifolium* (Rick et al., 1977, 1978), was examined to determine whether reduced exsertion (reflecting reproductive trait displacement) occurred in sympatry. While our results showed a trend towards reduced stigma exsertion in sympatry compared to allopatry (Table 4.2), this trait varied widely between populations and there were notable exceptions to the trend.

We identified a number of post-mating prezygotic IRBs that could act to prevent hybridization (Table 4.3). We find strong prezygotic interspecific barriers in sympatric crosses with red-fruited SC *S. pimpinellifolium*, a species that is widespread in Peru and Ecuador, including at seven of the 12 sympatric sites in this study, as well as at least 10 other sympatric sites not included in this study (Tables 4.1, Appendix S4.1). When *S. pimpinellifolium* was used as male in interspecific crosses, pollen tube rejection consistently occurred in styles of sympatric partner species (Fig. 4.2). Molecular mechanisms underlying this barrier, termed unilateral incompatibility (UI), involve pollen and pistil SI components that have been lost to mutation in red-fruited species including *S. pimpinellifolium* (Covey et al., 2010; Tovar Mendez et al., 2014; Li and Chetelat 2014). It should be noted that although UI is essentially 100% effective, it is by definition highly asymmetric. Another example of a post-mating, prezygotic IRB was observed, with slow relative growth of interspecific *S. arcanum* pollen tubes compared to conspecific ones in *S. habrochaites* pistils

(Fig. 4.3). While this behavior is not specific to sympatric accessions (Baek et al., 2015), in sympatry the slow relative growth of interspecific pollen tubes could constitute conspecific pollen preference.

In crosses with no clear prezygotic barriers, we detected strong postzygotic barriers to hybridization in 14 cases (Table 4.3). In two cases pollen tubes were able to reach ovaries but hybrid fruit did not develop, perhaps due to the lack of pollen tube targeting to ovules (Appendix S4.3). Frequently, when hybrid fruit formed, a reduction in hybrid fruit weight (Fig. 4.4) was correlated with presence of abnormal SLS within the fruits. Anatomic examination of SLS revealed that endosperm tissue was abnormal, and appeared to have collapsed (Figs. 4.6, Appendix S4.6, and S4.7). Although the distinctive walls of the endosperm cells were frequently visible, the cells generally appeared to be empty or to have clumped intracellular contents. Developmental arrest at the globular embryo stage is a common consequence endosperm failure in mutant or hybrid crosses (Cooper and Brink, 1945; Nowack et al., 2010). The molecular mechanisms underlying defects in hybrid seed development, including both genic incompatibilities and epigenetic effects, are being actively investigated (Josefsson et al., 2006; Fishman and Willis, 2006; Marfil et al., 2006; Bomblies et al., 2007; Michalak, 2009; Shivaprasad et al., 2012; Ng et al., 2012; Lafon-Placette and Köhler, 2015).

In several cases of interspecific crosses where either *S. habrochaites* or *S. pennellii* was the pistil parent, we observed aborted seeds in which there was overgrowth of the endothelium, the innermost layer of the integument that is normally only one cell layer thick. Such proliferation of the endothelium, accompanied by subnormal growth of the endosperm and embryo, has previously been observed in various incompatible crosses between members of the Solanaceae (Cooper and Brink, 1945; Sachet, 1948; Lee and Cooper, 1958; Wann and Johnson, 1963; Masuelli and Camadro, 1997). Among the possible explanations for this developmental pattern is lack of the normal pathway for transfer of nutrients from the maternal sporophyte to the embryo sac through specialized conducting cells between the chalazal pocket and vascular strands in the funiculus. Future studies of seed development in crosses between members of the tomato clade will focus on the formation of this connection, particularly in interspecific crosses with *S. habrochaites* or *S. pennellii* as female.

Functional hybrid seed formed in four of the 26 interspecific crosses that we performed (Table 4.3, Fig. 4.7). In these cases the F1 plants displayed an intermediate leaf and flower phenotype (Appendix S4.8) and molecular markers, when available, demonstrated hybrid formation (Appendix S4.9). Although hybrid plants in the wild have not been reported, in light of our results, a more thorough search for hybrids is justified, an effort that will become more straight-forward as species-specific molecular markers become available. Of course, it is also possible that hybrid plants may not survive in natural environments due to reduced fitness.

One of our most interesting observations is that the strength of IRBs between the same species pairs can vary significantly at different sympatric sites. For example, reciprocal crosses between accessions of *S. corneliomulleri* and *S. pennellii* from sympatric Site 8 (Siscaya, Perú, Fig. 4.7) produced viable seed, but the same interspecies crosses using accessions from Site 9 (Cacra, Perú, Appendix. S4.7, F and G) produced defective inviable seeds. Interspecific crosses using accessions of *S. pimpinellifolium* and *S. habrochaites* from sympatric Site 2 also produced viable seed (Fig. 4.7), but in crosses between the same species pair from Site 1 there were potential prezygotic and strong postzygotic IRBs (Appendix. S4.4, 4.6). While it is possible that reproductive character displacement could play a role in the enhancement of IRBs at different sympatric sites, further experimentation will be necessary to establish this.

Finally, it should be noted that reproductive barriers to gene flow are not only crucial for preserving species integrity; they are also essential for the completion of the process of speciation after the initial divergence of new lineages. With their fundamental role in the generation and maintenance of biodiversity, it will be of great interest to investigate the mechanisms underlying these barriers, and how they evolve during speciation.

Table 4.3. Summary of IRBs between tomato clade members in sympatric populations

Sympatric interspecific crosses			Prezygotic barriers			Postzygotic barriers			Hybridization
Site	Species cross	Mating system	PT rejection	Slow relative PT growth [a]	Fruit set fails	Seed development [b]			F1 Hybrid plants formed
						Type 1[c]	Type 2[d]	Type 3[e]	
1	*S. hab* x *S. pim*	SC-pop x SC	X						
2	*S. hab* x *S. pim*	SC-pop x SC	X						
3	*S. arc* x *S. pim*	SI x SC	X						
5	*S. hua* x *S. pim*	SI x SC	X						
6	*S. cor* x *S. pim*	SI x SC	X						
10	*S. cor* x *S. pim*	SI x SC	X						
4	*S. hab* x *S. arc*	SI xSI		X			X		
11	*S. cor* x *S. hab*	SI x SC-pop			X				
12	*S. neo* x *S. chm*	SC x SC			X				
3	*S. pim* x *S. arc*	SC x SI				X			
4	*S. arc* x *S. hab*	SI x SI				X			
5	*S. pim* x *S. hua*	SC x SI				X			
6	*S. cor* x *S. hab*	SI x SI				X			
7	*S. cor* x *S. hab*	SI x SC-pop				X			
9	*S. cor* x *S. pen*	SI x SI				X			
10	*S. pim* x *S. cor*	SC x SI				X			
6	*S. hab* x *S. cor*	SI x SI					X		
7	*S. hab* x *S. cor*	SC-pop x SI					X		
9	*S. pen* x *S. cor*	SI x SI					X		
11	*S. hab* x *S. cor*	SC-pop x SI					X		
1	*S. pim* x *S. hab*	SC x SC-pop						X	
1	*S. pim* x *S. hab*	SC x SC-pop							X
8	*S. pen* x *S.cor*	SI x SI							X
8	*S. cor* x *S. pen*	SI x SI							X
12	*S. chm* x *S. neo*	SC x SC							X

[a] Interspecific pollen tube growth was slower than conspecific pollen tube growth (pollen tubes reached ovaries eventually); [b] Most advanced stage of embryo development observed; [c] Seed development blocked at globular embryo stage; [d] Seed development blocked at the globular embryo stage with overgrowth of endothelium; [e] Seed development blocked at pre-heart embryo stage

CHAPTER 5

TESTING WHETHER A LOW ACTIVITY S-RNASE IS INVOLVED IN INTERSPECIFIC POLLEN TUBE REJECTION IN THE WILD TOMATO SPECIES *SOLANUM NEORICKII*

Introduction

In my previous work (Chapter 3; Baek et al., 2015) I demonstrated that while crosses between SI and SC tomato species followed the SI x SC rule in the tomato clade, unilateral incompatibility (UI) was also observed when pistils of SC species are pollinated using red-fruited SC species. Pollen tube rejection was not expected between two SC species since it was assumed that the loss of SI would also cause the loss of the ability to reject interspecific pollen tubes. One population of *S. neorickii*, a green-fruited SC species, rejected pollen tubes from the four SC red-fruited species, while no pollen tube rejection was observed in the reciprocal crosses, i.e. UI was exhibited. However, another population of *S. neorickii* did not show UI, as pollen tubes were not rejected in either direction. Specifically, my previous studies showed that the accession LA4023 of *S. neorickii* from Azuay, Ecuador, shows rejection of pollen tubes from the red-fruited SC species while the accession LA0247 from Huánuco, Peru, does not. Another accession, LA0735, from Huánuco, Peru, had previously been reported as accepting pollen tubes from *S. lycopersicum* (Rick et al., 1976).

Stylar secreted *S*-locus ribonuclease, S-RNase, is the known female determinant of self-incompatibility (SI). Five conserved regions and two hyper-variable regions (involved in allele-specific recognition) have been characterized in S-RNases (Ioerger et al., 1991). Self-incompatible species express S-RNases with high enzymatic activity (Huang et al., 1994; Kondo et al., 2002a). Mutations in the *S-RNase* gene can cause the loss of SI. For example, the replacement of hypervariable region of S-RNase in S_3 allele with the corresponding region in another allele, S_1, led to loss of specificity for the rejection of pollen with the S_3 allele (self-pollen) in transgenic *Petunia inflata* (Kao and McCubbin, 1996). Kondo et al. (2002a) tested S-RNase protein expression and enzymatic activity in styles of SC wild tomato species including SC

species/populations of wild tomatoes, and showed that lack S-RNase expression or low activity due to gene deletion, insertion of transposable elements in regulatory regions, or missense mutations. S-RNase expression in *S. neorickii* was also tested using accessions from two different groups: LA1322 located in Cusco, Peru and LA0247 located Huánuco, Peru. Kondo et al. (2002a) showed that LA1322 (Cusco, Peru) expresses a low activity S-RNase while LA0247 (Huánuco, Peru) lacks S-RNase expression. LA0247 (Huánuco, Peru) has a single nucleotide insertion in the coding region of the gene, resulting in a frameshift that would produce a truncated protein. The S-RNase expressed in the styles of LA1322 (Cusco, Peru) contains an intact coding region including the five conserved regions and active site amino acid residues (His and Cys) found in functional S-RNases from SI plants, so there is not a clear explanation for the low enzymatic activity of the LA1322 S-RNase. It is possible that other proteins might be involved in regulating S-RNase activity (Kondo et al., 2002a). My results suggest that the low activity S-RNase from *S. neorickii* (LA1322) may be insufficient for SI but could be involved in interspecific pollen tube rejection of red-fruited species in the styles of *S. neorickii*.

In this chapter I examine the hypothesis that there is a correlation between interspecific pollen tube rejection and different genetic backgrounds of four geographically distinct groups of *S. neorickii*, especially with regard to S-RNase expression. First, I assessed interspecific pollen-pistil interactions with pollen of red-fruited SC species including *S. lycopersicum, S. pimpinellifolium, S. galapagense,* and *S. cheesmaniae* on *S. neorickii* pistils to test whether geographically distinct groups of *S. neorickii* show variability in rejection of pollen tubes from red-fruited SC species. I then tested the correlation between interspecific pollen tube rejection and the presence of S-RNase protein in *S. neorickii* geographic groups and in F_2 plants of inter-group hybrids of *S. neorickii*. Based on my hypothesis it is expected that groups of *S. neorickii* with S-RNase will reject pollen tubes of red-fruited species, while groups not expressing S-RNase will fail to reject interspecific pollen tubes.

Material and Methods

a. Plant material

Protocols for seed acquisition and plant growth were followed as described in the previous chapter. Note: *S. neorickii* has previously been known as *L. minutum or L. parviflorum* (Chmielewski et al., 1964; Rick et al., 1976; Kondo et al., 2002a) and here is referred to with the currently accepted species name *S. neorickii*.

1) Grouping populations of *S. neorickii*

The current information on the range of *S. neorickii* by the Tomato Genetics Resource Center (TGRC; http://tgrc.ucdavis.edu/) shows four geographically distinct groups: 1) Azuay and Loja, Ecuador; 2) Amazonas, Peru; 3) Huánuco, Peru; and 4) Cusco and Apurimac, Peru. These groups are separated by about 800-1000 km while the range within a group is 200-300 km (Fig. 5.1). In these studies, three accessions from each group were selected as representatives of the groups (Table 5.1).

2) Obtaining inter-group hybrids and F_2 plants

Prior to producing inter-group hybrids, pollen tube growth was assessed in different groups of *S. neorickii* to confirm the presence or absence of reproductive barriers within *S. neorickii* according to the procedures described in Chapter 3. Reciprocal crosses between four groups of *S. neorickii* were performed to obtain inter-group hybrid seeds, with crosses between LA1322 (group D) as the female and LA0247 (group C) as the pollen donor (I assumed that directions of maternal and paternal parents in these crosses are equivalent). Hybrid fruits were left on plants for at least two months to allow fruit and seed maturation. Four seeds from a single fruit of the hybrid cross between groups D and C were planted to produce four F_1 lines (DxC-1, -2, -3 and -4). 20 F_2 seeds from each F_1 line were planted (total 80 plants). The F_2 plants for segregation studies were labeled using the hybrid line name and each F_2 plant was given an individual number (601- 620; e.g., DxC-1-612).

b. Interspecific pollen-pistil interactions

Interspecific crosses between representative accessions in the four groups of *S. neorickii* with pollen of red-fruited species, *S. lycopersicum, S. pimpinellifolium, S. galapagense,* and *S. cheesmaniae*, were performed according to the protocols in Chapter 3. I attempted to perform three replicated crosses from each of the three accessions in each *S. neorickii* group as females in interspecific crosses with each of

the red-fruited species as males. Crosses with pollen from *S. pimpinellifolium* and *S. lycopersicum* were tested with pistils of every *S. neorickii* accession. However, not all accessions were tested with pollen of *S. galapagense* and *S. cheesmaniae* due to pollen limitation. Since there is no noticeable difference in pollen tube growth among the four different red-fruited species in pistils of *S. neorickii,* pollen-pistil interactions were tested in inter-group hybrids (F1 and F2 generations) using *S. lycopersicum* and *S. pimipinellifolium* as representative of the red-fruited species.

Table 5.1. Accessions of *Solanum neorickii* used in this study

Group	Accessions	Site	Lat/Long
A	LA4023	Azuay, Ecuador	-2.783333/ -78.766667
	LA2862	Azuay, Ecuador	-3.550000/ -79.166667
	LA2113	Loja, Ecuador	-4.000000/ -79.290000
B	LA2190	Amazonas, Peru	-5.925000/ -78.066667
	LA2197	Amazonas, Peru	-6.107778/ -77.896111
	LA2200	Amazonas, Peru	-6.266667/ -77.733333
C	LA0247	Huánuco, Peru	-9.783333/ -76.583333
	LA2403	Huánuco, Peru	-10.166667/ -76.175000
	LA0735	Huánuco, Peru	-10.375000/ -76.208333
D	LA1322	Cusco, Peru	-13.450000/ -72.430000
	LA2639A	Apurimac, Peru	-13.558333/ -72.591667
	LA1319	Apurimac, Peru	-13.633889/ -72.881389

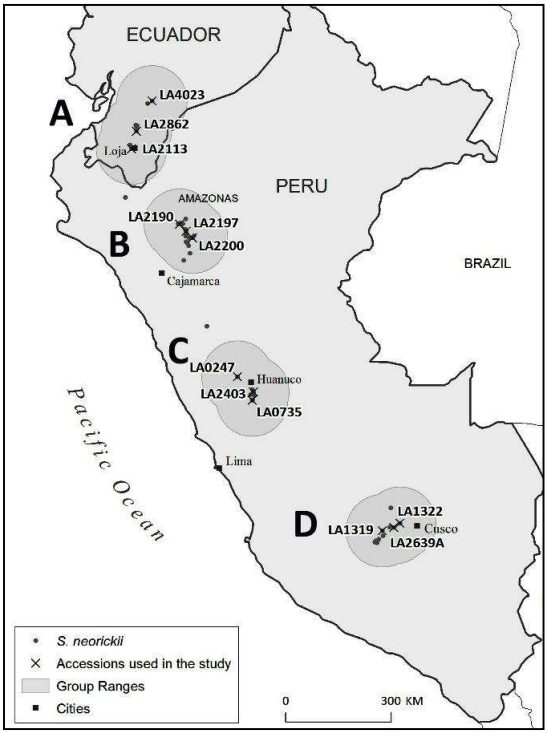

Figure 5.1. Groups A-D of *Solanum neorickii* used in this study. Accessions are listed and grouped according to geographical location (north to south) and the range of distributions.

c. S-RNase detection

Stylar protein was extracted from at least 20 mature, post-anthesis styles to test for S-RNase expression. Weighed styles were homogenized in 2x SDS buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 50 mM dithiothreitol, and 0.01% Bromophenol blue) at 10 uL per mg fresh weight. After grinding

styles in the buffer, samples were heated for 5 min in a 90 °C water bath and centrifuged at 14,000g for 10 min. The supernatant was collected and frozen until use.

Proteins were separated, blotted, and immunostained by my collaborator Dr. Alejandro Tovar-Mendez at University of Missouri- Columbia.

d. PCR amplification and sequencing of S-RNase alleles

Qiagen DNeasy plant kits (https://www.qiagen.com/us/) were used to purify genomic DNA. *Lpfsrn-1* and *Lpfsrn-2* in groups of *S. neorickii* were amplified using Pfx polymerase from ThermoFisher (https://www.thermofisher.com). For examination of the *Lpfsn-1* allele, the following primers were used to amplify a 730-bp product from the coding region: 5'neosrn-1-FP7 (5'-ATGGTTAAACCACAACTC ACAGCA-3') and 3'neosrn-1-RP7 (5'-TGTTGTTCAGCGAAAAAATATTTTTCCGG-3') using sequence registered in NCBI gene-bank accession number AB072475. Thermocycling conditions consisted of 32 cycles of 94 °C for 15 sec, 55 °C for 33 sec, and 68 °C for 1 min. For examination of the *Lpfsrn-2* allele, the following primers were used to amplify a 546-bp product: 5'neosrn-1-FP-1 (5'-GCGATGTAACCCCTTGAGG-3'), which lies where a base pair insertion results in a premature stop codon, and 3'neosrn-2-RP-1 (5'-CCAATCTCATTAAGTTCCACATTTCC-3') using sequence registered in NCBI gene-bank accession number. Thermocycling conditions were the same as for *Lpfsn-1*. The positions of the primers are show in Fig. 5.5. PCR products were separated on a 1% agarose gel and cloned into pJET for sequencing. To confirm quality of DNA, PCR primers designed to amplify a conserved positive control gene, *CAC* (Clathrin Adaptor Complexes medium subunit; SGN-U314153), were used: 5'CACFP (5'CCTCCGTTGTGATGTAACTGG-3') and 3'CACRP (5'-TTGGTGGAAA GTAACATCATCG-3'). This *CAC* primer set amplifies a 173-bp product from cDNA and a 610-bp product from gDNA (Expósito-Rodríguez et al., 2008).

For RT-PCR, total RNA was purified from mature pistils and leaves using a Qiagen RNeasy Plant Mini Kit and treated with a Qiagen RNase-Free DNase Kit. First strand cDNA templates were synthesized using a Bio-Rad iScript cDNA Synthesis Kit (http://www.bio-rad.com) using cycling conditions of 25 °C

for 5 min, 40 °C for 30 min, and 85 °C for 5 min. Econotaq plus Green Mastermix (Lucigen) were used to amplify cDNA with the same primer sets and thermocycling conditions of the gDNA methods. For cDNA samples, styles were pooled from multiple individuals in the same accessions (A2113, LA2639A, LA2403, and LA1319) or at least two different plants per accession were tested (LA4023, LA2862, LA0247, and LA1322). Sample preparations of F_2 populations were performed in only 16 plants. The quality of cDNA was also tested using *CAC* primers (described above). RT-PCR products were run on a 1.2% agarose gel to show size differences between cDNA and genomic DNA (control) due to the absence of an 80-bp intron.

Results

Assessment of pollen-pistil interactions between pollen of red-fruited species and pistils of different groups of Solanum neorickii

I performed cross-pollinations using pollen from red-fruited species onto pistils of three accessions from each group of *S. neorickii* to test whether interspecific prezygotic barriers differ in the four groups. As shown in Fig. 5.2 the peripheral group A (North, Ecuador) and group D (South, Cusco, Peru) reject pollen tubes from the SC red-fruited species, while group C (Huánuco, Peru) does not reject pollen tubes from the SC red-fruited species. Group B (Amazonas, Peru) shows variable pollen tube rejection depending on the individuals tested (Fig. 5.2).

Pollen tube growth of red-fruited species in pistils of each accession is shown in Fig. 5.3. In pistils of group A accessions (LA4023, LA2862, and LA2113), consistent rejection of pollen tubes from all red-fruited species occurred, with an average range of pollen tube growth of 2.1 to 2.7 mm, near the middle part of style.

Pistils of the three accessions of *S. neorickii* used as representatives of group B (LA2190, LA2197, and LA2200), showed variability between individuals in pollen tube rejection of red-fruited species. Two out of six crosses between styles of LA2190 and pollen with red-fruited species display pollen tube rejection, while pollen tube rejection was found in five out of six crosses with styles of LA2197. Half of

the crosses (three out of six) with styles of LA2200 show pollen tube rejection. Generally, individual plants showed consistent pollen-pistil interactions with the four red-fruited species. For example, pistils of LA2190-400 never rejected pollen tubes of red-fruited species while those of LA2190-521 always rejected pollen tubes. However, there was variability in pollen tube rejection in five individuals; LA2190-521, LA2197-540, -522, LA2200-400 and -621 pistils mostly rejected pollen tubes of red-fruited species, although occasionally pollen tubes were observed at the bottom of the styles in a few of these crosses (Appendix 1). In individuals where pollen tube rejection was observed, pollen tube growth of red-fruited species was inhibited at ~3 mm on average in the styles of three accessions in group B *S. neorickii*.

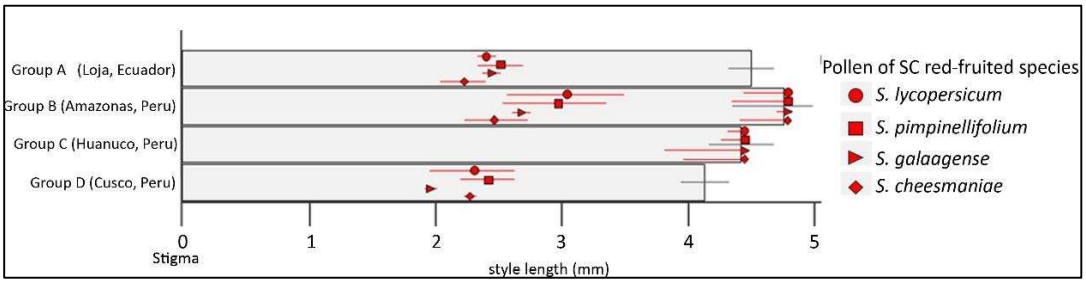

Figure 5.2. Length of pollen tubes from red-fruited species in pistils of four geographic groups of *S. neorickii* (group A-D, north to south). Pollen tube lengths are shown in millimeters and include the average of the majority of pollen tubes (symbols) and standard deviation (bars). Grey symbols show growth of pollen tubes to ovaries in some individuals of group B.

In group C, no pollen tube rejection was observed in pistils from any of the three accessions used, LA0247, LA2403, and LA0735 for pollen tubes of all four red-fruited species. This result is consistent with previous publications using LA0735 and LA0247 (Rick et al., 1967; Baek et al., 2015). In addition, the successful crosses between pistils of group C and pollen from *S. lycopersicum* and *S. pimpinellifolium* produced viable seeds and F_1 plants.

Pistils of the representative accessions of group D (LA1322, LA2639A, and LA1322) consistently rejected interspecific pollen tubes of red-fruited species at 2.3 mm on average.

In summary, the rejection of pollen tubes from red-fruited species differed in four geographic groups of *S. neorickii*. The peripheral groups (group A and group D) reject pollen tubes from red-fruited species while group C does not reject pollen tubes from red-fruited species. There is variability of pollen

tube rejection in individuals in group B. In all cases, pollen tube growth ceased after 2.4 mm on average to the middle of the style.

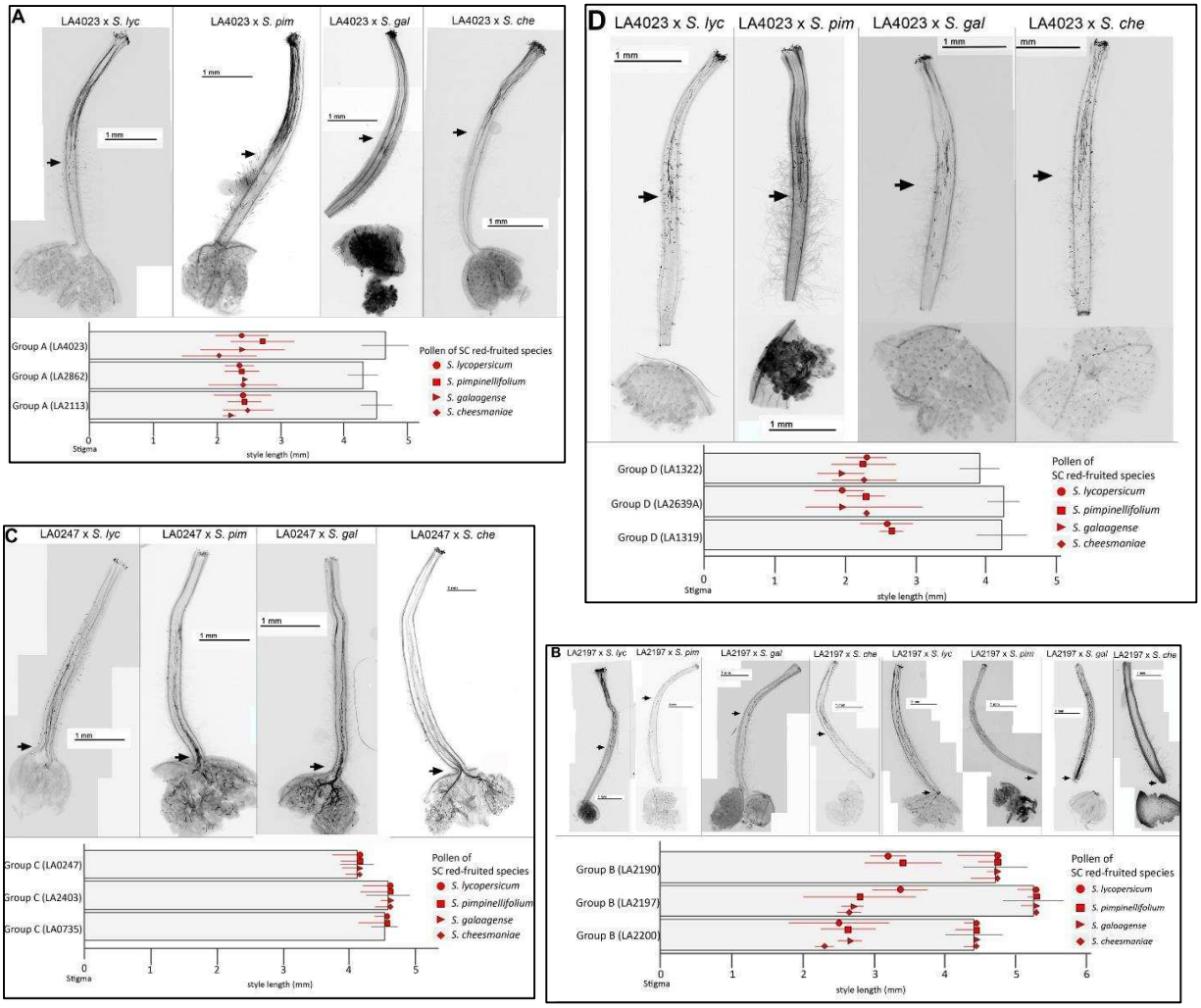

Figure 5.3. Pollen tube growth in crosses of pollen from red-fruited SC species onto pistils of four different geographic groups of *S. neorickii* (A-D). Top panel of each box shows representative images of crosses for each group of *S. neorickii* with pollen of red-fruited species; LA2862 is group A (top left), LA2197 is group B (top right), LA0247 is group C (bottom left), and LA1322 is group D (bottom right). Arrow indicates where majority of pollen tubes stop. Bar = 1mm. Bottom panel of each box shows the length of pollen tubes from red-fruited species in the pistils of three accessions from each group of *S. neorickii*. Shaded rectangle represents style length of *S. neorickii* and the average of majority pollen tube growth (mm) is indicated by symbols with standard deviation (bar).

Expression of pistil SI factor, S-RNase, in four groups of Solanum neorickii

Testing for S-RNase expression in styles of *S. neorickii* was performed in collaboration with Dr. Alejandro Tovar-Mendez, with immunoblots using an antibody raised to the conserved C2 domain of all S-RNases (Fig. 5.4). S-RNase was detected in accessions of group A (Ecuador, Peru) and group D (Cusco, Peru) but not group C (LA0247; Huánuco, Peru). Expression of S-RNase protein in group B appears to be segregating since S-RNase expression was observed in only some individuals in each accession. S-RNase expression correlated with pollen tube rejection in crosses with pollen from red-fruited species in group A, B and D. In contrast, pollen tubes of red-fruited species reached the ovaries in styles of group C and in the group B individuals lacking S-RNase expression.

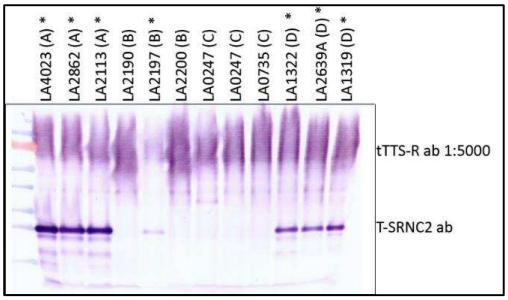

Figure 5.4. S-RNase in representative individual of accessions in four geographic groups (A-D) of *S. neorickii*. Immunostaining of stylar proteins by anti S-RNase C2 domain antibody. tTTS-R (Cheung et al., 1993) of blotted protein extracts acts as a loading control. * = accessions/individuals in which pollen tubes from red-fruited species are rejected. The expression of S-RNase in LA2197 was confirmed in a separate blot using a greater volume of extract (Fig. 5.14, bottom left panel).

S-RNase genes in Solanum neorickii

PCR amplification and sequence analysis were used to detect the presence of *S-RNase* genes in *S. neorickii*. In a previous study, Kondo et al. (2002a) found two different *S-RNase* alleles from two different accessions of *S. neorickii*. The *Lpfsrn-1* allele was identified in accession LA1322 (group D). While the sequence of the *Lpfsrn-1* allele indicates that it could encode an intact S-RNase protein, stylar extracts from LA1322 showed very low level of RNase activity. The *Lpfsrn-2* allele was found in accession LA0247 (group C), and the sequence of this allele shows a single base pair insertion that introduces a premature stop codon. To determine the distribution of the two alleles in the four groups of *S. neorickii*, specific primers for each allele were designed (Fig. 5.5) based on the sequences reported by Kondo et al. (2002a).

Consistent with the results of Kondo et al. (2002), all accessions in group D contain the *Lpfsrn-1* allele, and the *Lpfsrn-2* allele is present in all accessions in group C. Surprisingly, the *Lpfsrn-1* allele was detected in all 12 accessions of *S. neorickii* used in this study, including accessions that lack S-RNase expression (Fig. 5.5). Sequencing of cloned PCR products demonstrated that genomic DNA sequences of all the *Lpfsrn-1* PCR products are very similar, with only few base pair differences (Fig. 5.6). All coding sequences from the full length *Lpfsrn-1* PCR products could encode an intact S-RNase with five conserved regions and essential active site amino acids (His and Cys) found in functional S-RNases. Two base pair differences in coding sequences were found in one group A accession (LA2862) and three accessions in group B (LA2190, LA2197, and LA2200) which result in nonsynonymous, but conservative (L to I in the signal peptide and L to V in the C4 region) amino acid substitutions. All of the genomic DNA sequences show one intron with conserved splice site sequences as is typical of all characterized *S-RNase* genes (Fig. 5.6 and 5.7).

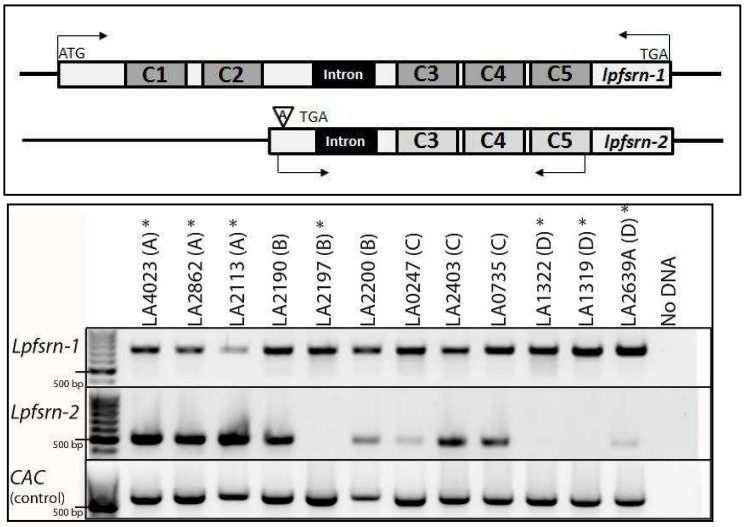

Figure 5.5. PCR amplification of *Lpfsrn-1* and *Lpfsrn-2* in genomic DNA of four geographic groups (A-D) of *S. neorickii*. Specific PCR primers were designed to anneal to the coding region of *Lpfsrn-1* (top schematic diagram). Specific primers designed for the *Lpfsrn-2* allele included a single base pair insertion for *Lpfsrn-2* (bottom schematic diagram). Amplification of a *Lpfsrn-1* product is shown in the top panel of gel figure; and *Lpfsrn-2* in the middle panel. The bottom panel shown amplification of a control gene (*CAC*; Clathrin Adaptor Complexes medium subunit). * = accessions in which pollen tubes from red-fruited species are rejected.

The *Lfsrn-2* allele previously identified in accession LA0247 (group C) by Kondo et al. (2002) was detected in all group C accessions. Surprisingly, the *Lpfsrn-2* allele was also detected in all accessions of

group A (LA4023, LA2862, and LA2113), in two accessions of group B (LA2190 and LA2200), and in one accession of group D (LA2639A) (Fig. 5.5). All sequencing results of the *Lpfsrn-2* allele from PCR products of each accession revealed the predicted single base pair insertion that results in a frameshift and premature stop codon, as previously reported (Fig. 5.8, Kondo et al., 2002).

Transcript expression of Lpfsrn-1 and Lpfsrn-2

The presence of the *Lpfsrn-1* allele in the genomes of all *S. neorickii* accessions was unexpected, because S-RNase protein expression was not detected in group C and in some individuals in group B. Since known S-RNases are expressed specifically in pistil (McClure et al., 1989; Kao and Tsukamoto, 2004), RT-PCR was performed to test for transcription of *Lpfsrn-1* in two different tissues (styles and leaves) in representative accessions for the A, C and D group. The expression of *Lpfsrn-1* was only observed in styles and not leaves in representative accessions from each group. Intriguingly, *Lpfsrn-1* transcript expression was greatly reduced in styles of LA0247 (group C) while styles of LA2862 (group A) and LA1322 (group D) exhibited high expression of *Lpfsrn-1* (Fig. 5.9). Therefore, although LA0247 has the full *Lpfsrn-1* coding region sequence, LA0247 has low *Lpfsrn-1* transcript expression and low or no S-RNase protein expression in style tissue.

To confirm the correlation of *Lpfsrn-1* expression and S-RNase protein expression, RT-PCR was performed in three groups (A, C and D) of *S. neorickii* (group B shows variability of S-RNase protein expression between individual plants, so it was not tested in RT-PCR due to the difficulty in getting sufficient RNA from a single individual). All accessions of groups A and D in which S-RNase protein was detected showed expression of *Lpfsrn-1* while the three accessions of group C lacked or showed greatly reduced expression of both *Lpfsrn-1* and S-RNase protein. These results correlated with pollen tube rejection in crosses with red-fruited species, in which pistils of groups A and D rejected pollen tubes from red-fruited species and group C did not.

The expression of *Lpfsrn-2* at the transcription level was also tested, and *Lpfsrn-2* does not appear to be transcribed in either styles or leaves from any group of *S. neorickii* (Fig. 5.9).

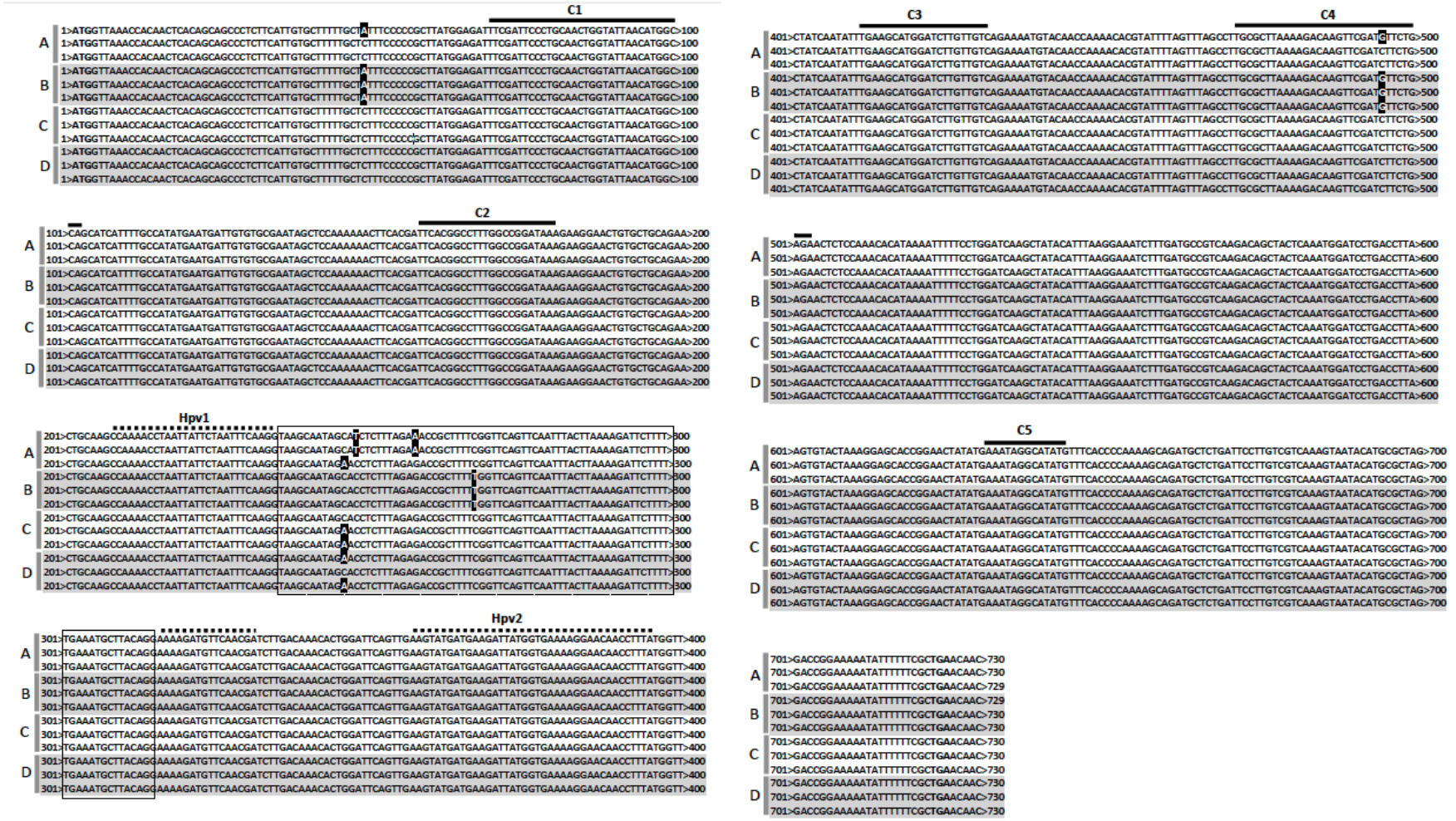

Figure 5.6. Alignment of *Lpfsrn-1* DNA sequences in 12 accessions of *S. neorickii*. Sequences are listed in the order of Table 5.1 (LA2862, LA4023, LA2113, LA2190, LA2197, LA2200, LA0247, LA2403, LA0735, LA1322, LA2639A, and LA1319). The black shaded regions show different base pairs among 12 accessions of *S. neorickii*. Sequences of group B and D are shaded in gray for visual convenience. The five conserved regions are indicated as C1 through C5 with a solid line above the sequences and the two hyper-variable regions are labeled as Hpv-1 and 2 with dotted lines above the sequences. The intron region is boxed. A, B, C, and D on the left side of the sequences represent the *S. neorickii* geographic groups.

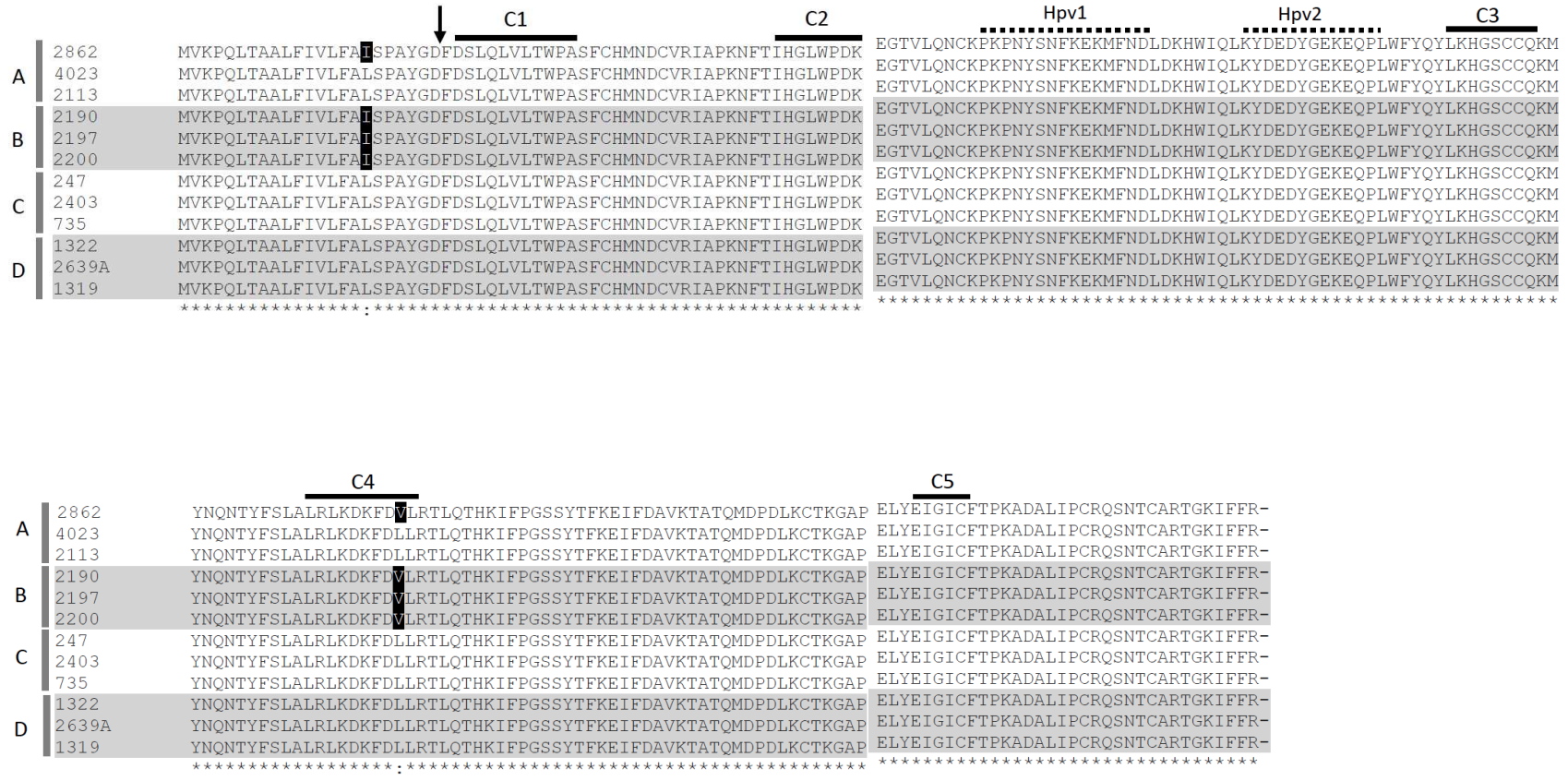

Figure 5.7. Deduced amino acid alignment of *Lpsrn-1* in *S. neorickii*. Accessions are labeled at the front of the sequences. The black shaded regions show different base pairs with the : symbol below the sequences among 12 accessions of *S. neorickii.* Sequences of group B and D are shaded in gray for visual convenience. The five conserved regions are indicated as C1 through C5 with a solid line above the sequences and the two hyper-variable regions are labeled as Hpv-1 and 2 with dotted lines above the sequences. A, B, C, and D on the left side of the sequences represent the *S. neorickii* geographic groups. *Indicates consensus sequences below the sequences. Arrow indicates the predicted signal peptide cleavage site.

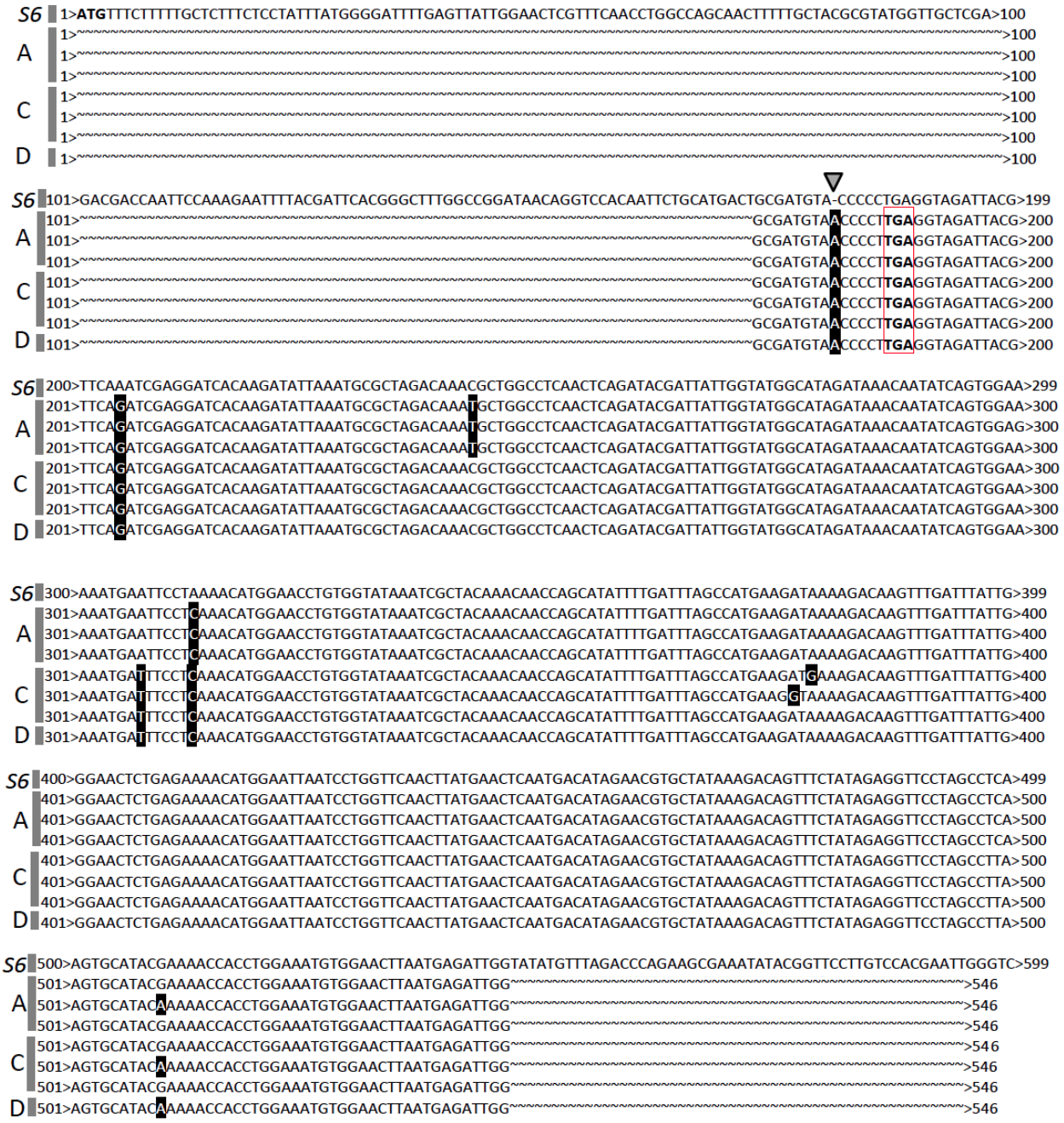

Figure 5.8. Sequences of *Lpfsrn-2* from genomic PCR after removing intron sequences in 7 accessions of *S. neorickii* aligned to the *S6* allele of SI *S. arcanum.* Sequences are listed in the following order: LA2163, LA2862, LA4023, LA2113, LA0247, LA2403, LA0735, and LA2639A. The black shaded regions show different base pairs. The triangle represents the single base pair insertion in *S. neorickii* and the red-box represents the premature stop codon. A, B, C, and D on the left side of the sequences represent the *S. neorickii* geographic groups.

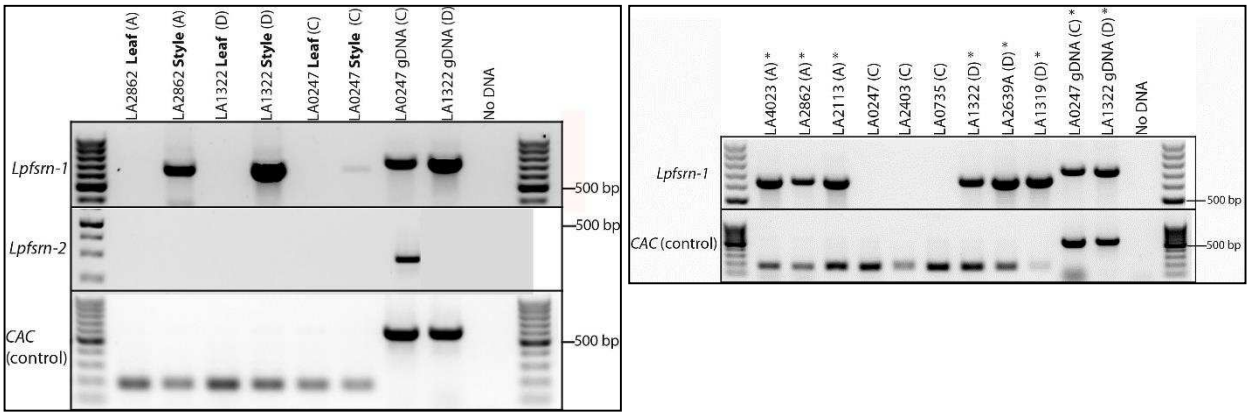

Figure 5.9. RT-PCR to test for the transcriptional level of *Lpfsrn-1* in style and leaves of *S. neorickii* (left), *Lpfsrn-1* in three groups of *S. neorickii* (right). * = accessions in which pollen tubes from red-fruited species are rejected.

Table 5.3. Summary of pollen tube rejection and S-RNase allele presence and expression in four geographic groups of *S. neorickii*.

	Accessions	Site	Pollen tubes of red-fruited species	S-RNase allele present	Transcriptional level of *Lpfsrn-1*	Expression of protein
A	LA4023	Azuay, Ecuador	Rejected	*Lpfsrn-1 -2*	High	Present
	LA2862	Azuay, Ecuador	Rejected	*Lpfsrn-1 -2*	High	Present
	LA2113	Loja, Ecuador	Rejected	*Lpfsrn-1 -2*	High	Present
B	LA2190	Amazonas, Peru	Variable	*Lpfsrn-1 -2*	NT	Variable
	LA2197	Amazonas, Peru	Variable	*Lpfsrn-1*	NT	Variable
	LA2200	Amazonas, Peru	Variable	*Lpfsrn-1 -2*	NT	Variable
C	LA0247	Huánuco, Peru	Not rejected	*Lpfsrn-1 -2*	Low/absent	Absent
	LA2403	Huánuco, Peru	Not rejected	*Lpfsrn-1 -2*	Low/absent	Absent
	LA0735	Huánuco, Peru	Not rejected	*Lpfsrn-1 -2*	Low/absent	Absent
D	LA1322	Cusco, Peru	Rejected	*Lpfsrn-1*	High	Present
	LA2639A	Apurimac, Peru	Rejected	*Lpfsrn-1 -2*	High	Present
	LA1319	Apurimac, Peru	Rejected	*Lpfsrn-1*	High	Present

NT= not tested.

Segregation of S-RNase and interspecific pollen tube rejection in inter-group F2 plants

Because Rick et al. (1976) reported some variability in the success of inter-group crosses in *S. neorickii*, pollen-pistil interactions in crosses between accessions from the four geographic groups of *S. neorickii* were assessed to test for the inter-group pollen tube growth using at least one accessions from each group. In reciprocal crosses, pollen tube rejection was not observed (Fig. 5.10). Successful crosses between groups of *S. neorickii* generated inter-group hybrids between two different parents, group C (LA0247) and group D (LA1322). The presence of S-RNase and the rejection of pollen tubes from red-fruited species were evaluated in the F_1 of inter-group hybrids to test for dominance and in the F_2 generation to test for the correlation between S-RNase and interspecific pollen tube rejection of red-fruited species. I also obtained F_1 hybrids between LA4023 group A and LA0247 group C, as well as between LA2862 group A and LA1322 group D for future studies.

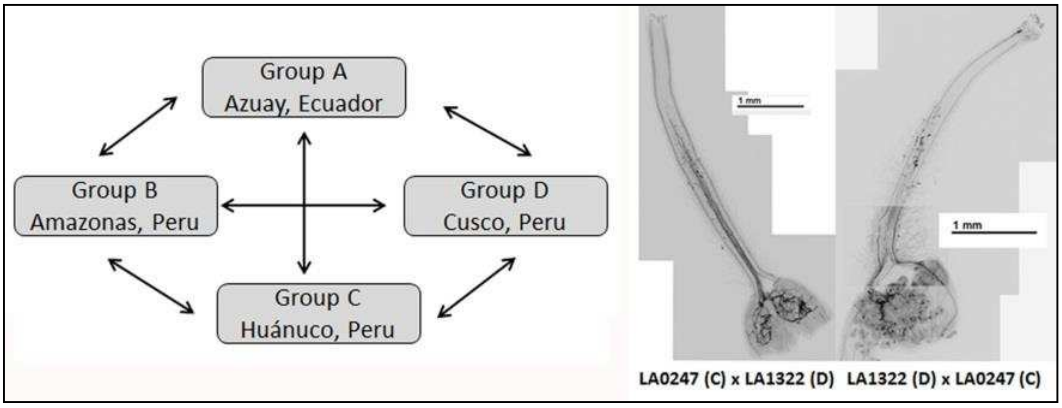

Figure 5.10. Pollen-pistil interactions between four groups of *S. neorickii*. Arrows indicate the compatible pollen tube growth (left). Representative images of pollen tube growth between groups C and D of *S. neorickii* (right).

Four individuals of the F_1 generation expressed S-RNase and rejected pollen tubes from red-fruited species in greenhouse conditions (Fig. 5.11 and Appendix S5.2.). However, it should be noted that variability of interspecific pollen tube rejection was observed in crosses done in the field. For example, pistils of DxC-2 F_1 line reject pollen tubes of red-fruited species in the greenhouse but half of crosses done in the field (three out of six crosses) showed pollen tubes of red-fruited species in the ovaries of DxC-2 F_1 line, which may reflect environmental influences on interspecific pollen tube rejection.

To assess the segregation of interspecific pollen tube rejection, 60 total F_2 plants were used in crosses as the female with pollen from *S. lycopersicum* and *S. pimpinellifolium* representing the red-fruited species. In 22 out of 60 plants, pollen tubes from both species of red-fruited species were rejected, and pollen tube rejection was not observed in 38 plants (Fig. 5.11 and Table 5.3). In some crosses, most of the pollen tubes were inhibited at about 80% the length of the styles, but a few pollen tubes (less than three) reached the ovaries. These crosses were denoted as "long tubes" (LT) in Table 5.4. In cases where a significant number of pollen tubes reached ovaries, pollen tubes were usually observed targeting the ovules (the second and the fourth images in Fig. 5.11). However, in a few individuals (DxC-2-611, 619, 625, and DxC-3-612) pollen tubes were observed at the bottom of the styles but not targeting the ovules (e.g., the last image on Fig. 5.11). If pollen tube rejection is controlled by a dominant allele of a single gene, we expected that 75% of the F_2 progeny should reject pollen of red-fruited species. However, the segregation of interspecific pollen tube rejection was 22 (reject) to 38 (pollen tubes at ovaries including LT; 63%) and the chi-square value is 7.019×10^{-12}, thus not supporting a simple Mendelian segregation ratio. If we classified the LT crosses as rejection, the ratio is 24 (reject) to 36 (pollen tubes at ovaries; 60%) with 3.825×10^{-10} of the chi-square value.

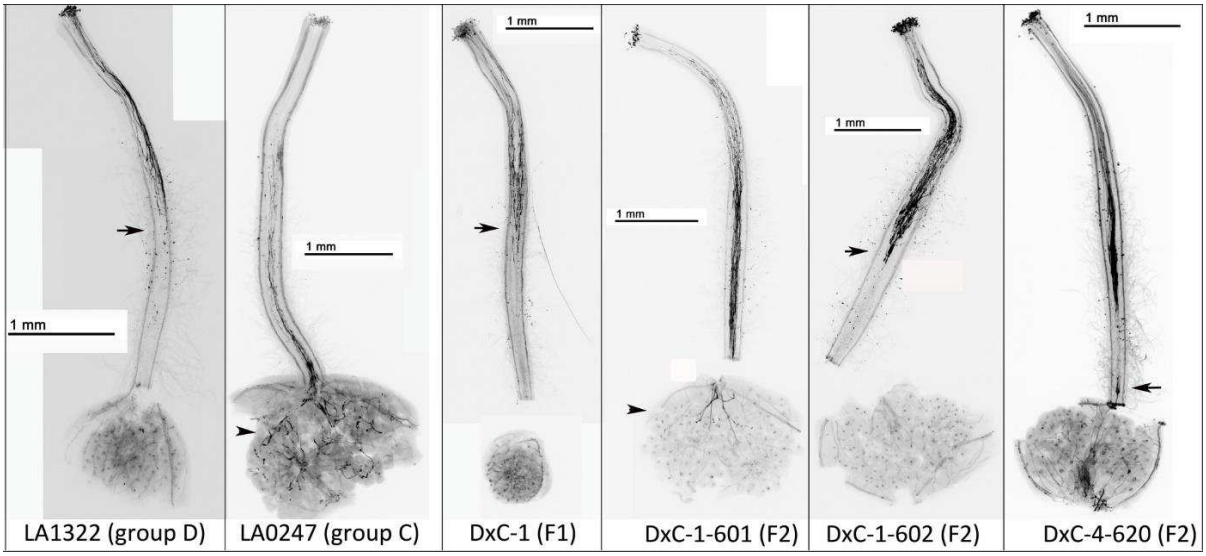

Figure 5.11. Interspecific pollen tube growth with pollen from red-fruited species in styles of *S. neorickii.* Representative images of crosses in the F_2 plants with pollen from red-fruited species (*S. lycopersicum* or *S. pimpinellifolium*). Plants used as the female are listed in the figures. Arrow indicates the point at which sthe majority of pollen tubes stop growing and arrowhead indicates pollen tubes in the ovaries.

69 F_2 individuals were tested for the presence of the *Lpfsrn-1* and *Lpfsrn-2* alleles. As expected, all individuals have the *Lpfsrn-1* allele since both parents possess the allele. Interestingly, 63 out of 69 individuals (91%) also contain the other allele, *Lpfsrn-2*, which is not the predicted in a Mendelian ratio of 75% (Fig. 5.12), suggesting that segregation distortion has occurred in F_1 self-pollinations. The presence of the *Lpfsrn-2* allele does not correlate with pollen tube rejection; individuals that do not possess the *Lpfsrn-2* allele can still reject pollen tubes of red-fruited species, consistent with observations of interspecific pollen tube rejection in LA1322 (group D) that lack the *Lpfsrn-2* allele (Fig 5.12 and Table 5.3).

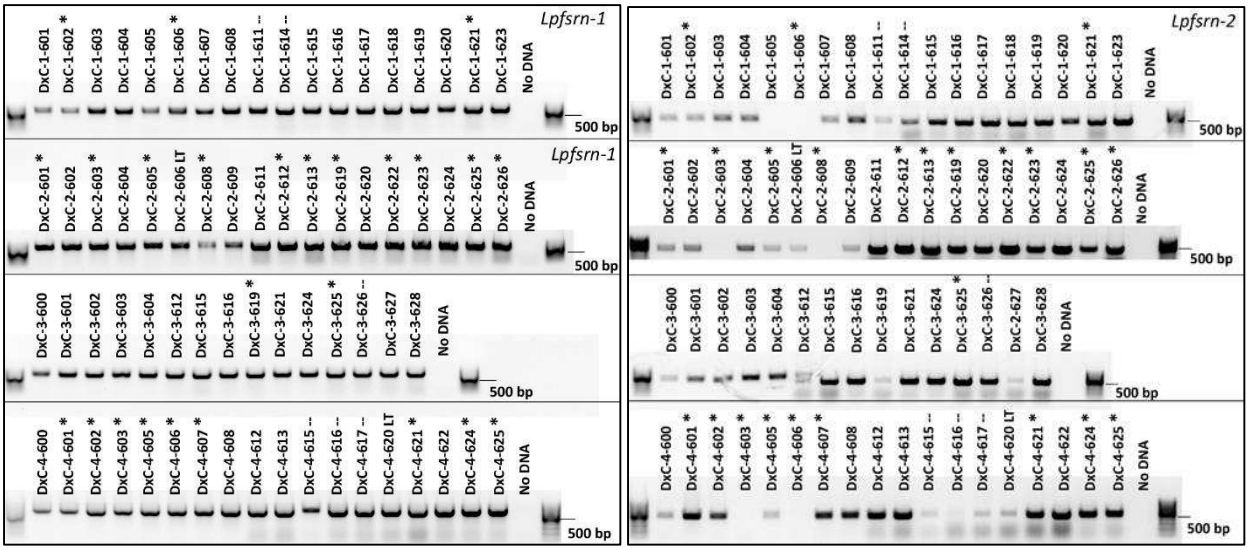

Figure 5.12. PCR amplification of *Lpfrsrn-1* (left) and *Lpfsrn-2* (right) in genomic DNA of F_2 plants. * = individuals in which pollen tubes from red-fruited species are rejected. -- = individuals were not tested for interspecific pollen tube rejection. LT= pollen tubes of red-fruited species were observed in the ovaries of these individuals.

Since *Lpfsrn-1* transcript expression was not observed in the parent LA0247 (group C), I tested for the expression of *Lpfsrn-1* in a few individuals of the F_2 population using RT-PCR. As predicted, *Lpfsrn-1* transcript expression was detected in the individuals that reject pollen tubes of red-fruited species and low expression (or absence of expression) was observed in the individuals that do not display interspecific pollen tube rejection (Fig. 5.13).

Stylar extracts of 56 F_2 individuals were used to assess S-RNase protein expression in order to examine whether S-RNase expression segregates with the ability to reject pollen tubes from red-fruited

species in pistils of *S. neorickii*. Individuals that rejected pollen tubes from red-fruited species also expressed S-RNase (Fig 5.14). However, only 15 of 29 individuals expressing S-RNase rejected pollen tubes from red-fruited species (Fig 5.14). In addition, S-RNase expression was still detected in two individuals with long pollen tube growth. [Note total 35 individuals express S-RNase and six of those plants were not tested for pollen tube rejection.] Interestingly, those 14 plants that express but do not reject pollen tubes from red-fruited species were grown from field condition.

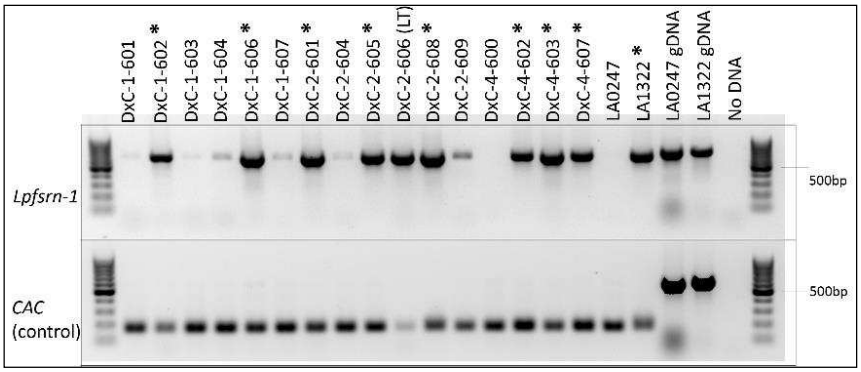

Figure 5.13. RT-PCR to test expression of *Lpfsrn-1* in stylar cDNA of the F_2 generation and genomic DNA as a control. * = accessions/individuals in which pollen tubes of red-fruited species are rejected. LT= pollen tubes of red-fruited species were observed at the bottom of styles not in the ovaries of these individuals.

As described earlier, the test of *Lpfsrn-1* transcript expression showed the correlation between the express and pollen tube rejection in 16 greenhouse-grown F_2 plants. *Lpfsrn-1* transcript expression correlated with S-RNase protein expression and interspecific pollen tube rejection (Fig. 5.13). However, given the field-grown 13 plants that expressed S-RNase but did not reject pollen tubes of red-fruited species (Fig. 5.14), it appears that either S-RNase expression may be required but not sufficient for this rejection, or that environmental effects can influence S-RNase function in pistils, as discussed below (Table 5.4).

The S-RNase expression segregation ratio in the F_2 population was about 1:1, not the expected 3:1 Mendelian segregation ratio, again suggesting that segregation distortion has occurred. Intriguingly, the observed segregation distortion is more apparent in two out of the four F_2 lines (Fig. 5.14). In the F_2 plants that are from DxC-1 and -3 F_1 lines, 40% of DxC-1 F_2 plants and 43% of DxC-3 F_2 plants express S-RNase

protein, while F_2 plants from the DxC-2 and DxC-4 F_2 lines express S-RNase at close to predicted Mendelian 3:1 ratio, 78% and 67 %, respectively.

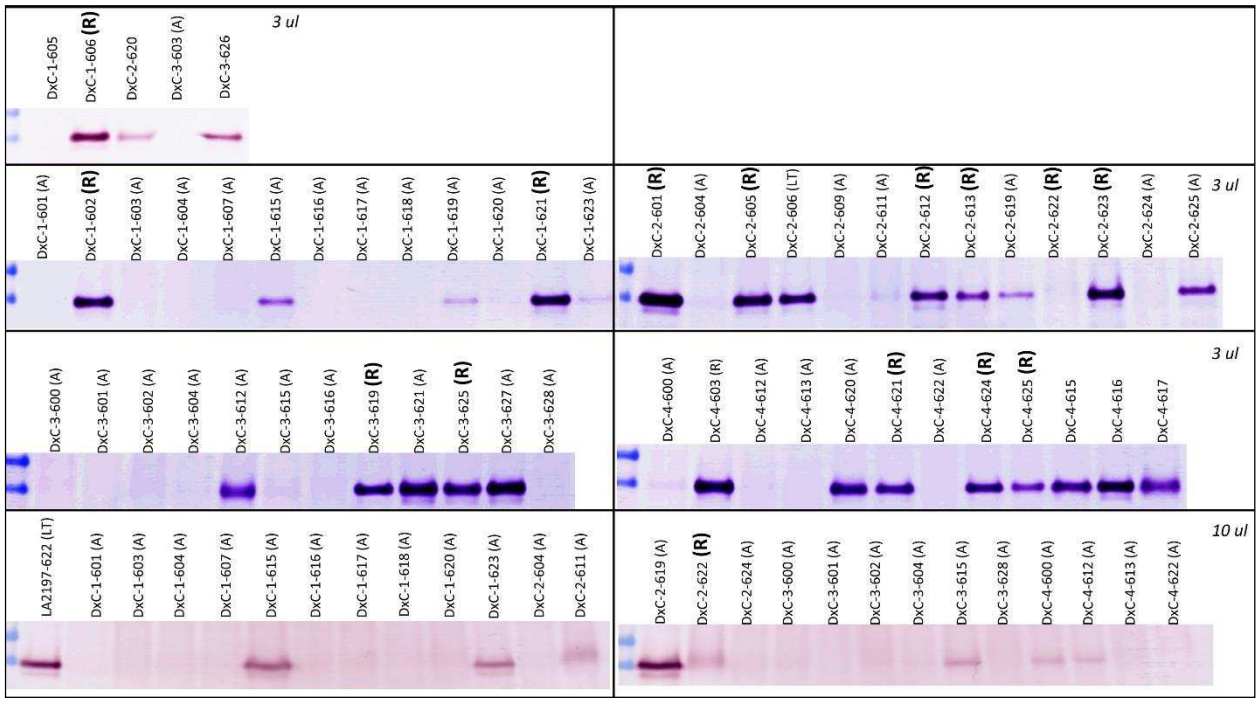

Figure 5.14. Immunostained S-RNase proteins in pistil extracts from the F_2 generation (provided by Alejandro Tovar-Mendez). Blots on the top two panels were performed with 3 µl of stylar extract, while 10 µl of stylar extract was loaded on the bottom panel. (A) represents pollen tubes of red-fruited species were accepted in pistils of these individuals; (R) represents interspecific pollen tube rejection; LT represents pollen tubes of red-fruited species were observed in the ovaries of these individuals

Table 5.4. Summary of results from F2 populations.

		PT	*Lpfsrn*			
			-1	*-2*	RNA	Protein
DxC-1	608	Accept	+	+		
	616	Accept	+	+		-
	617	Accept	+	+		-
	618	Accept	+	+		-
	620	Accept	+	+		-
	601	Accept	+	+	-	-
	603	Accept	+	+	-	-
	604	Accept	+	+	-	-
	607	Accept	+	+	-	-
	623	Accept	+	+		+
	615	Accept	+	+		+
	619	Accept	+	+		+
	621	Reject	+	+		+
	602	Reject	+	+	+	+
	606	Reject	+	-	+	+

		PT	*Lpfsrn*			
			-1	*-2*	RNA	protein
DxC-2	602	Accept	+	+		
	624	Accept	+	+		-
	604	Accept	+	+	-	-
	609	Accept	+	+	+ low	-
	611	Accept [a]	+	+		+
	619	Accept [a]	+	+		+
	625	Accept [a]	+	+		+
	606	LT	+	+	+	+
	626	Reject	+	+		
	622	Reject	+	+		+
	612	Reject	+	+		+
	613	Reject	+	+		+
	623	Reject	+	+		+
	601	Reject	+	+	+	+
	605	Reject	+	+	+	+
	608	Reject	+	-	+	

		PT	*Lpfsrn*			
			-1	-2	RNA	protein
DxC-3	624	Accept	+	+		
	600	Accept	+	+		-
	601	Accept	+	+		-
	602	Accept	+	+		-
	603	Accept	+	+		-
	604	Accept	+	+		-
	616	Accept	+	+		-
	628	Accept	+	+		-
	621	Accept	+	+		+
	612	Accept [a]	+	+		+
	615	Accept	+	+		+
	627	Accept	+	+		+
	619	Reject	+	+		+
	625	Reject	+	+		+

		PT	*Lpfsrn*			
			-1	*-2*	RNA	protein
DxC-4	608	Accept	+	+		
	613	Accept	+	+		-
	622	Accept	+	+		-
	612	Accept	+	+		+
	600	Accept	+	+	-	+
	620	LT	+	+		+
	601	Reject	+	+		
	605	Reject	+	+		
	606	Reject	+	-		
	621	Reject	+	+		+
	624	Reject [b]	+	+		+
	625	Reject	+	+		+
	603	Reject [b]	+	-	+	+
	602	Reject	+	+	+	
	607	Reject	+	+	+	

PT column summarizes results of pollen tube rejection; *Lpfsrn* column summaries presence of PCR products in genomic DNA; RNA column summarizes presence of the *Lpfsrn-1* transcript expression from RT-PCR with stylar cDNA; protein column summarizes presence of S-RNase protein expression.
+ represents the presence of the gene or its expression, - indicates the absence; in RNA column, either absence or low expression of transcript was indicated with -.
Blank cells represent those not tested.
[a] In these individuals, pollen tubes reach the ovaries but are never observed within ovaries targeting the ovules.
[b] Most of the crosses display interspecific pollen tube rejection, but one cross shows pollen tube acceptance.

Discussion

The role of SI factors in interspecific pollen tube rejection

Interspecific pollen tube rejection frequently follows the SI x SC rule, in which crosses between self-incompatible (SI) species and self-compatible species (SC) are successful when the SC species is female in a cross but not in the reciprocal cross, a phenomenon called unilateral incompatibility (UI). This suggests overlapping mechanisms between UI and SI (Lewis and Crowe, 1958; Mutschler and Liedl, 1994; Liedl et al., 1996; Murfett et al., 1996; Covey et al., 2010; Baek et al., 2015). Mechanisms of the gametophytic SI system in the Solanaceae have been well studied (McClure, 2004; McClure and Franklin-Tong, 2006; Kubo et al., 2010; Li and Chetelat, 2014), while interspecific pollen tube rejection is far less understood. In the tomato clade, the SI system uses *S* locus-encoded stylar S-RNase to recognize and reject self-pollen tubes. The relationship between SI and UI has been supported in several studies in the tomato clade. Pollen and pistil UI QTL have been mapped to the *S*-locus (Chetelat and Deverna, 1991; Bernacchi and Tanksley, 1997). In addition, *HT* genes (another pistil SI factor) map to major UI QTL on chromosome 12 (Covey et al., 2010). A more recent study directly tested the function of SI genes in UI, with the introduction of both *S-RNase* and *HT* into cultivated tomatoes creating an IRB leading to rejection of pollen tubes from red-fruited species (Tovar Mendez et al., 2014).

Low activity S-RNase alleles

Low enzymatic activity S-RNases are a feature of several SC taxa in Solanaceae (Huang et al., 1994; Golz et al., 1998; Kondo et al., 2002; Covey et al., 2010). For example, an expressed S-RNase in an SC population of *S. arcanum* (LA2157) has low enzymatic activity (Royo et al., 1994). In this SC *S. arcanum*, low S-RNase enzymatic activity was attributed to the loss of histidine residue known to be at the active site of the enzyme (Huang et al., 1994; Royo et al., 1994). This SC *S. arcanum* with low activity S-RNase exhibits a weak UI in crosses with pollen of red-fruited species (Baek et al., 2015). In addition, an SC population of *S. habrochaites* (LA1927) was found to express a low activity S-RNase and still display UI when pollinated with red-fruited *S. lycopersicum* (Covey et al., 2011). Therefore, although low enzymatic activity S-RNases might be insufficient for SI, they may be sufficient for rejecting interspecific

pollen tubes from red-fruited species. Kondo et al. (2002a) reported that S-RNase in LA1322 (group D *S. neorikcii*) has low enzymatic activity. Since the sequence of a putative *S-RNase* allele from this line had no obvious defects, it was proposed that the S-RNase in LA1322 maybe not functional due to abnormal post-translational modification. It is also possible that another factor may be involved in activity of S-RNase, in which as absence of an activator gene or the presence of an inhibitor gene could influence activity of S-RNase. Another alternative is that in interspecific pollen tubes are rejected by a redundant, non-S-RNase based UI mechanism (Murfett et al., 1996; Covey et al., 2010; Eberle et al., 2013; Baek et al., 2015).

Differential pollen tube rejection and S-RNase expression in four geographic groups of Solanum neorickii

Despite *S. neorickii* showing very low genetic diversity in the allozyme study performed by Rick et al. (1976), I have identified different groups of *S. neorickii* displaying variability in both S-RNase expression and interspecific pollen tube rejection with red-fruited species. In this study, differences in interspecific pollen tube rejection were observed in four different geographic groups of *S. neorickii*. Pistils of group A (Azuay, Ecuador) and group D (Cusco/Apurimac, Peru) accessions reject pollen tubes from red-fruited species, while group C (Huánuco, Peru) does not. This pattern of pollen tube rejection correlated with the expression of S-RNase protein; protein expression was detected in groups A and D but not in group C. Accessions in group B showed segregation of interspecific pollen tube rejection and protein expression; individuals expressing the protein also reject pollen tubes from red-fruited species. HT protein, which is also required for interspecific pollen rejection, was detected by immunoblotting in all *S. neorickii* accessions regardless of geographic group (data not shown). The correlation between the S-RNase protein expression and interspecific pollen tube rejection, in which accessions in groups A and D express the protein while accessions in group C do not, led me to hypothesize that the low activity S-RNase may be involved in interspecific pollen tube rejection.

Distribution of two S-RNase alleles, Lpfsrn-1 and Lpfsnr-2, and transcriptional expression

Kondo et al. (2002a) identified two putative *S-RNase* alleles in *S. neorickii*. The *Lpfsrn-2* allele from group C has a single base pair insertion in the coding region leading to a frame shift that would result

in a truncated protein. The *Lpfsrn-1* allele in LA1322 (group D) could encode a functional protein with the five conserved regions and essential active site amino acid residues (His and Cys) found in all S-RNase proteins. I tested for the presence of each allele by performing PCR with genomic DNA from accessions in the four geographic groups of *S. neorickii* to see if there was a relationship of either allele with interspecific pollen tube rejection. Surprisingly, the loss-of-function *Lpfsrn-2* allele was found in not only accessions of group C but also in group A, some accessions of group B, and one accession in group D (Fig. 5.5 and 5.8). Thus, the *Lpfsrn-2* allele is not correlated with either S-RNase expression or interspecific pollen tube rejection. At this time, we do not know whether *Lpfsrn-2* is pseudogene at the *S*-locus or is located elsewhere in the genome.

Also surprisingly, the *Lpfsrn-1* allele was detected in all S. *neorickii* accessions regardless of S-RNase protein expression. Since group C does not show protein expression even though the *Lpfsrn-1* allele is present in the genomes, I hypothesized that S-RNase protein was regulated at the level of transcription. Indeed, transcripts of *Lpfsrn-1* were detected in the A and D groups, while transcript abundance was greatly decreased in accessions of the C group (Fig. 5.9, Table 5.3). At this time we do not know why transcription is decreased in the C group, but it is possible that it may be due to a mutation in a regulatory region. An example of such a mutation is the insertion of a transposable element in the promoter region of an S-RNase gene in northern accession of SC *S. habrochaites* that has been associated with the absence of transcription and protein expression (Kondo et al., 2002; Covey et al., 2010). Alternatively, the C group may lack factor(s) required for *S-RNase* transcription. Further experiments need to be performed to confirm that the *Lpfsrn-1* allele maps to the S-locus and encodes the low activity S-RNase protein detected by immunoblotting.

Segregation of interspecific pollen tube rejection and S-RNase protein in an F2 generation.

The four different geographic groups of *S. neorickii* that differ in interspecific pollen tube rejection are fully inter-fertile (Fig. 5.10), so I was able to make inter-group F_1 hybrids between group D and C, and I selfed these to generate F_2 plants. I expected that interspecific pollen tube rejection and S-RNase expression were a dominant trait in the F_1 generation and this did seem to be the case in greenhouse crosses.

In some pollinations performed in the field, however, interspecific pollen tubes reached ovaries in plants expressing S-RNase, suggesting that the UI system may be labile in some environmental conditions (as discussed below).

Because F_1 parents were heterozygous, I expected a 3:1 ratio of the *Lpfsrn-2* allele, of S-RNase protein expression and of interspecific pollen tube rejection in F_2 plants. Instead I found unexpected ratios of all three in the F_2 generation. Since the *Lpfsrn-2* allele should have been heterozygous in F1 plants, it was expected that 75% of the F_2 plants would contain this allele. However, 63 out of 69 plants (91%) had the *Lpfsrn-2* allele, far different from the expected Mendelian 3:1 ratio. The segregation distortion that we observed for the *Lpfsrn-2* allele and S-RNase expression might be due to the selection against the *S*-locus from LA1322 (group D) on pollen side in selfs of F_1 plants. An example of this kind of selection was seen in a previous study (Rick and Chetelat, 1991) in the distorted segregation of the *S*-locus in F2 plants from crosses between northern and southern SC populations of *S. habrochaites*. Unexpectedly, there was a complete absence of plants homozygous for the southern population *S-RNase* allele, implying that pollen containing this allele either did not survive to maturation or was excluded by the pistil of the F1 hybrid. Our finding that a higher fraction of F_2 plants contain the *Lpfsrn-2* allele and the fraction of F_2 plants expressing S-RNase (60%) is lower than expected the 75%, is suggestive that this kind of selection is occurring in our studies as well. It should be noted, however, that in my study, only a total of 50-60 F_2 plants were analyzed, and therefore the sample size may be too small to observe the expected Mendelian ratios.

I hypothesized that interspecific pollen tube rejection would strictly correlate with S-RNase protein expression in *S. neorickii*. However, although S-RNase protein expression was detected in all F2 plants that reject pollen tubes of red-fruited species, some plants that express S-RNase protein did not reject interspecific pollen tubes. One possible explanation for this is that independently segregating factors in addition to S-RNase may be required for interspecific pollen tube rejection in *S. neorickii.* It has been observed in the tomato clade that more recently evolved SC populations of SI species still exhibit UI even

though they lack S-RNase expression (Murfett et al., 1996; Covey et al., 2010; Baek et al., 2015), suggesting factors other than S-RNase are involved in UI in some cases.

Another possible explanation is that environmental factors could influence interspecific pollen tube rejection. All of my crosses using greenhouse-grown plants were consistent with my hypothesis - that a low-activity S-RNase would be both necessary and sufficient for interspecific pollen tube rejection. However, my crosses conducted in summer fields in Colorado were not consistent with my hypothesis, since in these crosses I observed pollen tube rejection in only about half of the plants (11/22) expressing S-RNase. Temperature is one possible environmental factor that is known to impact pollen tube growth. For example, pistils of *Nicotiana tabacum* normally inhibit the growth of *N. obtusifolia* pollen tubes. However, pollen tubes of *N. obtusifolia* were not inhibited by pistils of *N. tabacum* when crosses were done at temperatures at or exceeding 35 °C (Eberle et al., 2013). Martin (1964) also observed variability in pollen pistil interactions in *S. habrochaites* and noted that "pseudo-compatibility" and apparent segregation ratios could be due to high temperatures. It is possible that since the enzymatic activity of S-RNase is already low in *S. neorickii*, environmental factors could further reduce activity to non-functional levels under field conditions, allowing interspecific pollen tubes to continue growth in styles that are expressing protein. Further tests such as testing interspecific pollen tubes in F_1 plants under controlled hot temperature condition would be required to validate this hypothesis.

Further implications

The differential genotypes and phenotypes in four *S. neorickii* geographic groups that I observed may be the result of differing selection pressures. The three groups of *S. neorickii* (A, B, and D) that can express S-RNase and reject interspecific pollen tubes overlap with the distribution ranges of other tomato clade species, including the red-fruited species *S. pimpinellifolium*, and the green-fruited *S. habrochaites* and *S. chmielewskii.* In these situations, there could be continuous selection for interspecific reproductive barriers to prevent hybridization. However, *S. neorickii* group C from the Huánuco, Peru area, which does not express S-RNase protein and accepts interspecific pollen tubes from red-fruited species (and indeed

makes fruits with viable seeds), shows no range overlap with other tomato clade species. In fact, the closest other wild species (SI *S. arcanum* and SI *S. peruvianum*) to the *S. neorickii* C group is at least 100 km away based on the information from TGRC.

In some cases, stronger interspecific reproductive barriers can be selected through a process called reinforcement (Dobzhansky 1940; Howard, 1993; Servedio and Noor, 2003; Kay and Schemske, 2009; Hopkins and Rausher, 2012) or reproductive character displacement (Levin and Kerster 1967; Schluter and McPhail, 1992; Fishman and Wyatt, 1999). Reinforcement results from selection favoring the strengthening of reproductive barriers to reduce the frequency of mating between species or hybrid formation. The term of reinforcement is restricted to cases where gene flow is still occuring between closely related taxa. On the other hand, reproductive character displacement is a selected pattern of differentiation in reproductive traits (i.e. floral morphology) between populations occurring in sympatry compared to those occurring in allopatry, when speciation between two taxa is complete, i.e. there is no gene flow (Butlin 1987; Hopkins et al. 2012). I propose that since the group C is isolated from other wild tomato species, the selection for interspecific reproductive barriers may have been reduced, leading to the weakening of IRBs.

In summary, results from this study support the idea that differences in interspecific pollen tube rejection in four geographic groups of *S. neorickii* may be related to a low activity S-RNase. A candidate *S-RNase* gene, *Lpfsrn-1*, is present in all groups of *S. neorickii*, but expression of *Lpfsrn-1* was detected only in accessions that express S-RNase protein and reject pollen tubes of red-fruited species. Although the test of segregation in S-RNase expression and IRBs in my F_2 population suggests that other either factor(s) or environmental effects may also be involved in regulating interspecific reproductive barriers, a low activity S-RNase may still be required to reject interspecific pollen tubes in *S. neorickii*. The finding an *S-RNase* allele that cannot function in the SI system still may be able to participate in interspecific pollen tube rejection is novel. Further experimentations will be required to prove this hypothesis.

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APPENDICES

Appendix S3.1. Accessions used in this study

Species	Mating system	Accession	Country (Province/Department)	Collection site	Latitude	Longitude
S. lycopersicum	SC	VF36 M82 LA1221				
S. pimpinellifolium	SC	LA1383	Perú (Amazonas)	Chachapoyas to Bagua	N/A	N/A
		LA2149	Perú (Cajamarca)	Puente Muyuna	–7.21806	–78.7878
		LA1590	Perú (La Libertad)	Virú to Tomaval	–8.37	–78.73
		LA1589	Perú (La Libertad)	Virú - Galunga	–8.39	–78.74
		LA3798	Perú (Ancash)	Río Pativilca	–10.65472	–77.4428
		LA1610	Perú (Lima)	Asia - El Piñon	–12.76667	–76.5167
S. galapagense	SC	LA1408	Ecuador (Galápagos Islands)	Isabela: SW volcano, Cape Berkeley	–0.04611	–91.55861
		LA0317	Ecuador (Galápagos Islands)	Bartolomé	–0.28333	–90.55
		LA0438	Ecuador (Galápagos Islands)	Isabela: coast at Villamil	–0.9775	–91.0211
S. cheesmaniae	SC	LA0426	Ecuador (Galápagos Islands)	Bartolomé: E of landing	–0.283333	–90.55
		LA0522	Ecuador (Galápagos Islands)	Fernandina: Outer slopes	–0.36667	–91.55
		LA0166	Ecuador (Galápagos Islands)	Santa Cruz: Barranco, N of Puerto Ayora	–0.75	–90.3167
		LA0421	Ecuador (Galápagos Islands)	San Cristobal: cliff E of Wreck Bay	–0.89778	–89.6094
S. neorickii	SC	LA4023	Ecuador (Azuay)	Paute	–2.78333	–78.7667
		LA0247	Perú (Huanuco)	Chavinillo	–9.78333	–76.5833
		LA2403	Perú (Huanuco)	Wandobamba	–10.16667	–76.175
S. chmielewskii	Facultative SC	LA1316	Perú (Ayacucho)	Ocros	–13.3925	–73.915
		LA1325	Perú (Apurimac)	Puente Cunyac	–13.56667	–72.5833
S. arcanum	SC	LA2157	Perú (Cajamarca)	Tunel Chotano	–6.50583	–78.8089
	SI	LA2163	Perú (Cajamarca)	Cochabamba to Yamaluc	–6.49444	–78.8983
		LA2150	Perú (Cajamarca)	Puente Muyuna	–7.21806	–78.7878
		LA1351	Perú (Cajamarca)	Rupe	–7.28333	–78.8167
		LA2327	Perú (Cajamarca)	Aguas Calientes	–7.45833	–78.1083

S. huaylasense	SI	LA3799	Perú (Ancash)	Río Pativilca	–10.65472	–77.4428
S. corneliomulleri	SI	LA1646	Perú (Lima)	Yaso	–11.56361	–76.7236
		LA1294	Perú (Lima)	Surco	–11.86667	–76.4417
		LA1609	Perú (Lima)	Asia - El Piñon	–12.76667	–76.5167
		LA1373	Perú (Lima)	Asia	–12.78694	–76.5786
		LA1694	Perú (Lima)	Cacra	–12.8125	–75.7836
		LA1722	Perú (Huancavelica)	Ticrapo Viejo	–13.43528	–75.4653
S. chilense	SI	LA3153	Perú (Moquegua)	Desvio Omate	–17.07167	–70.8494
		LA1962	Perú (Tacna)	Huaico Tacna	–17.95	–70.3167
		LA2773	Chile (Arica and Parinacota)	Zapahuira	–18.36667	–69.6333
		LA2755	Chile (Tarapacá)	Baños de Chusmisa	–19.68417	–69.1811
		LA2884	Chile (Antofagasta)	Ayaviri	–22.24778	–68.3608
		LA4330	Chile (Antofagasta)	Caspana	–22.35083	–68.3197
S. habrochaites	SC	LA0407	Ecuador (Guayas)	El Mirador, Guayaquil	–2.983333	–79.7667
	SI	LA1352	Perú (Cajamarca)	Rupe	–7.333611	–78.7969
		LA1986	Perú (La Libertad)	Casmiche	–7.977778	–78.6472
		LA1777	Perú (Ancash)	Rio Casma	–9.55	–77.59
		LA1778	Perú (Ancash)	Rio Casma	–9.56	–77.62
		LA1648	Perú (Lima)	Above Yaso	–11.55056	–76.715
S. pennellii	SC	LA0716	Perú (Arequipa)	Atico	–16.20944	–73.6222
	SI	LA2560	Perú (Ancash)	Santa to Huaraz	–8.66667	–78.3078
		LA0751	Perú (Lima)	Sisacaya	–12.025	–76.6417
		LA1340	Perú (Lima)	Capillucas	–12.83472	–75.9292

Notes: SC and SI refer to self-compatible and self-incompatible mating systems respectively. Accessions were provided by the Charles M. Rick Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/)

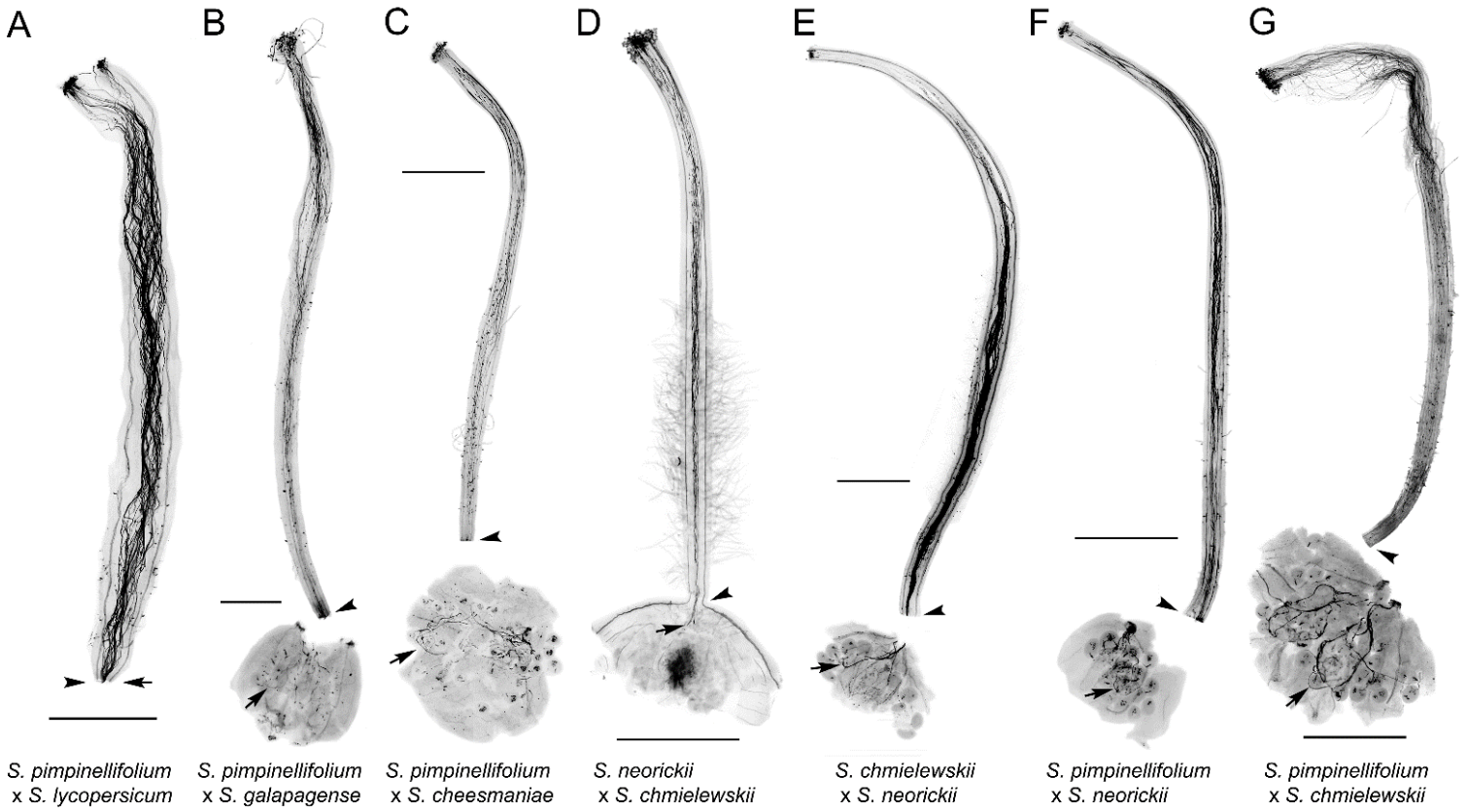
Appendix S3.2. Differences between lengths of the majority and the longest pollen tubes during UI rejection in interspecific crosses in the tomato clade.

	Male					
Female	*S. lyc (SC)*	*S. pim (SC)*	*S. gal (SC)*	*S. che (SC)*	*S. chm (SC)*	*S. neo (SC)*
S. chm (SC)	0.5 [a]	0.6 [a]	0.8 [a]	0.9 [a]	**SC**	Seed
S. neo (SC)	0.5 [b]	0.6 [b]	1.0 [b]	0.7 [b]	Seed	**SC**
S. arc (SI)	0.2	0.4	0.1	0.2	0.8	0.3
S. hua (SI)	0.2	0.2	0.5	0.4	0.4	0.5
S. cor (SI)/ S. per (SI)	0.2	0.3	0.3	0.1	0.4	0.3
S. chi (SI)	0.3	0.9	0.3	0.4	1.6	0.8
S. hab (SI)	0.2	0.1	0.4	0.4	0.9	0.6
S. pen (SI)	0.2	0.6	0.3	0.3	0.3	0.4

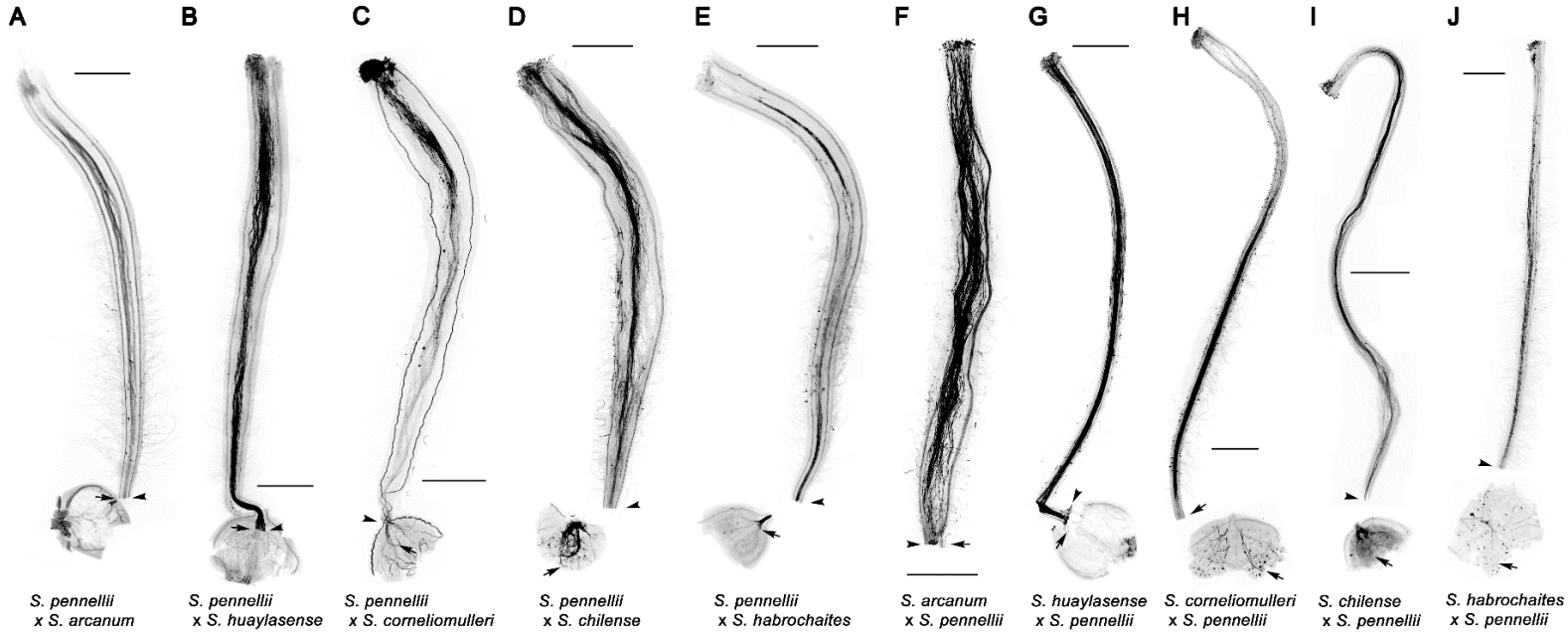
Notes: SC and SI refer to self-compatible and self-incompatible mating systems respectively. "Seed" means that seed was produced in interspecific crosses as previously reported (Rick et al., 1976). Numbers refer to the difference in lengths in mm between the majority of pollen tubes and the longest pollen tubes in cases of pollen tube rejection. *S. lyc= S. lycopersicum, S. pim= S. pimpinellifolium, S. gal= S. galapagense, S. che= S. cheesmaniae, S. chm= S. chmielewskii, S. neo= S. neorickii, S. arc= S. arcanum, S. hua= S. huaylasense, S. cor= S. corneliomulleri, S. per= S. peruvianum, S. chi=S. chilense, S. hab= S. habrochaites, S. pen= S. pennellii*

[a] Pollen tubes did not reach ovaries in some accessions of *S. chmielewskii.*

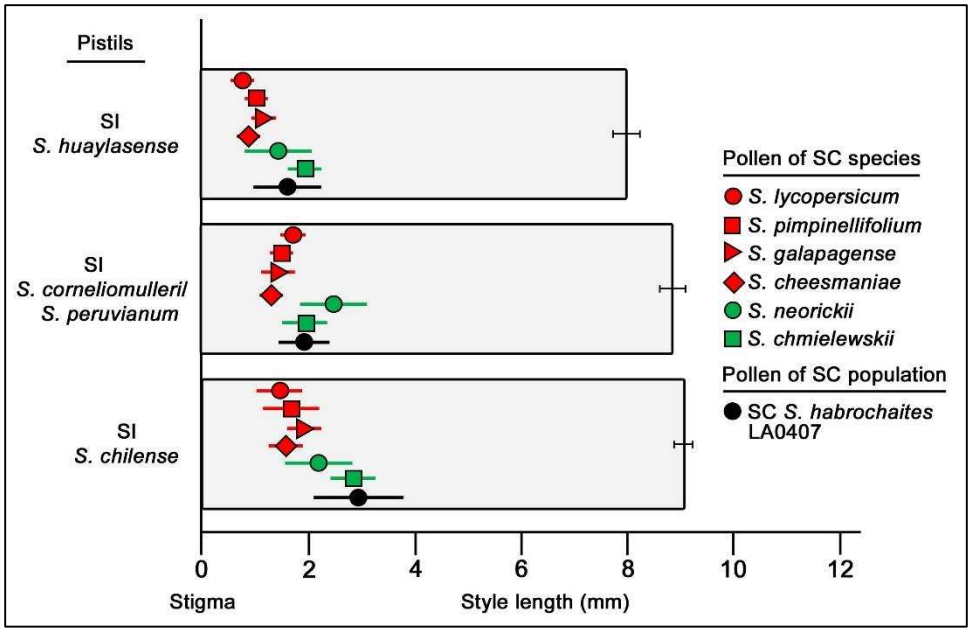
[b] Pollen tubes rejection occurred in some accessions of *S. neorickii*.



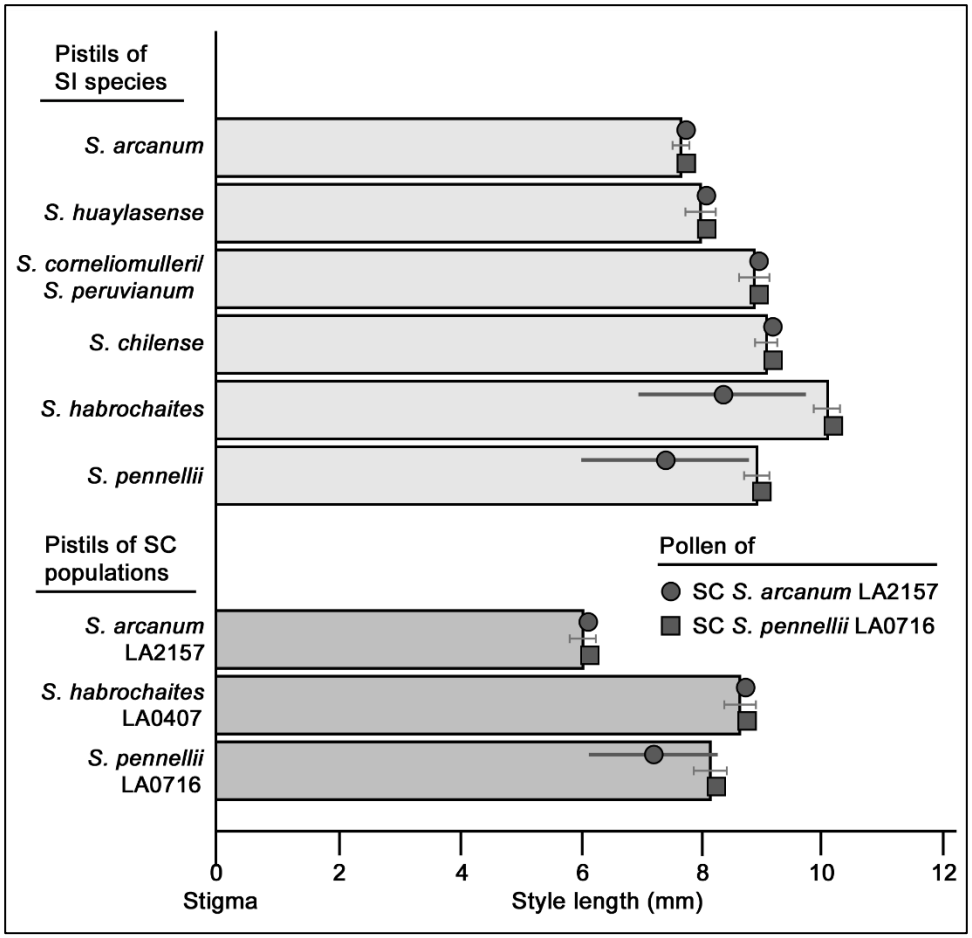
Appendix. S3.3. Representative images of pollen tube growth to ovaries in interspecific crosses between SC species. In all crosses the female parent is listed first. (A) SC *S. pimpinellifolium* X SC *S. lycopersicum* (B) SC *S. pimpinellifolium* X SC *S. galapagense* (C) SC *S. pimpinellifolium* X SC *S. cheesmaniae* (D) SC *S. neorickii* X SC *S. chmielewskii* (E) SC *S. chmielewskii* X SC *S. neorickii* (F) SC *S. pimpinellifolium* X SC *S. neorickii* (G) SC *S. pimpinellifolium* X SC *S. chmielewskii*. Pollen tubes are not rejected in any of these interspecific crosses. Arrowheads mark the end of the majority of pollen tubes and arrows mark the end of the longest pollen tubes in each pistil. Bars are 1mm.



Appendix S3.4. Representative images of pollen tube growth to ovaries in interspecific crosses between pairs of SI species. In all crosses the female parent is listed first. **(**A) SI *S. pennellii* X SI *S. arcanum* (B) SI *S. pennellii* X SI *S. huaylasense* (C) SI *S. pennellii* X SI *S. corneliomulleri* (D) SI *S. pennellii* X SI *S. chilense* (E) SI *S. pennellii* X SI *S. habrochaites* (F) SI *S. arcanum* X SI *S. pennellii* (G) SI *S. huaylasense* X SI *S. pennellii* (H) SI *S. corneliomulleri* X SI *S. pennellii* (I) SI *S. chilense* X SI *S. pennellii* (J) SI *S. habrochaites* X SI *S. pennellii*. Pollen tubes are not rejected in any of these interspecific crosses. Arrowheads mark the end of the majority of pollen tubes and arrows mark the end of the longest pollen tubes in each pistil. Bars are 1mm.



Appendix S3.5. Growth of pollen tubes from SC species in styles of SI *S. huaylasense*, *S. corneliomulleri*/*S. peruvianum* and *S. chilense*. All pollen tubes of all SC species are rejected in the upper third of styles (shaded rectangles), although pollen tubes of SC red-fruited species are rejected more rapidly than those of SC green-fruited species or an SC accession of *S. habrochaites* (differences in pollen tube lengths between SC red and green-fruited species are statistically tested in Microsoft Excel software 2011 (*office.microsoft.com/en-us/excel).*; in the styles of SI *S. huaylasense*, df = 1 and P = 0.003; in the styles of SI S. *corneliomulleri*, df = 1 and P = 0.0005; in the styles of SI *S. chilense*; df = 1 and P= 0.0001). Pollen tube lengths are shown in mm, with the averages of the majority of pollen tube lengths (symbols) and the standard deviations (bars).



Appendix S3.6. Growth of pollen tubes of SC *S. arcanum* LA2157 and SC *S. pennellii* LA0716 in styles of SI species and SC populations of SI species in the tomato clade. Pollen tubes of SC *S. pennellii* LA0716 (squares) reach ovaries within 48 h, except pollen tubes of SC *S. arcanum* LA2157 (circles), which require 72 to consistently reach ovaries in pistils of SI *S. habrochaites*, or SI or SC *S. pennellii*, as shown in Fig. 3.4. Style lengths are represented by shaded rectangles, light gray for SI species and darker gray for SC populations of SI species. Pollen tube lengths are shown in mm, with the averages of the majority of pollen tube lengths (symbols) and the standard deviations (bars).

Appendix S4.1. Documented sympatric sites, wild tomato species

Fernandina and Isabela Islands, Galapagos, Ecuador	*S. cheesmaniae* *S. galapagense*		See Darwin et al. (2003)
Hacienda Carrizal, Cajamarca, Peru	*S. pimpinellifolium* *S. arcanum* *S. habrochaites*	LA0398 Not available Not available	S 06 34 53/W 79 14 10
Rio Jequetepeque, Magdalena, Cajamarca, Peru	*S. pimpinellifolium* *S. arcanum*	LA0391 LA0390	S 07 14 39/W 78 40 54
Rio Jequetepeque, Tembladera, Cajamarca, Peru*	*S. arcanum* *S. pimpinellifolium*	LA2066 LA2389	S 07 15/W 79 08
Aricapampa, La Libertad, Peru	*S. habrochaites* *S. arcanum*	LA2329 LA1032	S 07 49 13/W 77 42 46
Mouth of Rio Rupac, Ancash, Peru	*S. arcanum* *S. neorickii*	LA1626 LA1626A	S 08 30 30/W77 22 0
Culebras #1, Ancash, Peru	*S. peruvianum* *S. pimpinellifolium*	LA0372 LA0373	S09 56 24/W 78 13 48
Huarmey, Anchash, Peru	*S. pimpinellifolium* *S. huaylasense*	LA1599 Not available?	S 10 03 30/W 78 11
Rio Huara, Navan, Lima, Peru	*S. corneliomulleri* *S. habrochaites*	LA1377 LA1378	S 10 54/W 76 59
Rio Huara, Quintay, Lima, Peru	*S. pimpinellifolium* *S. pennellii*	LA1520 LA1522	S 11 00 30/W 77 07
Irrigacion Santa Rosa, Lima, Peru	*S. peruvianum* *S. pennellii*	LA1518 LA1523	S 11 22/W 77 19 30
Rio Chillon, Trapiche, Lima, Peru	*S. pennellii* *S. peruvianum*	LA1277 LA1278	S 11 38 30/W 76 57 30
Rio Chillon, Santa Rosa de Quives. Lima, Peru	*S. pennellii* *S. corneliomulleri*	LA1299 LA1300	S 11 39/W 76 42 30
Rio Lurin, Palma, Lima, Peru*	*S. pennellii* *S. corneliomulleri* *S. pimpinellifolium*	New site Seeds not available	S 12 02 47/W 076 34 25
Rio Lurin, Pichicato, Lima, Peru	*S. pimpinellifolium* *S. pennellii*	LA1992 Not available	S 12 04/W 76 45
Marca to Chincha, Ica, Peru	*S. pennellii* *S. peruvianum*	LA1656 Not available	S 13 21/W 75 43
Tambo de Mora, Ica, Peru	*S. pimpinellifolium* *S. peruvianum*	LA1606 Not available	S 13 28/W 74 12
Rio Pisco, Pampano, Huancavelica, Peru	*S. pennellii* *S. corneliomulleri*	LA1303 LA1304	S 13 34/W 75 32
Quita Sol, Ica, Peru	*S. pennellii* *S. corneliomulleri*	LA1302 Not available	S 13 38/W 75 43
Rio Pisco, La Quinga, Ica, Peru	*S. pennellii* *S. corneliomulleri*	LA1724 LA1723	S 13 39/W 75 43
Rio Pachachaca, Sorocata, Apurimac, Peru	*S. chmielewskii* *S. neorickii*	LA1327 LA1326	S 13 44/W 72 56
Rio Aja , Ica, Peru	*S. peruvianum* *S. pimpinellifolium*	LA2835 LA2836	S 14 45/W 74 48
Nazca grade, Ica, Peru	*S. corneliomulleri*	LA3664	S 14 50/W 74 43

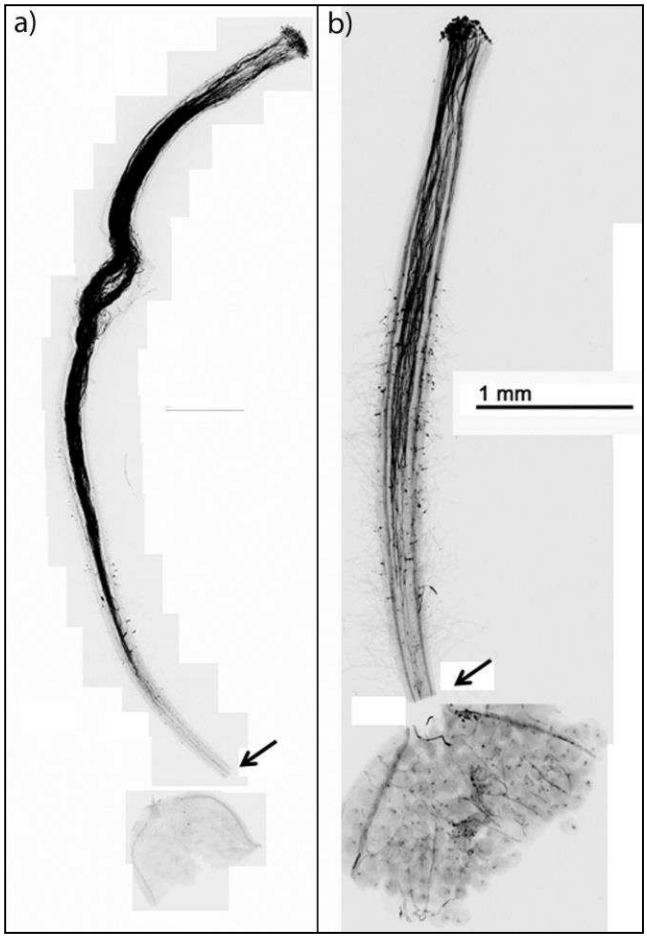
	S. pennellii	Not available	
Alta Chaparra, Arequipa, Peru	*S. chilense* *S. peruvianum*	LA3786 LA3787	S 15 34 25/W 73 44 30
Rio Chaparra, Areqipa, Peru	*S. corneliomulleri* *S. chilense*	LA3783 LA3784	S 15 43 52/W 73 51 02
Caraveli , Arequipa, Peru	*S. peruvianum* *S. pennellii*	LA3790 LA3791	S 15 47 16/W 73 23 38
Quebrada Huarangillo, Atico, Arequipa, Peru	*S. peruvianum* *S. chilense*	LA3779 LA3780	S 16 15 05/W 73 31 34
Pachica (Rio Camarones), Arica and Parinacota, Chile	*S. peruvianum* *S. chilense*	LA4128 LA4129	S 18 54 29/W 69 36 15
Esquina, Arica and Parinacota, Chile	*S. chilense* *S. peruvianum*	LA4132	S 18 55 33/W 69 33 2
Camina, Taracapa, Chile	*S. peruvianum* *S. chilense*	LA4125	S 19 18 22 /W 69 25 14

*Confirmed in 2009

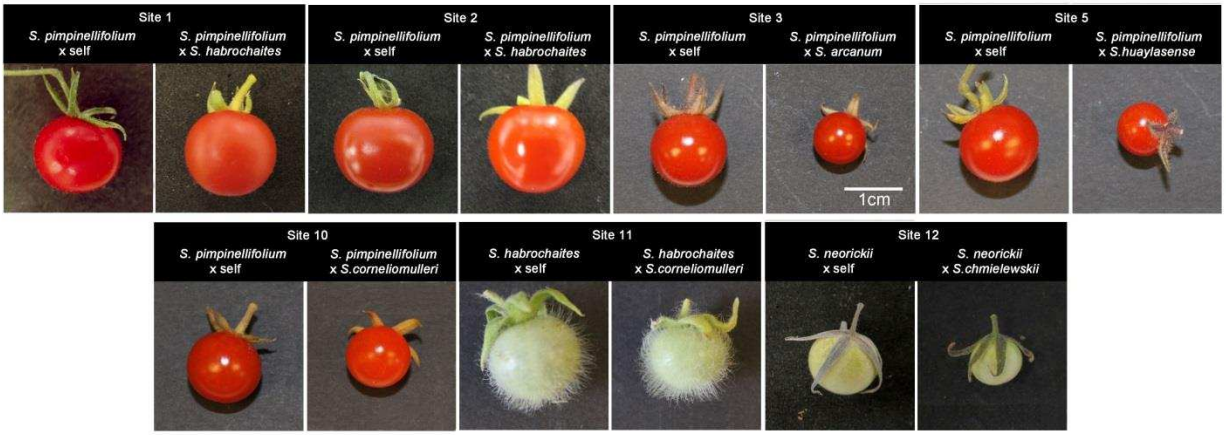
Appendix S4.2. Summary of fruit and seed/SLS data for intra- and interspecific crosses.

Site	Cross	Average fruit weight (g)	% of control weight	Average fruit diameter (mm)	% of control diameter	Average number of seeds/SLS per fruit	Average seed/SLS width (mm) [1]
1	SC *S. pim* self	0.7		10.7		22	1.69
	SC *S. pim* x SC *S. hab*	0.8	105.5	10.0	104.3	45	0.78, 1.09 [2]
2	SC *S. pim* self	1.2		11.7		42	1.60
	SC *S. pim* x SC *S. hab*	0.8	78.0	10.3	95.9	35	1.42
3	SC *S. pim* self	0.8		10.4		23	1.70
	SC *S. pim* x SI *S. arc*	0.4	45.5	8.1	80.7	21	0.73
4	SI *S. arc* sib	0.8		10.0		28	1.41
	SI *S. arc* x SI *S. hab*	0.1	51.7	5.9	59.0	25	0.65
	SI *S. hab* sib	1.7		14.4		36	1.59
	SI *S. hab* x SI *S. arc*	0.4	58.6	8.4	58.6	17	0.96
5	SC *S. pim* self	0.9		10.9		17	1.76
	SC *S. pim* x SI *S. hua*	0.2	20.3	6.3	69.0	6	0.82
6	SI *S. cor* sib	0.8		11.8		28	1.04
	SI *S. cor* x SI *S. hab*	0.1	17.1	6.2	48.1	12	0.39
	SI *S. hab* sib	1.6		14.5		39	1.53
	SI *S. hab* x SI *S. cor*	0.4	24.4	8.5	57.6	29	0.76
7	SI *S. cor* sib	0.5		8.7		36	1.39
	SI *S. cor* x SC *S. hab*	0.2	43.8	6.9	79.6	55	0.49
	SC *S. hab* self	1.7		13.2		38	1.52
	SC *S. hab* x SI *S. cor*	0.8	49.5	10.5	79.6	37	1.05
8	SI *S. cor* sib	0.6		8.9		22	1.53
	SI *S. cor* x SI *S. pen*	0.4	66.7	8.9	99.4	32	1.08
	SI *S. pen* sib	0.9		10.0		67	0.89
	SI *S. pen* x SI *S. cor*	0.7	81.0	9.9	104.1	64	0.74 [3]
9	SI *S. cor* sib	1.6		13.8		75	1.16
	SI *S. cor* x SI *S. pen*	0.6	34.7	9.4	72.5	32	0.69
	SI *S. pen* sib	0.8		9.4		81	0.86
	SI *S. pen* x SI *S. cor*	0.2	28.4	6.7	83.1	35	0.46
10	SC *S. pim* self	0.9		10.9		28	1.67
	SC *S. pim* x SI *S. cor*	0.5	53.9	8.9	84.8	25	0.84
11	SC *S. hab* self	1.5		12.9		38	1.50
	SC *S. hab* x SI *S. cor*	1.0	69.5	10.9	90.8	29	0.94
12	SC *S. chm* self	0.5		9.3		31	1.50
	SC *S. chm* x SC *S. neo*	0.4	81.0	10.6	133.5	21	1.70

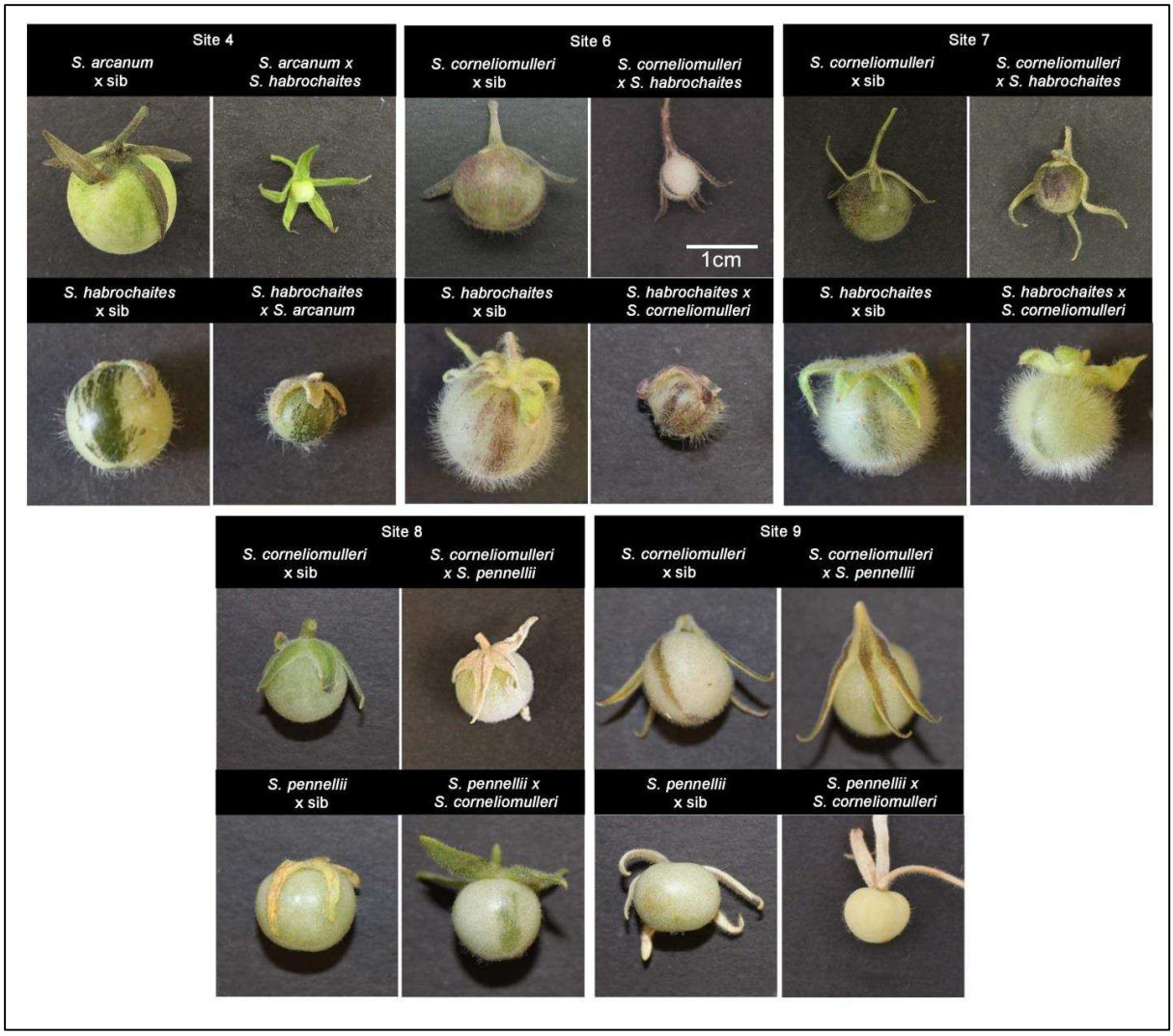
1 For fruit in which there were two distinct size classes of SLS, only the larger group is included in calculation of average seed/SLS width.
[2] Fruits from this interspecific cross contained <5% larger Type 3 SLS.
[3] When embryos protruded from the seed coat, only the width of the seed coat itself was measured



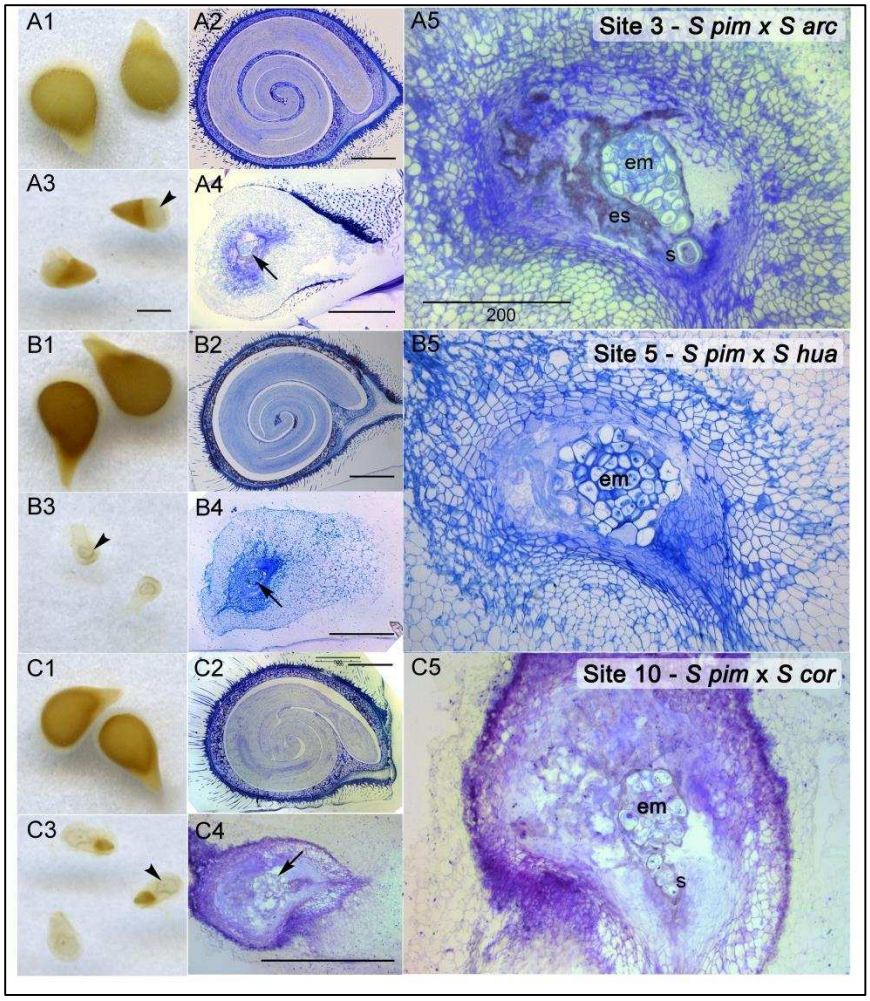
Appendix S4.3. Pollen tube growth in crosses of SI *S. corneliomulleri* x SC *S. habrochaites* at site 11 (a) and SC *S. neorickii* and SC *S. chmielewskii* (b) at site 12. Arrows indicate where the majority of pollen tube growth ended.



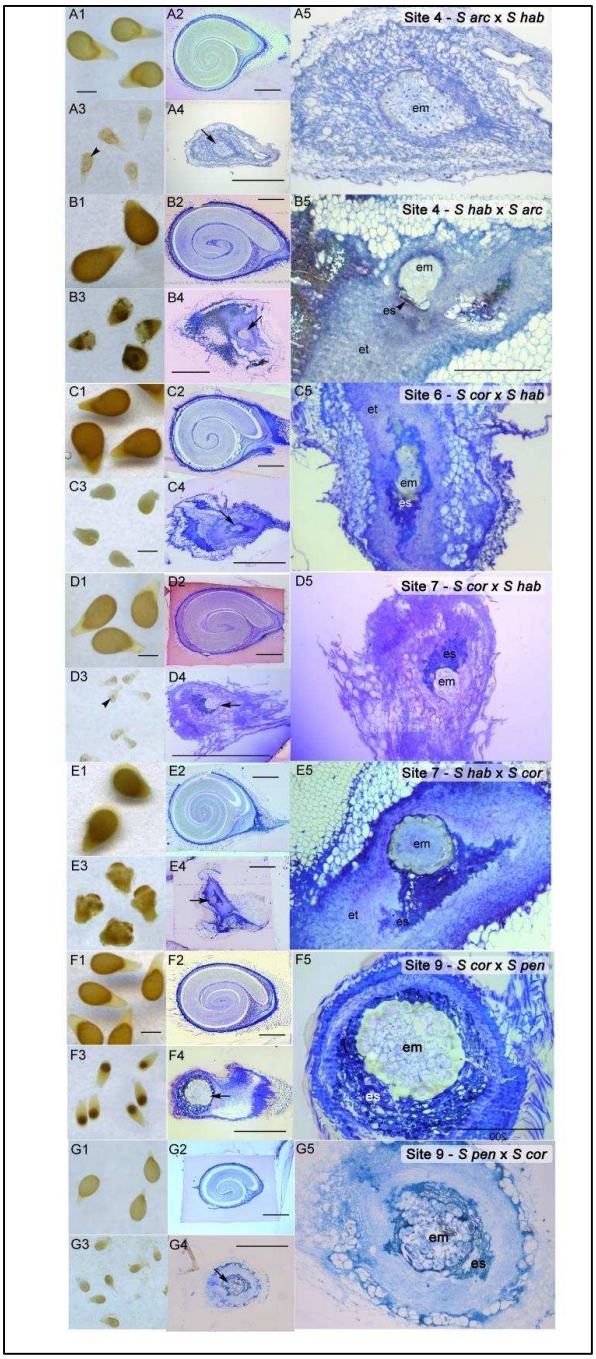
Appendix S4.4. Fruits resulting from control and interspecific crosses using SC species or populations as female, for which reciprocal crosses failed.



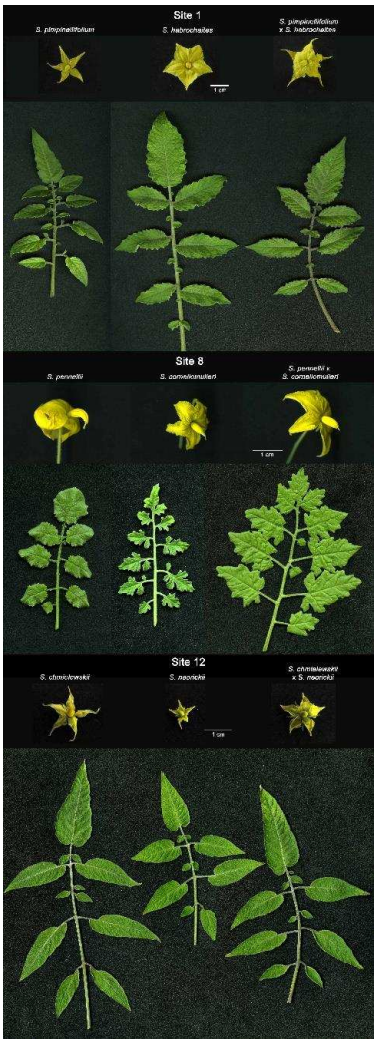
Appendix S4.5. Fruits resulting from control and reciprocal interspecies crosses.



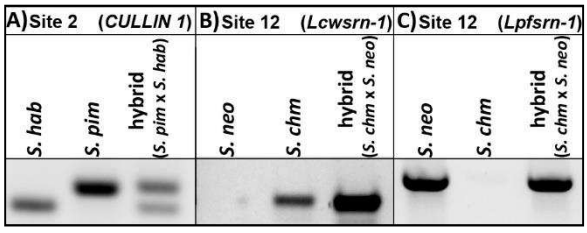
Appendix S4.6. Seeds from interspecific crosses using SC *S. pimpinellifolium* as female. A. Site 3, *S. pimpinellifolium* x *S. arcanum*; B. Site 5, *S. pimpinellifolium* x *S. huaylasense*; C. Site 10, *S. pimpinellifolium* x *S. corneliomulleri*; A1-C1, control seeds of the pistil parent for each cross; A2-C2, sagittal sections of control seeds; A3-C3, seeds and SLS in fruit resulting from the interspecific crosses; A4-C4, sagittal sections of seeds resulting from the interspecific crosses. A5-C5, enlargements of sections of interspecific seeds. Abbreviations: em, embryro; es, endosperm; s, suspensor. Arrowheads in A4-C4 indicate embryos.



Appendix S4.7. Seed structure in control and reciprocal interspecies crosses. A. Site 4, *S. arcanum* x *S. habrochaites*; B. Site 4, *S. habrochaites* x *S. arcanum*; C. Site 6, *S. corneliomulleri* x *S. habrochaites*; D. Site 7, *S. corneliomulleri* x *S. habrochaites*; E. Site 7, *S. habrochaites* x *S. corneliomulleri*; F. Site 9, *S. corneliomulleri* x *S. pennellii*; G. Site 9, *S. pennellii* x *S. corneliomulleri*. A1-G1, control seeds of the pistil parent for each cross; A2-G2, sagittal sections of control seeds; A3-G3, seeds and SLS in fruit resulting from the interspecific crosses; A4-G4, sagittal sections of seeds resulting from the interspecific crosses. A5-G5, enlargements of sections of interspecific seeds. Abbeviations: em, embryo; es, endosperm, et, endothelium; s, suspensor. Arrowheads in A4-C4 indicate embryos.



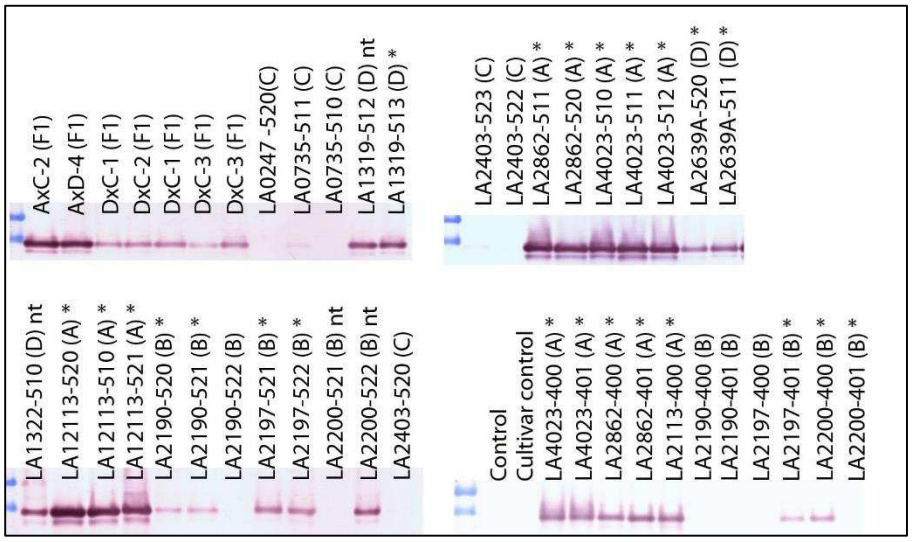
Appendix S4.8. Flowers and leaves of hybrids between accessions collected from sympatric sites 2, 8 and 12. Leaves are the first fully expanded leaves below the inflorescence. Order from left to right in all cases is: female of cross, male of cross, and hybrid.



Appendix S4.9. Confirmation of hybridization between sympatric pairs at sites 2 and site 12 using molecular markers. A. Site2, *CULLIN1* alleles – a 25 bp deletion is found in the *S. habrochaites* allele compared to that of *S. pimpinellifolium*. B. Site 12, detection of *Lcwsrn-1 S-RNase* allele in *S. chmielewskii* and a *S. chmielewskii* x *S. neorickii* hybrid. C. Site 12, detection of *Lpfsrn-1 S-RNase* allele in *S. neorickii* and a *S. chmielewskii* x *S. neorickii* hybrid.

Appendix 5.1. The summary of pollen tube rejection and protein expression in each individual in group B *S. neorickii*.

		S. lyc		S. pim		S. gal		S. che		Protein expression
		R	A	R	A	R	A	R	A	
LA2190	400		5		2		1		2	N
	401		3		4		3		4	N
	540		3							N
	522						1		1	N
	520	1		1						Y
	521	2		1	1					Y
LA2197	400		2				2		1	N
	622	3			1					Y
	540	3	1		1					
	521	2		2		1		1		Y
	401	2		3		2		2		Y
	522	1			1			1		Y
LA2200	622		5		5					N
	401		2		2		1		1	N
	540		1							
	400	2	1	2	2	1		1		Y
	503	2		1						
	621	1			1					



Appendix 5.2. S-RNase in four groups (A-D) of *S. neorickii*. Immunostaining of stylar proteins by anti S-RNase C2 domain antibody. * = accessions/individuals in which pollen tubes from red-fruited species are rejected. nt= individuals were not tested for pollen tube rejection.