RESEARCH ARTICLE

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Interspecific reproductive barriers between sympatric populations of wild tomato species (*Solanum* section *Lycopersicon*)[1]

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PREMISE OF THE STUDY: Interspecific reproductive barriers (IRBs) often prevent hybridization between closely related species in sympatry. In the tomato clade (*Solanum* section *Lycopersicon*), interspecific interactions between natural sympatric populations have not been evaluated previously. In this study, we assessed IRBs between members of the tomato clade from nine sympatric sites in Peru.

METHODS: Coflowering was assessed at sympatric sites in Peru. Using previously collected seeds from sympatric sites in Peru, we evaluated premating prezygotic (floral morphology), postmating prezygotic (pollen-tube growth), and postzygotic barriers (fruit and seed development) between sympatric species in common gardens. Pollen-tube growth and seed development were examined in reciprocal crosses between sympatric species.

KEY RESULTS: We confirmed coflowering of sympatric species at five sites in Peru. We found three types of postmating prezygotic IRBs during pollen–pistil interactions: (1) unilateral pollen-tube rejection between pistils of self-incompatible species and pollen of self-compatible species; (2) potential conspecific pollen precedence in a cross between two self-incompatible species; and (3) failure of pollen tubes to target ovules. In addition, we found strong postzygotic IRBs that prevented normal seed development in 11 interspecific crosses, resulting in seed-like structures containing globular embryos and aborted endosperm and, in some cases, overgrown endothelium. Viable seed and F_1 hybrid plants were recovered from three of 19 interspecific crosses.

CONCLUSIONS: We have identified diverse prezygotic and postzygotic IRBs that would prevent hybridization between sympatric wild tomato species, but interspecific hybridization is possible in a few cases.

KEY WORDS interspecific reproductive barriers; interspecific seed development; pollen–pistil interactions; postzygotic barriers; prezygotic barriers; self-incompatibility; *Solanum*; sympatry; unilateral incompatibility; wild tomato species

A variety of interspecific reproductive barriers (IRBs) contribute to maintaining species isolation (Dobzhansky, 1937; Mayr, 1942; Ramsey et al., 2003; Coyne and Orr, 2004; Rieseberg and Willis, 2007; Lowry et al., 2008; Widmer et al., 2009; Baack et al., 2015). Reproductive barriers between species can be classified according to the order of their action—premating, postmating prezygotic, and postzygotic (Mayr, 1963; Levin, 1971; Grant, 1981). In plants, premating IRBs can be due to geographic isolation (Mayr, 1963; Rice and Hostert, 1993), flowering phenology (Kiang and Hamrick, 1978; Martin and Willis, 2007; Fishman et al., 2014; Briscoe Runquist et al., 2014), 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), and floral characters related to pollinator preference, such as color or scent (Grant and Grant, 1965; Grant, 1994; Bradshaw et al., 1995; Bradshaw and Schemske, 2003; Ramsey et al., 2003; Hoballah et al., 2007; Cooley et al., 2008; Whitehead and Peakall, 2009; Hopkins and Rausher, 2012; Xu et al., 2012; Sheehan et al., 2016).

In cases where pollination is successful, postmating prezygotic barriers may contribute to reproductive isolation. In many species, interactions between pollen and stigmatic surfaces are critical for pollen adhesion and germination (Rougier et al., 1988; Zinkl et al., 1999; Fiebig et al., 2004; Dickinson et al., 2012). Interactions between

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pollen and style can also play a major role in restricting gene flow, particularly between self-incompatible (SI) and self-compatible (SC) species, which often demonstrate unilateral interspecific incompatibility. The general pattern of unilateral incompatibility follows the SI × SC rule, wherein crosses between SI-species females and SC-species males fail, but the reciprocal cross is often successful (Lewis and Crowe, 1958; Murfett et al., 1996; Onus and Pickersgill, 2004; Baek et al., 2015). Conspecific pollen precedence can also act as a postmating prezygotic reproductive barrier when interspecific pollen competes poorly against conspecific pollen (Arnold et al., 1993; Rieseberg et al., 1995; Carney et al., 1996; Howard, 1999; Aagaard et al., 2013; Swanson et al., 2016). Postmating prezygotic IRBs can also act within the ovary, when species-specific factors produced by the embryo sac are required for pollen-tube targeting to ovules for fertilization (Marton et al., 2005; Higashiyama et al., 2006; Escobar-Restrepo et al., 2007; Takeuchi and Higashiyama, 2012).

Postzygotic barriers that interfere with seed development or seed germination also restrict hybridization (Cooper and Brink, 1945; Scopece et al., 2008; Burkart-Waco et al., 2012; Ng et al., 2012; Baack et al., 2015; Lafon-Placette and Köhler, 2015; Oneal et al., 2016). Even if hybrid seeds germinate, hybrid lethality, necrosis (Sawant, 1956; Ramsey et al., 2003; Bomblies et al., 2007; Yamamoto et al., 2010), or sterility due to pollen inviability are often observed, ultimately preventing hybrid persistence (Henderson et al., 1959; Grant, 1971; Rieseberg et al., 1999; Fishman and Willis, 2001; Moyle and Graham, 2005; Sweigart et al., 2006; Kubo et al., 2008; Bomblies, 2010). Even in cases where F_1 plants are fertile, they may show reduced fitness in specific environments, and subsequent generations can experience hybrid breakdown due to low fitness (Stebbins, 1958; Rick et al., 1976; Rundle and Whitlock, 2001; Rhode and Cruzan, 2005; Baack et al., 2015).

Tomato clade species (*Solanum* section *Lycopersicon*) provide an excellent study system for IRBs, particularly because many sympatric species have been identified. The monophyletic tomato clade consists of domesticated *Solanum lycopersicum* and 12 wild species found in Ecuador, Peru, and Chile (Rick, 1979; Moyle, 2008; Peralta et al., 2008; Rodriguez et al., 2009). All species in the clade are $2n = 2x = 24$ diploids with a high degree of synteny, with conservation of chromosome structure among species (Ji and Chetelat, 2007; Peralta et al., 2008). The wild species exhibit a variety of mating systems, from autogamous SC to facultative SC to SI (Rick, 1979; Mutschler and Liedl, 1994; Peralta et al., 2008; Bedinger et al., 2011). The SI × SC rule is followed at the level of pollen–pistil interactions, as pollen tubes of SC species are rejected in pistils of SI species (Martin, 1961a, b, 1964; Hardon, 1967; Rick et al., 1976; Rick, 1979; Liedl et al., 1996; Bedinger et al., 2011; Baek et al., 2015). In the reciprocal SC × SI crosses, pollen–pistil barriers are generally not observed; however, significant postzygotic barriers, such as failure of fruit and/or seed formation, have been reported (Rick, 1979; Mutschler and Liedl, 1994). It should be noted that domesticated *S. lycopersicum* has substantially reduced IRBs compared to wild species, and, thus, hybrids can be generated by pollinating cultivars with closely related wild species. This has allowed important agronomic traits to be introduced to the cultivated species (McGuire and Rick, 1954; Hardon, 1967; Hogenboom, 1973, Rick et al., 1976; Rick, 1986; Rick and Chetelat, 1995; Tanksley and McCouch, 1997; Zamir, 2001).

Previous studies have tested broad patterns of species-level compatibility, irrespective of geography (Martin, 1961a; Mutschler and Liedl, 1994; Covey et al., 2010; Baek et al., 2015). However, many wild tomato species have overlapping ranges (Moyle, 2008; Peralta et al., 2008), and there are numerous reports of two or more tomato-clade species in sympatry. Yet, to our knowledge, hybrids have not been reported in natural populations. Therefore, an opportunity exists to test for interspecies barriers that are relevant in naturally occurring sympatric populations.

We assessed IRBs acting at different reproductive stages between sympatric wild tomato species at nine sites in Peru. We examined floral morphology, pollen–pistil interactions, and hybrid fruit and seed formation. We found strong prezygotic pollen–pistil IRBs in cases where pollen tubes of an SC species, *S. pimpinellifolium*, were rejected in styles of the sympatric partner. We also found one potential case of conspecific pollen precedence in crosses between two SI species, and two cases in which interspecific pollen tubes did not appear to target ovules. In addition, we found strong postzygotic seed-development IRBs in most cases when prezygotic barriers were not detected. Together, these barriers would likely prevent hybrid formation. However, we recovered healthy fertile hybrid plants in three of 19 interspecific sympatric crosses, which suggests that hybridization between sympatric wild tomato species could occur at a low frequency.

MATERIALS AND METHODS

***Plant material*—**Seeds of the wild species accessions used in this study were obtained from the Charles M. Rick Tomato Genetics Resource Center at the University of California, Davis (TGRC; http://tgrc.ucdavis.edu/). At the TGRC, every attempt is made to preserve the original genetic diversity present at the time of collection. Several measures are employed toward this goal. First, seeds are collected from several plants to adequately sample variation in the native population. Seeds are then regenerated at UC Davis, using sufficiently large populations to preserve most of this variability; for the outcrossing and facultative wild species, population sizes of at least 50 plants are used. Bulk pollen samples are collected from all plants in a population and then used for "mass sib" crosses onto all open flowers to maximize cross pollination. The interval between cycles of seed regeneration is maximized to reduce the opportunity for inbreeding, drift, and selection. Seeds are stored for at least 10 yr at low temperatures and low humidity to maintain viability. After 10 yr, seed germination response is tested every 2–3 yr. Accessions are regenerated only when the germination rate drops below 80%. Many species can be stored 20 yr or longer before grow-outs are needed.

In the present investigation, plants were either (1) grown in greenhouses at Colorado State University (CSU) or the University of California (UC) Davis, in ProMix-BX soil, with 16 h light at 26°C and 8 h dark at 18°C; or (2) grown in outdoor agricultural fields.

***Sympatric sites*—**Sympatry has been documented for collections of wild tomato species curated at the TGRC (Appendix S1, see Supplemental Data with the online version of this article). Eight of these previously reported sites were visited in 2009, and the continued presence of sympatric species was verified at five sites (Table 1). In addition, a new site at Palma, Peru, was found containing three species, *S. pennellii*, *S. corneliomulleri*, and S. *pimpinellifolium*. For these studies, sympatry was operationally defined as focal species growing within 20 m of each other. While potential pollinators may range over distances greater than 1 km, this stringent definition ensured that no physical barrier would prevent potential pollinators

TABLE 1. Sympatric sites for wild tomato (*Solanum*) species used in this study.

No.	Site	Mating system, species	TGRC accessions	Latitude/longitude	Coflowering
1	Puente Muyuna, Rio Jequetepeque, Cajamarca, Peru [a]	SC, *S. pimpinellifolium*	LA2149	S 07 13/W 078 47 13	Yes [a]
		SI, *S. arcanum*	LA2150		
2	Chilete-Rupe Cajamarca, Peru [a]	SI, *S. arcanum*	LA1351	S 07 17 14/W 078 49 15	Yes [a]
		SI, *S. habrochaites*	LA1352		
3	Above Yaso Rio Chillón, Lima, Peru [a]	SC, *S. pimpinellifolium*	n.a.	S 11 34 18/W 076 43 38	Yes [a]
		SI, *S. corneliomulleri*	LA1646		
		SI, *S. habrochaites*	LA1648		
4	Surco Rio Rimac, Lima, Peru [b]	SI, *S. corneliomulleri*	LA1294	S 11 52 32/W 076 25 42	Yes [c]
		SC, *S. habrochaites*	LA1295		
5	Sisacaya Rio Lurin, Lima, Peru [b]	SI, *S. corneliomulleri*	LA0752 [d]	S 12 01 16/W 076 38 05	Yes [c]
		SI, *S. pennellii*	LA0751		
6	Cacra Rio Cañete, Lima, Peru [a]	SC, *S. pimpinellifolium*	n.a.	S 12 49 07 /W 075 51 40	Yes [a]
		SI, *S. corneliomulleri*	LA1694		
		SI, *S. pennellii*	LA1340		
7	Asia-El Piñon Lima, Peru [b]	SC, *S. pimpinellifolium*	LA1610	S 12 46 56/W 076 33 27	Yes [c]
		SI, *S. corneliomulleri*	LA1609		
8	Ticrapo Rio Pisco, Huancavelica, Peru [a]	SI, *S. corneliomulleri*	LA1722	S 13 22 56/W 075 25 55	Yes [a]
		SC, *S. habrochaites*	LA1721		
9	Puente Cunyac, Apurimac, Peru	SC *S. neorickii*	LA2639A	S 13 33 30/ W 72 35 30	Yes [c]
		SC *S. chmielewskii*	LA2639B		

Notes: SC = self-compatible; SI = self-incompatible; TGRC = Tomato Genetics Resource Center, University of California, Davis; n.a. = seeds not available from TGRC.
[a] Confirmed in 2009.
[b] Single species found at site in 2009.
[c] TGRC field notes; photos and/or flowering noted, fruits collected on same date.
[d] The status of this accession is somewhat ambiguous, and it has been recently reclassified as *S. chilense* at TGRC (http://tgrc.ucdavis.edu).

from visiting multiple species and effecting cross-pollination. IRBs are more likely to play a role in maintaining species barriers between such strictly defined sympatric species pairs than between more widely separated populations.

One cross between sympatric species was performed on site in Peru (Fig. 2A), but, since it was not possible to export seed, all other crosses were performed in greenhouses and research fields at CSU and UC Davis, using material available through the TGRC. For stigma exsertion and pollen-tube rejection experiments, we were interested in comparing sympatric populations to allopatric populations to determine whether sympatric occurrence might lead to increased IRBs. We define "allopatric" conservatively, in that the population of interest was required to occur at least 25 km away from any documented site of collection for other species of the tomato clade. We determined occurrence based on database searches of TGRC and the Germplasm Resources Information Network (GRIN, curated by the U.S. Department of Agriculture), the literature, and our brief survey in the summer of 2009. However, it is acknowledged that information on co-occurrence of tomato-clade members could be incomplete and may change on a temporal scale.

***Stigma exsertion measurements*—**Stigma exsertion was measured in flowers from at least three individuals of each accession at bud break (anthesis). Flowers with sepals and petals removed were imaged using either an EPSON Perfection V700 photo scanner (Epson America, Long Beach, CA, USA) at 2400 dpi or a Nikon SMZ1500 (Nikon Instruments, Melville, NY, USA) dissecting microscope with Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) connected with a Nikon DMX1200 digital camera (Nikon Instruments, Melville, NY, USA). Stigma exsertion above the stamens was measured using ImageJ 1.33 (National Institutes of Health, Bethesda, MD, USA). 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. Differences in stigma exsertion between allopatric and sympatric sites were examined using a two-sample *t*-test.

***Pollinations*—**In greenhouses or fields at CSU floral buds of the female parent were emasculated 1 d before bud break and allowed to mature 24 h before pollination. Pollen was obtained from mature flowers of male parents by vibrating anther cones into gelatin capsules using tooth polishers, and pollen was applied to stigmas. In crosses not performed in greenhouses, inflorescences were covered with fine-mesh nylon net bags after emasculation to prevent insect pollination.

***Pollen-tube growth analysis*—**Pollinations for pollen-tube growth analysis were performed as described above, and pistils were harvested into fixative (3:1 ethanol:acetic acid) 48 h postpollination. For crosses performed in Peru at Centro Internacional de la Papa using sympatric species at site 3 (Rio Chillón, Lima), pollen was collected from *S. pimpinellifolium*, and inflorescence branches of *S. corneliomulleri* were collected and submerged immediately in water. Inflorescence flower buds were emasculated and pollinated within 6 h after collection, and pollinated pistils were harvested into fixative after 24 h. In all cases, pollinated pistils were fixed, stained with Aniline Blue Fluorochrome (Biosupplies Australia, Bundoora, Victoria, Australia), and imaged as previously described (Covey et al., 2010; Baek et al., 2015). Pollen-tube rejection was characterized by measuring the point where the majority of pollen tubes were arrested (i.e., no more than three pollen tubes passed) and by the distance traversed by the longest pollen tube. All measurements and analysis of pollen tubes were performed as described in Baek et al. (2015). Differences in pollen-tube growth between sympatric crosses and allopatric crosses were examined using a two-sample *t*-test.

***Fruit analysis*—**Fruit were allowed to mature until they were soft and ripe (≥50 d). For comparisons between interspecific hybrid and control fruit (intraspecific pollinated), fruits were weighed, and the

height (longitudinal dimension) and diameter (longest transverse dimension) were measured using a digital caliper. Differences in relative hybrid fruit mass compared to controls were examined using a two-sample *t*-test.

***Seed measurements*—**All seeds and seed-like structures (SLS) were removed from each intraspecific control and interspecific fruit and counted, including all SLS that were larger than unpollinated ovules. Prior to selecting a sample of seeds to be embedded for microscopy, the gelatinous placental tissue was dissected away from the seeds/SLS, and all seeds and SLS from each fruit were imaged using an EPSON Perfection V700 photo scanner (Epson America, Long Beach, California, USA) at 2400 dpi.

Seed/SLS measurements were obtained using MicroMeasure software developed at Colorado State University (Fort Collins, Colorado, USA). For intraspecific controls, seeds were measured from at least two fruits when possible, and measurements were obtained from at least 10 seeds per fruit. For interspecific hybrids, all of the seeds and SLS in each fruit were measured. 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. Although both seed/SLS widths and lengths were measured, width was chosen for statistical comparisons because it was more difficult to accurately determine length due to variable amounts of funicular tissue remaining with the seeds/SLS after dissection. Differences in relative hybrid seed width compared to intraspecific controls were examined using a two-sample *t*-test.

***Seed fixation and microscopy*—**Halved fruits or seed-containing pulp were fixed in 2.5% glutaraldehyde, 3.7% formaldehyde, and 0.1 M sodium cacodylate buffer, pH 7.3, and stored at 4°C. After fixation for at least 24 h, seeds/SLS were extracted from the pulp. Before further processing, seed coats of mature seeds (and some more-developed SLS) were opened on one or both lateral surfaces and, where possible, part of the seed coat was 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, Redding, California, USA). A mild vacuum was used to facilitate penetration during both fixation and infiltration. Following polymerization of the embedding resin, seeds and SLS were sectioned using a diamond knife and Reichert-Jung Ultracut E Ultramicrotome (Leica Biosystems, Buffalo Grove, Illinois, USA). Sagittal or cross sections 1–5 μm in thickness were mounted on glass microscope slides and stained with toluidine blue, and cover slips were mounted using Cytoseal 60 mountant (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA). Sections were imaged using a Leica DM5500 B microscope, Leica DFC450 color camera, and Leica Application Suite Version 4.1 image capture software (Leica Microsystems, Buffalo Grove, Illinois, USA). Figures were prepared using Adobe Photoshop (Adobe Systems, San Jose, California, USA). Since we were primarily interested in determining the greatest degree of development possible for hybrid embryos, as well as the stage(s) at which seed development failed, we examined the largest, most developed seeds or SLS from hybrid fruits. We also examined representative seeds from intraspecific control fruits.

***Molecular marker tests for hybridization*—**To confirm hybridization between species at sympatric site 9 (*S. neorickii* and *S. chmielewskii*), we performed polymerase chain reaction (PCR) assays using DNA isolated from leaves of parent and putative hybrid plants, using established species-specific markers. Genomic DNA was prepared using the "shorty prep" method: briefly, a small piece of leaf was placed in a 1.5 mL tube containing 500 μL of extraction buffer (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 at the maximum speed for 5 min, and 300 μL of supernatant was mixed with 400 μL isopropanol to precipitate DNA. DNA was pelleted, washed in 1 mL of 70% EtOH, and resuspended in 50 μL of 10 mM Tris, pH 8. Previously identified species-specific *S-RNase* alleles in *S. neorickii* (*Lpfsrn-1*) and *S. chmielewskii* (*Lcwsrn-1*) (Kondo et al., 2002) were used to design primers 5′neosrn-1-FP7: 5′-ATGGTTAAACCACAACTCACAGCA-3′ and 3′neosrn-1-RP7: 5′-TGTTGTTCAGCGAAAAAATATTTTTCCGG-3′for the *S. neorickii S-RNase Lpfsn-1* allele (GenBank sequence AB072475); and primers 5′chmsrn-FP: 5′- CAAGTCCGTAATACTGAATAACTGC-3′ and 3′chmsrn-2-RP-1: 5′- GGAAATGTGGAACTTAATGAGATTGG -3′ for the *S. chmielewskii S-RNase Lcwsrn-1* allele (GenBank sequence AB072477).

PCR was performed using Econotaq Plus Green Mastermix (Lucigen, Middleton, Wisconsin, USA), 0.5 μM of each primer, and ~80 ng of genomic DNA per 20 μL reaction (95°C 90 s; 35 cycles of 95°C 30 s, 55°C 30 s, 72°C 30 s; 72°C for 3 min). PCR products were visualized by ethidium bromide staining after separation in a 1% agarose gel.

***Estimating the strength of reproductive isolating barriers*—**To estimate the strength of tested IRBs for each sympatric pair we used the linear formulation of Sobel and Chen (2014). For simplicity, the basic equations used to calculate each reproductive isolation (RI) value are given below. These values were transformed into the RI metric of Sobel and Chen (2014), and the absolute contribution of each barrier was determined. The prepollination RI index was calculated as $RI_{flowering} = 1 - (n$ tomato-clade species flowering at sympatric site / total n of tomato-clade species at sympatric site). Indices for three post-pollination prezygotic barriers were calculated as follows: $RI_{pollen-style} = 1 - (n$ styles with heterospecific pollen tubes accepted / n styles with conspecific pollen tubes accepted); $RI_{pollen-ovule} = 1 - (n$ images of heterospecific pollen tubes targeting ovules / n images of conspecific pollen tubes targeting ovules); $RI_{pollen\ growth\ rate} = 1 -$ (heterospecific pollen-tube length at 48 h / conspecific pollen-tube length at 48 h). Finally, the RI index for hybrid seed development was calculated as: $RI_{seed\ development} = 1 - (n$ approximately normal-size heterospecific seed per fruit / n normal-size intraspecific seed per fruit).

RESULTS

***Incidence of sympatric populations*—**Sympatric sites with two or more wild tomato species have been documented at 36 sites, including the nine sites in this study (http://tgrc.ucdavis.edu/; Darwin et al., 2003; Table 1 and Appendix S1). To our knowledge, hybrids have not been reported in natural sympatric populations, suggesting that IRBs are likely important in species maintenance at these sites. At the nine sites represented in this study (Table 1 and Fig. 1), seven different species were found in different sympatric pairings.

Species with varied mating systems were found in sympatry (Fig. 1). For example, three different pairs of SI species (*S. arcanum* and *S. habrochaites*; *S. corneliomulleri* and *S. habrochaites*; *S. pennellii*

FIGURE 1 Sympatric sites in this study (black circle = self-compatible [SC] species present at site; white circle = self-incompatible [SI] species present at site; hatched circle = SC population of a generally SI species at site). Sites are numbered 1–9 from north to south.

and *S. corneliomulleri*) were found at four sites: 2, 3, 5, and 6. SC populations of *S. habrochaites*, a generally SI species, were found in sympatry with SI *S. corneliomulleri* at sites 4 and 8. *Solanum pimpinellifolium*, an SC species, was found in sympatry with four different SI species (*S. arcanum*, *S. corneliomulleri*, *S. habrochaites*, and *S. pennellii*) at four sites: 1, 3, 6, and 7. Finally, two SC species, *S. chmielewskii* and *S. neorickii*, were found in sympatry with each other at site 9 (Rick et al., 1976).

Premating prezygotic barriers—At all nine sympatric sites, coflowering was either confirmed by direct or recorded observation or inferred from concurrent seed collection (Table 1; http://tgrc.ucdavis.edu/). Thus, flowering phenology is unlikely to contribute to RI at these sympatric sites (Appendix S2, see Supplemental Data with the online version of this article). At two sites, we were able to capture and identify the same bee species on both resident species of wild tomato (data not shown). However, since pollinators were not studied in detail, we were not able to evaluate the importance of pollinator visitation as an IRB at these sites.

Since stigma exsertion is positively correlated with the degree of outcrossing in *S. pimpinellifolium* (Rick et al., 1977), we hypothesized that reduced stigma exsertion would be selected for in sympatry to reduce interspecific cross-pollination. We measured this trait in *S. pimpinellifolium* populations and compared populations from sympatric and allopatric sites. When possible, we also noted whether these were classified as autogamous (selfing) or facultative (outcrossing) SC accessions (Rick et al., 1977). Table 2 shows that *S. pimpinellifolium* stigma exsertion in allopatric populations averaged 1.15 mm, whereas in sympatric populations stigma exsertion averaged 0.73 mm. However, the range of stigma exsertion was wide for both allopatric and sympatric populations studied, and average stigma exsertion was not significantly different between allopatric and sympatric groups ($t = 0.83$, df = 6, $P = 0.44$).

Postmating prezygotic barriers (pollen–pistil interactions)—To assess postmating prezygotic barriers, reciprocal crosses were performed between sympatric species, and pollen-tube growth was evaluated. In total, pollen-tube growth was examined in 19 reciprocal crosses between sympatric species pairs. We expected to find active rejection of pollen tubes only in SI × SC crosses of sympatric pairs (at sites 1, 3, and 7; note: *S. pimpinellifolium* from site 6 was not available through the TGRC), as predicted by the SI × SC rule and data from Baek et al. (2015). As predicted, pollen tubes of SC *S. pimpinellifolium* were always rejected in pistils of their sympatric SI species partner: SI *S. arcanum* at site 1, and SI *S. corneliomulleri* at sites 3 and 7, as shown in Fig. 2A. *S. pimpinellifolium* pollen-tube rejection occurred at an average of 1.4 mm from the stigma in styles of SI species (Fig. 2B, black circles). When the reciprocal crosses were performed, and SC *S. pimpinellifolium* was used as female, pollen tubes of the SI species partner consistently reached ovaries (data not shown). Therefore, a strong asymmetric postmating prezygotic IRB acts when SC *S. pimpinellifolium* is the pollen donor on pistils of SI species, but not in the reciprocal cross (Table 3 and Appendix S2).

To determine whether pollen tubes from sympatric *S. pimpinellifolium* accessions are rejected more rapidly in styles of sympatric SI partner species than pollen tubes from an allopatric

TABLE 2. Stigma exsertion of *Solanum pimpinellifolium* at allopatric and sympatric sites.

Site type	Location	Accession	Stigma exsertion (mm)
Allopatric	Miramar, Peru	LA1683	1.93 [a]
			(1.5 outcrossing) [b]
	Chanchape, Peru	LA1380	0.03 [a]
	Malpaso, Peru	LA2538	1.06 [a]
	Patapo-La Cria, Peru	LA2536	1.51 [a]
	Patapo, Peru	LA2535	2.21 [a]
	Virú–Galunga, Peru	LA1589	0.19 [c]
			(0.3 selfing) [b]
			Average 1.16 ± 0.90 [d]
Sympatric	1. Puente Muyuna, Peru	LA2149	0.59 [c]
	8. Asia-El Piñon, Peru	LA1610	0.20 [c]
			(0.3 selfing) [b]
	Tembladera, Peru [e]	LA2389	1.40 [c]
			Average 0.73 ± 0.61 [d]

[a] Measurements performed at University of California, Davis.
[b] Measurements and mating type by Rick et al. (1977).
[c] Measurements performed at Colorado State University.
[d] Average and standard deviation are shown for each site type. Measurements from Rick et al. (1977) are not included in the averages. No significant difference was found between site types.
[e] The sympatric partner of LA2389 was not available through TGRC.

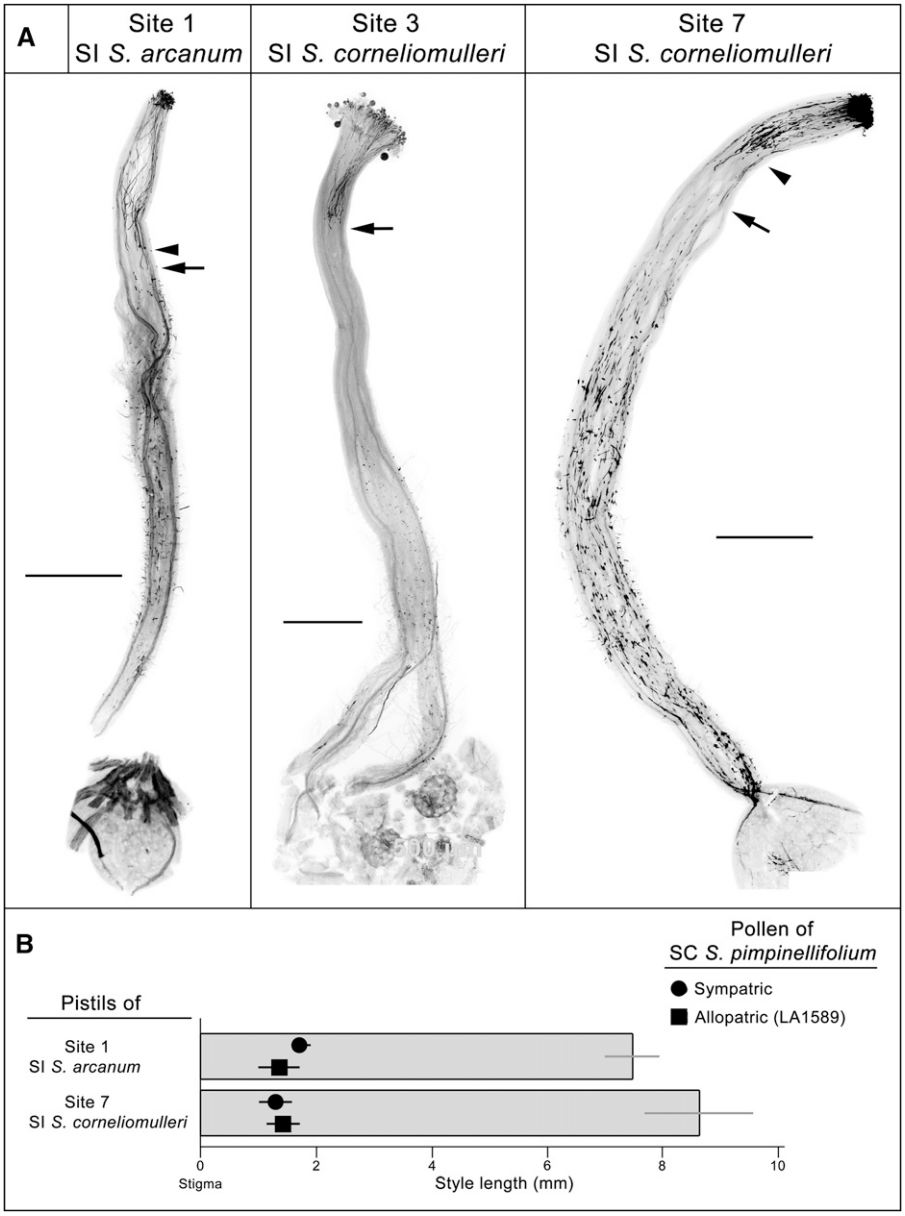

FIGURE 2 Rejection of self-compatible (SC) *Solanum pimpinellifolium* pollen tubes by pistils of sympatric partner species. (A) Representative images of sympatric *S. pimpinellifolium* pollen-tube growth in pistils of sympatric partner species: (left to right) self-incompatible (SI) *S. arcanum* at site 1 and SI *S. corneliomulleri* at sites 3 and 7. Arrows indicate the tip of the longest pollen tube, and arrowheads indicate where the majority of pollen tubes stop. (B) Sympatric and allopatric pollen-tube growth in pistils (represented by shaded bars) with stigmas to the left and ovaries to the right. Lengths are shown in millimeters with standard deviations (linear bars). Pollen-tube lengths are shown for sympatric (circles) and allopatric (LA1589; squares) SC *S. pimpinellifolium* accessions after 48 h growth. Scale bars = 1 mm.

S. pimpinellifolium accession, we evaluated growth of *S. pimpinellifolium* LA1589 pollen tubes (Fig. 2B, black squares) in styles of SI species that are sympatric partners of other *S. pimpinellifolium* accessions. We did not detect a significant difference between sympatric and allopatric *S. pimpinellifolium* pollen-tube growth ($t = 1.938$, df =11, $P = 0.079$) in the styles of these SI species.

Pollen-tube rejection in styles was not observed in either direction in crosses among all other species in sympatric populations. These included SI species pairs at sites 2, 3, 5, and 6; SI *S. corneliomulleri* and SC populations of SI *S. habrochaites* at sites 4 and 8; and the SC/SC pair *S. neorickii* and *S. chmielewskii* at site 9.

We observed differences in pollen-tube growth rates in crosses between the sympatric pair at site 2 (Fig. 3). SI *S. arcanum* pollen tubes grew to only 79% of the style length in sympatric partner SI *S. habrochaites* in 48 h, whereas sibling conspecific pollen tubes of SI *S. habrochaites* reached the ovaries by 48 h. This pollen–pistil interaction does not constitute a complete prezygotic barrier like those described above (Appendix S2), because by 72 h, *S. arcanum* pollen tubes had reached ovaries in the *S. habrochaites* partner (Fig. 3).

In two crosses, SI *S. corneliomulleri* × an SC population of SI *S. habrochaites* from site 8 and SC *S. neorickii* × SC *S. chmielewskii* from site 9, pollen tubes reached ovaries, but in all cases they did not appear to target ovules (Appendix S3, see Supplemental Data with the online version of this article). Further, fruit production failed after multiple attempts (>35 and >26 attempts, respectively). This suggests that a strong postmating prezygotic IRB acts at the level of ovule targeting in these interspecific crosses (Appendix S2).

Postzygotic barriers—*Fruit development*—Fruit and seed formation were assessed for the cases in which complete prezygotic barriers were not detected and appropriate seed was available from the TGRC. In these 14 crosses between sympatric species pairs, hybrid fruits containing seeds or seed-like structures (SLS) were produced. Size and mass of hybrid fruits were compared to those of intraspecific control fruits (Appendix S4, see Supplemental Data with the online version of this article). In general, hybrid fruits were substantially smaller compared to intraspecific controls ($t = 9.305$, df = 13, $P = 4.12E-07$; Fig. 4). For example, the masses of hybrid fruits made with accessions from sites 3 and 6 were reduced to, on average, ~25% of intraspecific controls, and hybrid fruits made with accessions from sites 1, 2, 4, and 7 showed a ~50% reduction in mass compared to intraspecific controls. Representative images of intraspecific control and hybrid fruits produced from the 14 crosses in which hybrid fruits were obtained are shown in Appendices S5 and S6 (see Supplemental Data with the online version of this article).

Seed development—Fruits formed after interspecific crosses contained seeds or SLS of varying sizes and degrees of maturity. Because seed development was clearly compromised in many cases, we characterized developing seeds of self-pollinated *S. pimpinellifolium*

TABLE 3. Summary of IRBs between tomato-clade (*Solanum* section *Lycopersicon*) members in sympatric populations.

Sympatric interspecific crosses			Prezygotic barriers			Postzygotic barriers: Seed development [b]		Hybridization
Site no.	Species cross	Mating system	PT rejection	Slow relative PT growth [a]	Fruit set fails	Type 1 [c]	Type 2 [d]	F_1 hybrid plants formed
1	*S. arc* × *S. pim*	SI × SC	X					
3	*S. cor* × *S. pim*	SI × SC	X					
7	*S. cor* × *S. pim*	SI × SC	X					
2	*S. hab* × *S. arc*	SI × SI		X			X	
8	*S. cor* × *S. hab*	SI × SC-pop			X			
9	*S. neo* × *S. chm*	SC × SC			X			
1	*S. pim* × *S. arc*	SC × SI				X		
2	*S. arc* × *S. hab*	SI × SI				X		
3	*S. cor* × *S. hab*	SI × SI				X		
4	*S. cor* × *S. hab*	SI × SC-pop				X		
6	*S. cor* × *S. pen*	SI × SI				X		
7	*S. pim* × *S. cor*	SC × SI				X		
3	*S. hab* × *S. cor*	SI × SI					X	
4	*S. hab* × *S. cor*	SC-pop × SI					X	
6	*S. pen* × *S. cor*	SI × SI					X	
8	*S. hab* × *S. cor*	SC-pop × SI					X	
5	*S. pen* × *S.cor*	SI × SI						X
5	*S. cor* × *S. pen*	SI × SI						X
9	*S. chm* × *S. neo*	SC × SC						X

Notes. PT = pollen tube; SI = self-incompatibility; SC = self-compatibility; SC-pop = self-compatible population; *S. arc* = *S. arcanum*; *S. chm* = *S. chmielewskii*; *S. cor* = *S. corneliomulleri*; *S. hab* = *S. habrochaites*; *S. neo* = *S. neorickii*; *S. pen* = *S. pennellii*; *S. pim* = *S. pimpinellifolium*.

[a] Interspecific pollen-tube growth was slower than conspecific pollen-tube growth (pollen tubes reach ovaries eventually and fruit is formed).

[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.

to provide a reference. Normal seed structure at 10 d 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. 5.

At 10 d postpollination (Fig. 5A), the embryo sac is surrounded by a single integument (int), consisting of a single innermost layer of endothelial cells (et), numerous parenchymal layers, and an epidermis (ep). The globular stage embryo (em) 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. 5B), the fully developed embryo assumes a spiral form, with the two cotyledons (cot) curled within the hypocotyl (hyp) and radicle (rad). The embryo and the surrounding endosperm (es) 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 (testa) with surface pseudohairs (ph).

We also examined the structure of normal mature seeds formed from intraspecific crosses in the maternal species of each interspecific cross, as further controls (Figs. 6 and 7; Appendices S7 and S8, see Supplemental Data with the online version of this article). The widest differences in seed morphology were seen between *S. pennellii* and *S. pimpinellifolium*. Seeds varied in size from approximately 0.9 mm × 1.7 mm (width × length) in *S. pennellii* to approximately 1.7 mm × 2.9 mm in *S. pimpinellifolium*, with sizes of the other species ranging between these two extremes (seed width data included in Appendix S4). Seed thickness ranged from approximately 0.5 mm to 1.0 mm (data not shown). Seed coat color of control seeds ranged from yellow through brown. Pseudohairs derived from the cell walls of the outer seed-coat layer covered the surface to a greater (*S. pimpinellifolium*) or lesser (*S. pennellii*) extent. Longer pseudohairs sometimes formed a tuft at the distal end of the seed body (*S. pennellii*) or completely surrounded the seed-coat margins (*S. pimpinellifolium*). The internal seed structure, including a spiral mature embryo, of all intraspecific controls were very similar to that of *S. pimpinellifolium* (Fig. 5B).

Seed development was abnormal in 11 of 14 interspecific crosses examined, resulting in complete RI in many sympatric pairs (Appendix S2). The number of total seed/SLS formed in interspecific hybrid fruit was significantly less than that of intraspecific controls (t = 4.4, df = 13, P = 0.000657; Appendix S4). Abnormal interspecific hybrid SLS were much smaller than the control seeds described above (t = 8.77, df = 13, P = 8.02 E-07; SLS

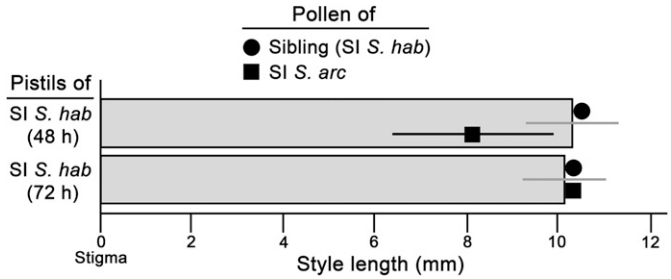

FIGURE 3 Slower growth of interspecific vs. conspecific pollen tubes in self-incompatible (SI) *Solanum habrochaites* pistils at site 2, Chilete-Rupe, Peru. Shaded bars represent pistils, and pollen-tube growth is from left to right. Circles indicate conspecific sibling (i.e., compatible) *S. habrochaites* LA1352 pollen tubes; squares, interspecific SI *S. arcanum* LA1351 pollen tubes. Shown are results after 48 h and 72 h growth. *S. hab* = *S. habrochaites*; *S. arc* = *S. arcanum*.

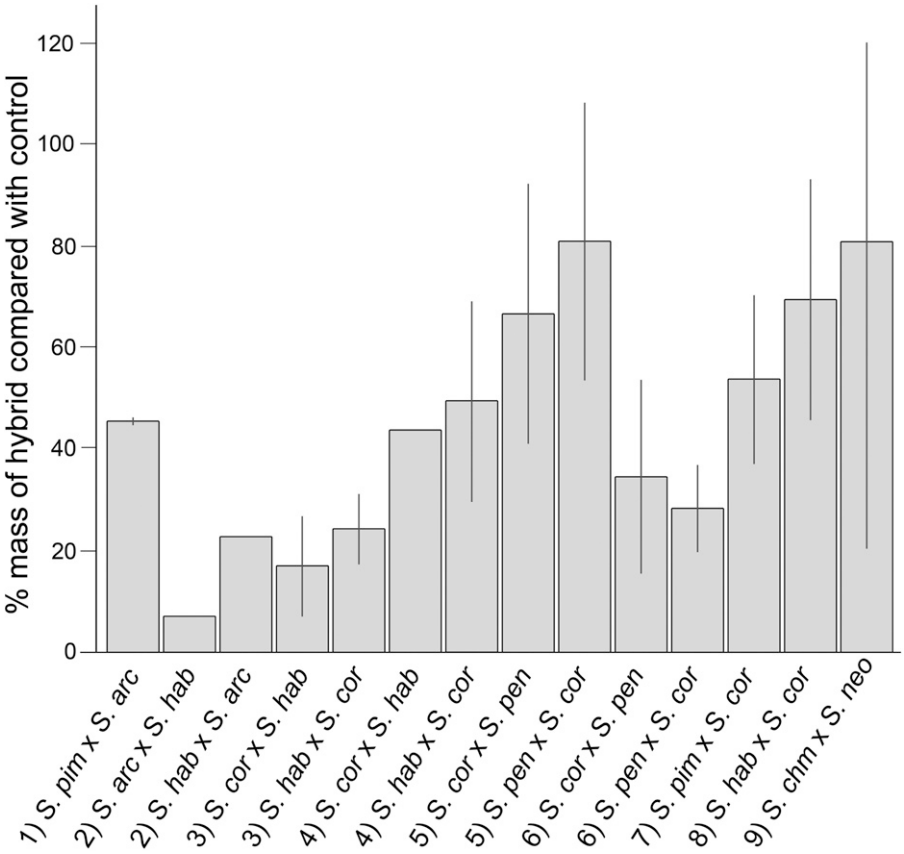

FIGURE 4 Hybrid fruit mass as percentage of intraspecific control. Data represent the 14 crosses between sympatric species pairs where fruit set occurred. Only a single fruit was obtained after multiple pollination attempts for each cross using accessions from site 2 and for *Solanum corneliomulleri* × *S. habrochaites* accessions from site 4. Average relative masses and standard deviations are shown. *S. arc* = *S. arcanum; S. chm* = *S. chmielewskii; S. cor* = *S. corneliomulleri; S. hab* = *S. habrochaites; S. neo* = *S. neorickii; S. pen* = *S. pennellii; S. pim* = *S. pimpinellifolium*.

were 57% of the control seed width on average; Appendix S4) and were usually pale and translucent. In many SLS, the outline of the embryo sac was visible through the integument, with a darker dot in the center indicating the position of the embryo (e.g., Appendix S7: B3). Figure 6 illustrates two different abnormal hybrid seed phenotypes that we observed. Type 1 (six of 14 crosses; Fig. 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 pseudohairs. Type 2 (five of 14 crosses) was observed when *S. habrochaites* or *S. pennellii* was the female in interspecific crosses (Fig. 6B). 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 conspicuous multilayered endothelium, rather than the single cell layer of endothelium found in normal seeds, was a distinguishing feature of this phenotype. When *S. habrochaites* was the female parent, the overgrown endothelium appeared densely stained, completely surrounded the embryo sac, and often occupied a large part of the seed interior. When *S. pennellii* was the female parent, the thickened endothelial layer showed lesser staining and tended to be discontinuous (Appendix S8), with endosperm cells occasionally appearing to lie outside it (data not shown).

Formation of normal or nearly normal hybrid seeds and plants—Normal or nearly normal seeds were produced in fruits of three of 14 interspecific crosses (Fig. 7). These putative hybrid seeds resembled control intraspecific seeds, in that they had well-developed seeds coats with normal pigmentation. Hybrid seed width was not significantly different from that of control seeds (t = 0.837, df = 2, P = 0.4904), and seed-coat pseudohairs generally resembled those on control seeds of the pistil parent (Appendices S7 and S8). Upon sectioning, these seeds were found to contain fully developed embryos, with normal endosperm and a single endothelial layer. Interestingly, hybrid *S. pennellii* × *S corneliomulleri* (site 5) embryos often erupted from the seed coat (Fig. 7: B4), perhaps because of the smaller seed-coat size typical of the *S. pennellii* maternal parent (Fig. 7: B1). Interspecific fruits also contained less-developed SLS with globular embryos or postglobular embryos at torpedo, walking-stick, or early-spiral stages. On average, 42% (site 9), 61% (site 5, *S. pennellii* × *S. corneliomulleri*), and 74% (site 5, *S. corneliomulleri* × *S. pennellii*) of seeds found in interspecific fruits were near normal in size (Appendix S4).

Some of the largest, fully or nearly fully developed, seeds from these three crosses germinated and produced F_1 plants. Both leaves and flowers of these putatively hybrid plants were intermediate in phenotype (Appendix S9, see Supplemental Data with the online version of this article). Molecular markers for species at site 9 confirmed hybridity (Appendix S10, see Supplemental Data with the online version of this article); species-specific molecular markers were not available for species at site 5.

DISCUSSION

Reproductive isolation between plant species can be regarded as arising from a series of barriers affecting prepollination and postpollination processes (Ramsey et al., 2003; Rieseberg and Willis, 2007; Lowry et al., 2008; Widmer et al., 2009; Sobel et al., 2010; Baack et al., 2015). In previous studies focused on barriers between species in sympatry, prezygotic barriers including ecological differentiation (Martin and Willis, 2007), differences in flowering phenology (Kenney and Sweigart, 2016), floral morphology (Grossenbacher and Whittall, 2011), pollinator preference (Kay and Sargent, 2009; Dell'Olivo et al., 2011; Whitehead and Peakall, 2014; Sheehan et al., 2016), and pollen–pistil interactions (Rieseberg et al., 1995; Carney et al., 1996; Klips, 1999; Pellegrino et al., 2010) predominated. However, postzygotic barriers can also play an

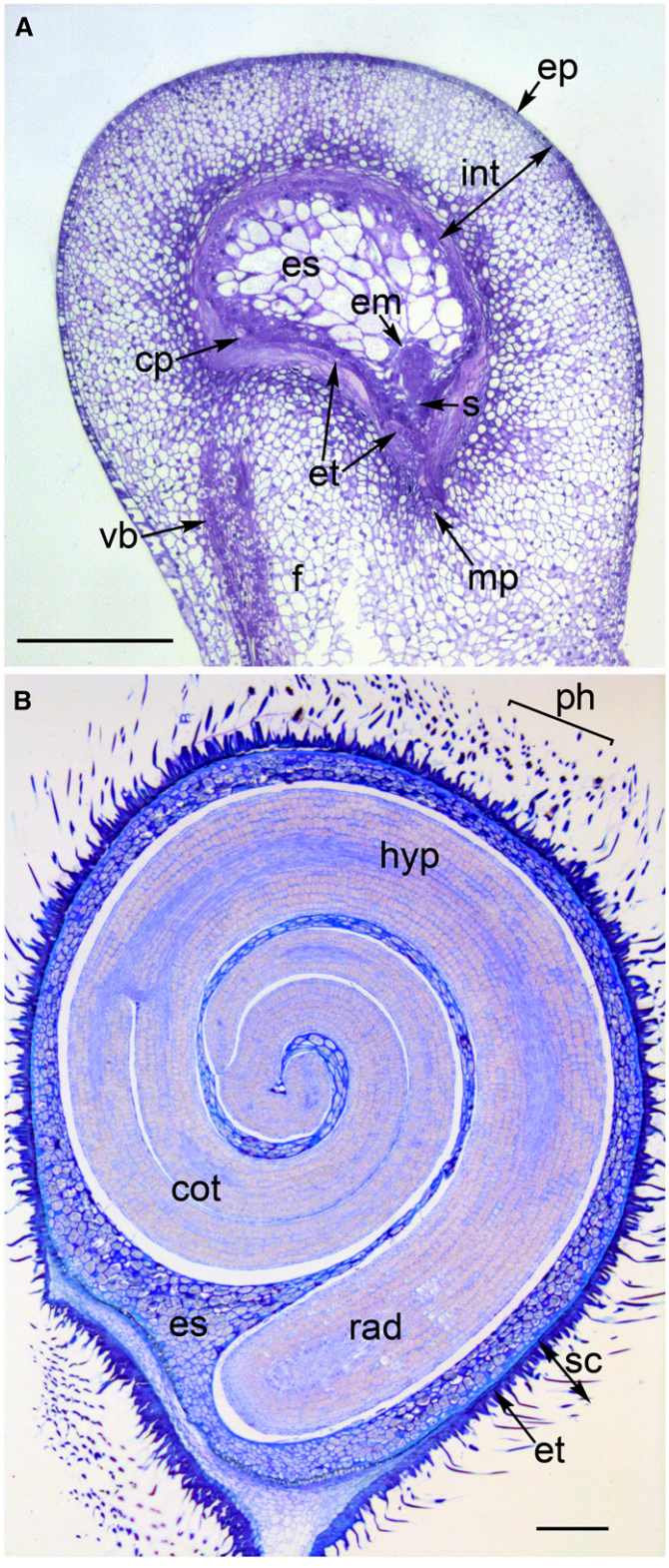

FIGURE 5 Normal seed development in self-pollinated *Solanum pimpinellifolium* LA2149. (A) 10 d postpollination. (B) Mature seed. Abbreviations: cot, cotyledon; cp, chalazal pocket; em, embryo; es, endosperm; ep, epidermis; et, endothelium; f, funiculus; ph, pseudohairs; hyp, hypocotyl; int, integument; mp, micropyle; rad, radicle; s, suspensor; sc, seed coat; vb, vascular bundle. Scale bars = 200 µm.

important role in the isolation of sympatric species (Costa et al., 2007; Jewell et al., 2012; Oneal et al., 2016).

The tomato clade is very recently diverged, with relatively minor differences in floral morphology between species (Peralta et al., 2008; Rodriguez et al., 2009; Pease et al., 2016). Consistent with the low seasonal variability of the tropical environments that favors more-or-less continuous flowering, we confirmed that the species at our sympatric sites coflower (Table 1). Members of subfamily Solanoideae, including the tomato clade with their solanoid flowers, share a buzz pollination syndrome (Rick et al., 1978; Knapp, 2010; De Luca and Vallejo-Marin, 2013). Yet, even with opportunities for hybridization through shared geography, floral morphology, and phenology, and potentially shared pollinators, tomato-clade hybrids have not been reported at sympatric sites, which suggests that RI is nevertheless effective. We chose nine tomato-clade sympatric sites and analyzed 19 interspecific crosses with a focus on pollen–pistil interactions and early postzygotic processes. Our results are summarized in Table 3 and Appendix S2.

We initially investigated whether stigma exsertion might act as a premating prezygotic IRB at some sympatric sites. Because stigma exsertion has been correlated with outcrossing rates in *S. pimpinellifolium* (Rick et al., 1978), we hypothesized that this species would show reduced stigma exsertion when it co-occurs with other tomato-clade species. However, we found no significant difference in *S. pimpinellifolium* stigma exsertion between sympatric and allopatric populations (Table 2), which suggests that reduced stigma exsertion in this species has not been selected for in sympatry.

We identified three different types of postmating prezygotic IRBs in six interspecific crosses tested. First, we observed interspecific pollen-tube rejection of SC *S. pimpinellifolium* in styles of all three SI sympatric partners tested (sites 1, 3, and 7; Fig. 2). *Solanum pimpinellifolium* is widespread in Peru and Ecuador and has been documented to occur in at least 15 sympatric sites, including four sites in our study (Table 1 and Appendix S1). The molecular mechanisms underlying this barrier (i.e., unilateral incompatibility) involve pollen SI components that have been lost to mutation in *S. pimpinellifolium* (Li and Chetelat, 2014; Li and Chetelat, 2015). We found no differences when comparing pollen-tube growth of sympatric vs. allopatric populations of *S. pimpinellifolium* in styles of SI sympatric species. This suggests that (in the cases studied) a stronger stylar barrier against pollen of the *S. pimpinellifolium* sympatric partner has not been selected for in these SI species. However, as mentioned above, the pollen–pistil barrier of unilateral incompatibility is entirely effective in preventing fertilization of SI species by *S. pimpinellifolium* (Appendix S2).

We observed a second type of postmating prezygotic barrier in crosses between sympatric species from site 2: slow relative growth of SI *S. arcanum* pollen tubes in SI *S. habrochaites* pistils compared to conspecific pollen tubes (Fig. 3). Although this postmating prezygotic IRB is not specific to sympatric accessions (Baek et al., 2015), the slow relative growth of interspecific pollen tubes in this case could result in conspecific pollen precedence, making a small contribution to total RI (Appendix S2; Rieseberg et al., 1995; Howard, 1999; Fishman et al., 2008; Aagaard et al., 2013; Swanson et al., 2016). Experiments using a mixture of conspecific and heterospecific pollen will be required to definitively assess whether conspecific pollen precedence occurs in crosses between this sympatric pair.

Finally, in two cases (SI *S. corneliomulleri* × SC *S. habrochaites* at site 8 and SC *S. neorickii* × SC *S. chmielewskii* at site 9), pollen tubes

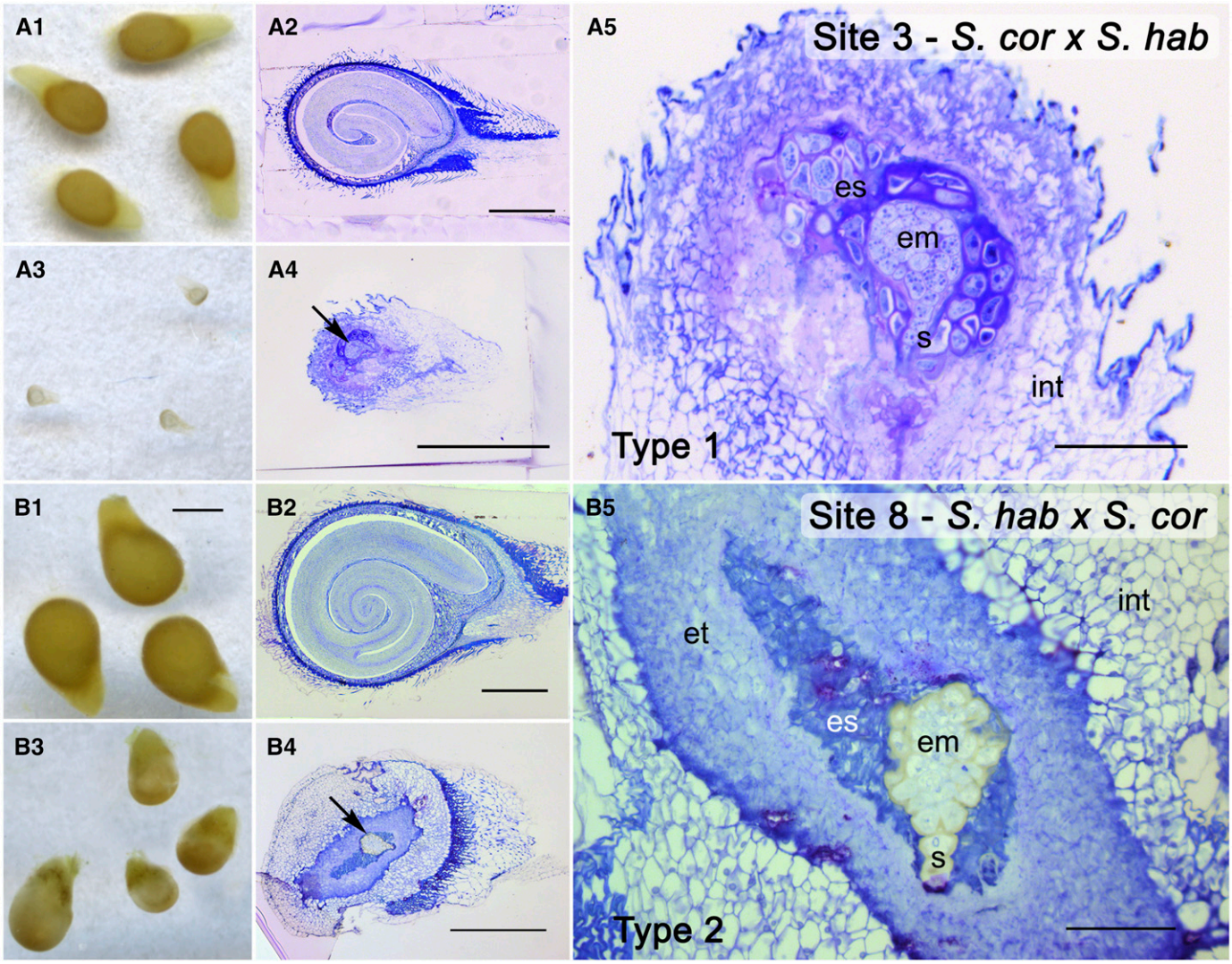

FIGURE 6 Examples of two phenotypes of hybrid SLS: (A) type 1 phenotype, site 3, *Solanum corneliomulleri* (*S. cor*) × *S. habrochaites* (*S. hab*); and (B) type 2 phenotype, site 8, *S. habrochaites* × *S. corneliomulleri*. (A1, B1) Intraspecific control seeds of the pistil parent for each cross. (A2, B2) Sagittal sections of control seeds. (A3, B3) SLS from the interspecific crosses. (A4, B4) Sagittal sections of SLS resulting from the interspecific crosses. (A5, B5) Enlargements of sections of interspecific SLS. Abbreviations: em, embryo; es, endosperm; et, endothelium; int, integument; s, suspensor. Arrowheads in A4 and B4 indicate embryos. Scale bar in B1 = 1 mm (also for A1, A3, B3). Scale bars in A2, A4, B2, B4 = 500 µm. Scale bars in A5, B5 = 100 µm.

were able to grow through the style and reach ovaries but may not target ovules (Appendix S3). This result was surprising since Rick et al. (1976) reported that *S. neorickii* and *S. chmielewskii* are interfertile. However, different populations of *S. neorickii* show differences in interspecific pollen rejection (Baek et al., 2015), which suggests that IRBs may differ between the population of *S. neorickii* used by Rick et al. (1976) and the more southern LA2639A sympatric population in our study. It will be interesting to pursue whether lack of ovule targeting that we observed involves factors similar to the small, cysteine-rich LURE proteins secreted by synergid cells (Kanaoka and Higashiyama, 2015) or their pollen receptors (Takeuchi and Higashiyama, 2016; Wang et al., 2016) that are involved in species-specific pollen-tube–ovule communication (Higashiyama et al., 2006; Takeuchi and Higashiyama, 2012; Lindner et al., 2015).

Very few studies have investigated RI between sympatric species within the Solanaceae. However, an in-depth analysis of two sympatric wild *Petunia* (Solanaceae) species found that prezygotic reproductive barriers were almost exclusively responsible for RI (Dell'Olivo et al., 2011). By contrast, we found strong postzygotic barriers to hybridization in over half of our interspecific crosses (11 of 19 cases; Table 3 and Appendix S2). In our studies, a reduction in hybrid fruit mass was correlated with the presence of abnormal SLS within fruits (Fig. 4). Anatomic examination of SLS revealed globular embryos and endosperm cells that generally appeared to be empty or to have clumped intracellular contents (Fig. 6; Appendices S7 and S8). In angiosperms, arrest in early embryo development is commonly associated with endosperm failure in interploidy or interspecific crosses (Cooper and Brink, 1945; Nowack et al., 2010; Ishikawa et al., 2011; Oneal et al., 2016). The molecular mechanisms underlying defects in hybrid seed development, including both genic incompatibilities and epigenetic effects, are under active investigation in numerous plant species (Fishman and Willis, 2006;

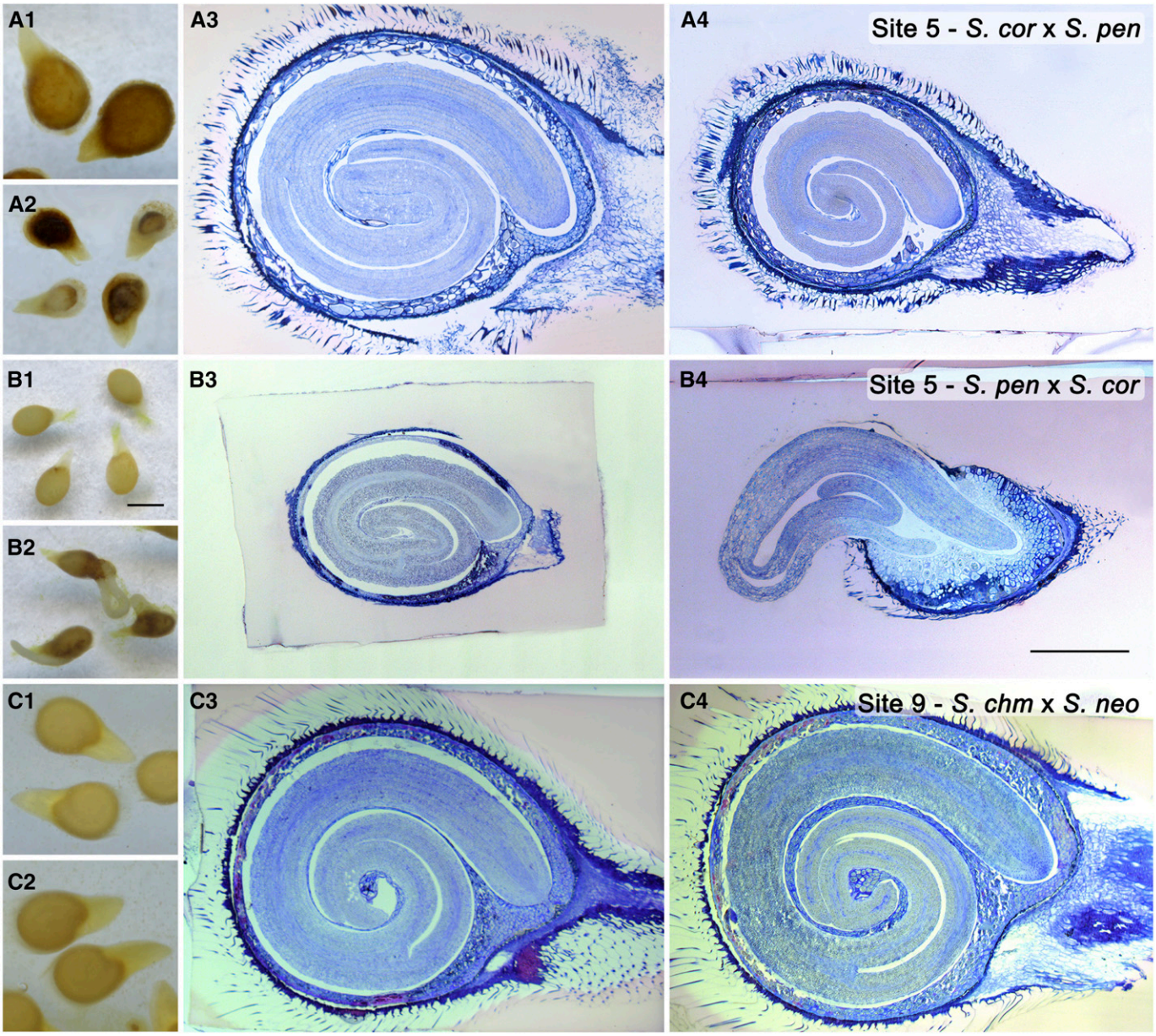

FIGURE 7 Normal or nearly normal hybrid seeds produced by interspecific crosses at two sympatric sites: (A) site 5, *Solanum corneliomulleri* (*S. cor*) × *S. pennellii* (*S. pen*); (B) site 5, *S. pennellii* × *S. corneliomulleri*; and (C) site 9, *S. chmielewskii* (*S. chm*) × *S. neorickii* (*S. neo*). (A1–C1) Intraspecific control seeds of the pistil parent for each cross. (A2–C2) Seeds and SLS in fruit resulting from the interspecific crosses. (A3–C3) Sagittal sections of seeds from intraspecific crosses. (A4–C4) Sagittal sections of seeds resulting from the interspecific crosses. Scale bar in B1 = 1 mm (also for A1, C1, A2–C2). Scale bar in B4 = 500 µm (also for A3–C3, A4, C4).

Josefsson et al., 2006; Marfil et al., 2006; Bomblies et al., 2007; Michalak, 2009; Ng et al., 2012; Shivaprasad et al., 2012; Lafon-Placette and Köhler, 2015, 2016).

In five cases where *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 sporophytic integument that is normally only one cell layer thick. Similar proliferation of the endothelium, accompanied by subnormal growth of the endosperm and embryo, has previously been observed in 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). A similar pattern of endosperm failure and endothelial overgrowth in hybrid seeds has been reported for *Medicago sativa* (Brink and Cooper, 1939, 1940), *Oenothera*, various orchard fruits, and grapes (for reviews of older literature concerning the latter groups, see Cooper and Brink, 1940; Brink and Cooper, 1941). This phenotype may result from poor nutrient transfer from the maternal sporophyte to the embryo sac. In this scenario, endothelial overgrowth may interfere with the formation or functioning of specialized conducting cells between the chalazal pocket and vascular strands in the funiculus (Cooper and Brink, 1940, 1945; Brink and Cooper, 1941). Future studies of seed

development in crosses between members of the tomato clade will focus on the formation of the sporophyte–endosperm connection, particularly in interspecific crosses with *S. habrochaites* or *S. pennellii* as female.

A significant proportion of normal-sized seed formed in three of the 19 interspecific crosses (Table 3 and Fig. 7). In these three interspecific crosses, the number of normal-sized hybrid seed per fruit was less than the number of intraspecific seed per fruit (Appendix S4), resulting in a contribution to RI (Appendix S2). Future studies determining the frequency of hybrid fruit formation and viability of hybrid vs. intraspecific seed will provide more complete information on postzygotic RI in these species pairs. F_1 plants resulting from the germination of interspecific seed displayed intermediate leaf and flower phenotypes (Appendix S9); and molecular markers, when available, confirmed hybrid formation (Appendix S10). Although hybrid plants have not been reported in the wild, our results suggest that a more thorough search for hybrids is justified at some sympatric sites. This effort will be facilitated as more species-specific molecular markers become available. Of course, it is possible that hybrids would not persist in natural settings, owing to low fitness.

In summary, we found multiple types of prezygotic and postzygotic reproductive barriers that could prevent hybridization between species in sympatry and that are likely to result in complete RI (Table 3, Appendix S2). Reproductive barriers to gene flow are not only crucial for preserving species integrity; they are also essential for the completion of speciation after the initial divergence of new lineages. Considering their fundamental role in the generation and maintenance of biodiversity, it will be of great interest to determine the mechanisms underlying these barriers and how they evolve during speciation.

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