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# A TALE OF TWO CONTINENTS: BAKER'S RULE AND THE MAINTENANCE OF SELF-INCOMPATIBILITY IN *LYCIUM* (SOLANACEAE)

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**Over 50 years ago, Baker (1955, 1967) suggested that self-compatible species were more likely than self-incompatible species to establish new populations on oceanic islands. His logic was straightforward and rested on the assumption that colonization was infrequent; thus, mate limitation favored the establishment of self-fertilizing individuals. In support of Baker's rule, many authors have documented high frequencies of self-compatibility on islands, and recent work has solidified the generality of Baker's ideas. The genus *Lycium* (Solanaceae) has ca. 80 species distributed worldwide, and phylogenetic studies suggest that *Lycium* originated in South America and dispersed to the Old World a single time. Previous analyses of the *S-RNase* gene, which controls the stylar component of self-incompatibility, have shown that gametophytically controlled self-incompatibility is ancestral within the genus, making *Lycium* a good model for investigating Baker's assertions concerning reproductive assurance following oceanic dispersal. *Lycium* is also useful for investigations of reproductive evolution, given that species vary both in sexual expression and the presence of self-incompatibility. A model for the evolution of gender dimorphism suggests that polyploidy breaks down self-incompatibility, leading to the evolution of gender dimorphism, which arises as an alternative outcrossing mechanism. There is a perfect association of dimorphic gender expression, polyploidy, and self-compatibility (vs. cosexuality, diploidy, and self-incompatibility) among North American *Lycium*. Although the association between ploidy level and gender expression also holds for African *Lycium*, to date no studies of mating systems have been initiated in Old World species. Here, using controlled pollinations, we document strong self-incompatibility in two cosexual, diploid species of African *Lycium*. Further, we sequence the *S-RNase* gene in 15 individuals from five cosexual, diploid species of African *Lycium* and recover 24 putative alleles. Genealogical analyses indicate reduced transgeneric diversity of S-RNases in the Old World compared to the New World. We suggest that genetic diversity at this locus was reduced as a result of a founder event, but, despite the bottleneck, self-incompatibility was maintained in the Old World. Maximum-likelihood analyses of codon substitution patterns indicate that positive Darwinian selection has been relatively strong in the Old World, suggesting the rediversification of S-RNases following a bottleneck. The present data thus provide a dramatic exception to Baker's rule, in addition to supporting a key assumption of the Miller and Venable (2000) model, namely that self-incompatibility is associated with diploidy and cosexuality.**

**KEY WORDS:** **Baker's rule, gametophytic self-incompatibility, genetic bottleneck, mating systems, molecular adaptation, *S-RNase* gene, Solanaceae, transgeneric evolution.**

Both the presence of physiological self-incompatibility and the deployment of sexual function within and among individuals are of profound importance in governing patterns of mating in plant populations (Barrett 2002). Such features control levels of heterozygosity within individuals and genetic diversity in populations, influence the evolution of species interactions, and help maintain reproductive barriers between species. Historically, the most useful systems for studying reproductive evolution are those in which self-incompatibility or gender expression varies either within taxa (e.g., Case and Barrett 2004; Yeung et al. 2005; Barrett and Case 2006) or among closely related species (e.g., Goodwillie 1999; Miller and Venable 2000, 2002; Obbard et al. 2006).

Genetically controlled self-incompatibility systems have evolved repeatedly among angiosperm species and are well established as mechanisms to avoid self-fertilization and prevent inbreeding depression in plants (de Nettancourt 1977; Matton et al. 1994; Castric and Vekemans 2004). In gametophytic self-incompatibility (GSI), two separate but tightly linked genes at the *S*-locus control the recognition specificity. The first is the *S-RNase* gene, which is expressed in the pistil. The second, more recently described gene, is called *SLF* (Qiao et al. 2004; Sijacic et al. 2004) or *SFB* (Ushijima et al. 2003) and is expressed in the haploid pollen grain. When the haploid *S*-genotype of the pollen grain matches that of either of the two S-RNases expressed in the pistil of the maternal parent, pollen tube growth is terminated and fertilization fails. Given this genetic control, offspring following successful fertilization are necessarily heterozygous. Strong negative frequency-dependence shelters rare alleles from extinction and creates strong selection for novel alleles; both of these factors, the magnitudes of which depend on population size, result in the maintenance of large numbers of *S*-alleles within self-incompatible populations. Several researchers have used allelic diversity at the *S-RNase* gene to characterize mating systems in natural populations (Richman et al. 1996a; Stone and Pierce 2005; Savage and Miller 2006). These studies report heterozygosity and high allelic diversity at this locus, as expected under GSI.

Baker (1955, 1967) was the first to note that self-compatible species are more likely to be successful island colonizers than obligate outcrossers that require pollen transfer between plants (i.e., self-incompatible species). In support of "Baker's Law" (as coined by Stebbins 1957, but see Baker 1967), a higher frequency of self-compatibility, as opposed to self-incompatibility, has been documented in island floras. For example, Anderson et al. (2001) reported that 80% of cosexual species tested in the Juan Fernandez Islands were self-compatible. Likewise, surveys of New Zealand (Webb and Kelly 1993) and the Galapagos Islands (McMullen 1987, 1990) document a similar trend. More recent empirical work has continued to emphasize the generality of Baker's rule in both plants (Schueller 2004; Busch 2005; Flinn 2006) and animals (Trouve et al. 2005), with various exceptions (Carr et al. 1986; Sun and Ritland 1998; Brennan et al. 2005, 2006). Baker also noted the similarity of invasive species to colonizers, and thus predicted invasive species to be disproportionately self-compatible. In southern Africa, Rambuda and Johnson (2004) found that of 17 invasive species tested, all were self-compatible, and nearly three-quarters were capable of autonomous self-fertilization. Pannell and Barrett (1998) have also modeled the generality of Baker's rule and shown that his ideas apply to plant metapopulations in which both colonization and extinction are frequent.

*Lycium* (Solanaceae) is a genus of ca. 80 species with a cosmopolitan distribution and centers of diversity in Argentina and Chile, southwestern North America, and southern Africa. Recent phylogenetic studies of *Lycium* (Levin and Miller 2005; Levin et al. 2007) strongly support a South American origin of the genus and the monophyly of all Old World species (Fig. 1A). Thus, *Lycium* dispersed to the Old World (Africa and Asia) a single time. Extending Baker's (1955, 1967) logic to the dispersal of *Lycium* worldwide, the expectation is that the establishment of *Lycium* in the Old World was facilitated by the presence of self-compatibility in the original colonists.

Members of *Lycium* vary in sexual function; whereas most species are cosexual and produce hermaphroditic flowers, others have gender dimorphism ranging from separate female and hermaphroditic plants (Miller and Venable 2002, 2003) to complete separation of males and females (Venter 2000, 2007). The evolution of gender dimorphism in *Lycium* is especially interesting because it has occurred on a phylogenetic background of self-incompatibility (Bianchi et al. 2000; Miller and Venable 2000, 2002; Richman 2000; Aguilar and Bernardello 2001; see also Fig. 1A). Indeed, molecular sequence data for the *S-RNase* gene indicate the presence of shared ancestral polymorphism across several *Lycium* species and other genera in Solanaceae, demonstrating the long-term maintenance of self-incompatibility (Richman and Kohn 2000; Igic et al. 2003; Savage and Miller 2006). The presence of both gender dimorphism and self-incompatibility presents a challenge to explanations for the evolution of single-sexed plants by an outcrossing advantage, given that all plants in the population are already outcrossing as a result of GSI.

To address this redundancy, Miller and Venable (2000) proposed that gender dimorphism in *Lycium* evolves following polyploidy, which acts as a trigger for the transition to dimorphism because it disrupts the preexisting self-incompatibility system. The breakdown of self-incompatibility eventually leads to the establishment of single-sexed mutants due to an outcrossing advantage. Consistent with this pathway, all species of *Lycium* with gender dimorphism ($n = 3$ species in North America; $n = 7$ species in Africa) are polyploid, whereas cosexual species are diploid (Miller and Venable 2000; Venter 2000). Remarkably, the association between gender dimorphism and polyploidy is also present among populations of a single species, *Lycium*

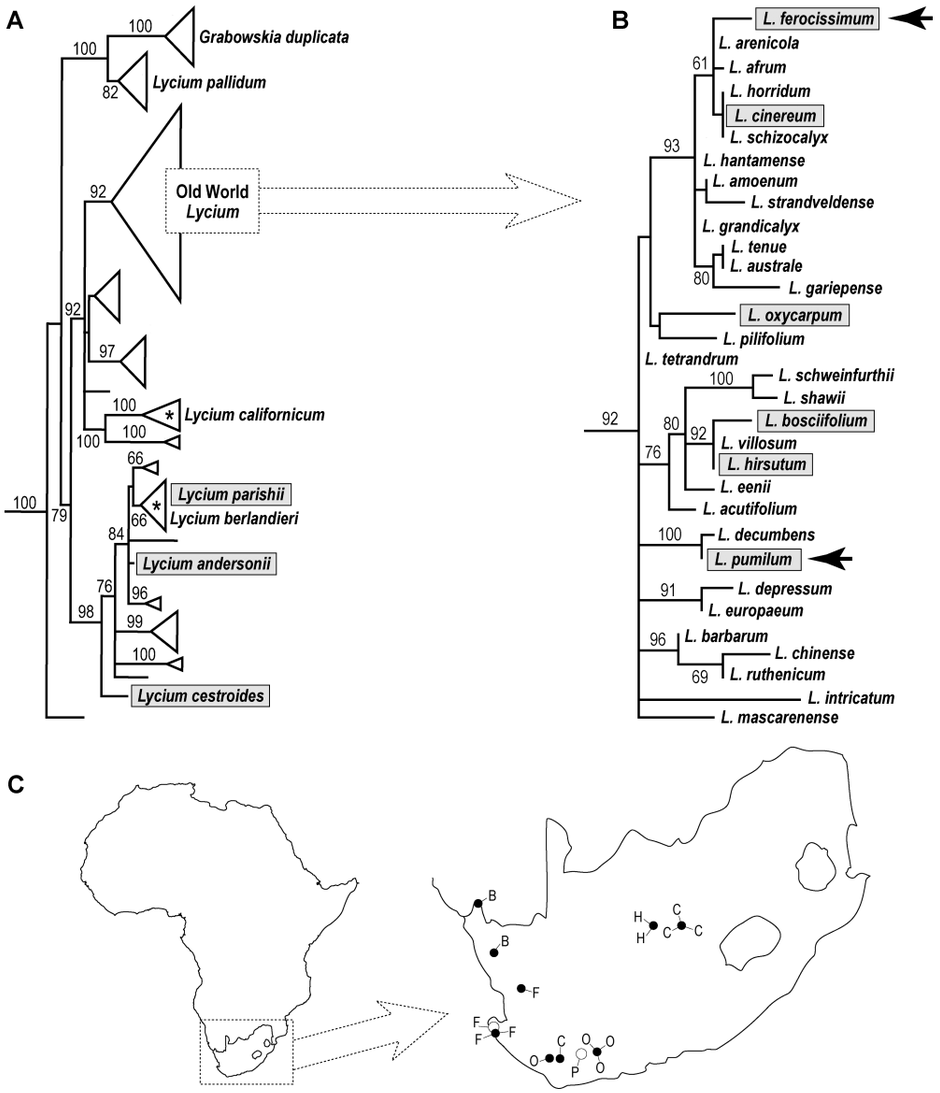


**Figure 1.** Schematic illustrating evolutionary relationships among members of tribe Lycieae (A) and Old World species of *Lycium* (B) from Levin et al. (2007); ML bootstrap values greater than 60% are indicated. Taxon names in (A) are shown only for species known to be self-incompatible (*L. andersonii*, Richman 2000; *L. cestroides*, Aguilar and Bernardello 2001; *Grabowskia duplicata*, Bianchi et al. 2000; *L. berlandieri*, *L. pallidum*, and *L. parishii*, Miller and Venable 2002; and a diploid population of *L. californicum*, Yeung et al. 2005 and JR Kohn, Univ. California, San Diego, pers. comm.). Self-compatibility is known only from two related species in one clade (*L. exsertum* and *L. fremontii*) and a polyploid population of *L. californicum* (Miller and Venable 2002), the positions of which are indicated with asterisks in (A). Taxa included in the present study are in shaded boxes, and black arrows indicate African species for which controlled pollinations were done. Maps (C) show the geographic distributions of South African species and populations in this study; letters are the first letter of the species name (see B), and locations are represented by either open circles (populations in which controlled pollinations were carried out) or closed circles (sampling localities for individuals used in *S-RNase* studies).

*californicum*; in this taxon cosexual populations are diploid whereas dimorphic populations are polyploid (Yeung et al. 2005). A central assumption of the Miller and Venable (2000) model is the association of self-incompatibility with both diploidy and cosexuality. Although self-incompatibility is documented in diploid, cosexual American *Lycium* (Fig. 1A; Richman 2000; Miller and Venable 2002; Savage and Miller 2006), no studies of mating systems in African species have been conducted to date.

Here, we use controlled pollinations to assess the compatibility status for two diploid, cosexual species in Africa, *Lycium ferocissimum* and *L. pumilum*. In addition, we investigate allelic diversity and patterns of selection at the pistil *S-RNase* locus for

all alleles retrieved from 15 individuals of five diploid species of southern African *Lycium*. Using these data we address both Baker's assertion that self-compatibility facilitates establishment, as well as the model proposed by Miller and Venable (2000), which assumes self-incompatibility in diploid, cosexual *Lycium*.

## Materials and Methods

### STUDY SYSTEM

*Lycium* (Solanaceae) is a genus of ca. 80 species of predominantly insect-pollinated, perennial shrubs. Within *Lycium*, Levin and Miller (2005) and Levin et al. (2007) have documented a single dispersal event from the Americas to the Old World (Fig. 1A). The sister group of *Lycium* is *Nolana* (Olmstead et al. 1999; Levin and Miller 2005), which is estimated to be ca. 10 MY old (Tago-Nakazawa and Dillon 1999); thus, both the origin of *Lycium* and its dispersal to the Old World are relatively recent.

We conducted controlled pollination experiments in natural populations of two African diploid, cosexual species, *L. ferocissimum* and *L. pumilum*; the species phylogeny of Old World *Lycium* (Levin et al. 2007; Fig. 1B) indicates that these two species are in different clades. We also collected material from five diploid, cosexual species (including *L. ferocissimum*) for analyses of the stylar *S-RNase* gene. These taxa were collected over a wide geographic range and are present in several clades within Old World *Lycium* (Figs. 1B,C).

### CONTROLLED POLLINATIONS

To determine if *L. ferocissimum* and *L. pumilum* are self-compatible, we compared fruit and seed production of flowers pollinated with either self or outcross pollen in natural populations of these species. Pollinations for *L. ferocissimum* were carried out in West Coast National Park in the Western Cape province, South Africa (33°7.116′S, 18°3.506′E) during 5–8 September 2006. We pollinated *L. pumilum* from 21–24 September 2006 in Groenefontein Nature Reserve, Western Cape province, South Africa (33° 38.266′S, 21°39.129′E).

On each plant, unopened buds were covered with fine mesh bags to prevent insect visitation. Over the next several mornings, plants were revisited and open flowers with undehisced anthers were emasculated and pollinated with either self (collected from the same plant) or outcross pollen. Outcross pollen was collected from multiple (>10) donors, and each flower in the outcross treatment was pollinated using a minimum of three flowers from this pool. Both pollination treatments delivered sufficient pollen for full seed set. Following pollinations, flowers were re-covered with mesh bags to prevent insect visitation. We pollinated a total of 174 self and 163 outcross flowers on 16 plants of *L. ferocissimum* and 165 self and 136 outcross flowers on 14 plants of *L. pumilum*. In addition, we marked 179 and 177 newly opened, unmanipulated and uncovered flowers to assess natural levels of fruit and seed production in *L. ferocissimum* and *L. pumilum*, respectively. Both populations were revisited and flowers were checked for fruit production. Developing fruit were kept covered with fine mesh bags to prevent birds from removing fruit. A total of 172 fruit were collected on 12 November 2006 for *L. ferocissimum*, and 104 fruit were collected from *L. pumilum* on 10 November 2006. To calculate average seed number in the outcross, self, and control treatments, respectively, seed number was counted for all 115, 14, and 43 fruit in *L. ferocissimum* and all 71, 8, and 25 fruit in *L. pumilum*.

Data for fruit production were analyzed using a generalized linear model with a binomial error distribution (individual flowers either succeeded or failed at producing a fruit) and a logit link function (PROC GENMOD, SAS Institute 1989). Each flower was treated independently, and the model included the effects of plant, pollination treatment, and the interaction of plant by pollination treatment. Seed number was analyzed using a general linear model in SAS (PROC GLM, SAS Institute 1989); analyses included the effects of plant, pollination treatment, and the plant by pollination treatment interaction.

### S-RNASE DIVERSITY

We collected 10–20 styles from five diploid species of African *Lycium*, including four individuals each of *L. oxycarpum* and *L. cinereum*, three individuals of *L. ferocissimum*, and two individuals each from *L. hirsutum* and *L. bosciifolium* (Fig. 1C). Styles from mature buds and first-day flowers were preserved in RNAlater® (Ambion, Inc., Austin, TX) and stored initially at 4°C before being transferred to −20°C.

For each individual, we obtained stylar mRNA using the RNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA) and synthesized cDNA with the First Strand cDNA Synthesis Kit (EMD Biosciences, Inc., Madison, WI). The initial amplification used degenerate primers PR1 and PR3 (Richman et al. 1995) to amplify a portion of the *S-RNase* gene between conserved regions C2 and C5, previously identified by Ioerger et al. (1991) and following conditions in Savage and Miller (2006). Amplification products were cloned into the pT7Blue vector using the Perfectly Blunt Cloning Kit (EMD Biosciences, Inc.). As we do not explicitly demonstrate whether specific sequence variants encode for different specificities, rather than referring to these variants as "alleles" at the *S-RNase* locus, we will use the terms "sequence variants" or "putative alleles."

Individual colonies were amplified using the PCR primers and conditions described above. Colony PCR products were screened for restriction fragment length polymorphisms (RFLPs). Approximately 12 colonies were selected from each individual for RFLP analysis, and all colonies with unique RFLP banding patterns were amplified with vector primers U19 (5′-GTT TTC

CCA GTC ACG ACG T-3′) and R20 (5′-CAG CTA TGA CCA TGA TTA CG-3′) and sequenced on an Applied Biosystems Automated 3730 DNA Analyzer by the Biotechnology Resource Center at Cornell University (Ithaca, NY). We sequenced an average of seven colonies for each of the 15 accessions. A total of 24 S-RNases were isolated from Old World *Lycium* (GenBank accession numbers: EU074803–EU074826).

To compare Old World and New World *S-RNase* diversity, we included 24 previously published alleles for North American *Lycium parishii* (Savage and Miller 2006; DQ367853-DQ367876) and 11 alleles of North American *L. andersonii*. Only those *L. andersonii* sequences that spanned regions C2–C5 were included in the alignment (Richman 2000; AF05343–4, AF105347–9, AF105353, AF105355, AF105358–9, AF105362–3). In addition, to increase sampling of S-RNases from New World *Lycium*, we sequenced four individuals of the South American species *Lycium cestroides*. These four individuals had a total of six *S-RNase* putative alleles (Genbank accession numbers: EU074797–EU074802).

Sequences were aligned by eye using Ioerger et al. (1991) as a guide and confirmed by comparing multiple sequenced colonies both within and among individuals. All *Lycium* S-RNases were included in a multiple alignment that included 55 S-RNases from previously published studies of Solanaceae: *Petunia axillaris* (AF239907–10, AY180048, AY180050), *Petunia integrifolia* (AF301167–8, AF301171–3, AF301176–7, AF301180), *Physalis cinerascens* (AF058930–1, AF058933, AF058935–7, AF058940), *Physalis crassifolia* (L46653, L46656–8, L46663, L46668–9, L46672–3), *Solanum carolinense* (L40539–46), *S. chacoense* (AF176533, L36666, S69589, X56896–7), *Witheringia maculata* (AF102066–7, AF102070–3), *W. solanacea* (AY454099, AY454105, AY454111, AY454113–4, AY454117). The *S-RNase* S2 from *Antirrhinum hispanicum* (X96465) was used as the outgroup.

Average pairwise distances for S-RNases from Old and New World *Lycium* were calculated in PAUP* (Swofford 2002). We constructed gene genealogies of the complete 120 S-RNase dataset using both maximum likelihood and Bayesian approaches. Maximum likelihood (ML) model parameters were determined using the Akaike information criterion in Modeltest version 3.7 (Posada and Crandall 1998). The best-fit model (GTR + I + G) was used in an ML analysis in PAUP* using the heuristic search option, TBR branch swapping, MulTrees option in effect, and a single neighbor-joining tree as a starting topology. The Bayesian analysis was run in MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) and included four simultaneous Markov chain Monte Carlo (MCMC) chains, each starting from a random tree, with a general time reversible substitution model and gamma-distributed rate variation across sites. Two million generations were run, with a tree saved every 100 generations; trees preceding the stabilization of likelihood values were excluded in the construction of a consensus tree in PAUP*.

The number of transgeneric lineages (TGLs) for Old World and New World *Lycium* was determined for both the ML and Bayesian topologies; we define a TGL as the most recent node including sequences from *Lycium* and another genus. However, because the number of TGLs is sensitive to the number of sequences included, it is possible that a greater number of TGLs will be inferred for New World *Lycium* as a result of a larger sample (41 New World S-RNases vs. 24 Old World S-RNases). In addition, estimates of TGLs are also sensitive to uncertainty in the genealogy. To investigate these concerns, we used a resampling approach in which we randomly selected 24 (of the 41 available) New World S-RNases. These 24 randomly selected New World sequences, all 24 Old World sequences, and the 55 S-RNase Solanaceae-wide dataset were then used to construct a neighbor-joining tree in PAUP*, and the number of TGLs for New World and Old World *Lycium* was determined. This procedure was repeated 100 times.

### SELECTION ANALYSES OF THE S-RNASE GENE

As positive selection occurs in particular regions of the *S-RNase* gene (Savage and Miller 2006), we investigated patterns of selection using site-specific codon models as implemented in the *codeml* package in PAML version 3.15 (Yang 1997). Specifically, we used *codeml* for estimates of the $d_N/d_S$ rate ratio ($\omega$) for New World and Old World *Lycium* S-RNases. Likelihood ratio tests (LRTs) were used to compare null models that assume nearly neutral evolution (M1a and M7) with more complex models that incorporate positive selection (M2a and M8; models in Yang et al. 2000 and Wong et al. 2004). The use of both LRTs (M1a vs. M2a and M7 vs. M8) is the recommended procedure for investigating positive selection using PAML (Yang and Bielawski 2000; Wong et al. 2004), and similarity between analyses can be used to assess robustness. The M1a model assumes two amino acid site classes; one site class fixes $\omega$ at one, whereas $\omega$ for the second site class is estimated under the constraint that it varies between zero and one (models in Wong et al. 2004). Thus, in the M1a model, sites are either evolving neutrally or nearly neutrally ($\omega_1 = 1$) or are subject to purifying selection (i.e., under the constraint, $0 < \omega_0 < 1$). The M2a positive selection model has a third site class ($\omega_2$) that is also estimated; $\omega_2$ can exceed one and is the $d_N/d_S$ rate ratio of those sites under positive selection. We also compared models M7 ($\beta$) and M8 ($\beta$ & $\omega$>1; models in Yang et al. 2000). Both M7 and M8 assume a $\beta$-distribution for $0 \leq \omega \leq 1$, but model M8 includes an additional parameter that incorporates positive selection. Where LRTs indicated that the models incorporating positive selection (M2a and M8) fit the data significantly better than their corresponding null models (M1a and M7) and the $d_N/d_S$ rate ratio associated with positive selection ($\omega_2$ in model

M2a and $\omega_s$ in model M8) was greater than one, diversifying selection was inferred for sites in the sequences. The empirical Bayesian probabilities (Yang et al. 2005) were then used to determine the number and identity of sites under positive selection.

# Results

## CONTROLLED POLLINATIONS

For both *L. ferocissimum* and *L. pumilum*, outcross pollination resulted in greater fruit and seed production compared to self pollination. There was a significant main effect of pollination treatment on fruit production for both species (*L. ferocissimum*, $\chi^2 = 110.9$, df = 2, $P < 0.0001$; *L. pumilum*, $\chi^2 = 72.2$, df = 2, $P < 0.0001$). In *L. ferocissimum*, 75% of flowers pollinated with outcross pollen set fruit compared to only 8% following self pollination. Likewise, in *L. pumilum* 60% of flowers formed fruit following outcross pollination, whereas only 5% of self pollinations resulted in fruit production (Fig. 2A). There was also a main effect of plant for both species (*L. ferocissimum*, $\chi^2 = 58.3$, df = 15, $P < 0.0001$; *L. pumilum*, $\chi^2 = 41.1$, df = 13, $P < 0.0001$), indicating that some plants had higher fruit production than others, regardless of pollination treatment. Outcross pollination was also over 2.5 times more effective at fruit production compared to unmanipulated control flowers (0.75 vs. 0.27 for *L. ferocissimum* and 0.60 vs. 0.18 for *L. pumilum*; Fig. 2A). There was a significant plant by pollination treatment for *L. ferocissimum* ($\chi^2 = 51.6$, df = 30, $P = 0.009$), but not *L. pumilum* ($\chi^2 = 26.9$, df = 26, $P = 0.414$). Comparison of the plant by pollination treatment means for *L. ferocissimum* revealed that the outcross pollination treatment was more successful than the self pollination treatment for all genotypes (the difference between outcross and self pollination treatments ranged from 0.48–1.0). The only exception, and the source of the interaction, was a single individual in which fruit set for both the outcross and self pollination treatments was low (both treatments, 0.09; control fruit set was 0.20 for this plant); removal of this individual and reanalysis resulted in a nonsignificant interaction.

For seed production in both species, there were significant main effects for both pollination treatment (*L. ferocissimum*, $F_{2,39.2} = 22.3$, $P < 0.0001$; *L. pumilum*, $F_{2,30.2} = 60.9$, $P < 0.0001$) and plant (*L. ferocissimum*, $F_{15,21.2} = 2.3$, $P < 0.039$; *L. pumilum*, $F_{13,17.7} = 4.5$, $P < 0.002$). On average, outcrossed fruits produced roughly five and a half times more seed than selfed fruits for *L. ferocissimum* (43.1 vs. 7.9) and over seven and a half times as many for *L. pumilum* (14.7 vs. 1.9; Fig. 2B). Seed set in unmanipulated control fruits was significantly higher than for self fruits, but lower than outcrossed seed number in both species (Fig. 2B).

## S-RNASE DIVERSITY

Sequences ranged from 363 to 384 bp in length and 30 unique *S-RNase* sequence variants were identified. Twenty-four putative alleles were recovered among the 15 individuals of Old World *Lycium*, and six putative alleles were designated in the four New World *L. cestroides* individuals (Table 1). Consistent with the presence of self-incompatibility, 13 of 15 Old World individuals and all of the South American individuals were heterozygous at the *S-RNase* locus.

Old World S-RNases were more similar to each other than were comparisons within New World S-RNases. Specifically, average pairwise amino acid distances were 0.34 and 0.53 for Old World and New World S-RNases, respectively. There was a group of similar S-RNases among the Old World putative alleles (16 sequences with average AA distance of 0.075). Although average divergence was low in this group, all had unique combinations of amino acids (average difference among these sequence variants was 9.4 amino acids). The only exception was the comparison between *OXYC6* and *CINE4*, which differed at only two synonymous sites; however, these differences were confirmed using multiple independent clones.

Results of the ML and Bayesian analyses were similar, and the ML genealogy is presented in Fig. 3. The 65 *Lycium* S-RNases occur in 11 TGLs; however, New World and Old World sequences were not distributed equally among these lineages.

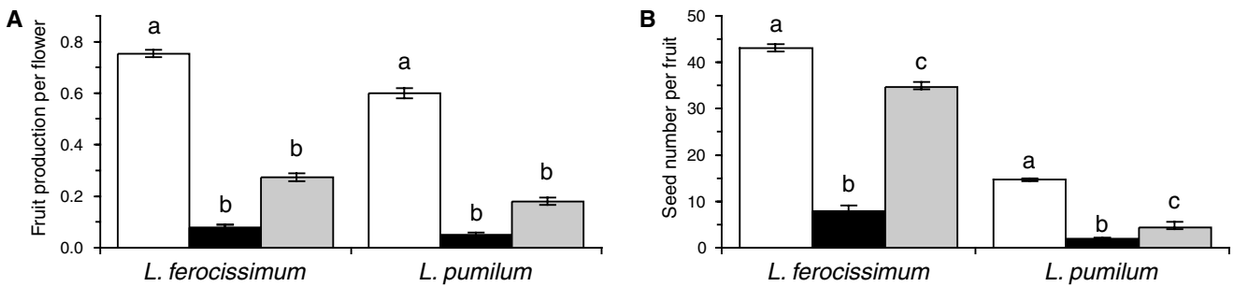


**Figure 2.** Means (±1 SE) for fruit production per flower (A) and seed number per fruit (B) in *Lycium ferocissimum* and *L. pumilum* following either outcross (open bars) or self (closed bars) pollination or in unmanipulated controls (shaded bars). For within-species comparisons of fruit or seed production, means sharing the same superscript do not differ significantly.

**Table 1.** ***S-RNase*** **genotypes for individual** ***Lycium*** **sequenced in the present study.**

| Species | Genotype |
|---|---|
| ***Old World*** | |
| *L. bosciifolium* | *BOSC1 / BOSC4* |
| *L. bosciifolium* | *BOSC2 / BOSC3* |
| *L. cinereum* | *CINE1 / CINE2* |
| *L. cinereum* | *CINE1 / CINE5* |
| *L. cinereum* | *CINE3 / CINE4* |
| *L. cinereum* | *CINE6* |
| *L. ferocissimum* | *FERO1 / FERO2* |
| *L. ferocissimum* | *FERO2 / FERO5* |
| *L. ferocissimum* | *FERO3 / FERO4* |
| *L. hirsutum* | *HIRS1 / HIRS2* |
| *L. hirsutum* | *HIRS3* |
| *L. oxycarpum* | *OXYC1 / OXYC5* |
| *L. oxycarpum* | *OXYC2 / OXYC3* |
| *L. oxycarpum* | *OXYC2 / OXYC3* |
| *L. oxycarpum* | *OXYC4 / OXYC6* |
| ***New World*** | |
| *L. cestroides* | *CESTa / CESTb* |
| *L. cestroides* | *CESTa / CESTf* |
| *L. cestroides* | *CESTb / CESTe* |
| *L. cestroides* | *CESTc / CESTd* |

S-RNases isolated from New World species were present in all of the TGLs, whereas those from Old World taxa were present in only four TGLs (Fig. 3). Resampling using equal numbers of New and Old World S-RNases reinforced this finding; the number of S-RNase TGLs in the Old World was only half the number found in the New World (Fig. 4).

### SELECTION ANALYSES OF THE S-RNASE GENE

Selection analyses indicated that the model of positive selection (M2a) fits the data significantly better than the model of nearly neutral (M1a) evolution for both the Old World and New World samples (Old World: LRT = 48.08, $P < 0.0001$; New World: LRT = 15.14, $P < 0.001$; Table 2). Likewise, the second set of LRTs showed the same pattern, with the model incorporating positive selection (M8) fitting the data significantly better than the null model (M7) (Old World: LRT = 51.83, $P < 0.0001$; New World: LRT = 16.56, $P < 0.001$).

Not surprisingly, the majority of positively selected sites for both datasets were located in the hypervariable regions of the *S-RNase* gene (Fig. 5). However, the number of positively selected sites among Old World S-RNases was three to four times higher than the estimate for New World S-RNases (Fig. 5). In addition, the $d_N/d_S$ rate ratio for those sites in the positive selection class ($\omega_2$ for the M2a model and $\omega_s$ for the M8 model; Table 2) ranged from 4.4 to 4.7 for Old World putative alleles, but from only 1.4 to 1.9 for the New World dataset.

## Discussion

### CONTROLLED POLLINATIONS

The two South African species of *Lycium* studied here, *L. ferocissimum* and *L. pumilum*, are strongly self-incompatible based on fruit and seed production in controlled crosses (Fig. 2). In our pollinations, outcrossing resulted in a 51- (*L. pumilum*) or 93-fold (*L. ferocissimum*) increase in seed production per flower compared to selfing. The present data for African species indicate stronger self-incompatibility than recorded previously for North American species of *Lycium* (Miller and Venable 2000, 2002). Miller and Venable (2002) documented a 14- to 15-fold advantage of outcross, compared to self pollen, for three North American species. It is not clear why the relative success of outcross and self pollen should vary so dramatically among species. Data from two of three North American species suggest higher fruit set following self-pollination (Miller and Venable 2002) than reported here for the South African species. An influencing factor includes the timing of pollination, which is known to affect self fruit set in self-incompatible species (Stone 2004; Travers et al. 2004). Additionally, in the present study we pooled pollen from > 10 individuals to serve as outcross pollen donors, whereas Miller and Venable (2002) in some cases used as few as two outcross donors. As the genotypes of individuals were unknown at the time of pollination in both studies, it is possible that the larger pool of outcross pollen in the present study increased the likelihood of compatible crosses among outcross pollinations. Paschke et al. (2002) experimentally investigated the effects of pollen load diversity in self-incompatible *Cochlearia bavarica* (Brassicaceae) and reported that increasing the number of pollen donors (i.e., pollen diversity) was associated with higher reproductive success.

Although the presence of self-incompatibility in African *Lycium* appears to contradict Baker's rule (1955, 1967), there is ample support for Baker's assertions in the literature. Much of this evidence involves broadscale comparative surveys of island floras, and many such studies document the relative rarity of self-incompatibility compared to self-compatibility on islands (McMullen 1987, 1990; Webb and Kelly 1993; Anderson et al. 2001; Bernardello et al. 2001). Likewise, Rambuda and Johnson (2004) document self-fertility among alien invasive species in South Africa. However, evidence within species or among closely related species provides more powerful tests of Baker's assertions. In *Lycopersicon hirsutum-Solanum habrochaites*, populations are self-incompatible in the center of the species range, but self-compatibility is thought to have evolved independently in peripheral northern and southern populations (Rick et al. 1979; Rick and Chetelat 1991). More recently, Busch (2005) and Ortiz et al. (2006) have found increased selfing rates in peripheral populations, as opposed to central populations, consistent with the hypothesis that self-fertility facilitates

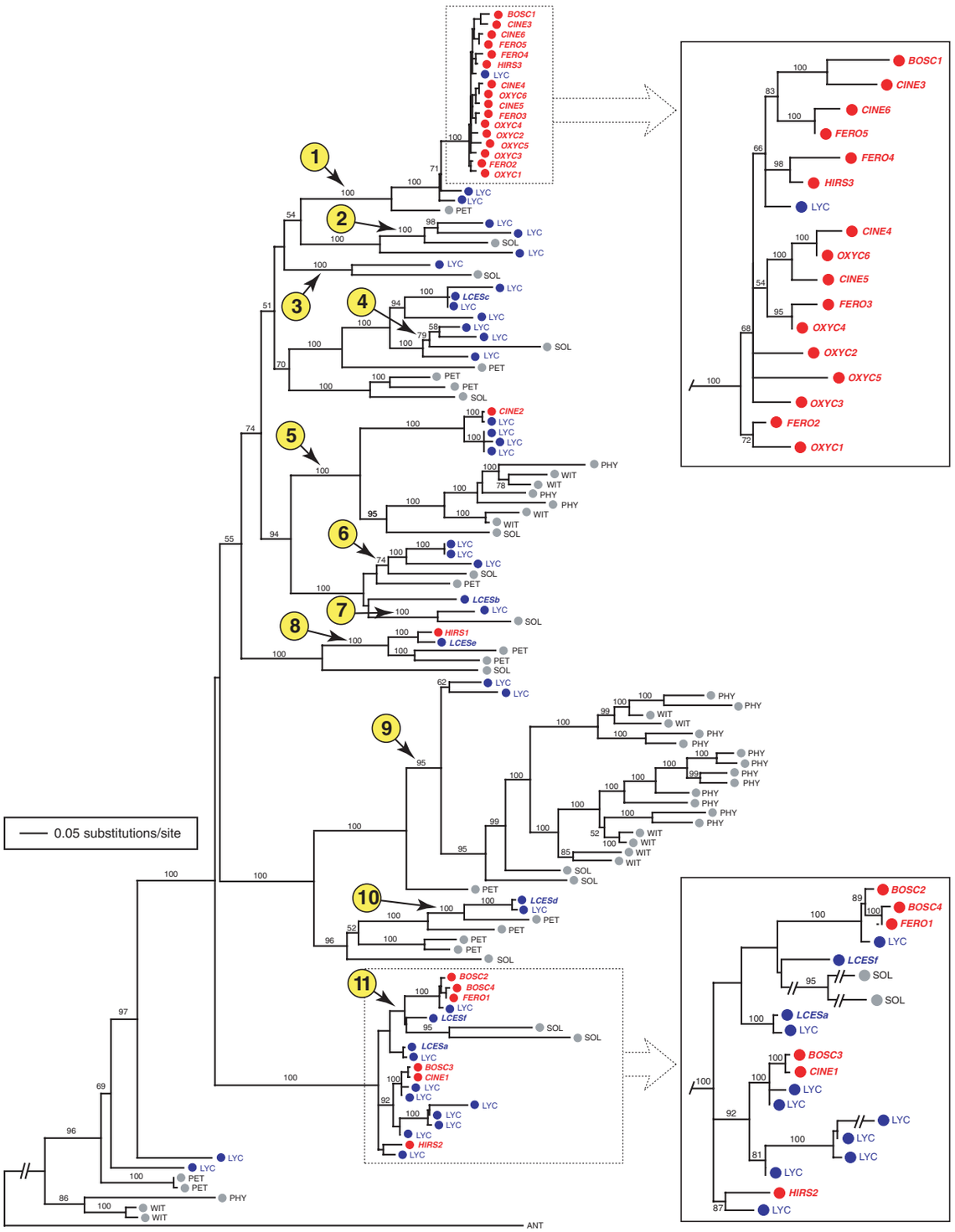


**Figure 3.** Relationships among S-RNases isolated from Old World (red circles) and New World (blue circles) *Lycium* species, and four additional genera in Solanaceae. The single tree from an ML analysis of the 120 S-RNase dataset using *Antirrhinum* (ANT) as an outgroup is shown with Bayesian posterior probabilities. Transgeneric lineages for *Lycium* are indicated using circled numbers. Sequences generated in the present study are in boldface, italic type and are labeled with their species and putative allele identity following Table 1. Previously obtained New World *Lycium* alleles from Richman (2000) and Savage and Miller (2006) are labeled LYC. S-RNases from *Petunia* (PET), *Physalis* (PHY), *Solanum* (SOL), and *Witheringia* (WIT) are from Genbank (see Materials and Methods).

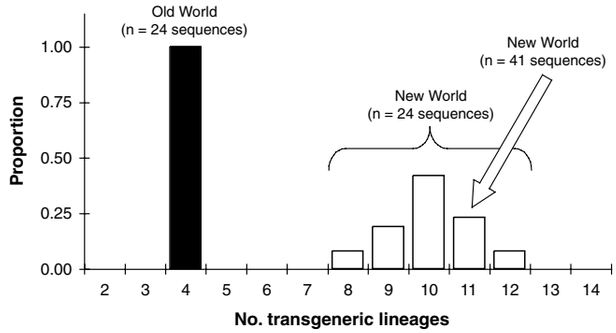


**Figure 4.** The distribution of the number of transgeneric lineages as a proportion of the topologies inferred from 100 resampled datasets. Each resampled dataset included all 24 Old World sequences and a partial sample ($n = 24$) of randomly chosen New World S-RNases. Open bars are the number of TGLs for New World sequences and the closed bar is the number recovered for Old World sequences in all resampled datasets. The open arrow indicates the number of TGLs recovered for New World S-RNases when all 41 New World S-RNases were included (see Fig. 3).

colonization and range expansion. Likewise, Schuller (2004) investigated the capacity for self-fertilization in island and mainland populations of self-compatible *Nicotiana glauca*. Her data suggest that enhanced self-fertilization in island (vs. mainland) populations is a result of the establishment success of highly self-fertilizing genotypes, as opposed to selection for selfing following colonization. A recent study by Flinn (2006) also documents the association of self-fertilization ability and colonization history; specifically, among three fern species those with frequent colonization had the highest selfing rates.

In contrast, only a handful of studies (including the results presented here) demonstrate clear exceptions to Baker's rule. For example, Brennan et al. (2005, 2006) have documented the maintenance of sporophytic self-incompatibility in *Senecio squalidus* (Asteraceae) following its establishment and expansion into Britain. Brennan et al. (2002) also suggested that the presence of pseudo-incompatibility in some genotypes might have aided this expansion. Sun and Ritland (1998) estimated high outcrossing rates for introduced populations of *Centaurea solstitialis* (Asteraceae), although the genetic control of incompatibility in this system is unknown. Other groups that have maintained self-incompatibility after colonization include the silversword alliance radiation in Hawaii (Carr et al. 1986) and the colonizing, clonal species *Ipomoea pes-caprae* (Convolvulaceae; Devall and Thien

**Table 2.** Likelihood ratio tests comparing models of neutral evolution (M1a and M7) with corresponding models that incorporate positive selection (M2a and M8) for New World and Old World *Lycium* S-RNases. For models M1a and M2a, values of $p_0$, $p_1$, and $p_2$ are the proportion of sites inferred to be evolving under purifying selection, neutral evolution, and positive selection, respectively, and $\omega_0$, $\omega_1$, and $\omega_2$ are their corresponding $d_N/d_S$ rate ratios. For models M7 and M8, the β distribution, $\beta(p,q)$, describes the distribution of the $d_N/d_S$ rate ratio between zero and one, and $p_0$ is the proportion of sites within this distribution. In these models, $p_1$ is the proportion of sites inferred to be under positive selection, and $\omega_s$ is the $d_N/d_S$ rate ratio for those sites.

| | Model | λ | 2Δλ | Parameters |
|---|---|---|---|---|
| **New World *Lycium*** | M1a: nearly neutral | −7430.833 | | $p_0 = 0.449$ ($\omega_0 = 0.202$); $p_1 = 0.551$ ($\omega_1 = 1$) |
| | M2a: positive selection | −7423.265 | 15.14*** | $p_0 = 0.432$ ($\omega_0 = 0.213$); $p_1 = 0.466$ ($\omega_1 = 1$) |
| | | | | $p_2 = 0.102$ ($\omega_2 = 1.88$) |
| | M7: β | −7410.260 | | $p = 0.554$; $q = 0.427$ |
| | M8: β & ω>1 | −7401.981 | 16.56*** | $p_0 = 0.790$; $p = 0.664$; $q = 0.732$ |
| | | | | $p_1 = 0.210$ ($\omega_s = 1.43$) |
| **Old World *Lycium*** | M1a: nearly neutral | −2425.514 | | $p_0 = 0.373$ ($\omega_0 = 0.103$); $p_1 = 0.627$ ($\omega_1 = 1$) |
| | M2a: positive selection | −2401.474 | 48.08**** | $p_0 = 0.295$ ($\omega_0 = 0.105$); $p_1 = 0.506$ ($\omega_1 = 1$) |
| | | | | $p_2 = 0.199$ ($\omega_2 = 4.71$) |
| | M7: β | −2429.138 | | $p = 0.333$; $q = 0.195$ |
| | M8: β & ω>1 | −2403.221 | 51.83**** | $p_0 = 0.780$; $p = 0.332$; $q = 0.190$ |
| | | | | $p_1 = 0.220$ ($\omega_s = 4.43$) |

****$P < 0.0001$, df = 2, ***$P < 0.001$, df = 2.

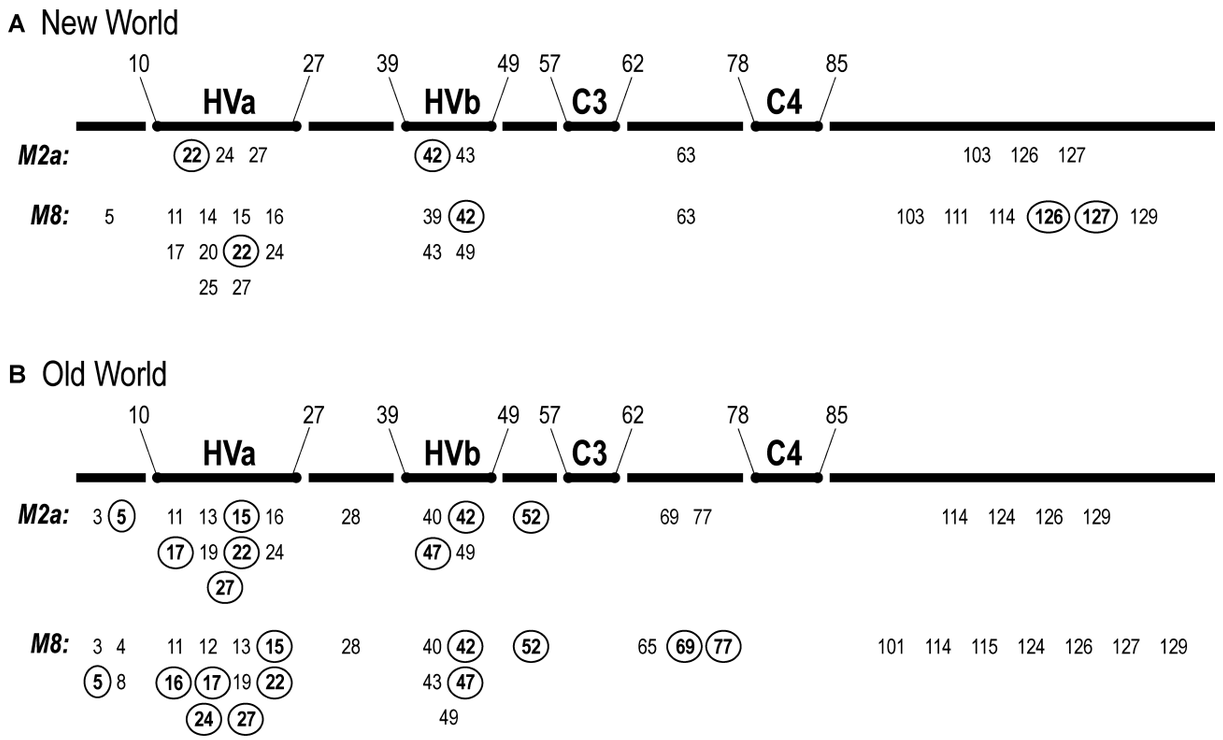


**Figure 5. Site-specific selection at the *S-RNase* locus in New World (A) and Old World (B) *Lycium*. The sequenced region of the *S-RNase* gene is drawn to scale, and the hypervariable (HVa and HVb) and conserved regions (C3 and C4) identified previously by Ioerger et al. (1991) are indicated using numbers above the diagrams. Numbers below the diagrams are those amino acid sites inferred to be under positive selection (models M2a and M8). Circled numbers are sites with a posterior probability greater than or equal to 0.95, whereas all other positions had 0.50 $\leq$ probabilities $<$ 0.95.**

1992). It is notable that published exceptions are from species with sporophytically controlled self-incompatibility systems as found in Asteraceae and Convolvulaceae. Our data for *Lycium* also suggest the maintenance of self-incompatibility following long-distance dispersal, and to our knowledge this is the only documented exception to Baker's rule in a species with gametophytically controlled self-incompatibility. Although it is not clear if differences between the two incompatibility systems should be expected, some authors have suggested that selection for increased dominance interactions among alleles can allow for greater mate availability under sporophytically controlled self-incompatibilty (Byers and Meagher 1992; Brennan et al. 2003), whereas in gametophytic systems, dominance among incompatibility alleles is unknown.

## S-RNASE DIVERSITY

We isolated 24 unique *S-RNase* sequences from 15 individuals and five species of African *Lycium*. Of the 19 individuals genotyped in the present study, 17 were heterozygous (Table 1). The putative alleles recovered for the African taxa were more similar (average pairwise amino acid identity 66%) than equivalent comparisons among New World alleles (48% among North American *L. parishii* alleles, Savage and Miller 2006; 44% among South American *L. cestroides*, this study). However, the estimate of nucleotide diversity ($\pi$) for Old World alleles (MEGA ver. 3.1, Kumar et al. 2004) was 0.330 and of similar magnitude to $\pi$ calculated for other species with GSI (ranges from 0.208 to 0.463 in Table 1 of Castric and Vekemans 2004).

Four of the five *L. ferocissimum* putative alleles are included in a large group of closely related Old World S-RNases (top clade in Fig. 3); these four sequences differ from each other at an average of 8.7 (range: 7–12) amino acid sites. Although quite similar, the majority of their differences lie within the hypervariable regions, which are known to be associated with specificity determination (Ioerger et al. 1991; Ida et al. 2001). Further, Matton et al. (1999) and Wang et al. (2001) have noted that changes in as few as five amino acid sites are sufficient to generate novel specificities. Our *S-RNase* data for *L. ferocissimum*, coupled with the crossing data demonstrating self-incompatibility in this species (Fig. 2), provide strong evidence that similar *S-RNase* sequence variants can have distinct functional phenotypes. That said, we obtained only partial *S-RNase* sequences, and more extensive sequencing could reveal additional differences.

## TRANSGENERIC EVOLUTION

Igic et al. (2003) documented that GSI is the ancestral condition in Solanaceae; in fact, GSI is ancestral for nearly three-quarters of eudicot lineages (Igic and Kohn 2001; Steinbachs and Holsinger 2002). GSI is a complex character requiring the coordinated action of several genes (McClure 2006) and, as such, it has been argued that this trait is more likely to be lost than gained over evolutionary time (Igic et al. 2006). The regain of self-incompatibility following its loss has never been documented in Solanaceae (Igic et al. 2006; see also Takebayashi and Morrell 2001), despite many empirical studies of the *S-RNase* locus among genera in this family (Ioerger et al. 1990; Richman et al. 1996a,b; Richman and Kohn 2000; Lu 2001; Wang et al. 2001; Igic et al. 2003; Stone and Pierce 2005; Savage and Miller 2006). Following the loss of self-incompatibility, polymorphism at the *S*-locus is expected to decline rapidly, because functionally distinct alleles are rendered selectively neutral (e.g., Igic et al. 2003, 2006; Charlesworth and Vekemans 2005). Empirical studies of *S-RNase* diversity following the transition to self-compatibility support this loss of allelic diversity (Golz et al. 1998; Kondo et al. 2002). If self-incompatibility were to be regained following its loss, then S-RNases in descendant taxa would lack transgeneric polymorphism (Igic et al. 2006).

In contrast, our finding of multiple transgeneric *S-RNase* lineages in Old World *Lycium* is consistent with the presence of self-incompatibility in the colonists. The majority of Solanaceae studied to date possess deep coalescence of S-RNases with many TGLs (Richman and Kohn 2000; Igic et al. 2003). Consistent with previous studies of self-incompatible *Lycium* species (Richman 2000; Savage and Miller 2006), *Lycium* S-RNases in the present study are distributed among many TGLs. However, S-RNases from New and Old World species are not distributed evenly. Whereas S-RNases isolated from New World species are present in all of the TGLs (11 TGLs in Fig. 3), those from Old World taxa are present in only four TGLs (Fig. 3). Further, this result is robust to subtleties in topology, as well as differences in sampling between geographic regions. Our results show clearly that the number of S-RNase TGLs in the Old World is at most half (4 TGLs) of the number recovered for New World sequences (mean = 10.04, 95% CI 9.8–10.2; Fig. 4). Further, Old World S-RNases are nested within S-RNases from New World *Lycium* (Fig. 3), a pattern expected if dispersal to and colonization of the Old World occurred relatively recently. Figure 3 shows that there are six "transgeographic" lineages (i.e., well-supported lineages including both Old and New World *Lycium* S-RNases), which implies a minimum of three colonists (each with two different New World allelic lineages). However, we note that uncertainty in the genealogy, as well as limited *S-RNase* sampling, will affect this estimate; additional sampling and simulations may allow for more robust estimates of the initial population size of colonists.

This pattern of reduced TGLs in Old World *Lycium* is similar to that observed in *Physalis* (Richman and Kohn 2000) and *Witheringia* (Stone and Pierce 2005) species, which together share a reduced set of TGLs (see also Fig. 3). Richman and colleagues (1996b) have suggested that a population bottleneck occurred in the common ancestor of *Physalis* and *Witheringia*, a result consistent with their close evolutionary relationship (Olmstead et al. 1999). Despite the loss of considerable transgeneric diversity in these taxa, allelic diversity persists and demonstrates the maintenance of GSI following a bottleneck (Richman et al. 1996b).

## SELECTION ANALYSES OF THE S-RNASE GENE

Positive selection can be difficult to detect in coding genes, given that most sites are expected to be under purifying selection and relatively few sites are expected to undergo positive selection. The site-specific analyses implemented in PAML have proven useful for inference of positive selection in the *S-RNase* gene (Takebayashi et al. 2003; Savage and Miller 2006; Igic et al. 2007) and other coding regions (Yang and Bielawski 2000). Our selection analyses indicate positive selection at certain sites in the *S-RNase* gene for both Old and New World S-RNases (Table 2; Fig. 5). Interestingly, comparison of the Old and New World datasets indicates relatively intense positive selection in Old World alleles. Specifically, the proportion of sites falling into the positive selection class was higher for the Old World compared to New World S-RNases (model M2a: $p_2 = 0.199$ vs. 0.102, respectively; Table 2). Further, the $d_N/d_S$ rate ratio was higher for Old World compared to New World alleles (model M2a: $\omega_2 = 4.71$ vs. 1.88, respectively; model M8: $\omega = 4.43$ vs. 1.43; Table 2). Thus, it appears that intense positive selection has occurred among Old World S-RNases. Such a result may be indicative of the rediversification of Old World S-RNases following colonization and establishment.

Given that the average amino acid pairwise distance was only 7.5% among the similar group of Old World S-RNases (top clade in Fig. 3), we also used PAML to analyze S-RNases in this clade to examine patterns of selection in these closely related sequences. Our analysis rejects the hypothesis that Old World S-RNases in this large clade are evolving neutrally (M1a vs. M2a: LRT = 38.245, $P < 0.0001$; M2a parameters, $p_2 = 0.09$ and $\omega_2 = 12.26$; M7 vs. M8: LRT = 38.280, $P < 0.0001$; M8 parameters, $p_1 = 0.089$ and $\omega_s = 12.43$). Thus, despite their similarity, these S-RNases have an excess of nonsynonymous substitutions as measured by the $d_N/d_S$ rate ratio. As across the dataset for all Old World *Lycium*, analysis of the large group of Old World sequences suggests positive selection favoring new specificities, consistent with the rediversification of alleles following a founder event.

## CONCLUSIONS

Baker (1955, 1967) was the first to suggest that self-fertility facilitates the successful establishment of species following long-distance dispersal. Although the transition from

self-incompatibility to self-compatibility has occurred often in the history of Solanaceae (Igic et al. 2006), our data for *Lycium* demonstrate an exception to Baker's ideas, indicating instead the maintenance of GSI following dispersal from the New World to the Old World. However, the transgeneric diversity of S-RNases in Old World *Lycium* is limited in comparison to New World alleles, a result consistent with a genetic bottleneck coincident with dispersal of *Lycium* to the Old World. These data are some of the first from a natural population to suggest that allelic differences in as few as 7–8 amino acids are sufficient for generation of novel allele specificities.

It is interesting to speculate about factors that may have facilitated the maintenance of self-incompatibility in Old World *Lycium*. Species of *Lycium* are typically shrubs that produce many-seeded yellow to red (sometimes black) fleshy berries, which are generally bird dispersed. Although repeated migration between southwestern North America and Argentina and Chile has been suggested (Levin and Miller 2005), all Old World *Lycium* sampled to date comprise a monophyletic group. In addition, all *Lycium* are long-lived perennials, and Bowers (2005) and Bowers et al. (1995) have recorded considerable longevity (120–211 years) for species of southwestern North American *Lycium*. These factors may well have aided the retention of self-incompatibility in *Lycium*, as a single multiseeded fruit could carry several incompatibility alleles; likewise, the presence of longevity increases opportunities for mating among colonists.

Although there have been a number of studies documenting self-incompatibility in American *Lycium* (Richman 2000; Aguilar and Bernardello 2001; Miller and Venable 2002; Savage and Miller 2006), this study is the first to examine breeding systems among Old World *Lycium* species. The finding of self-incompatibility in two diploid, cosexual African *Lycium* species, *L. ferocissimum* and *L. pumilum*, is consistent with a model for the evolution of gender dimorphism proposed by Miller and Venable (2000), which assumes self-incompatibility in cosexual, diploid species. In this model, gender dimorphism evolves following the loss of self-incompatibility in diploid, cosexual species. Polyploidy is implicated as the trigger for the loss of self-incompatibility, and considerable support for the breakdown of incompatibility with polyploidy exists in families with GSI (see references in Miller and Venable 2000, 2002, and Table 1 in Mable 2004). Among American species of *Lycium*, available data are consistent with the evolution of gender dimorphism as proposed by Miller and Venable (2000). Results of the present study suggest that this model may also explain the distribution of cosexual and dimorphic species in African *Lycium*.

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**Appendix. Raw data associated with Fig. 2. Values are average fruit production per flower or average seed number per fruit produced on *Lycium ferocissimum* and *L. pumilum* following outcross or self pollination or in unmanipulated controls. Values in parentheses are standard errors. Sample sizes are given and correspond to either the number of flowers for each treatment (fruit production) or the number of fruits in which seeds were counted (seed number).**

| Species | Fruit production | | | Seed number | | |
|---|---|---|---|---|---|---|
| | Outcross | Self | Control | Outcross | Self | Control |
| *Lycium ferocissimum* | 0.75 (0.03) | 0.08 (0.02) | 0.27 (0.03) | 43.1 (1.5) | 7.9 (2.5) | 34.6 (2.3) |
| | $n = 163$ | $n = 174$ | $n = 179$ | $n = 115$ | $n = 14$ | $n = 43$ |
| *Lycium pumilum* | 0.60 (0.04) | 0.05 (0.02) | 0.18 (0.03) | 14.7 (0.6) | 1.9 (0.7) | 4.4 (0.8) |
| | $n = 136$ | $n = 165$ | $n = 177$ | $n = 71$ | $n = 8$ | $n = 25$ |