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ASSOCIATION OF PLOIDY AND SEXUAL SYSTEM IN *LYCIUM CALIFORNICUM* (SOLANACEAE)

KAI YEUNG,[1] JILL S. MILLER,[2] ANNA E. SAVAGE,[2] BRIAN C. HUSBAND,[3] BORIS IGIC,[1] AND JOSHUA R. KOHN[1,4]
[1]*Section of Ecology, Behavior, and Evolution, Division of Biological Sciences, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0116*
[2]*Department of Biology, Amherst College, Amherst, Massachusetts 01002*
[3]*Department of Integrative Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada*

Abstract.—In North American *Lycium* (Solanaceae), the evolution of gender dimorphism has been proposed as a means of restoring outcrossing after polyploidization causes the loss of self-incompatibility. Previous studies of this process in *Lycium* focused on comparisons between species that differ in ploidy. We examined intraspecific variation in floral morphology and DNA content in populations of *L. californicum* to determine correlations between sexual system and cytotype. We also used nuclear ITS and GBSSI sequence data to determine whether diploid and polyploid forms represent the same phylogenetic species, and the phylogeographic relationships among populations and ploidy levels. Within populations, no variation in ploidy was found, although among populations there was a perfect correspondence between sexual system and cytotype. Diploid populations were all hermaphroditic, whereas tetraploid populations were all gender dimorphic. There was no clear geographic pattern to the occurrence of diploid and tetraploid forms. Phylogenetic analysis confirms that *L. californicum*, regardless of ploidy, forms a monophyletic group within the genus *Lycium*. Sequences from diploid and polyploid individuals did not form reciprocally monophyletic clades, indicating either multiple gains of polyploidy, ongoing gene flow between cytotypes, or lack of lineage sorting since the evolution of polyploidy. The correspondence between ploidy and sex expression is consistent with the hypothesis that polyploidization triggers the evolution of gender dimorphism in this and other *Lycium* species.

Key words.—DNA content, gender dimorphism, *Lycium*, *Lycium californicum*, polyploidy, self-incompatibility.

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The evolution of gender dimorphism has significant implications for a plant's life history, invasiveness, biotic interactions, physiology, and morphology (Geber et al. 1999). Although cosexuality (hermaphroditism) is far more common, gender dimorphism is widespread, occurring in nearly half of all angiosperm families (Renner and Ricklefs 1995). Relatively few empirical studies have focused on polyploidy as a possible cause for transition to a dimorphic sexual system. This trigger of gender dimorphism was proposed in studies of North American *Lycium* (Miller and Venable 2000, 2002; Miller 2002). Of the approximately eighteen North American species, three (*L. exsertum*, *L. fremontii*, and *L. californicum*) are polyploid (Lewis 1961; Chiang-Cabrera 1981), gender dimorphic (Miller and Venable 2000), and self-compatible (SC; Miller and Venable 2002). All other North American *Lycium* are cosexual and at least some are self-incompatible (Richman 2000; Miller and Venable 2002). Of the cosexual species, all 10 examined were found to be diploid (Chiang-Cabrera 1981). Following these observations, a model was proposed in which the ancestor of the dimorphic species acquired polyploidy which caused the loss of self-incompatibility (SI; Miller and Venable 2000). In the Solanaceae, polyploidy, or any duplication of the S-locus, often causes the breakdown of gametophytic self-incompatibility (GSI; Golz et al. 2001; Mable 2004). Collapse of SI was hypothesized to result in heightened selfing and the expression of inbreeding depression, providing the selective force for the evolution of gender dimorphism (Miller and Venable 2000). In phylogenetic analyses using morphological characters and sequence data from the internal transcribed spacer region of nuclear ribosomal DNA (nr-ITS; Miller and Venable 2000, Miller 2002), the three dimorphic, polyploid species of *Lycium* were thought to form a monophyletic group. Thus, polyploidy and gender dimorphism were hypothesized to have arisen a single time in North American *Lycium* (Miller and Venable 2000; Miller 2002).

More recently, in an analysis including both a broader taxonomic sample of *Lycium* species and sequence data from both a chloroplast (*trnT–trnF*) and a nuclear (granule-bound starch synthase I; GBSSI) locus, Levin and Miller (2005) found that the placement of *L. californicum* disagrees with previous studies. Specifically, the GBSSI data place *L. californicum* apart from the other North American dimorphic species with strong support, suggesting two independent origins of both polyploidy and gender dimorphism in North American *Lycium*.

Prior to the study reported here, *L. californicum* was thought to be predominantly polyploid and gender dimorphic (Miller and Venable 2000, 2002, 2003). The only exception was a single report of a diploid accession from northcentral Mexico (Chiang-Cabrera 1981) whose gender expression was not studied. Examination of a *L. californicum* population at the University of California's Scripps Coastal Reserve in La Jolla, California (population LJ, Table 1) revealed that this population has substantial variation at the S-locus, and observations of pollen tube growth following self- and cross-pollination showed it to be strongly self-incompatible (K. Yeung et al., unpubl. ms). In addition, all plants appeared hermaphroditic with no apparent gender dimorphism. Because S-locus variation is expected to be rendered neutral and collapse following the loss of self-incompatibility (Igic et al. 2003), the La Jolla population raised several questions that we explore here. First, is there variation among populations in gender expression with some populations being

[4] Corresponding author: E-mail jkohn@ucsd.edu.

TABLE 1. Location, DNA content (mean ± SD) per 2C nuclei, and inferred ploidy levels in thirteen populations of *Lycium californicum*. *Lycium andersonii* and *L. fremontii*, reported to be diploid and octoploid respectively (Chiang-Cabrera 1981), were sampled for comparison.

Population	General location	Coordinates	Base DNA content	Inferred ploidy	Plants sampled
LJ	La Jolla, CA	N 32°50′02″ W 17°16′53″	2.81 ± 0.14	2X	25
TJ	Tijuana River Estuary, CA	N 32°34′04.8″ W 117°07′23.7″	2.65 ± 0.14	2X	6
SR	S. of Rosarito, Baja California	N 32°15′9″ W 116°58′27.7″	3.03	2X	1
SSC	S. of Colonet, Baja California	N 30°52′43.2″ W 116°05′35.7″	2.95 ± 0.11	2X	15
NCR	Campo Costa Rica, Baja California	N 30°11′41.0″ W 115°47′25.3″	2.78 ± 0.33	2X	20
ElR	El Rosario, Baja California	N 30°05′09.7″ W 115°41′18.9″	3.00	2X	1
ND	E. of El Rosario, Baja California	N 30°05′10.5″ W 115°38′49.3″	5.99 ± 0.30	4X	19
TS	E. of El Rosario, Baja California	N 30°04′20.2″ W 115°37′26.4″	5.94 ± 0.12	4X	20
CD	SW. of Catavina, Baja California	N 29°36′33.2 W 114°35′03.0″	3.11 ± 11	2X	5
SD	W. of Bahia de Los Angeles, Baja California	N 29°01′55.9″ W 113°50′27.1″	3.15	2X	1
NB	W. of Bahia de Los Angeles, Baja California	N 29°01′59.6″ W 113°49′09.2″	3.14 ± 0.16	2X	7
HR	Houser Road, Tucson AZ	N 32°46′48″ W 111°37′48″	5.93 ± 0.06	4X	4
OP	Organ Pipe National Monument, AZ	N 32°14′38.5″ W 112°68′37″	3.15 ± 0.07	2X	15
L. andersonii	Houser Road, Tucson AZ	N 32°46′48″ W 111°37′48″	3.06 ± 0.08	2X	2
L. fremontii	Houser Road, Tucson AZ	N 32°46′48″ W 111°37′48″	13.82 ± 0.58	8X	2

monomorphic (hermaphroditic) and others dimorphic (gynodioecious or dioecious)? Second, is there also variation among populations in ploidy and does ploidy and sex expression covary as predicted, with diploid populations monomorphic and tetraploid populations dimorphic? Third, what is the geographic distribution of gender and ploidy? Fourth, do diploid and polyploid populations represent a monophyletic species or group relative to other members of *Lycium* and what are the phylogenetic relationships among sequences from diploid and polyploid individuals and populations?

MATERIALS AND METHODS

Lycium californicum is a perennial shrub found in sage scrub habitat on Pacific coastal bluffs, in saline inland flats, and in semideserts. Its range extends from Santa Monica, California, southward to the tip of Baja California, eastward to southcentral Arizona, and in mainland Mexico along the western coast and in the northcentral states of Coahuila, Zacatecas, and San Luis Potosi (Chiang-Cabera 1981). Bushes are typically <2 m tall but spread laterally to form large (often >2 m wide) individuals. Flowers are white to lavender (Hickman 1993).

Young leaves of *Lycium californicum* were collected between 26 March and 4 April 2004 from 13 field locations in coastal and desert regions in Southern California, northern and central Baja California, and southern Arizona (Table 1). Plants were sampled haphazardly with a minimum distance of 1 m between individuals. Leaves were stored at 2°C until processed. *Lycium fremontii* and *L. andersonii* leaves were collected from a single site in Arizona (population HR, Table 1) where they co-occur with *L. californicum*. *Lycium andersonii* is reported to be diploid (Chiang-Cabera 1981) and self-incompatible (Richman 2000), whereas *L. fremontii* is octoploid (Chiang-Cabera 1981) and gender dimorphic (Miller and Venable 2000, 2002, 2003).

To determine DNA content, the flow cytometry protocol of Dart et al. (2004) was used. Isolated nuclei from each individual were stained with the fluorescent dye propidium iodide and DNA content assessed as the magnitude of fluorescence. To estimate the base DNA content of samples, the ratio of the position of the first peak of fluorescence for *Lycium* to the first peak of fluorescence for *Sorghum* Pioneer 8695 was calculated and multiplied by the DNA content of *S. bicolor* (1.74 pg/2c; Johnston et al. 1999) used as either an internal or external standard. In assigning ploidy, those populations with similar DNA content to *L. andersonii* were classified as diploids whereas those populations having twice that DNA content were classified as tetraploids.

We inferred population gender expression by quantifying variation in floral morphology for eight of the 13 populations where ploidy level was determined. In the remaining populations, few plants were blooming when collections were made, prohibiting the determination of population sexual system. A total of 583 freshly blooming flowers from 151 individuals were collected between 22 January 2003, and 4 April 2004. Flowers were preserved in ethanol and seven

floral characters were measured using a dissecting scope with an ocular micrometer: anther width, anther length, filament length, corolla tube length, total flower length, stigma width, and style length. These are the same features measured in a previous study of a dimorphic population (Miller and Venable 2003) which we resampled here (population HR, Table 1). An average of four flowers from each plant was used to generate plant mean floral measurements for analysis.

Among-plant correlation matrices of floral characters were generated to infer population sexual system. A pattern of significant negative correlations between male and female floral characters indicates gender dimorphism (Miller and Venable 2003) because female plants produce large female organs and small sterile anthers, whereas males produce reduced female organs and functional anthers. Nonsignificant or positive correlations indicate gender monomorphism. We also plotted the anther length distributions for each population with the prediction that gender dimorphic populations would show bimodal distributions while monomorphic populations would be unimodal.

To determine whether diploid and polyploid accessions of *Lycium californicum* represent a monophyletic group, and the phylogenetic relationships among accessions from different populations, we sequenced both the nr-ITS region and part of the granule-bound starch synthase gene (GBSSI). Both markers are commonly used in plant phylogenetic studies and GBSSI appears to be a single copy gene in Solanaceae (Mason-Gamer et al. 1998). Eleven diploid and seven polyploid accessions from a total 10 populations were included for *L. californicum*. In addition, 17 new world *Lycium* species were included as outgroups (for species names, see Fig. 2).

DNA extractions were performed using either the Qiagen DNeasy plant mini kit (Qiagen, Inc., Valencia, CA) or following Miller (2002). Amplification of the ITS region followed conditions cited in Miller (2002) and involved two primer pairs: N-nc18s10 and C26A from Wen and Zimmer (1996) or ITS4A 5′-GTCCACTGAACCTTATCATTTAG-3′ (L. Bohs, University of Utah, Salt Lake City, UT) and IT-Sleu1 from Bohs and Olmstead (2001). For the nuclear GBSSI gene, the 3′ end of exon 3 through the 5′ end of exon 8 was amplified following conditions in Levin and Miller (2005) and using the 622-B primer from Peralta and Spooner (2001) and a modified version of their 1555-CR primer (primer CR; Levin and Miller, in press). Amplification products were cleaned using either PEG precipitation and ethanol cleanup (Morgan and Soltis 1993) or the QIAquick PCR purification kit (Qiagen, Inc.). ITS cycle-sequencing was done with both amplification primers N-nc18s10 and C26A; when amplification was done with ITS4a and ITSleu1, sequencing was done with the internal primers ITS4 (White et al. 1990) and ITS5HP (Hershkovitz and Zimmer 1996). Cycle-sequencing of GBSSI was done with both amplification primers 622-B and CR. Sequencing was completed on an ABI automated sequencer by the Biotechnology Resource Center, Cornell University, Ithaca, New York. In total, 684 bases of ITS and 964 of GBSSI were combined for analysis. Genbank accession numbers for the sequences in the present study are: DQ124618–53 for ITS; DQ124501–3, DQ124508–13, DQ124520–1, DQ124524, DQ124529–32, DQ124534–5, DQ124543, DQ124546, DQ124549, and DQ127263–77 for GBSSI.

Phylogenetic analyses were performed using maximum-parsimony, maximum-likelihood, and Bayesian methods as implemented in PAUP* version 4.0b10 (Swofford 2002) and Mr. Bayes version 3.0b4 (Huelsenbeck and Ronquist 2001). Parsimony analyses were conducted using heuristic searches with 1000 random addition sequence replicates and TBR branch-swapping. Due to large numbers of equal length trees, each addition replicate was limited to 200 trees that were greater than or equal to the shortest trees for each replicate (Zimmer et al. 2002). Internal support for branching relationships was determined using bootstrap analysis (Felsenstein 1985). Bootstrap values were determined from 500 full heuristic bootstrap replicates, each with 10 random addition sequence replicates. The MulTrees option was not in effect. Maximum-likelihood model parameters were determined using the Akaike information criterion implemented in Modeltest version 3.5 (Posada and Crandall 1998). The best model (HKY + I + G) was used in a likelihood analysis in PAUP* with 20 random addition heuristic search replicates, TBR branch-swapping, and the MulTrees option in effect. Bayesian analysis was conducted using four simultaneous Markov chain Monte Carlo (MCMC) chains each starting from a random tree. We used a general time reversible substitution model and gamma-distributed rate variation across sites. Two million generations were run and a tree every 100 generations was saved. Trees that preceded stabilization of likelihood values were excluded, and the remaining trees were used to construct the 50% majority rule consensus tree in PAUP*. We report Bayesian posterior probabilities.

Results

Leaf tissue of *L. andersonii*, a representative diploid, was found to have base DNA content of 3.06 ± 0.08 (mean±SE picograms/2C; Table 1). *Lycium californicum* populations LJ, TJ, SR, SSC, NCR, CD, SD, NB, and OP had similar base DNA content to *L. andersonii* (population mean estimates ranged from 2.65 to 3.15 picograms/2C; Table 1) and were therefore classified as diploids. Populations HR, TS, and ND, had twice the DNA content per 2C nuclei (means ranged from 5.93–5.99 picograms/2C; Table 1) and were classified as tetraploids. *Lycium fremontii* had over 4.5 times the DNA content of *L. andersonii* (13.82 ± 0.58; Table 1), close to that expected for an octoploid. Although *L. californicum* populations vary in cytotype, no variation in ploidy was found within any population. Estimates from individuals assigned alternative ploidy levels always differed by more than eight standard deviations (Table 1).

The occurrence of populations of *L. californicum* with different ploidy levels does not seem to follow any obvious geographic pattern. Diploid populations were found in sage scrub habitats along the Pacific coast of southern California and northern Baja California (populations LJ, TJ, SR, SSC, NCR, and ElR) and also in the desert regions of northcentral Baja California (populations CD, SD, and NB). Tetraploid populations ND and TS were found close (12–14 km) to the Pacific coast and within 17 km of diploid populations in Baja

California. In the Sonoran desert of Arizona, both diploid (OP) and tetraploid (HR) populations occur (Table 1).

Diploid populations exhibited positive correlations between most floral characters including male (anther width, anther length, and filament length) by female (stigma width and style length) pairings. The few negative correlations were small in magnitude and nonsignificant (Fig. 1a). In contrast, correlations between male and female characters were generally large and negative for tetraploid populations (Fig. 1a). Male flowers in tetraploid populations generally had larger corollas since corolla length was positively correlated with male, and negatively correlated with female, characters. Tetraploid populations exhibited larger ranges of anther lengths than did diploid populations (Fig. 1b), as well as gaps in anther size distributions between 0.8 mm and 1.1 mm. Tetraploid plants with anthers <0.8 mm were females, plants with anthers >1.1 mm were males (Fig. 1b).

Parsimony, likelihood, and Bayesian analyses all recovered similar topologies (Fig. 2). Dimorphic and monomorphic *L. californicum* accessions together formed a monophyletic clade with strong support (1.0 Bayesian posterior probability, 98% parsimony bootstrap). Five of seven tetraploid accessions were monophyletic in the likelihood and Bayesian analyses, despite coming from widely geographically separated populations (Tucson, AZ, and near the west coast of Baja California). Diploid populations OP (Arizona) and CD (central Baja) group together and are allied with the clade of five tetraploid accessions. Phylogenetic groupings within *L. californicum* must be taken as provisional, however, as little to no bootstrap support was found among *L. californicum* accessions using maximum parsimony.

Much of the variation found in the species could be recovered from a small sample within a single population. The three sequences from the tetraploid population TS are dispersed among three different clades as distinguished by Bayesian methods. Conversely, the two accessions from population HR and the three accessions from population LJ showed little within-population variation. As found by Levin and Miller (2005), the evolution of polyploidy and gender dimorphism in *L. californicum* apparently represent independent transitions from diploidy and monomorphism relative to *L. fremontii* and *L. exsertum*.

Discussion

Variation in Ploidy and Sexual System

To the extent sampled, each population is of uniform ploidy, with some populations diploid and others tetraploid (Table 1). In every case, analysis of floral characters indicates that tetraploid populations are dimorphic while diploid ones are monomorphic. This is consistent with the ploidy-triggered gender transition pathway of Miller and Venable (2000) because gender dimorphism was never observed in diploid populations.

Absence of variation in ploidy within populations is in agreement with theoretical and experimental data which indicate that populations at equilibrium largely fix on a single ploidy level (Levin 1975; Fowler & Levin 1984; Felber 1991; Burton and Husband 1999; Husband 2000). If matings between cytotypes have low fertility, the minority form will tend to be excluded (Levin 1975). If there is ecological differentiation among cytotypes, populations might also tend to be of uniform ploidy.

Our study of ploidy only partly agrees with prior karyotypic analysis of *L. californicum* using pollen mother-cell staining (Chiang-Cabrera 1981). That study found haploid chromosome numbers of 12 and 24 in *L. californicum*. However, only one diploid population in the species was reported, located in western mainland Mexico beyond the range sampled in this study. Chiang-Cabrera (1981) also reported a tetraploid population just south of Rosarito, Baja California, Mexico, whereas we found the same population, or a population in the immediate vicinity (population SR; Table 1), to be diploid. Most of the populations sampled by Chiang-Cabrera (1981) were located beyond the range of those studied here, so the extent of discrepancy between ploidy estimates from our study and Chiang-Cabrera's (1981) is not yet known. Clearly more extensive sampling, from a greater fraction of the range of the species, will be needed in order to uncover any geographic or ecological patterns of ploidy variation.

Phylogenetic Analysis

The phylogeny generated from nuclear ITS and GBSSI sequences suggests at least two instances of parallel evolution of polyploidy and gender dimorphism in North American *Lycium*, at least once in *L. californicum* and once in the ancestor of *L. exsertum*, and *L. fremontii* (see also Levin and Miller 2005). Our phylogeny reveals that although *L. californicum* is monophyletic, the different ploidy levels within this taxon may not form reciprocally monophyletic groups. There is some interdigitation of monomorphic and dimorphic accessions (Fig. 2), and different accessions from a single population (TS) contain much of the variation found in the species. These observations can be explained by three nonexclusive hypotheses: (1) There may have been multiple transitions of both ploidy and gender in *L. californicum*; (2) There may be exchange of genes between populations of different ploidy (reviewed in Ramsey and Schemske 1998); and (3) The interdigitation of sequences from populations of different cytotype and sexual system may be due to low divergence time resulting in incomplete lineage sorting since the origin of polyploid, dimorphic, *L. californicum*.

Miller and Venable's (2000) hypothesis is consistent with the perfect association between cytotype and population sex expression. The hypothesis is also supported by the observation that hermaphrodites in the diploid LJ population are strongly SI (Yeung et al., unpubl. ms) whereas those in the dimorphic population HR are self-compatible (Miller and Venable 2002). Nevertheless, there remain serious difficulties in understanding the sequence of events that lead from diploid, self-incompatible, cosexual populations to polyploid, self-compatible, and sexually dimorphic ones. The model assumes selfing avoidance following the loss of self-incompatibility drives the evolution of gender dimorphism in tetraploids (Miller and Venable 2000). Most models of the evolution of dioecy to ensure outcrossing require both high levels of selfing and high inbreeding depression (Schultz 1994). Polyploids are usually predicted to suffer less from inbreed-

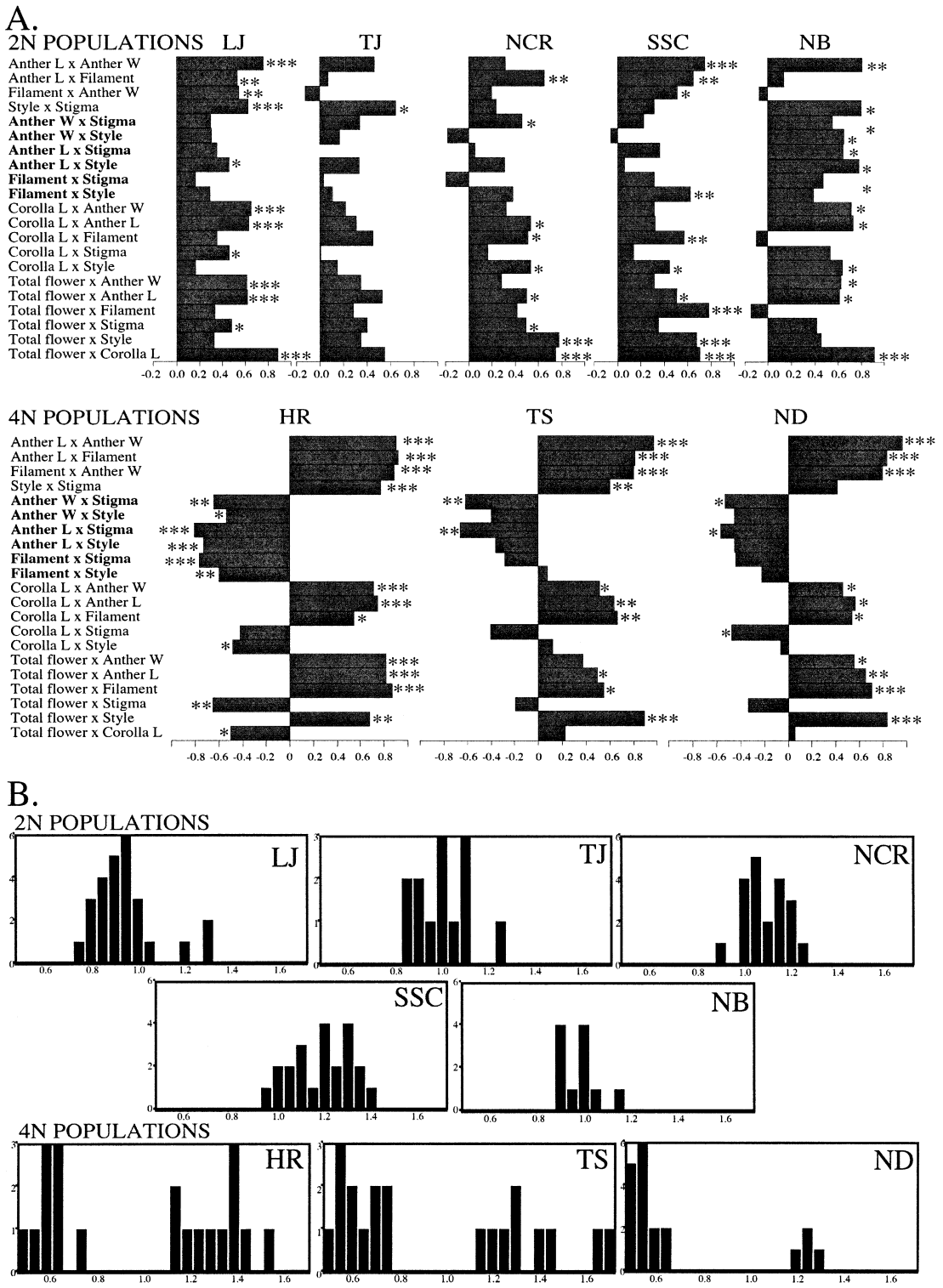

FIG. 1. (A) Pairwise correlations of nine morphological characters (Anther L, anther length; Anther W, anther width; Filament, filament length; Stigma, stigma width; Style, style length; Corolla L, corolla length; Total flower, total flower length) in diploid (2X) and tetraploid (4X) populations. Significance is denoted at three levels of probability: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Numbers of individuals and flowers measured per diploid (2*n*) population are: LJ, 26, 123; TJ, 13, 43; NCR, 21, 78; SSC, 22, 85; and NB 11, 30. Note axis range is −0.2 to 1. In tetraploid (4*n*) populations, numbers of individuals and flowers measured per population are: HR, 20, 81; TS, 20, 70; and ND 19, 73. Note axis range is −0.8 to 1. Correlations between male and female organs are in boldface. (B) Frequency distributions of average anther lengths (mm) for diploid and tetraploid populations of *Lycium californicum*. In tetraploid populations, there is both increased variation and an absence of individuals with intermediate anther lengths, indicative of gender dimorphism.

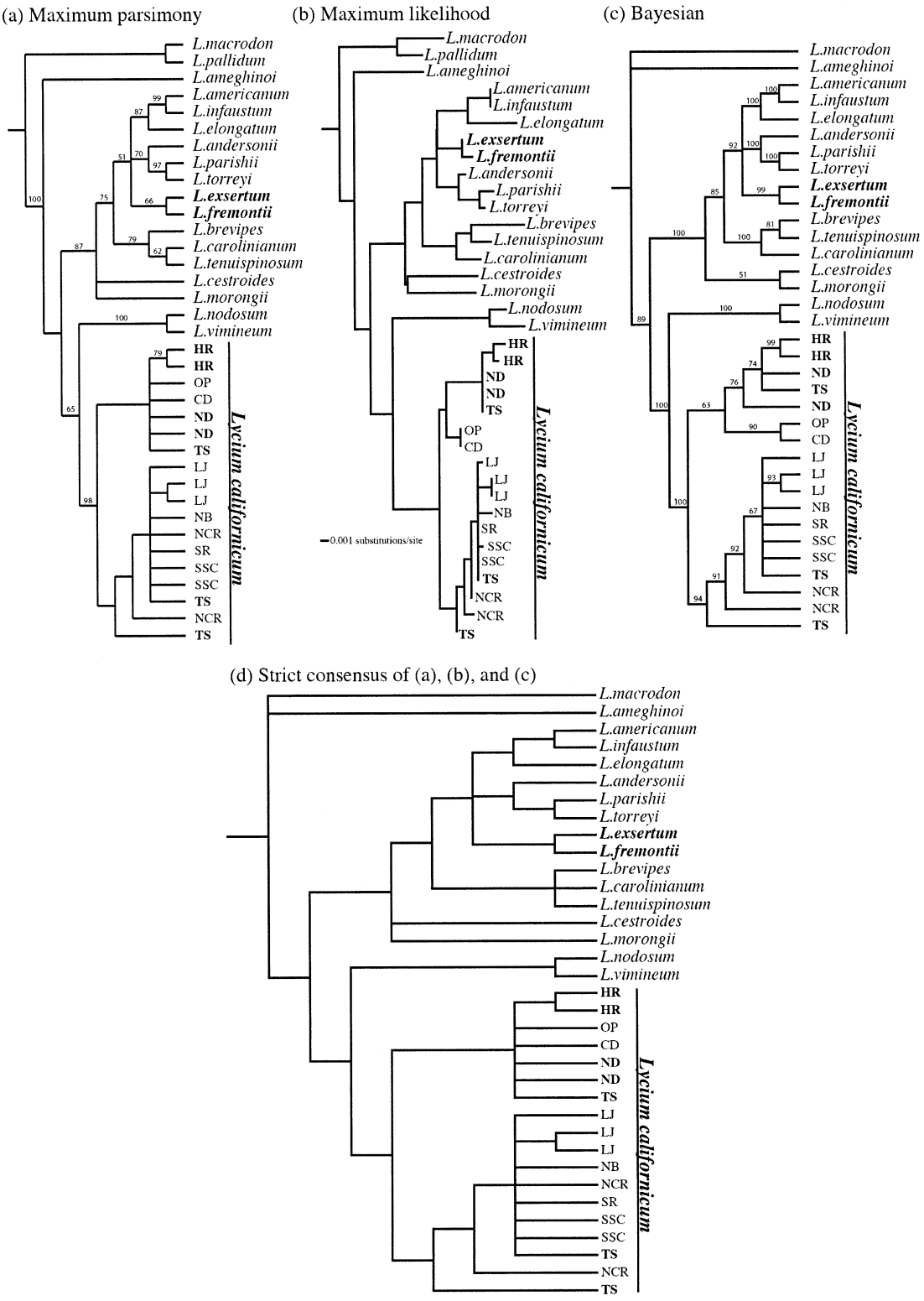

FIG. 2. Phylogeny of 18 New World *Lycium* species including 18 accessions from 10 populations of *L. californicum*. *Lycium californicum* accessions are identified by population as in Table 1. Polyploid species and populations of *L. californicum* in boldface. (a) Maximum parsimony cladogram with bootstrap values >50% indicated. (b) Maximum-likelihood phylogram. (c) Bayesian cladogram with posterior probabilities indicated. (d) Strict consensus of trees from 2a–c.

ing depression than are diploids (but see Ronfort 1999), but may still harbor sufficient genetic load for inbreeding depression to be above the 0.5 threshold value necessary to favor dioecy in many models (Husband and Schemske 1997). However, if levels of selfing and inbreeding depression are high when tetraploidy originates, it is difficult to imagine how tetraploidy offers sufficient selective advantage to favor its spread in the face of this large fitness cost. Conversely, if levels of inbreeding depression were not high in the diploid, self-incompatible ancestral population, it is difficult to imagine why self-compatibility would not have evolved in this ancestor (Porcher and Lande 2005). The process would appear more likely to occur if the gain of polyploidy were accompanied by the lowering of inbreeding depression from > 0.5 in the diploid to < 0.5 in the neopolyploid. Subsequent fixation and mutation accumulation could increase the value of inbreeding depression, favoring the evolution of gender dimorphism. Further phylogeographic studies can determine whether polyploidy and gender dimorphism arose one or many times in this species. Experimental studies are needed to measure the selective forces involved.

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