Rapid recent radiation of S-RNase lineages in *Witheringia solanacea* (Solanaceae)

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Strong frequency-dependent selection as found in the self-incompatibility loci of flowering plants maintains allelic lineages for extremely long time scales, such that allelic genealogies can shed insight into long-term demographic patterns of species. Effective mutation rate, as well as demographic change such as population bottlenecks, can influence genealogical structure. In addition, loss of functionality at the self-incompatibility locus is likely to affect radiation rates. Partial sequences for 21 S-RNase alleles of the mid-elevation tropical species *Witheringia solanacea* were obtained in order to compare their substitution rates and genealogy with those of *Witheringia maculata* and two species in the closely related genus *Physalis*. Sequences for *W. solanacea* fell into the three clades within the Solanaceae already identified for the genus. Terminal branch lengths for *W. solanacea*, scaled to the total depth of its

phylogeny, were intermediate between the unusually short terminal branches of *W. maculata* and those of the two *Physalis* species. In contrast to the *Physalis* species, where interspecific d_N/d_S for closely related alleles exceeded 1.0 to the same degree as did intraspecific d_N/d_S , in *Witheringia* only intraspecific comparisons showed an excess of non-synonymous substitutions, suggesting postspeciation radiation of alleles. Alleles associated with lowered S-RNase production and self-compatibility showed extremely short terminal branches. In summary, it appears that rapid recent diversification of alleles characterizes the *Witheringia* lineages. In some cases, this rapid diversification can be attributed to relaxed constraints due to breakdown of self-incompatibility.

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Introduction

The power of negative frequency-dependent selection to maintain large numbers of alleles has long been recognized, with self-incompatibility (SI) alleles (S-alleles) of flowering plants providing the classical example. Because only pollen bearing S-alleles distinct from those of the maternal plant can sire seeds, rare alleles are favored. Recent advances in molecular biology and in analytical techniques have promoted the re-invigoration of classical questions about equilibrium allele number, population structure, and mutation rates of S-alleles (reviewed by Clark, 1993), and have raised new questions as well. Allele genealogies can be scrutinized for departures from null expectation based on coalescent theory (Takahata, 1990; Vekemans and Slatkin, 1994). Population size should be revealed not only by number of alleles, but also by divergence time, with lineages from species with larger populations coalescing deeper in the tree. Differences in effective mutation rate (the effective rate of mutation to new S-alleles) will also impact tree structure, with high effective mutation rates causing both high allele number and shallow coalescence (Uyenoyama, 1997). Much current work seeks to discern genealogical impacts of the

interaction between effective mutation rate and demographic history.

The Solanaceae has been the focus of much of the work on S-allele genealogies from natural populations. Its SI response is characterized by an S-RNase that is expressed in the style of open flowers. The S-RNase degrades RNA of pollen that bears an allele not discernable as different from either of the two maternal alleles (McClure *et al.*, 1990). Richman and Kohn have obtained partial S-RNase sequences from natural populations of five species (Richman *et al.*, 1995; Richman and Kohn, 1999, 2000). Two of these species, *Lycium andersonii* and *Solanum carolinense*, each possess about a dozen trans-generic lineages and show unexpectedly long terminal branches when scaled to the total depth of the tree (Richman and Kohn, 2000). The other three species, from the closely related genera *Physalis* and *Witheringia*, together occupy only three lineages, representing a relatively shallow coalescence. Demographic effects may be responsible for the contrasts in tree shape between *Physalis*/*Witheringia* and other taxa (Richman and Kohn, 1999, 2000). A severe bottleneck along the lineage that led to *Physalis* and *Witheringia* may have drastically reduced the number of trans-generic lineages. Radiation of alleles subsequent to the bottleneck would result in relatively short terminal branches, in comparison with other genera that had not recently lost basal lineages.

In addition to demography, effective mutation rate and selective retention of alleles are also expected to influence tree shape. Uyenoyama (1997) modeled S-allele genealogies and found that divergence time among

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alleles is related linearly to effective population size. Effective mutation rate had an even more pronounced impact on divergence times, with low mutation rates increasing divergence times according to a power relationship. A third factor that potentially works with demography and effective mutation rate to influence tree-shape characteristics is the selective retention of alleles. Richman and Kohn (1999) suggest that more divergent alleles may be selectively maintained, perhaps because they are more likely to be recognized as nonself than are closely related alleles.

A fourth factor that could affect genealogical tree-shape characteristics, but that has not yet been considered in this light, is breakdown of functional SI of particular alleles. Breakdown of SI is an extremely common evolutionary trajectory in flowering plants (Stebbins, 1974), and can be caused either by mutations linked to the *S*-locus or by modifier genes (Stone, 2002). *S*-RNase alleles cosegregating with self-compatibility (SC), unlike their functional predecessors, are not subject to frequency-dependent selection. Lineages of such alleles will no longer persist for the extremely long time scales of functional alleles but, instead, may be expected to conform to neutral expectation (Vekemans and Slatkin, 1994). Therefore, in species where nonfunctional *S*-RNases have been able to invade, their diversification could be more rapid than those of functional *S*-RNases, resulting in relatively short terminal branch lengths. Counteracting the release from frequency-dependent selection will be the extremely low effective population sizes of selfing lineages, which may deter diversification of these neutral alleles. Theoretical models have not yet treated the effects of polymorphism for SI on diversification rates of nonfunctional mating system alleles, but it is clear that effective mutation rate will itself be influenced by the breeding system.

It will not be simple to untangle the contribution of demography, effective mutation rates, selective retention, and mating system to tree-shape characteristics of *S*-alleles. A potentially promising tactic is to focus intensively on particular genera with anomalous tree-shape characteristics. An intriguing finding of earlier work was the report of unusually short terminal branch lengths in *Witheringia maculata*, a tropical cloud forest gap species (Richman and Kohn, 2000). According to the view that effective mutation rates are important, short branch lengths reveal rapid diversification due to an unusually high effective mutation rate. However, Richman and Kohn concluded that *W. maculata* displayed an extremely low diversification rate in comparison with *Physalis*, based on the low total number of alleles recovered and the inferred small population size. An examination of a second species in the genus will reveal whether short terminal branches are characteristic of the genus, due perhaps to similar effective mutation rates, or whether terminal branch lengths differ among congeners, perhaps due to differences in effective population size. Sequence comparisons among congeners can also provide information about timing of diversification with respect to speciation. If most diversification precedes speciation, the ratio of nonsynonymous to synonymous substitutions (d_N/d_S) should be similar for inter- and intraspecific paired comparisons (Richman and Kohn, 1999). In contrast, if much diversification has occurred postspeciation, d_N/d_S should be greater in intraspecific than in interspecific comparisons.

The objective of this research was to examine genealogical characteristics and nucleotide substitution rates for a second species of *Witheringia*, to see whether low diversification and evolutionary rates characterize postbottleneck recovery of this tropical genus. We selected *Witheringia solanacea*, which, like *W. maculata*, would have been restricted to canopy gap habitats until recent human habitat alterations have expanded suitable habitat to include roadsides and other disturbed areas. *W. solanacea* occupies slightly lower elevations than does *W. maculata*, and so in spite of its historically fugitive life history, could be expected to have a larger population size. Accessions of *W. solanacea* polymorphic for SI had previously been identified by Bohs (1999). Relictual *S*-RNases from known self-compatible individuals were sequenced and included in an allele genealogy with *S*-RNases from functional alleles. The following specific questions were addressed:

- Does *W. solanacea* have more *S*-alleles than *W. maculata*, as might be expected from its larger altitudinal range?
- Are terminal branch lengths in *W. solanacea* unusually short as found in *W. maculata*, suggesting similarly high effective mutation rates (to new *S* alleles)?
- Do nonsynonymous substitution rates in *Witheringia* indicate a lack of postspeciation diversification as found in the closely related *Physalis*?
- Are nonfunctional *S*-RNases characterized by shorter terminal branches than are functional alleles?

Materials and methods

Sampling from wild populations

W. solanacea L' Her. is a morphologically variable shrub occurring from southern Mexico to Central America and the Caribbean to South America (D'Arcy, 1973), reportedly at elevations from sea level to 2000 m (Standley, 1937). Individual plants were collected at a distance of at least 5 m from other individuals and transported as cuttings to the greenhouse at Colby College. See Table 1 for locations of collections. Five populations were sampled. Four of these populations consist of two pairs, one population each of relatively low and high elevations, in the southwest (Las Alturas and Las Cruces) and northwest (Monteverde and San Luis). Plants were identified as SC or SI based on observations of pollen tube growth following hand pollinations, and/or repeated autonomous production of progeny in a pollinator-free greenhouse. Most plants from these four populations rarely produce selfed seeds (Bohs, 1999; Stone, in preparation). A fifth population, Vara Blanca, occurs in north-central Costa Rica, and consists of a mixture of self-incompatible and self-compatible individuals (Bohs, 1999; Stone, in preparation). At all sites but Monteverde plants occurred in a compact area, and small sample sizes reflect either small population sizes or conservative sampling due to efforts to ensure that different ramets of the same genet were not inadvertently collected. Specimens from all populations have been verified as belonging to *W. solanacea* by taxonomist Lynn Bohs, and vouchers are deposited in the herbarium at the University of Costa Rica (Stone collection numbers 1500–1510).

Table 1 Locations, habitats, sampling effort, and numbers of alleles found for populations of *W. solanacea* in Costa Rica

Population	Location	Elevation (m)	Habitat	N1	N2
Las Alturas (LA)	8°57'N, 82°51'W	1600	Forest plantation	2	2
Las Cruces (LC)	8°47'N, 82°57'W	1180	Weedy garden	3	2
Monteverde (MV)	10°18'N, 84°49'W	1330–1540	Roadside	10	6
San Luis (SL)	10°17'N, 84°48'W	1100	Roadside	3	1
Vara Blanca (VB)	10°13'N, 84°10'W	1360	Roadside; cliff by waterfall	3	0

N1 = number of individuals sampled; N2 = number for which two alleles were found. Samples included one self-compatible individual at Monteverde and two self-compatible individuals at Vara Blanca.

Sequencing of S-RNases

Partial S-RNase sequences were obtained, essentially as described by Richman *et al* (1995). Styles collected 1–2 days before anthesis were processed immediately. Four to six styles were ground in liquid nitrogen, and Qiagen's RNeasy Plant Mini Kit was used to obtain stylar mRNA. Invitrogen's cDNA cycle kit was used to generate cDNA. Degenerate primers PR1 and PR3 (Richman *et al*, 1995), based on conserved regions C2 and C5 (Ioerger *et al*, 1991), were used to amplify approximately 490 base pairs of the S-RNase gene. Thermal cycler conditions were modified for use with a thermocycler with a heated lid (94°C, 15 s; 45°C, 60 s; 72°C, 60 s). PCR products were cloned and then sequenced using an ABI Prism 310 capillary sequencer. Forward and reverse strands were compared using Sequencher. At least two separate inserts were sequenced for all but two alleles (S11 and S18). These two alleles each differed by several amino acids from other alleles (S7 and S4, respectively), which were each sequenced from four or more inserts, and which nestle within the large *Physalis/Witheringia* clades.

Allele number

Individuals with functional gametophytic SI systems are expected to possess two different S-alleles, but sometimes PCR with degenerate primers is successful in recovering only one of the two. Individuals that have nonfunctional SI systems may also yield a single allele; these are more likely to be true homozygotes. For the following analyses, we treated alleles identically regardless of whether they were found in SI or SC individuals. We applied the Mantel statistic across all samples to see whether alleles were represented in equal frequency (Mantel, 1974; Richman *et al*, 1995). If alleles are represented in equal frequency, Paxman's (1963) formula can be used to estimate the total number of alleles in a population by

$$n = N(1 - (1 - 2/N)^r)$$

where n is the number of alleles found, r is the number of plants sampled, and N is the estimated total number of alleles in the population. We estimated total allele number for the Monteverde population, and also for the combined Monteverde and San Luis populations, which are separated by 2.5 km. We counted plants where only a single allele was amplified as representing half of an individual with respect to sampling effort.

Genealogy construction and analysis

The relationship among S-RNase sequences from *W. solanacea* and other S-RNases was examined using an interspecific phylogeny. To include relevant closely related species, we obtained sequences from GenBank for available *Physalis cinerascens*, *Physalis crassifolia* and *W. maculata* sequences, discarding from our analysis those sequences that span only regions C2–C4 and retaining those that span C2–C5. In order to provide context for the *Physalis* and *Witheringia* species, we included sequences from natural populations of *S. carolinense* and *L. andersonii*, which had been used in the analysis that revealed *Witheringia* to cluster within the three *Physalis* clades (Richman and Kohn, 2000). In addition, we chose to include a manageable number of sequences from species that had not appeared with *Witheringia* in any prior analysis. To select these sequences, we sampled at random throughout the Solanaceae by obtaining S-RNase sequences submitted to GenBank before the year 2000, for species where four or more sequences were deposited (*Nicotiana glauca* S2, S3, S6, S7; *Petunia hybrida* S1, S3, SB1, SB2, and *Solanum chacoense* S2, S3, S11, S13). Solanaceous S-like RNases LeLE and LeLX (Kock *et al*, 1995) and Nalata NE (Dodds *et al*, 1996) were used as the outgroup. ClustalX was used to align inferred amino-acid sequences, using the BLOSUM series substitution matrices, a gap origination penalty of 10 and a gap extension penalty of 0.5. The alignment was checked by eye to ensure that conserved motifs and cysteine residues were properly aligned (Ioerger *et al*, 1991). Small insertions/deletions combined with highly variable sequences created problems aligning the ultimate seven to nine amino acids; for analysis, the terminal 4–5 amino acids of the sequences were truncated following the conserved cysteine at amino-acid position 119/120 (position 160 of Ioerger *et al*, 1991). PHYLIP v3.573c (Felsenstein, 1995) was used to construct a neighbor-joining tree. Distances were first calculated using PROTDIST with the Dayhoff PAM 001 amino-acid substitution matrix. A bootstrap tree was based on 100 re-samplings.

Intraspecific phylogenies for both *Witheringia* species were built to allow analysis of scaled terminal branch lengths. For the intraspecific phylogenies, DNA sequences rather than predicted amino-acid sequences were used, due to the closer relationships among alleles. Kimura's two-parameter distance as implemented by PAUP (Swofford, 1998) was used to create the matrix for the neighbor-joining tree, with *S. carolinense* S9 used as the outgroup. We used KITSCH (Felsenstein, 1995) to calculate terminal branch lengths, after using the branch program of LINTREE (Takezaki *et al*, 1995) to ensure that

the genealogy's sequences did not deviate significantly from clocklike behavior. Relative terminal branch lengths were assessed by

$$R_{SD} = S(1 - 1/n)/D$$

where S is the sum of terminal branch lengths, D the total depth of tree, and n the number of alleles in the sample.

Significance of R_{SD} was evaluated by comparison with Uyenoyama's (1997) simulated expectations. Testing of the assumption of a molecular clock and calculation of R_{SD} was also carried out for S-RNase sequences of *W. maculata*, using DNA sequences from GenBank (accession numbers AF102065–AF102075).

Nucleotide substitution rates

Closely related pairs of alleles were chosen for analysis of synonymous and nonsynonymous substitution rates. For comparisons within each *Witheringia* species, we focused on sister alleles that occupied paired terminal branches on the species-level phylogeny. For *W. maculata*, there were four such pairs, and for *W. solanacea*, there were five. For interspecific comparisons, we chose sister alleles occupying paired terminal branches on the full phylogeny. There were five interspecific pairs. We wanted to focus on substitutions for functional S-alleles, so we excluded the known nonfunctional alleles S12 and S23, with their single (nonsynonymous) substitution, leaving four pairs of sister alleles for the within *W. solanacea* comparison. Number of synonymous and nonsynonymous substitutions, and number of potentially synonymous and nonsynonymous sites were calculated using SITES (Hey and Wakely, 1997). Synonymous and nonsynonymous substitution rates were calculated using the Nei and Gojobori (1986) method. Paired t -tests were used to compare nonsynonymous substitution rates within and outside of hypervariable regions (Ioerger *et al.*, 1991) for both intraspecific and interspecific comparisons.

Results

Allelic diversity and genealogy

In our sample of 21 plants, we found a total of 21 different alleles (GenBank accession numbers AY454099–AY454121). For our sample of 18 outcrossing plants, two alleles were amplified in each of 11 individuals, and one allele was amplified for each of the remaining seven individuals (Table 1). For the two self-compatible plants from Vara Blanca and one self-compatible plant found in Monteverde, only one allele each was amplified. Alleles

sampled from the largest population, Monteverde, were found at San Luis, Las Cruces, and Las Alturas as well (Table 2). Alleles were represented in approximately equal frequency, as expected under frequency-dependent selection, and assessed using the Mantel statistic ($\chi^2 = 17.5$, $P > 0.5$). Because the hypothesis of equal frequencies was not rejected, we were able to use Paxman's (1963) formula to estimate the total number of alleles. For the Monteverde population only, $n = 12$ alleles were recovered from $r = 8$ individuals, and the estimated number of alleles in the population is 24. For Monteverde pooled with San Luis, $n = 15$ alleles were recovered from $r = 10$ individuals, and the estimated number of alleles in the combined populations is 30. Extension of the analysis to include the entire area sampled yields $n = 21$ alleles, $r = 16$ individuals, and an estimated total of 31 alleles. If self-compatible plants are excluded, estimated allele numbers are 21 for Monteverde only, and 35 for Monteverde plus San Luis and for the entire range sampled.

S-RNase sequences for *W. solanacea* clustered in the three *Physalis*/*Witheringia* clades already identified by Richman and Kohn (1999, 2000; Figure 1). For convenience, Clade 1 in the figure can be arbitrarily divided into three subclades, the central one of which has strong bootstrap support. *W. solanacea* sequences were not distributed evenly across the three clades (Table 2). Nine of them occur in clade 3, along with nine *W. maculata* and *Physalis* sequences (Figure 1). *W. solanacea* alleles also dominate in clade 2, where they represent three of the five alleles, and in subclade 1B. In contrast, only one *W. solanacea* sequence appears among the 17 sequences in clade 1A, which is dominated by *Physalis* species. Lineage sorting among *Physalis* and *Witheringia* indicates substantial diversification of alleles after the divergence of the two genera.

Terminal branch lengths

The ordinary least squares method of Takezaki *et al.* (1995) failed to reject deviation from a molecular clock for S-RNase genealogies of both *W. maculata* ($Q = 11.5$, $df = 10$, $P > 0.1$) and *W. solanacea* ($Q = 20.2$, $df = 20$, $P > 0.1$). The scaled ratio of terminal branch length to total depth, R_{SD} , of 1.81 for *W. maculata* did not differ significantly from the expected value of 1.40 as predicted by simulation (Uyenoyama, 1997). R_{SD} for *W. solanacea* was much larger, 2.73, similar to the values found for *Physalis* species, which show a marginally significant trend toward long terminal branches (Uyenoyama, 1997; Richman and Kohn, 2000). The two *W. solanacea* alleles

Table 2 Allele frequencies by clade

Clade	Alleles	Frequencies	Populations
1a	S19	1	LA
1b	S1, S2, S8, S9	4, 1, 1, 1	MV, MV, MV, MV
1c	S12, S13, S14, S23	2, 2, 2, 2	MV and SL, MV and LC, MV and LC, VB
2	S7, S11, S21	1, 1, 1	MV, MV, LC
3	S6, S22, S5, S4, S18	1, 1, 2, 1, 1	MV, VB, MV and SL, MV and LC, LA
3	S3, S15	3, 1	LA and SL, SL
3	S16, S20	1, 1	MV, LC

See Figure 1 for clade designations and Table 1 for population codes. Frequency is number of occurrences out of the 31 allele copies sequenced.

Figure 1 Neighbor-joining tree showing relationships among S-RNase amino-acid sequences spanning regions C2–C5 for *W. solanacea*, *W. maculata*, *P. cinerascens*, *P. crassifolia*, and other members of the Solanaceae (*L. andersonii*, *N. alata* S2, S3, S6, S7; *P. hybrida* S1, S3, SB1, SB2; *S. carolinense*; *S. chacoense* S2, S3, S11, S12, S13). Sequences for all species other than *W. solanacea* were obtained from GenBank. The outgroup consists of S-like RNases from *Lycopersicon* and *Nicotiana*.

associated with SC, S12 and S23, have the shortest terminal branch lengths of any Wsol alleles (0.0014, as compared with an average of 0.059). Wsol branches S4, S7, and S14 that appear to be of zero length on the amino-acid phylogeny have an average terminal branch length of 0.014 on the species-specific phylogeny: longer than that of the SC alleles because of the length of the branch connecting them to the nearest intraspecific allele.

Substitutions among closely related alleles

Several *W. solanacea* S-RNase sequences were very similar to other sequences, both intra- and interspecifically (Figure 2). Wsol S12 and Wsol S23 differ for only a single replacement substitution at consensus amino-acid position 128. This substitution was validated by independent sequences obtained on two different dates and for two different plants for each allele. For the case of S12, the sequence was found in one plant from Monteverde and another at lower elevation in San Luis. For the case of S23, the sequence was found for two different plants from Vara Blanca. Other pairs of similar sequences within *W. solanacea* differed by at least three nucleotides. Interspecific sister alleles and sister alleles for *W. solanacea* can be seen on the phylogeny in Figure 1. Two pairs of the sister alleles for *W. maculata* are less apparent on the full phylogeny: S7 and S8 occur amidst numerous *Physalis* sequences in Clade 1A, and S1 and S10 occur interdigitated with *W. solanacea* S13 and S14 in Clade 1C.

The *Witheringia* S-RNase alleles in Clade 1C (Wsol S12, S13, S14, S23, and Wmac S1, S10) showed a nonconser-

vative amino-acid substitution from aspartic acid to glycine in position 118 of conserved region C4 of Ioeiger *et al* (1991). Allele S23 occurs in the highly selfing plants of Vara Blanca, which exhibit extremely low stylar RNase production (Stone, in preparation). The closely related allele S12 appears to be in homozygous state (based on lack of amplification of a second allele) in an autogamous plant from Monteverde. The alleles S13 and S14 have been found only in heterozygous state. It is not yet clear whether the mutation in C4 is itself responsible for low S-RNase activity and SC, whether other mutations leading to SC have relaxed selection pressure on this replacement substitution, or whether it is a coincidence that SC appears in the lineage with this mutation.

Synonymous and nonsynonymous substitution rates in *Witheringia*

Synonymous substitution rates for closely related alleles in intraspecific comparisons are equal to those in interspecific comparisons (Table 3; Mann-Whitney $U = 26.5$, $n_1 = 8$, $n_2 = 5$, $P > 0.10$). In contrast, nonsynonymous substitution rates for closely related pairs of intraspecific alleles exceed those for interspecific allele pairs (Table 3; Mann-Whitney $U = 40$, $n_1 = 8$, $n_2 = 5$, $P < 0.01$). As a result, the d_N/d_S ratio differs between intra- and interspecific comparisons, with d_N/d_S exceeding 1 for closely related alleles within species and about 0.5 for closely related alleles between species (Table 3). Nonsynonymous substitutions for intraspecific comparisons occur with higher frequency in regions previously designated as hypervariable than in the remainder of the

Figure 2 Nonsynonymous (N) and synonymous (S) substitutions for closely related intra- and interspecific alleles of *W. solanacea* and *W. maculata*. Numbering system for amino-acid residues corresponds to Ioeiger *et al* (1991). Previously identified hypervariable and conserved regions are identified by boxes. Multiple substitutions occurred for some amino-acid residues, and these are indicated with a single symbol.

Table 3 Mean (standard error) of synonymous and nonsynonymous substitution rates for sister alleles

Allele pairs	N	d_s	d_N	d_N/d_s
<i>W. solanacea</i>	4	0.045 (0.020)	0.052 (0.021)	1.245 (0.377)
<i>W. maculata</i>	4	0.066 (0.012)	0.078 (0.019)	1.247 (0.242)
Interspecific	5	0.036 (0.008)	0.011 (0.005)*	0.385 (0.078)*

N is number of sister alleles available for comparison. An asterisk indicates that the value differs from the others in the same column at $P < 0.01$.

sequence (Figure 2; paired t -test, $P = 0.005$, $df = 7$). For interspecific comparisons, in contrast, nonsynonymous substitutions occur at equal rates inside and outside of the hypervariable regions (paired t -test, $P > 0.1$, $df = 5$).

Discussion

Ecological correlates of allele number

W. solanacea has a similar life history to the previously investigated *W. maculata*: both historically occupied gaps in tropical forest canopies and therefore have had historically small, fugitive, populations. Estimated allele number of 24 for *W. solanacea* at Monteverde is larger than the 14.5 estimated for *W. maculata* at the same site. The larger altitudinal amplitude of *W. solanacea*, which is most commonly found between 1000 and 1500 m, may be responsible for its large effective population size relative to *W. maculata*, which is restricted to elevations above 1500 m. The effect of altitudinal range can be seen within our sample, with the inclusion of low elevation San Luis alleles resulting in an increase of estimated allele number from 24 to 30. Whether or not San Luis and Monteverde represent a panmictic population is an open question. Pollen is carried primarily by halictid bees (Stone, personal observation) and is unlikely to travel the 2.5 km separating the two areas. Gene flow by seed, however, is quite likely. *W. solanacea* fruits year-round; one of its primary dispersers at high elevations is *Myadestes melanops* (the Black-faced Solitaire; Murray, 1988), which migrates altitudinally into lowland forests when food is scarce in its montane breeding habitat (Stiles *et al.*, 1989; Loiselle and Blake, 1991).

Although increased altitudinal range may explain differences in allele number between the two species of *Witheringia*, it remains puzzling how the effective population size of *W. solanacea* can be so much larger than that of several temperate fugitive species. For example, horsenettle (*S. carolinense*), being found over the entire eastern United States, has a much larger distribution than *W. solanacea*, but has a predicted allele number of only 11–18, with almost complete overlap of alleles found in two populations separated by 240 km. Allele number for *W. solanacea*, in spite of its apparently weedy lifestyle, is intermediate between horsenettle and that found in temperate species such as *P. crassifolia* and *L. andersonii*, which are considered to display relatively large, stable population sizes (Richman and Kohn, 2000). It will be interesting to see whether high allele number characterizes tropical mid- to low-elevation species, where relatively stable climatic conditions may allow persistence of high allele number even for those species with fugitive life history strategies.

An issue in interpretation of allele number as obtained solely by molecular techniques is the question of whether different sequences necessarily correspond to different specificities, especially as here, when individuals from a broad geographic range are compared. Within a population, small N_e of specificities should eliminate multiple functionally equivalent variants (Takahata, 1990). Across populations, however, it is possible that slightly different amino-acid sequences could represent alternative forms of equivalent specificities. Hand crossing experiments are required to determine whether or not two closely related alleles are functionally equivalent. In the absence of compatibility data from crossing experiments, co-occurrence of closely related alleles within a population can be used to infer functional nonequivalence. For three of the four pairs of closely related SI alleles reported here, both sequences were found segregating within a single population. For one pair of closely related alleles (Wsol S4, Wsol S18), the two alleles were not found together in any population. Wsol S4 was found in Monteverde and 260 km away in Las Cruces, and Wsol S18 was found only at Las Alturas, about 23 km from Las Cruces. These alleles differ only by one synonymous and two nonsynonymous substitutions, and may represent a single functional allele. If the two alleles are functionally equivalent, allele diversification rate would be slightly lower than estimated here, although inferences about the excess of nonsynonymous substitutions in *Witheringia* would remain unchanged.

Short terminal branch lengths are not a general characteristic of *Witheringia*

Previous work has shown that whereas S-RNase alleles from *Lycium* and *Solanum* have extremely long terminal branches compared to neutral expectation, S-RNase alleles from *Physalis* have terminal branch lengths that are only marginally longer than predicted (Uyenoyama, 1997; Richman and Kohn, 2000). Explanations for long terminal branches include the presence of sheltered load reducing diversification rate (Uyenoyama, 1997, 2003) and selective retention of divergent alleles (Richman and Kohn, 2000). The S-RNase genealogy of *W. maculata* is unique in showing scaled terminal branch lengths that easily met neutral expectation (Uyenoyama, 1997; Richman and Kohn, 2000). An objective of this study was to see whether unusually short terminal branches would prove to be characteristic of the genus *Witheringia*, suggesting that the forces causing deviation from neutral expectation did not apply there. We found that scaled terminal branches for *W. solanacea* were not particularly short, and are similar to those found in *Physalis*. The shape of the S-RNase genealogy for *W. maculata* remains anomalous.

Substitution rates indicate postspeciation radiation

Intraspecific comparisons of closely related alleles for both *Witheringia* species show an excess of nonsynonymous substitutions, whereas interspecific comparisons among *Witheringia* species do not. This observation, together with the concentration of nonsynonymous substitutions in previously identified hypervariable regions thought to be associated with allelic recognition, suggests that selection has promoted diversification of new alleles postspeciation. In contrast, for *P. cinerascens* and *P. crassifolia*, interspecific and intraspecific d_N/d_S ratios are indistinguishable, at about 1.6–1.8 (Richman and Kohn, 1999). The inference from these data is that the lineages that led to the two *Physalis* species examined had already radiated into the currently observed lineages prior to the speciation event that separated them. Thus, although both *Witheringia* and *Physalis* shared the same demographic bottleneck, speciation within the genera occurred at different times with respect to allele diversification, at least for the pairs of species considered. A higher rate of postspeciation allelic diversification for *Witheringia* is perhaps unsurprising, as cpDNA sequences suggest that *W. solanacea* is basal to *Physalis* (Olmstead *et al.*, 1999). Still, the extent of lineage sorting between the genera is extensive, showing substantial radiation since their divergence; this radiation postdates speciation for these *Witheringia* but not for these *Physalis* species.

Breakdown of SI permits short-term allelic diversification

The shortest terminal branches on the *W. solanacea* S-RNase genealogy were the two that have been characterized as associated with SC, as is expected when variants within these allele classes are subject to neutral rather than inverse frequency-dependent selection (Vekemans and Slatkin, 1994). Classical theory dictates that an allele conferring SC should either be able to spread to fixation, if inbreeding depression can be purged, or should be rapidly lost, if not (eg, Charlesworth and Charlesworth, 1979; Lande and Schemske, 1985). Recent theory suggests, in contrast, that SI and SC alleles may stably coexist under certain conditions (Uyenoyama *et al.*, 2001; Porcher and Lande, 2005). It is interesting that the lineage representing alleles S12 and S23 has persisted long enough to diversify, suggesting that parameter combinations required for a stable polymorphism may occur in nature. That the two SC alleles were not found cooccurring in any single population is hardly surprising due to their small effective population size, but even species-level polymorphism suggests a relatively long residence time. More functional studies will be necessary to assess the influence of loss of SI on diversification rates, and to evaluate the degree to which SC alleles can persist in predominantly SI populations. Breakdown of SI needs to be considered along with effects of demography and effective mutation rate on diversification of S-RNase lineages.

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