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Research

Mating system transitions in *Solanum habrochaites* impact interactions between populations and species

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Summary

- In plants, transitions in mating system from outcrossing to self-fertilization are common; however, the impact of these transitions on interspecific and interpopulation reproductive barriers is not fully understood. We examined the consequences of mating system transition for reproductive barriers in 19 populations of the wild tomato species *Solanum habrochaites*.
- We identified *S. habrochaites* populations with self-incompatible (SI), self-compatible (SC) and mixed population (MP) mating systems, and characterized pollen–pistil interactions among *S. habrochaites* populations and between *S. habrochaites* and other tomato species. We examined the relationship between mating system, floral morphology, interspecific and interpopulation compatibility and pistil SI factors.
- We documented five distinct phenotypic groups by combining reproductive behavior with molecular data. Transitions from SI to MP were not associated with weakened interspecific reproductive barriers or loss of known pistil SI factors. However, transitions to SC at the northern range margin were accompanied by loss of S-RNase, smaller flowers, and weakened (or absent) interspecific pollen−pistil barriers. Finally, we identified a subset of SC populations that exhibited a partial interpopulation reproductive barrier with central SI populations.
- Our results support the hypothesis that shifts in mating system, followed by additional loss-of-function mutations, impact reproductive barriers within and between species.

Introduction

Plants exhibit an extraordinary range of mating system strategies that influence outcrossing and reproductive success (Darwin, 1876), ultimately shaping the genetic and demographic structure of populations (Stebbins, 1974; Richards, 1986; Holsinger, 2000; Barrett, 2002; Coyne & Orr, 2004; Goldberg *et al.*, 2010). In many plant species, outcrossing is enforced by self-incompatibility (SI), a complex genetic mechanism in which an individual rejects its own (self) and closely related pollen, preventing self-fertilization (Lewis, 1944; de Nettancourt, 1977, 2001; Takayama & Isogai, 2005; McClure & Franklin-Tong, 2006). Obligatory outcrossing ensures high levels of genetic diversity, the basis for adaptation in changing environments, and reduces the potential costs associated with inbreeding (Lande & Schemske, 1985; de Nettancourt, 2001; Charlesworth & Willis, 2009; Goldberg *et al.*, 2010). Despite the advantages of outcrossing, the transition from SI to self-compatibility (SC) is common in plant species (Ornduff, 1969; Stebbins, 1974; Richards, 1986; Barrett, 2002) while the reverse transition (SC → SI) is exceedingly rare (Igic *et al.*, 2006, 2008; Goldberg & Igic, 2012).

The loss of SI is often associated with an increase in self-fertilization and reduced heterozygosity, which can reduce fitness through inbreeding depression (Jain, 1976; Hamrick & Godt, 1989, 1996; Mable & Adam, 2007; Charlesworth & Willis, 2009). Shifts from outcrossing to self-fertilization can also be accompanied by changes in floral phenology and morphology, including changes in flowering time, floral longevity, floral size, herkogamy and dichogamy (Ornduff, 1969; Goodwillie *et al.*, 2010; Sicard & Lenhard, 2011; Kalisz *et al.*, 2012; Vallejo-Marin *et al.*, 2014). Increased self-fertilization can limit gene flow between populations and reduce genetic diversity (Allard, 1975; Schoen & Brown, 1991; Charlesworth, 2003; Martin & Willis, 2007). However, the cost of self-fertilization can be mitigated through the purging of maladaptive alleles (Schemske & Lande, 1985; Pujol *et al.*, 2009; Szoevenyi *et al.*, 2014), and SC individuals may benefit from reproductive assurance when mates or pollinators are limiting, particularly at species range margins (Baker, 1955, 1967; Stebbins, 1957; Lloyd, 1992; Pannell & Barrett, 1998; Kalisz *et al.*, 2004; Busch & Schoen, 2008; Pannell *et al.*, 2015). Additionally, transitions from SI to SC are known to influence compatibility both within and between species that differ in mating system (Lewis & Crowe, 1958; Martin, 1961, 1963; Hogenboom, 1973; Baek *et al.*, 2015). Understanding the

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sequence of genetic and morphological changes that occur during the loss of SI can further clarify the dynamics and evolution of mating system transitions and the role these transitions may ultimately play in speciation (Martin & Willis, 2007; Rieseberg & Willis, 2007; Wright *et al.*, 2013). Here, we investigated how the loss of SI along a geographic gradient influences interspecific and interpopulation reproductive barriers in a species of wild tomato.

The tomato clade (*Solanum* section *Lycopersicon*) is ideal for elucidating the mechanistic aspects of mating system transitions and their linkage to interspecific and interpopulation compatibilities. It is a young clade that diverged from an SI common ancestor (Igic & Kohn, 2001; Igic *et al.*, 2006) over the course of *c.* 2.5 million yr (Pease *et al.*, 2016). Within the 13-member clade, six SC species are recognized, and SC populations have been identified in several of the seven extant SI species, suggesting that mating systems in the clade are dynamic (Peralta *et al.*, 2008). In the Solanaceae, SI is genetically based and controlled by the polymorphic *S* locus, which encodes both male and female determinants of specificity. This type of SI is determined gametophytically: if the single *S* allele of the male gametophyte matches either of the two *S* alleles of the diploid pistil, pollen is rejected (McClure *et al.*, 1989, 2011; Igic & Kohn, 2001; de Nettancourt, 2001; Kao & Tsukamoto, 2004; McClure & Franklin-Tong, 2006). The *S* locus encodes pistil-expressed S-RNase which determines the female specificity of the SI response, but additional factors unlinked to the *S* locus, such as HT protein and the 120-kDa glycoprotein, are also required for the rejection of self-pollen (McClure *et al.*, 1989, 1999; Murfett *et al.*, 1994; Hancock *et al.*, 2005). Pollen-expressed factors that determine SI include *S*-locus-encoded F-box (SLF) proteins (Entani *et al.*, 2003; Qiao *et al.*, 2004; Sijacic *et al.*, 2004; Kubo *et al.*, 2010, 2015; Williams *et al.*, 2014a,b) as well as non-*S*-locus pollen-expressed E3 ubiquitin ligase components such as Cullin1 and S-phase kinase-associated protein 1 (Qiao *et al.*, 2004; Hua & Kao, 2006; Li & Chetelat, 2010, 2014; Entani *et al.*, 2014; Li *et al.*, 2014).

Pollen–pistil interspecific reproductive barriers (IRBs) are mechanistically linked to SI. Unilateral incompatibility (UI), in which fertilization occurs in only one direction, is a type of IRB that is common between SI species and related SC species (Lewis & Crowe, 1958; Pandey, 1962, 1981; Levin, 1971; Bedinger *et al.*, 2011; Baek *et al.*, 2015). In a number of plant families, UI follows the SI × SC rule: interspecies crosses fail when an SI species is used as female and an SC species is used as male, but the reciprocal cross is compatible (Lewis & Crowe, 1958; Hogenboom, 1973; Murfett *et al.*, 1996; Baek *et al.*, 2015). SI factors including S-RNase, HT protein, Cullin1 and SLF proteins have been shown to participate in this type of IRB (Covey *et al.*, 2010; Li & Chetelat, 2010, 2015; Tovar-Méndez *et al.*, 2014). However, while it is clear that UI and SI mechanisms share significant overlap, it should be noted that additional UI mechanisms exist that are not dependent on S-RNases (Murfett *et al.*, 1996; Kondo *et al.*, 2002; Covey *et al.*, 2010; Eberle *et al.*, 2013; Baek *et al.*, 2015).

The wild tomato species *Solanum habrochaites* is particularly suitable for the study of how mating system transitions affect pollen–pistil incompatibilities between and within species. *Solanum habrochaites* has undergone at least two independent transitions from the ancestral SI mating system to an SC mating system at both its northern and its southern species range margins (Martin, 1963; Rick *et al.*, 1979; Rick & Chetelat, 1991). Previous studies identified one northern SC population that lacks expression of S-RNase (Covey *et al.*, 2010), presumably as a result of the insertion of a transposable element (TE) in the promoter region (Kondo *et al.*, 2002). UI has been demonstrated between *S. habrochaites* and SC species in the tomato clade, wherein fruit is only formed when the SC species is used as female in reciprocal crosses (Mutschler & Liedl, 1994; Sacks & St Clair, 1998). *Solanum habrochaites* pistils reject pollen tubes of *Solanum lycopersicum*, although, interestingly, SC population LA0407 displayed weakened IRBs compared with SI population LA1777 (Covey *et al.*, 2010; Baek *et al.*, 2015). UI has also been observed between marginal SC populations and central SI populations of *S. habrochaites* (Martin, 1961, 1963, 1964).

We hypothesized that the transition from SI to SC would be associated with specific mutations in SI factors and changes in floral morphology, and that the transition would influence both interspecific and interpopulation pollen–pistil compatibilities. To address these hypotheses, we utilized morphological, functional and molecular techniques within a biogeographic context. We focused on *S. habrochaites* populations at the northern range margin that vary in mating system (SI, SC or mixed SI/SC population) and identified differences in floral characters. We assessed pollen–pistil interactions of these populations with other species in the tomato clade and with each other. Further, we ascertained whether known pistil SI factors (S-RNase and HT proteins) are present, and identified a loss-of-function *S-RNase* allele in a subset of populations.

Materials and Methods

Solanum habrochaites plant material and growth

Solanum habrochaites (S. Knapp & D. M. Spooner) is a wild relative of cultivated tomato (*Solanum lycopersicum*) that ranges from south-central Peru, near Nazca to central Ecuador. The *S. habrochaites* accessions (referred to hereafter as populations) used in this study (Table 1) represent populations from the center to the northern margin of the species range, from Río Casma, Peru to Jipijapa, Ecuador. Seeds from all populations were acquired from the C. M. Rick Tomato Genetic Resource Center (TGRC) at the University of California, Davis (http://www.tgrc.ucdavis.edu) or the United States Department of Agriculture (USDA). Eco-geographic groups (A–F) were established by Sifres *et al.* (2011), and are based on a strong correlation between genetic differentiation and geographic distance (Table 1).

Seeds were sterilized according to recommendations of the TGRC (TGRC 2016) and were planted into 4-inch pots containing ProMix-BX soil (Premier Tech Horticulture, Quakertown, PA, USA) with 16 h 26°C : 8 h 18°C, light : dark

Table 1 Populations of *Solanum habrochaites* used in this study

Population/accession	Source	Collection site	Province/department	Country	Lat/Long	Altitude (m)	Group*
LA1624	TGRC	Jipijapa	Manabí	Ecuador	−1.300/−80.583	300	**A**
PI 129157	USDA	West of Baños	Tungurahua	Ecuador	−1.400/−78.45†	1800†	A
LA1625	TGRC	South of Jipijapa	Manabí	Ecuador	−1.500/−80.517	300	A
LA1266	TGRC	West of Pallatanga	Chimborazo	Ecuador	−2.011/−78.975	1000	**A**
LA1264	TGRC	East of Bucay	Chimborazo	Ecuador	−2.167/−79.100	200	A
LA0407	TGRC	Guayaquil	Guayas	Ecuador	−2.178/−79.914	70	**A**
LA1223	TGRC	Alausí	Chimborazo	Ecuador	−2.196/−78.850	2200	A
LA2119	TGRC	Saraguro	Loja	Ecuador	−3.622/−79.238	2600	B
LA2106	TGRC	Yambra – La Providencia	Loja	Ecuador	−4.203/−79.230	1700	B
LA2101	TGRC	San Pedro de Cariamanga	Loja	Ecuador	−4.332/−79.562	1800	B/C
LA2868	TGRC	Arenillas	El Oro	Ecuador	−3.757/−80.049	540	C
LA2864	TGRC	Sozoranga	Loja	Ecuador	−4.333/−79.783	1650	**C**
LA2099	TGRC	Sabiango – Sozoranga	Loja	Ecuador	−4.353/−79.802	1000	C
LA2098	TGRC	Sabiango	Loja	Ecuador	−4.365/−79.813	700	C
LA2175	TGRC	Timbaruca	Cajamarca	Peru	−5.142/−79.008	1150	D
LA1391	TGRC	Bagua – Olmos	Cajamarca	Peru	−6.028/−79.022‡	N/A	**D**
LA2314	TGRC	San Francisco	Amazonas	Peru	−6.417/−77.867	1650	**D**
LA1353	TGRC	Contumaza	Cajamarca	Peru	−7.367/−78.800	2650	**E**
LA1777	TGRC	Río Casma	Ancash	Peru	−9.550/−77.667	3216	**F**

*Eco-geographic/genetic grouping according to Sifres *et al.* (2011). Letters in bold indicate populations used both in this study and in Sifres *et al.* (2011); grouping of other populations is inferred by location. †Lat/Long and altitude from a nearby collection, TGRC LA0128. ‡Lat/Long estimated from the described location mid-way between Olmos and Bagua.

for *c.* 2 months. Plants were transplanted to outdoor agricultural fields at Colorado State University (May–September 2011–2015) in order to obtain sufficient flowers for multiple crosses.

Verification of mating system

We verified the mating system of each population by assessing self-pollen tube growth in the style and self-fruit set. For measures of pollen tube growth, we conducted controlled self-pollinations on three to 20 individuals in each population. Because some populations from eco-geographic groups C–F were previously described as SI or SI/SC (TGRC, 2016), intrapopulation pollinations served as positive controls in eight of nine of these populations. Flower staging was as described previously (Chalivendra *et al.*, 2013). Buds were emasculated at stage −1 (1 d before anthesis) and hand pollinated with self-pollen 24 h later at stage 0 (bud break). Pollinations were covered with nylon mesh bags after emasculation to prevent pollen deposition by natural pollinators. To assess pollen tube growth in crosses, pollinated pistils were collected 48 h post-pollination, placed directly into fixative (3 : 1 ethanol : acetic acid), cleared with 5 M NaOH and stained with aniline blue fluorochrome (ABF) as previously described (Covey *et al.*, 2010). Pistils were examined with a Leica DM 5500B microscope (http://www.leica.com) at ×5 magnification using a 4′,6-diamidino-2-phenylindole (DAPI) emission filter. Images were composited, inverted and contrast-adjusted to visualize pollen tubes. Individual crosses were classified as compatible if three or more pollen tubes reached the base of the style after 48 h; however, in the majority of images analyzed (> 96%) substantially more pollen tubes reached ovaries in crosses scored as compatible. After scoring individuals for compatibility, population averages were calculated.

Fruit set from self-pollinations was assessed in all populations by pollinating open flowers that had been bagged before opening. In some cases, additional pollinations were conducted by pollinating emasculated flowers. For each population, two to seven individual plants were tested, and multiple pollination attempts for fruit were conducted for most (85%) individuals. Fruits were allowed to ripen for at least 2 months and seeds were collected. Per cent self-fruit set (per attempt) was calculated for each individual and used to calculate population means. To assess germination, pooled seeds from multiple fruits (when applicable) were sterilized for 10 min in 1% sodium hypochlorite, rinsed three times with water and placed on sterile germination paper in sealed petri dishes. Germination (radicle emergence) was quantified after 1 wk.

Floral morphology

To characterize changes in floral morphology associated with mating system, we examined corolla diameter and stigma exsertion in a minimum of three individuals in each population. Measurements were taken with electronic digital calipers beginning at stage 0 and up to stage +4, depending on floral longevity. When multiple measurements of the same developmental stage were made on a single individual, these values were averaged before statistical analyses. Mean corolla diameter and mean stigma exsertion at each stage were compared between groups of populations (grouping based either on mating system or on the five proposed reproductive groups; see later, Fig. 7) using separate analysis of variance (ANOVA) with a Tukey adjustment for multiple testing (R statistics program; www.r-project.org). For overall model statistics and Tukey's post hoc tests, see Supporting Information Tables S1 and S2. Style lengths for each individual were

determined from images of interspecific crosses as described in the next subsection.

Interspecific and interpopulation compatibility

Interspecific pollen tube rejection between *S. habrochaites* populations and SC *Solanum neorickii* (D. Spooner, G. J. Anderson and R. K. Jansen) (LA4023, LA0247, LA2197 and LA2862) or SC *Solanum lycopersicum* L. (cultivated tomato; LA4354, LA1221, LA0490 and LA3475) was assessed using pollen tube growth assays. Pollen tubes of these two SC species are rejected by pistils of SI *S. habrochaites* LA1777. However, while pollen tubes of *S. lycopersicum* are also rejected by pistils of *S. habrochaites* SC LA0407, those of *S. neorickii* are not (Baek *et al.*, 2015). Because of the observed differences in pollen tube rejection, we adopted these two SC species as pollen parents. Further, because gradations in the strength of pistil IRBs are linked to mating system (Baek *et al.*, 2015), we determined the distance interspecific pollen tubes grew through styles. Emasculations, pollinations, and scoring of compatibility were conducted following the methods described above (see 'Verification of mating system' subsection). The lengths of styles (from top of the stigmas to the top of the ovary) and the point at which the majority of pollen tubes stopped growing (point in the style where no more than three pollen tubes pass; mean values are given in Table S3) were measured using ImageJ v.1.47 (http://rsb.info.nih.gov/ij/). Pollen tube measurement data were converted to percentage of style length and averaged for each population. For crosses using *S. lycopersicum* pollen, images were measured from two to five individuals for all 19 *S. habrochaites* populations. For crosses using *S. neorickii* pollen, images were measured from two to five individuals for each *S. habrochaites* population for 12 populations. In cases where fewer images were available, between one and four images were measured for a single individual female per population. To avoid pseudoreplication in instances where images were obtained from multiple flowers on a single individual, data were collapsed to a single mean value before calculating population means. Where appropriate, means were compared between groups of populations for each type of interspecific cross using ANOVA as described above (see the Floral morphology subsection; for statistics see Tables S4, S5).

Interpopulation pollen tube rejection was also assessed using pollen tube growth assays. Pollen of *S. habrochaites* SC population LA0407 (eco-geographic group A) was utilized to assess pistil functionality of *S. habrochaites* populations, whereas pistils of SI population LA1777 (eco-geographic group F) were used to assess pollen functionality. These populations were chosen as testers based on previous studies that demonstrate UI between SC LA0407 and SI LA1777 (Baek *et al.*, 2015). For the purposes of our study, we considered LA1777 to be representative of a population with fully functional pistils and pollen, whereas LA0407 is representative of populations that have lost pollen side factors required to traverse SI styles. Additional crosses between the five proposed reproductive groups (see Fig. 7, later, for description) were subsequently performed to characterize additional interpopulation reproductive barriers.

S-RNase and HT-protein detection

We assessed S-RNase and HT-protein expression in pooled stylar extracts from two to four plants from each population. Styles were pooled from stages −1 to +1, weighed and immediately homogenized in 2× Laemmli buffer (8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.008% bromophenol blue and 0.25 M Tris-HCl, pH 6.8) at 10 µl per mg fresh weight. Samples were boiled for 5 min and centrifuged for 10 min (14 000 ***g***) and the supernatant was collected. For sample preparation, equal volumes of supernatant were pooled for all individuals within a population, except when particular individuals were of interest. Proteins equivalent to 0.2 mg (S-RNase) or 1.5 mg (HT protein) fresh weight per lane were separated, blotted, and immunostained as previously described (Covey *et al.*, 2010). The S-RNase C2 domain antibody was prepared as previously described (Chalivendra *et al.*, 2013). Affinity purified HT-protein antibody was prepared against the peptide LEANEIHNTELNNPTLQKKGGC-amide (21st Century Biochemicals; http://www.21stcenturybio.com/). Antibodies were used at a 1 : 5000 dilution.

Detection of the *Lycopersicon hirsutum L glabratum SRNase 1* (*LhgSRN-1*) region

To determine whether northern SC populations of *S. habrochaites* share a known loss of function *S-RNase* allele, we used a PCR-based assay. Genomic DNA was prepared using the Qiagen DNeasy mini-prep kit (Qiagen). PCR primers were designed to amplify a 460-bp region of GenBank sequence AB072478.1 (Kondo *et al.*, 2002), which includes the 5′ flanking region of *LhgSRN-1* that harbors a TE. The forward primer (KON502F2: 5′-GACCTACGTGGCACTATCTTG-3′) lies within the middle segment of the TE and the reverse primer (KON502 R3: 5′-CATCCCAATTGACGGTATGGG-3′) lies 56 bp upstream of the *LhgSRN-1* coding region. Primers were also designed to amplify the full *LhgSRN-1* coding region from genomic DNA including the intron. The forward primer (AB7F: 5′-**ATG**ATTAAAACACAGCACACGTTATC-3′) was designed based on GenBank sequence AB072478.1 (Kondo *et al.*, 2002), but as this sequence is not full length, the reverse primer (AB10 R: 5′-**TTA**AGGAAAGAAAATTTCCGTATTTCC-3′) was designed based on a homolog of *LhgSRN-1*, GenBank sequence AB072464 (*Solanum peruvianum* L. S22-RNase; 98% identity to *LhgSRN-1*). Sequencing the 790-bp product confirmed 99.5% identity to *LhgSRN-1*. Positive control primers were designed to amplify a 341-bp single-copy product (GenBank, XM_004235953; 2749F: 5′-TGGTTTCCTTAGAGGGACCTT-3′; 2749R: 5′-CCTTAAGTGCTTCCATCTCTG-3′; Van Deynze *et al.*, 2007). PCR was performed using Econotaq Plus Green Mastermix (Lucigen, Middleton, WI, USA), 0.5 µM of each primer and *c.* 80 ng of genomic DNA per 20-µl reaction (95°C for 90 s; 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; 72°C for 3 min). PCR products were separated on a 1% agarose gel and visualized using ethidium bromide staining. Genomic DNA from at least three individuals was tested per population.

Results

Mating systems of *S. habrochaites*

The results of the mating system assessment revealed three general phenotypes: populations in which a majority of individuals displayed SI, populations in which the majority of individuals displayed SC, and populations containing both SI and SC individuals as well as individuals displaying variability in mating system (hereafter referred to as mixed populations (MPs)). Eco-geographic groups A and B were found to have predominately SC mating systems. Within eco-geographic group A, five of seven populations tested displayed SC phenotypes for all individuals tested (Fig. 1, black and gray bars). The remaining two populations in group A (LA1624 and LA1625) displayed SC phenotypes in only 66% of individuals. Group C populations either displayed strong SI (LA2868 and LA2864), or MP (LA2098 and LA2099) wherein the majority of individuals tested displayed SI, but 8–38% of individuals were capable of accepting self-pollen tubes. Group D populations also displayed MP. Somewhat surprisingly, single SC individuals were discovered in both eco-geographic groups E and F, which are reported to be SI (Rick *et al.*, 1979; Covey *et al.*, 2010; TGRC, 2016).

Self-fruit set results (Table 2) were largely consistent with the self-pollen tube growth phenotypes. However, when divergence was seen between the two assays, we utilized fruit set data to decisively classify populations as SC, SI or MP. A and B group populations typically set fruit and produced viable seed, although the frequency of self-fruit set per pollination was lower in the northernmost populations (PI 121957, LA1624 and LA1625). In addition, LA1624 and LA1625 exhibited the lowest overall rates of seed germination (47–55%; Table 2). Population LA2101 was collected from a location between the B and C eco-geographic groups. However, based on pollen tube growth and fruit set, the behavior of LA2101 most closely resembles group B and it is hereafter regarded as a member of this group.

Some populations from the C and D groups, collected near the Ecuador–Peru border, showed divergent behavior in our two mating system assays. For instance, in LA2099 (C), 28% of individuals tested showed pollen tube growth to the ovary; however, fruit production was extremely rare and the single ripe fruit recovered in this study contained underdeveloped seeds. Pollen tubes reached the ovaries in 75% of LA2175 (D) individuals, yet only 14% of pollination attempts resulted in self-fruit, all of which set viable seed. Because substantial numbers of pollen tubes reached ovaries in these populations, the discrepancy between the assays did not appear to be attributable to insufficient fertilization and subsequent fruit abortion. These populations were classified as MP for the purposes of this study, because at least one individual was capable of setting fruit; however, further tests are required to clarify the dynamics of the SI/SC system in these populations.

Populations LA2868 and LA2864 (C) were classified as SI. Like LA2314 (D), LA1353 (E) and LA1777 (F), they did not produce self-fruit and over 88% of individuals did not accept self-pollen tubes. Intrapopulation positive controls were successful for MP and SI populations (data not shown), further verifying mating system assessment.

Floral morphology in *S. habrochaites*

We found that floral longevity differed between populations of *S. habrochaites*, with SC populations exhibiting shorter longevity (+1 to +2) than SI/MP populations (+2 to +4) (data not shown),

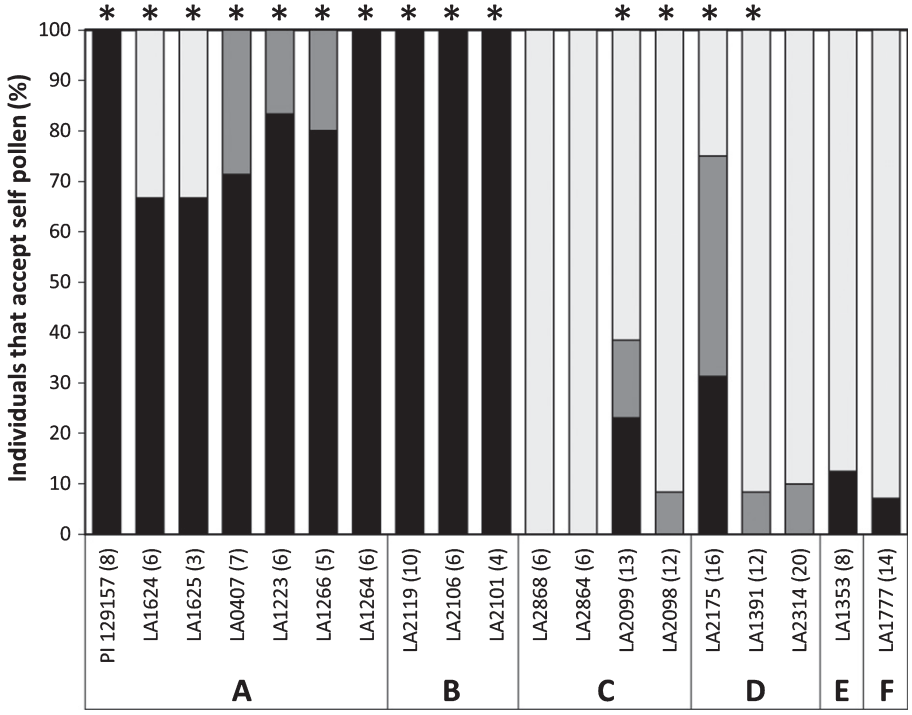

Fig. 1 Percentage of *Solanum habrochaites* individuals tested in which self-pollen tubes (PT) reached the base of the style. Pollen tube growth for each selfed individual was scored as compatible (black bars), incompatible (white bars) or variable (gray bars). The number of individuals tested is shown in parentheses. Eco-geographic group (A–F) and population number are noted at the base of the plot. *, population contains individuals that set fruit after self-pollination.

Table 2 Fruit set and seed germination in self-pollinations of *Solanum habrochaites*

Accession/population	Group*	Individuals setting self-fruit, % ($n^{\dagger}$)	Self-fruit per attempt, % ($n^{\dagger}$)	Germination of self-seed, % ($n^{\ddagger}$)	Mating system
PI 129157	A	100 (3)	67 (3)	100 (25)	SC
LA1624	A	100 (4)	32 (4)	47 (15)	SC
LA1625	A	100 (3)	67 (3)	55 (11)	SC
LA0407	A	100 (4)	94 (4)	90 (20)	SC
LA1223	A	83 (6)	72 (6)	75 (20)	SC
LA1266	A	100 (3)	87 (3)	87 (30)	SC
LA1264	A	100 (2)	100 (2)	90 (10)	SC
LA2119	B	100 (7)	85 (7)	77 (30)	SC
LA2106	B	100 (5)	87 (5)	85 (40)	SC
LA2101	B	100 (3)	95 (3)	63 (30)	SC
LA2868	C	0 (3)	0 (3)	na	SI
LA2864	C	0 (3)	0 (3)	na	SI
LA2099	C	14 (7)	2 (7)	Undeveloped	MP
LA2098	C	67 (3)	15 (3)	90 (10)	MP
LA2175	D	60 (5)	14 (5)	100 (9)	MP
LA1391	D	33 (3)	6 (3)	Undeveloped	MP
LA2314	D	0 (4)	0 (4)	na	SI
LA1353	E	0 (3)	0 (3)	na	SI
LA1777	F	0 (5)	0 (4)	na	SI

Percentages of individuals that set fruit after self-pollination and the average of individual rates of fruit set within a population are shown; $n^{\dagger}$, number of individuals. Self-seeds were germinated to determine viability; $n^{\ddagger}$, number of seeds. Mating systems were classified as self-compatible (SC), self-incompatible (SI), or mixed populations (MP) based on fruit set. *Eco-geographic grouping from Sifres *et al.* (2011). na, not applicable.

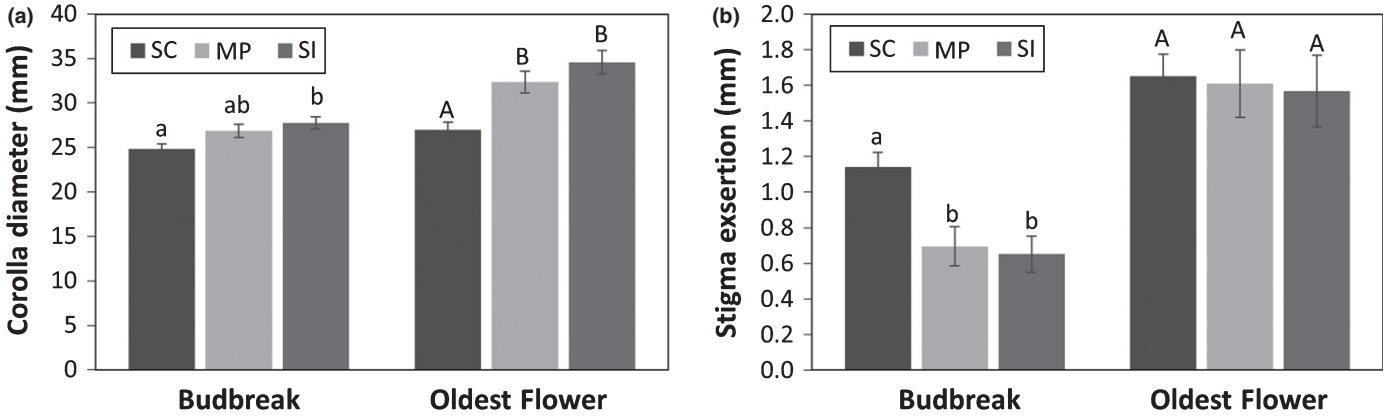

Fig. 2 Corolla diameter and stigma exsertion in *Solanum habrochaites*. (a) Corolla diameter and (b) stigma exsertion were measured in *S. habrochaites* populations at bud break and in the oldest open flower. Populations were grouped by mating system and least squared means and ± SE were calculated. Differences between groups were analyzed within each time-point using ANOVA. Significant differences within a time-point ($P < 0.05$) are represented by different letters. SC, self-compatible; MP, mixed population; SI, self-incompatible.

and that flower size tended to increase with age. Therefore, we measured floral traits at two time-points within a flower's lifespan; bud break (day 0) and just before senescence. Mating system affected corolla diameter at both time-points (Fig. 2a; Table S1; bud break: $F_{2,79} = 5.76$; $P = 0.0046$; oldest flowers: $F_{2,63} = 14.79$; $P < 0.0001$). Regardless of flower age, the corolla diameter of SC populations was significantly smaller than that of the SI populations (Tukey's honestly significant difference (HSD); bud break: $P = 0.0046$; oldest flowers: $P < 0.0001$). However, SC populations only differed significantly from MP populations in the oldest flowers (Tukey's HSD; $P = 0.0015$). At bud break, mating system impacted stigma exsertion (Fig. 2b; Table S1; $F_{2,79} = 8.69$; $P = 0.0039$), and stigma exsertion was significantly greater in SC populations in comparison to both MP and SI populations (Tukey's HSD; $P < 0.005$). However, in the oldest flowers, no significant differences in stigma exsertion were identified. We also found that, on average, style length was greater in SI populations than SC populations (Fig. S1; Table S1). Although we saw significant effects of mating system on floral characteristics, there was also variation between populations within mating system groups (Fig. S2).

Interspecific reproductive barriers

Results of interspecific pollinations demonstrated that *S. habrochaites* mating system impacts the strength of IRBs

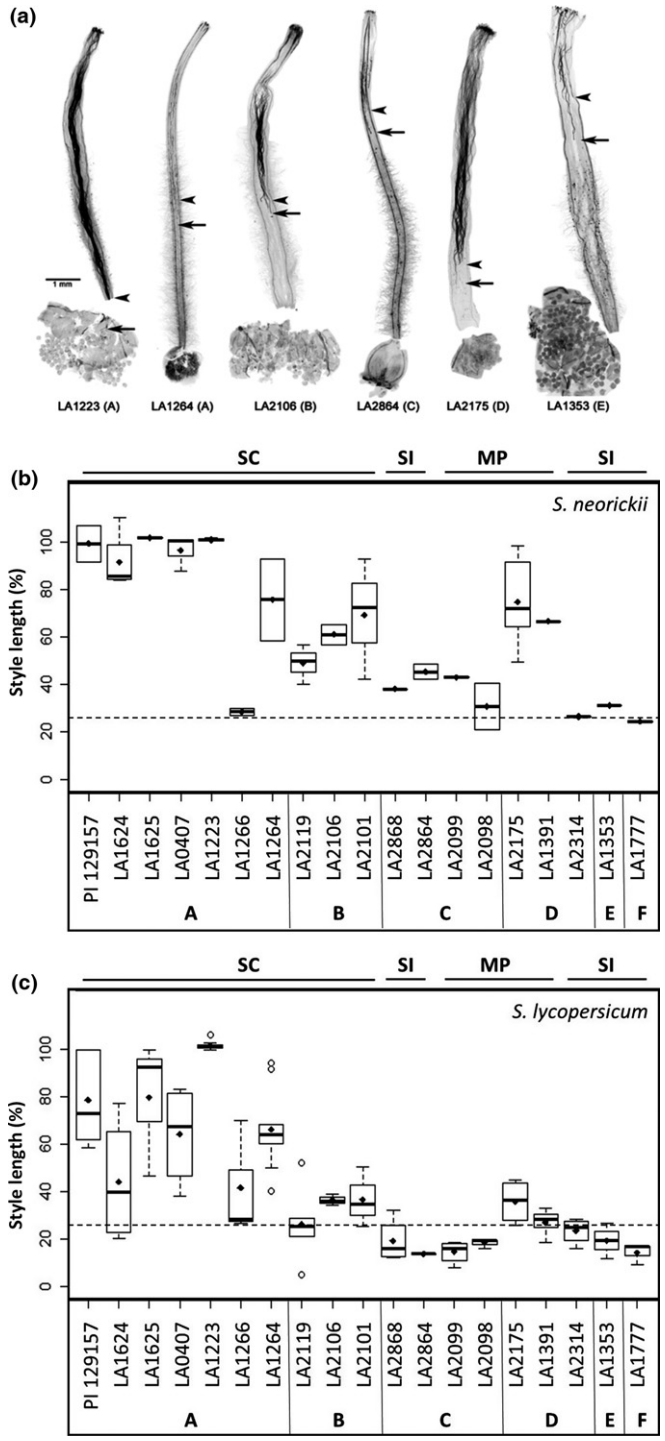

Fig. 3 Interspecific pollen tube growth in pistils of *Solanum habrochaites* populations. (a) Representative images of *S. habrochaites* pistils pollinated by *S. neorickii*. Arrows, longest pollen tube; arrowheads, majority of pollen tubes; bar, 1 mm. (b) *Solanum neorickii* and (c) *S. lycopersicum* mean (closed dots) and median (lines) pollen tube growth in *S. habrochaites* pistils expressed as per cent of style length traversed. First and third quartiles (boxes), maximum and minimum values (whiskers) and outliers (open dots) are shown. The dashed line at 25% of style length is shown for reference. Mating system (SC, self-compatible; SI, self-incompatible; MP, mixed population), eco-geographic group (A–F) and population numbers are shown.

(Fig. 3; Table S4; *S. neorickii*: $F_{2,38} = 8.17$; $P = 0.00112$; *S. lycopersicum*: $F_{2,58} = 25.01$; $P < 0.0001$). When SC *S. neorickii* was used as the pollen parent, pistils of SI populations rejected interspecific pollen tubes more rapidly than SC populations (Fig. 3a,b; Tukey's HSD; $P = 0.0009$). In all crosses with SI populations, the majority of *S. neorickii* pollen tubes traversed 35% of the style length (3.45 mm), whereas in SC populations, they traversed 78% of the style length (6.75 mm) (Fig. 3b; Table S3). MP and SC group B populations varied in the strength of rejection of *S. neorickii* pollen tubes, with the majority of pollen tubes traversing 35–65% of the style. Pistils of the five most northern SC group A populations (PI 129157, LA1624, LA1625, LA0407 and LA1223) were highly permissive, frequently allowing *S. neorickii* pollen tubes to traverse the entire style.

Among all populations, SC *S. lycopersicum* pollen tubes were rejected more rapidly than those of SC *S. neorickii* (Fig. 3b,c; mean ± SE, 40.7 ± 6.1% and 60.8 ± 6.3% of style length traversed, respectively; $t(36) = 2.3$; $P = 0.027$). Again, pistils of SI *S. habrochaites* populations rejected *S. lycopersicum* pollen tubes more rapidly than those of SC populations (Fig. 3c; Tukey's HSD; $P < 0.0001$). In most SI and MP populations, *S. lycopersicum* pollen tubes were rejected in the upper 25% of the style (2.18 mm), whereas in SC populations *S. lycopersicum* pollen tubes grew to an average of 57% of style length (5.13 mm) (Fig. 3c; Table S3). In populations from SC group B, *S. lycopersicum* pollen tubes grew to only 26–37% of the style length (3.07 mm), whereas six of the seven northern populations (group A) rejected *S. lycopersicum* pollen tubes after substantial growth. Population LA1223 (A) was exceptional, being the only population in which *S. lycopersicum* pollen tubes consistently reached ovaries.

Interpopulation reproductive barriers

Crosses between *S. habrochaites* populations indicated that mating system also impacts interpopulation compatibility (Fig. 4). We used *S. habrochaites* SC LA0407 pollen as a pistil-side tester and SI LA1777 pistils as a pollen-side tester because an interpopulation barrier had been previously demonstrated between these two populations (Baek *et al.*, 2015). As shown in Fig. 4(a), pistils of all SI populations consistently rejected LA0407 pollen tubes, and between 80 and 100% of individuals from MP populations were capable of rejecting LA0407 pollen tubes (Fig. 4a, white and gray bars). In pistils of the SC A and B groups, pollinations with LA0407 were overall compatible, with only a few individuals in populations LA1223 and LA1264 displaying incompatibility. Additional crosses confirmed this general pattern, in that SC females were highly compatible with pollen of other populations regardless of mating system (Table S6). The rejection of LA0407 pollen tubes by populations displaying SI and MP mating systems suggests that a pollen-side factor(s) required for growth in SI and MP pistils has been lost to mutation in LA0407.

Fig. 4(b) shows that pollen tubes from the northernmost *S. habrochaites* populations (PI 129157, LA1624 and LA1625, group A) were typically rejected by pistils of LA1777. Pollen

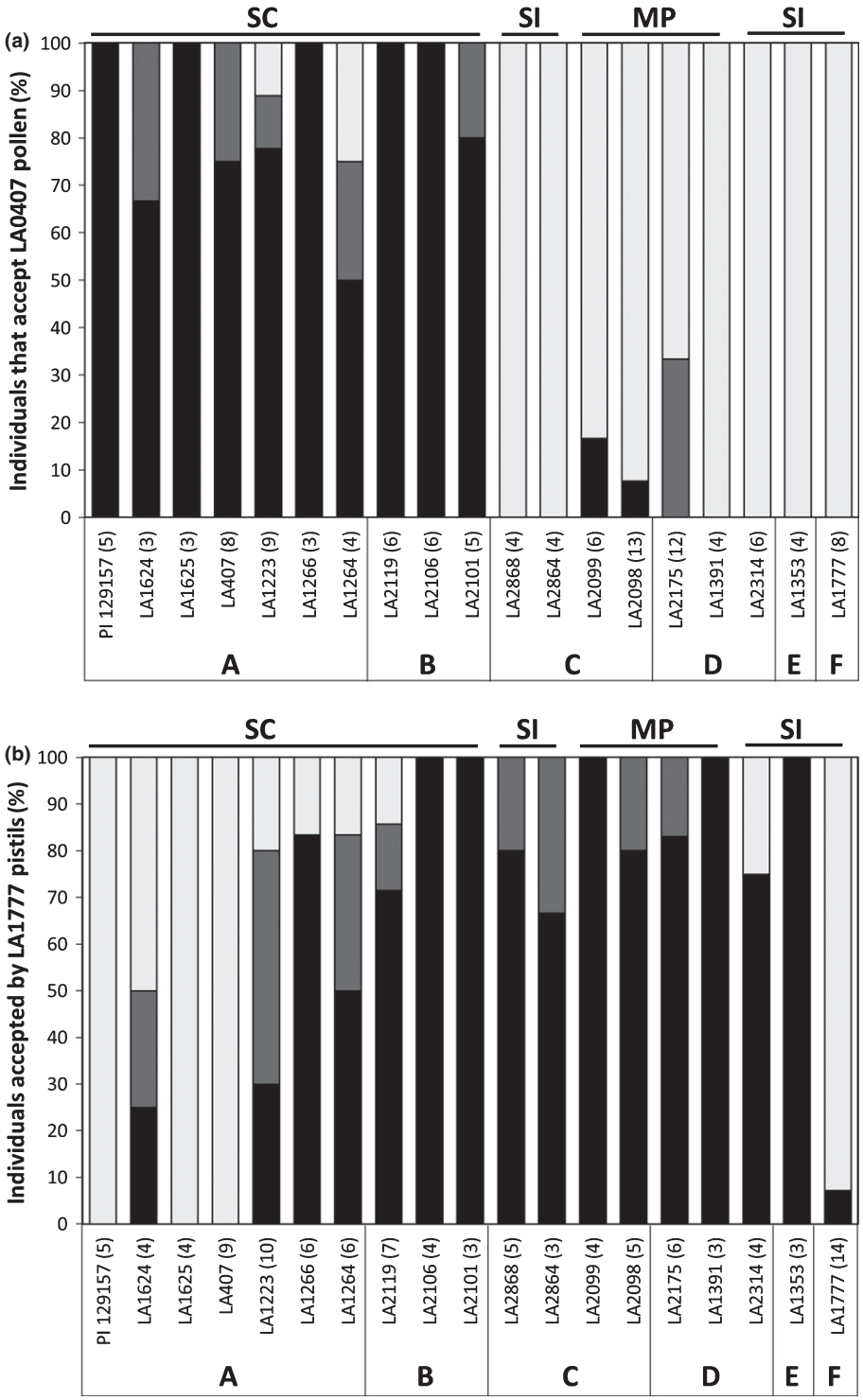

Fig. 4 Interpopulation compatibility in *Solanum habrochaites*. Growth of pollen tubes from LA0407 in pistils of each population (a), and growth of pollen tubes from each population in pistils of LA1777 (b) were scored as compatible (black bars), incompatible (white bars) or variable (gray bars). Data are depicted as per cent of individuals, with number of individuals tested shown in parentheses. LA0407 pistils (i.e. self-pollinations) were used as a control in (a) and pollen of LA0407 and LA1777 was used as controls in (b). Mating system (SC, self-compatible; SI, self-incompatible; MP, mixed population), eco-geographic group (A–F) and population numbers are shown.

tubes of LA0407 (A) were rejected by LA1777, confirming previous results (Baek *et al.*, 2015). However, other group A populations from a similar latitude (LA1264, LA1266 and LA1223) generally showed pollen tube growth to the base of the LA1777 style (Fig. 4b). Within these three populations, individual responses were variable, especially for population LA1223. In populations from groups B, C, D and E, pollen tubes reached the base of the LA1777 style in over 75% of individuals tested.

Pistil SI factors in *S. habrochaites*

Our pollination results demonstrate that both self- and interspecific pollen tube rejection is altered in some SC populations, suggesting that pistil factors required for SI and/or UI may have been lost in populations at the northern margin of the *S. habrochaites* species range. Therefore, we probed stylar proteins from each population with antibodies specific for

S-RNase and HT protein, two proteins implicated in both SI and UI (Murfett *et al.*, 1996; McClure *et al.*, 1999; Beecher *et al.*, 2001; Kondo *et al.*, 2002; Hancock *et al.*, 2005; Tovar-Méndez *et al.*, 2014).

Fig. 5 shows that S-RNase protein was not detected in populations from groups A or B, consistent with the SC phenotype of these groups. By contrast, S-RNase was detected in all group C and D populations, including individuals in MP populations in which self-pollen tubes were always compatible (double asterisk, Fig. 5). Thus, the lack of SI in groups A and B may be attributable to the lack of S-RNase protein, but this cannot explain the behavior of the populations from groups C and D that display MP phenotypes and express S-RNase protein. HT protein was detected in all populations tested except for LA1223.

As northern SC *S. habrochaites* LA0407 is known to harbor a specific transcriptionally inactive *S-RNase* allele, we tested for its presence in our study populations. The *LhgSRN-1* allele, which was first identified in the SC *S. habrochaites* population LS502 (Kondo *et al.*, 2002) and later in LA0407 (Covey *et al.*, 2010), harbors a TE in the 5′ flanking region that is thought to be responsible for its transcriptional inactivity (Kondo *et al.*, 2002; Covey *et al.*, 2010). Fig. 6 shows that specific TE and coding region *LhgSRN-1* products were amplified from five group A populations that lack S-RNase protein. As *LhgSRN-1* and the associated TE were not detected in other SC populations, including two from group A (LA1264 and LA1266) and all from group B (LA2119, LA2106 and LA2010), the lack of S-RNase protein accumulation in these populations must be attributable to different mutations.

Discussion

The transition from SI to SC has been widely observed in plant species, but the role of this transition in interspecific and interpopulation reproductive barriers is understudied. *Solanum habrochaites* is an exceptional model system in which to address this gap, as it includes both SI and SC populations that display variation in IRBs (Martin, 1961; Covey *et al.*, 2010; Baek *et al.*, 2015) and interpopulation compatibility (Martin, 1961, 1963; Rick & Chetelat, 1991; Baek *et al.*, 2015). The genetic structure of *S. habrochaites* correlates well with eco-geographic regions of occurrence and is consistent with isolation by distance (Sifres *et al.*, 2011), providing a useful framework for our study of reproductive characters. In Solanaceae, several genetic factors involved in the gametophytic SI mating system have been characterized (Kao & Tsukamoto, 2004; McClure & Franklin-Tong, 2006; McClure *et al.*, 2011), allowing us to link the loss of genetic factors with pollen–pistil incompatibility. Here, we conducted a detailed investigation of mating system transitions and their consequences for interspecific and interpopulation pollen–pistil interactions in 19 populations from the northern *S. habrochaites* range margin. Fig. 7 shows that these populations fall into five proposed groups (SI, MP, SC-1, SC-2 and SC-3) based on reproductive phenotype, as described in more detail in the following subsections.

SI to SC transition in *S. habrochaites* northern range

We found SI, the ancestral state of *S. habrochaites* (Rick *et al.*, 1979), in populations from central Peru to southern Ecuador in eco-geographic groups C (LA2864 and LA2868), D (LA2314), E (LA1353) and F (LA1777). All of these SI populations expressed S-RNase and HT protein (Fig. 4). MP populations were found near the Ecuador−Peru border and included accessions in eco-geographic groups C (LA2098 and LA2099) and D (LA2175 and LA1391). Although some individuals from these populations showed self-compatibility in either or both of our mating system assays (Fig. 1; Table 2), all individuals tested expressed S-RNase and HT protein (Fig. 5). Thus, breakdown of SI in these individuals is not a result of a complete loss of either of these SI proteins. It is possible that mutations in *S-RNase* genes affecting activity, specificity or glycosylation status (Green, 1994; Kowyama *et al.*, 1994; Royo *et al.*, 1994; Qin *et al.*, 2006; Soulard *et al.*, 2013) or changes in pollen side factors (Kubo *et al.*, 2015) could explain the 'leaky' SI in MP populations.

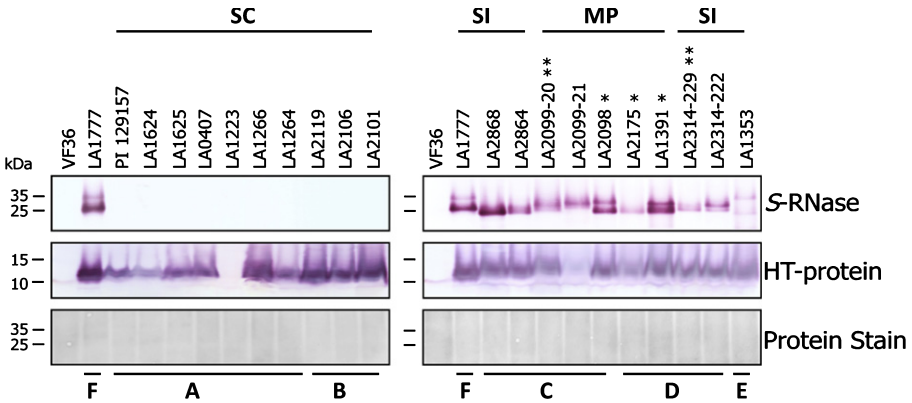

Fig. 5 S-RNase and HT proteins in *Solanum habrochaites* populations. Stylar proteins were separated in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), blotted and immunostained with S-RNase C2 domain (upper panels) or with anti HTA/B-protein (middle panels) antibodies. Protein stain is shown in the lower panels as a loading control. *, population/individual in which self pollen tubes sometimes reach ovaries; **, individuals in which pollen tubes always reach ovaries. Mating system (SC, self-compatible; SI, self-incompatible; MP, mixed population), eco-geographic group (A–F) and population numbers are shown.

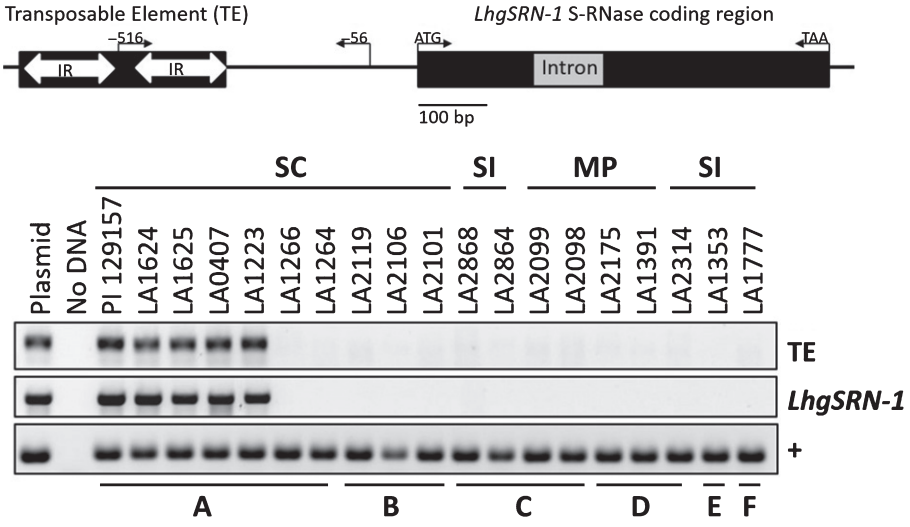

Fig. 6 PCR detection of *Lycopersicon hirsutum L glabratum SRNase 1* (*LhgSRN-1*) in *Solanum habrochaites* populations. Locations of specific PCR primers for the transposable element (TE), 5′ flanking region and coding region of the *LhgSRN-1 S-RNase* allele (top schematic) are shown. Genomic DNA was PCR amplified with primers based on the upstream TE area, *LhgSRN-1* and a conserved single-copy positive control (+). Mating system (SC, self-compatible; SI, self-incompatible; MP, mixed population), eco-geographic group (A–F) and population numbers are shown.

Population/ Accession	Map key	Proposed groups	Eco-geographic group	Self fruit	S-RNase protein	TE - *LhgSRN1*	HT-A protein	Accepts pollen of: *S. lyco*	Accepts pollen of: *S. neo*	Accepts pollen of: LA0407	Pollen accepted by LA1777
PI 129157	★	SC-2	A	X		X	X		X	X	
LA1624	★	SC-2	A	X		X	X		X	X	
LA1625	★	SC-2	A	X		X	X		X	X	
LA407	★	SC-2	A	X		X	X		X	SC	
LA1223	☆	SC-3	A	X		X		X	X	X	X
LA1266	●	SC-1	A	X			X			X	X
LA1264	●	SC-1	A	X			X			X	X
LA2119	●	SC-1	B	X			X			X	X
LA2106	●	SC-1	B	X			X			X	X
LA2101	●	SC-1	B	X			X			X	X
LA2099	▨	MP	C	X	X		X				X
LA2098	▨	MP	C	X	X		X				X
LA2175	▨	MP	D	X	X		X				X
LA1391	▨	MP	D	X	X		X				X
LA2314	○	SI	D		X		X				X
LA2868	○	SI	C		X		X				X
LA2864	○	SI	C		X		X				X
LA1353	○	SI	E		X		X				X
LA1777	○	SI	F		X		X				SI

Fig. 7 Summary of reproductive barriers in northern *Solanum habrochaites* populations. Proposed population groups are based on results from self, interspecific and interpopulation crosses and genetic analyses. X indicates that a population displays the indicated property: in interpopulation crosses (LA0407 and LA1777), >50% of individuals were compatible; in interspecific crosses (*S. lycopersicum* (*S. lyco*) and *S. neorickii* (*S. neo*)) at least one individual accepts interspecific pollen; for proteins (S-RNase and HT protein) and molecular studies (*TE-LhgSRN-1 S-RNase* allele) X indicates presence. Mating system (SC, self-compatible; MP, mixed population; SI, self-incompatible), eco-geographic group (A–F) and population numbers are shown.

Populations from eco-geographic groups A and B were consistently SC and individuals exhibited high rates of self-fruit set. The two most northern populations (LA1624 and LA1625) showed lower rates of self-pollen tube acceptance (66% compared to other SC populations (100%), which might be linked to factors involving pollen function. Interestingly, we

found that LA1624 and LA1625 also displayed low levels of self-seed germination, possibly as a result of inbreeding depression. Other SC populations had high self-seed germination rates (> 75%), similar to results obtained by Martin (1963, 1964), suggesting that the majority of northern SC *S. habrochaites* populations may suffer only low levels of inbreeding depression.

Although we did not directly measure outcrossing rates, we found that the SI → SC transition resulted in smaller flower size (Fig. 2a; Table S1), reduced floral longevity and shorter style length (Fig. S1; Table S1), particularly in SC-2 populations (Fig. 8a; Table S5). This reduction in floral display could reflect higher selfing rates, consistent with previous studies of *S. habrochaites* that demonstrated reduced heterozygosity in SC populations at the species range margins (Rick *et al.*, 1979; Sifres *et al.*, 2011). Interestingly, we did not find reductions in stigma exsertion associated with SI → SC (Fig. 2b; Table S1), suggesting that, while *S. habrochaites* SC populations are capable of self-fertilization, changes in stigma exsertion have not evolved as a result of selection for more effective autogamous self-fertilization.

All SC populations lacked expression of S-RNase protein, yet only a subgroup (SC-2 and SC-3) harbor the *LhgSRN-1* allele containing a TE insertion (Figs 6, 7) that presumably prohibits expression of the downstream *S-RNase* gene (Kondo *et al.*, 2002; Covey *et al.*, 2010). The SC-1 populations do not contain the *LhgSRN-1*/TE allele, so a different mutation(s) must abolish S-RNase protein accumulation in this group. Thus, SI → SC transitions, whether caused by loss of S-RNase protein or another pistil factor, probably occurred at least two times at the northern margin of the *S. habrochaites* range.

The lability of SI at the northern margin of *S. habrochaites* occurs in the geographic context of the Huancabamba Depression near the Ecuador–Peru border, where the Andes cordillera breaks up. As a result of habitat fragmentation, this region defines range limits for a number of plant and animal species, and is considered a speciation hotspot (Weigend, 2002). Range expansion into dispersed microhabitats in this region could result in mate limitation, where reproductive assurance associated with SC would be advantageous (Baker, 1967; Pannell & Barrett, 1998; Busch & Schoen, 2008; Pannell *et al.*, 2015).

Mating system transitions reduce the strength of IRBs

Because SI is mechanistically linked to UI in the tomato clade (Tovar-Méndez *et al.*, 2014; Li & Chetelat, 2015), we hypothesized that the transition to SC would lead to a reduction in the strength of interspecific pollen tube rejection. Moreover, we expected that rejection of *S. lycopersicum* pollen would be stronger than that of *S. neorickii*, as observed in previous studies (Baek *et al.*, 2015). Both of these expectations were met; however, we identified important differences between and within mating system groups (Figs 7, 8; Tables S4, S5).

We found that pistils of all SI and MP populations rejected *S. lycopersicum* pollen in the top 25% of the style (Fig. 8b), similar to previous observations in SI LA1777 (Covey *et al.*, 2010; Baek *et al.*, 2015). Rapid rejection of *S. lycopersicum* pollen tubes in these populations is probably linked to the expression of S-RNase and HT proteins, consistent with transgenic studies (Tovar-Méndez *et al.*, 2014). All SI populations also showed strong rejection of *S. neorickii* pollen tubes, which traversed only 30% of the style (Fig. 8c). However, on average, *S. neorickii* pollen tubes grew over two times farther in styles of MP populations compared with SI populations. This difference in the strength of *S. neorickii* pollen tube rejection suggests that an additional pistil factor(s) may be missing or compromised in some MP populations. Interestingly, transgenic studies also show that S-RNase and HT protein are not sufficient for rejection of *S. neorickii* pollen (Tovar-Méndez *et al.*, 2014).

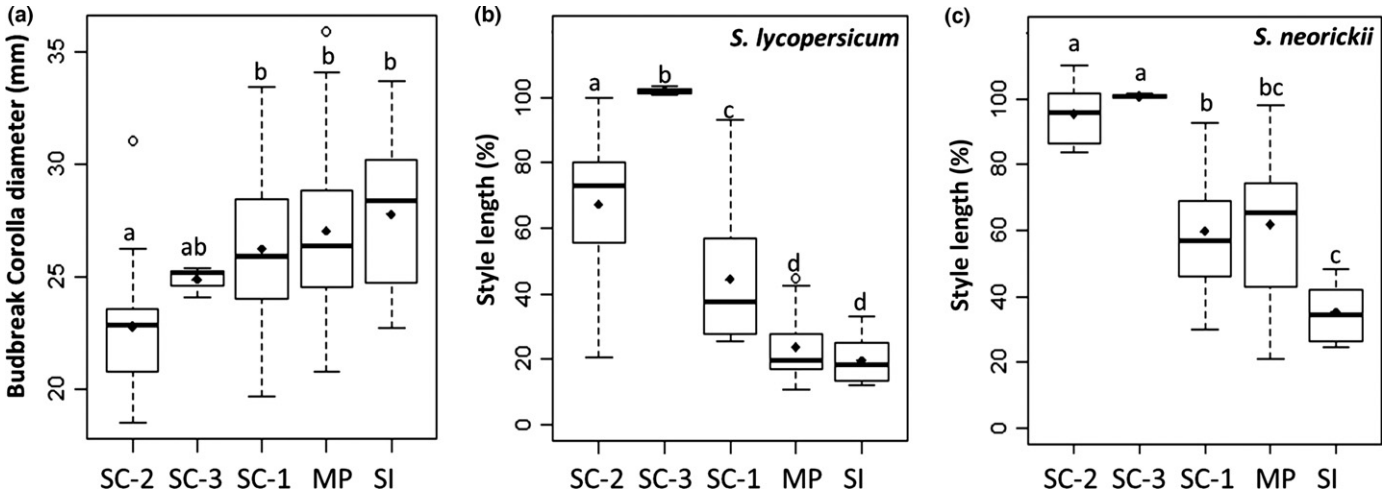

Fig. 8 Phenotypes of proposed *Solanum habrochaites* groups. Groups from Fig. 7 are noted at the base of the plot and are shown as they occur from north (left) to south (right). Phenotypes include corolla diameter at bud break (a); (b) *S. lycopersicum* and (c) *S. neorickii* pollen tube growth in *S. habrochaites* pistils. Data were analyzed using ANOVA and are presented as mean (dots) and median (lines) values, the first and third quartiles (box) and maximum and minimum values (whiskers). Significant differences between groups ($P < 0.05$) are represented by different letters. Mating system: SC, self-compatible; MP, mixed population; SI, self-incompatible.

The SC-1, SC-2 and SC-3 populations displayed very different interspecific pollen tube rejection phenotypes (Fig. 8; Table S5). SC-1 populations rejected pollen tubes from *S. lycopersicum* at 40% style length on average (Fig. 8b) and rejected *S. neorickii* pollen tubes at 60% of style length, similar to that of MP populations (Fig. 8c). As none of the SC-1 populations express S-RNase (Fig. 5), they display S-RNase-independent interspecific pollen tube rejection, distinct from the S-RNase-dependent mechanism.

SC-2 populations rejected *S. lycopersicum* pollen tubes later than those of SC-1, at 65% of style length on average (Fig. 8b; Tukey's HSD; $P = 0.0043$). However, pollen tubes of *S. neorickii* grew to the ovary in at least one individual from each SC-2 population, and on average *S. neorickii* pollen tubes were able to traverse 95% of style length (Fig. 8c). This difference in pollen tube rejection, relative to the SC-1 populations, suggests that pistil factors involved in IRBs in addition to S-RNase have been lost as a result of mutation in the SC-2 populations.

Population LA1223, the sole member of SC-3, was the only population that accepted interspecific pollen tubes from both *S. neorickii* and *S. lycopersicum* (Fig. 8b,c), and it was also the only population lacking expression of HT protein (Fig. 5). These results suggest that HT protein can play a key role in interspecific pollen tube rejection in the absence of S-RNase (SC-1 and SC-2). However, as SC-2 populations express HT protein it is clearly not sufficient for the rejection of *S. neorickii* pollen, consistent with transgenic results (Tovar-Méndez *et al.*, 2014).

The observed breakdown of IRBs within the different SC groups may have consequences for gene flow between species at *S. habrochaites* range margins. Although the particular SC populations used in this study have not been observed in sympatry with other wild tomato species, the range of SC *S. habrochaites* as a whole overlaps with that of cultivated and feral *S. lycopersicum*, and with the ranges of wild species *Solanum pimpinellifolium* L. and *S. neorickii* (Moyle, 2008; TGRC, 2016). Our results show that IRBs, particularly within SC-3, have been sufficiently weakened to the extent that interspecific hybridization could be permitted. Field studies will be required to determine whether hybridization is observed in nature.

Mating system transitions increase interpopulation barriers

Previous studies have demonstrated that SC *S. habrochaites* populations at both the northern and southern range margins are partially reproductively isolated from central SI populations (Martin, 1961, 1963, 1964; Baek *et al.*, 2015). In our study, all SI populations rejected pollen tubes from the SC-2 population LA0407 (Fig. 3a). A few MP individuals showed compatibility with LA0407 pollen, but a more extensive study would be required to determine whether differences in interpopulation barriers are correlated with SC in MP populations. It is noteworthy that the interpopulation pollen–pistil barriers that we observe apply to SI and SC populations collected in relatively close proximity. For example, these barriers occur in crosses between LA0407 (SC-2) and LA2868 (SI) which were collected only *c.* 250 km apart in western Ecuador.

Pollen of LA0407 was compatible on pistils of SC populations, suggesting that it lacks a pollen-side factor(s) required to traverse SI and most MP styles. We discovered that pollen tubes of all SC-2 populations were rejected on our SI pistil tester LA1777 (Fig. 3b), whereas pollen from other SC populations (SC-1 and some individuals of SC-3) was not. Our results reflect initial findings reported by Martin (1961, 1963), and our work has further defined the geographic boundaries in which this interpopulation barrier occurs. Overall, our results suggest that the mutational loss of pollen-side factors in some but not all SC populations can introduce asymmetric interpopulation reproductive barriers.

Conclusions

A common view is that reproductive isolation results from gradual genetic divergence after the initial geographic or ecological isolation of populations. This leads to the prediction that more genetically distinct populations will be more reproductively isolated than closely related populations. However, we found diverse reproductive phenotypes (SC-1, SC-2 and SC-3; Fig. 7) within a single closely related eco-geographic group of *S. habrochaites* (group A; Sifres *et al.*, 2011).

We propose the following mechanism to explain how reproductive isolation can evolve between SC and SI populations via changes at few loci of large effect, and the potential consequences for an incipient SC lineage. SI → SC transitions can clearly be caused by mutations leading to loss of pistil SI factors such as S-RNase and this may result in SC populations that expand at the species range margin. Thus, a mating system transition directly promotes both geographic and reproductive isolation between a migrating SC population and its ancestral SI population. The mechanistic linkage between SI and UI means that pistil-side SI mutations also result in weakening (*–S-RNase* in SC-1 and SC-2 populations) or even elimination (*–HT* in SC-3 populations) of interspecies reproductive barriers, increasing the potential for interspecific gene flow. The lack of selection for an intact SI system allows loss-of-function alleles in pollen SI factors to become fixed in some SC populations (SC-2). Because pollen SI factors are required for resistance to cytotoxic S-RNases in SI pistils, this generates a unidirectional barrier that would further decrease gene flow between SC and SI populations. Thus, reproductive changes associated with a mating system transition can act in concert to promote the divergence of a new SC lineage from progenitor SI populations at the species range margin.

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Author contributions

A.K.B. and A.M.R. contributed to the design of the research, data analysis, collection and interpretation, and writing the manuscript. S.A.S. contributed to the design of the research, data analysis, collection and interpretation, and editing the manuscript. A.T-M. contributed to data analysis, collection and interpretation, and editing the manuscript. B.M. contributed to data interpretation and editing the manuscript. P.A.B. contributed to the design of the research, data collection and interpretation, and writing the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Style lengths of day +2 flowers in populations of *Solanum habrochaites*.

Fig. S2 Corolla diameter and stigma exsertion in populations of *Solanum habrochaites*.

Table S1 Effects of mating system on floral morphology in *Solanum habrochaites*

Table S2 Effects of five proposed reproductive groupings on floral morphology in *Solanum habrochaites*

Table S3 Mean length (mm) of interspecific pollen tubes in pistils of *Solanum habrochaites* populations

Table S4 Effects of mating system on interspecifc pollen tube rejection in *Solanum habrochaites*

Table S5 Effects of five proposed reproductive groupings on interspecifc pollen tube rejection in *Solanum habrochaites*

Table S6 Interpopulation compatibility among proposed groups of *Solanum habrochaites*

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