RELATIONSHIPS WITHIN TRIBE LYCIEAE (SOLANACEAE): PARAPHYLHY OF LYCIUM AND MULTIPLE ORIGINS OF GENDER DIMORPHISM

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We infer phylogenetic relationships among Lycium, Grabowskia, and the monotypic Phrodus microphyllus, using DNA sequence data from the nuclear granule-bound starch synthase gene (GBSSI, waxy) and the chloroplast region trnT-trnF. This is the first comprehensive molecular phylogenetic study of tribe Lycieae (Solanaceae). In addition to providing an understanding of evolutionary relationships, we use the phylogenetic hypotheses to frame our studies of breeding system transitions, floral and fruit evolution, and biogeographical patterns within Lycieae. Whereas Lycium is distributed worldwide, Phrodus and the majority of Grabowskia species are restricted to South America. Tribe Lycieae is strongly supported as monophyletic, but Lycium likely includes both Grabowskia and Phrodus. Results also suggest a single dispersal event from the Americas to the Old World, and frequent dispersal between North and South America. The diversity of fruit types in Lycieae is discussed in light of dispersal patterns and recent work on fruit evolution across Solanaceae. Dimorphic gender expression has been studied previously within Lycium, and results indicate that transitions in sexual expression are convergent, occurring multiple times in North America (a revised estimate from previous studies) and southern Africa.

Key words: GBSSI; gender dimorphism; Grabowskia; Lycium; Phrodus; Solanaceae; trnT-trnF; waxy.

Tribe Lycieae A.T. Hunziker (Solanaceae) includes Lycium (ca. 80 spp.), Grabowskia (four spp.) and Phrodus (one sp.) (Hunziker, 2001). In recent years, the genus Lycium has received considerable attention; there have been studies of breeding system evolution (Miller and Venable, 2000, 2002), sexual dimorphism (Miller and Venable, 2003), species interactions (Nogales et al., 1998), phylogenetics (Bernardello and Chiang-Cabrera, 1998; Fukuda et al., 2001; Miller, 2002), and self-incompatibility systems (Richman, 2000; Richman and Kohn, 2000). Lycieae is an ideal group within which to study evolutionary relationships, and a phylogenetic hypothesis will provide the necessary framework for future evolutionary, ecological, and developmental studies. In the present study, such a hypothesis will be immediately applicable to questions regarding the evolution of gender dimorphism, fruit evolution, and the biogeography of the tribe.

Within Lycieae, both Grabowskia and Phrodus are predominantly South American, with Phrodus endemic to Chile, and most Grabowskia species limited to Argentina and adjacent countries. However, G. boerhaviaefolia is fairly widespread, occurring in a small area of southern Mexico, the Galapagos Islands, Peru, Bolivia, Chile, and Argentina (Hunziker, 1997, 2001). In contrast to the more restricted distribution of Grabowskia and Phrodus, the large genus Lycium is distributed in temperate and subtropical regions worldwide. The genus is disjunct between the northern and southern hemispheres, since Lycium is absent from both the Old and New World tropics. Areas of greatest species richness are in South America (Hitchcock, 1932; Bernardello, 1986), southwestern North America (Hitchcock, 1932; Chiang-Cabrera, 1981), and southern Africa (Venter, 2000), with fewer species in Eurasia (Feinbrun, 1968; Zhang et al., 1994) and at least two taxa found primarily on islands (Hitchcock, 1932; Yamazaki, 1991; Nogales et al., 1998). Accordingly, previous taxonomic treatments of Lycium have been regional in focus (Hitchcock, 1932; Feinbrun, 1968; Chiang-Cabrera, 1981; Bernardello, 1986; Venter, 2000). As in Grabowskia and Phrodus, Lycium species are long-lived perennial shrubs or small trees, and the majority inhabit arid to semiarid environments, though some are halophytic and found in coastal saline habitats. Plants are usually hermaphroditic with perfect flowers; however, there are a few species in North America and southern Africa that are dimorphic in gender expression (Miller and Venable, 2000, 2002; Minne et al., 1994; Venter, 2000, 2003a, b).

Fruit type has been the main character used to distinguish Grabowskia and Phrodus from Lycium, with fleshy fruits containing two pyrenes of 1–2 seeds each in Grabowskia and mucilaginous, multi-seeded berries with an accrescent calyx and two hard sclerified regions at the berry apex in Phrodus (Bernardello and Hunziker, 1987; Hunziker and Bernardello, 1995; Bernardello and Chiang-Cabrera, 1998; Hunziker, 2001). Red, fleshy, multi-seeded (>10) berries are the most common fruit type in Lycium, but a few species in the Americas produce drupaceous fruits with a hardened endocarp and two seeds (Miller, 2002). Additionally, several other taxa possess modified berries that are partially sclerified and have a reduced number of seeds (Miller, 2002).

Recently there has been much interest in phylogenetic relationships within Lycieae (Bernardello and Chiang-Cabrera, 1998; Fukuda et al., 2001; Miller, 2002). A morphological study including American Lycium species shows very little resolution of relationships and implies that North and South

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American *Lycium* do not comprise reciprocally monophyletic lineages (Bernardello and Chiang-Cabrera, 1998). Further, Bernardello and Chiang-Cabrera (1998) suggest that *Grabowska* and *Phrodus* may not be distinct from *Lycium*. The monophyly of *Lycium* has also been questioned by Olmstead et al. (1999) in a Solanaceae-wide study using chloroplast DNA data and sampling one *Grabowska* species as well as five *Lycium* species.

The first phylogenetic study of worldwide *Lycium* species was conducted by Fukuda et al. (2001), who examined evolutionary relationships among 23 *Lycium* species using chloroplast DNA sequence data from *matk* and *trnT-trnF*. A somewhat larger sampling of *Lycium* (25 species) and three *Grabowska* species was included in Miller’s (2002) study of the genus based on nuclear ribosomal ITS data. In accordance with Bernardello and Chiang-Cabrera (1998) and Olmstead et al. (1999), results of Miller (2002) suggest that *Grabowska* is likely nested within *Lycium*. In addition, all three previous phylogenetic studies (Bernardello and Chiang-Cabrera, 1998; Fukuda et al., 2001; Miller, 2002) found little support for the existing infrageneric classification (Chiang-Cabrera, 1981; Chiang, 1983; Bernardello, 1986, 1987). Further, Fukuda et al. (2001) and Miller (2002) conflict as to whether Old World *Lycium* comprise a monophyletic group; increased taxon sampling is needed to resolve this conflict.

Because the close relatives of Liceae are restricted to South America (Di Fulvio, 1961; Tago-Nakazawa and Dillon, 1999; Hunziker, 2001), and species richness within Solanaceae, Liceae, and *Lycium* is very high in that area of the world, the origin of all these taxonomic groups is thought to be in South America (Hitchcock, 1932; Bernardello, 1987; D’Arcy, 1991; Hunziker, 2001). South America has the greatest diversity of *Lycium*, with ca. 30 species (Bernardello, 1986). Both Fukuda et al. (2001) and Miller (2002) sampled *Lycium* from throughout its biogeographic range, but both suffered from an underrepresentation of South American species. Of the 23 species of Liceae included in Fukuda et al. (2001), only four were South American, and only six South American species (including three *Grabowska* species) of 28 total species of Liceae were included in Miller (2002). Because both Fukuda et al. (2001) and Miller (2002) did not find the North and South American taxa to comprise reciprocally monophyletic groups, it is necessary in this study to include more South American taxa to better understand evolutionary relationships within the genus as a whole.

Across the genus *Lycium*, dimorphic gender expression is known in three species in North America and six species in southern Africa. Inclusion by Miller (2002) of a wide sample of North American taxa, including all three of the American dimorphic species, allowed inference of a single evolutionary transition to gender dimorphism in North America. However, recently it has been shown that one of these species, *L. californicum*, is not uniformly dimorphic; rather there are both monomorphic and dimorphic populations (Yeung et al., 2005; Miller and Levin, unpublished data). Thus, these results demand the reconsideration of a single origin of dimorphism in North America. Here we include three accessions of *L. californicum* from both dimorphic and monomorphic populations to further investigate gender dimorphism in North American *Lycium*. The number of times that dimorphism has evolved among the southern African species is as yet unknown; both Fukuda et al. (2001) and Miller (2002) only included one dimorphic African taxon in their analyses, suggesting the need for increased taxon sampling.

These previous DNA sequence-based studies (Fukuda et al., 2001; Miller, 2002) provide a good start for understanding relationships within Liceae and the genus *Lycium*; however, less than a third of the total *Lycium* species were included in these analyses, and neither included *Phrodus microphyllus*. Even collectively, both studies contained only 37 of ca. 80 *Lycium* species and only five South American species. Further, weak resolution limited many conclusions regarding relationships within the genus. Thus, in the present study we examine relationships among a larger set of Liceae, with broad geographic sampling including the addition of many South American and African taxa, as well as the inclusion of multiple *Grabowska* species and *Phrodus microphyllus*. Evolutionary relationships are inferred using DNA sequence data from both the nuclear (GBSSI or waxy) and chloroplast (*trnT-trnF*) genomes. The specific goals of this study are to (1) test the monophyly of tribe Liceae and the genus *Lycium*, (2) examine phylogenetic relationships within *Lycium*, (3) reexamine the number of times that dimorphism has evolved in *Lycium*, and (4) better understand biogeographical patterns within the genus and, specifically, determine whether species from the same geographic region are monophyletic. We also discuss fruit evolution in light of phylogenetic relationships.

**MATERIALS AND METHODS**

**Taxon sampling**—Included in this study are 48 species of *Lycium* (60%) from across its geographic range, including 16 North American species (one species ranges into the Pacific islands), 13 South American species, 14 African species, and five Eurasian and Australian species. In addition to multiple accessions of the three American dimorphic taxa, we also include five (one is an undescribed species) dimorphic taxa from Africa to help determine the number of times that dimorphism has evolved within the genus. We have included sampling of the other taxa in tribe Liceae, including three *Grabowska* species and the monotypic *Phrodus microphyllus*. Also included are representatives of those genera thought to be close relatives of Liceae, including *Notana, Sclerophyslax, and Jaborosa* (Olmstead et al., 1999; R. Olmstead, University of Washington, personal communication). All 65 taxa with voucher information and GenBank accession numbers are listed in the Appendix.

**DNA extraction, amplification, and sequencing**—Total genomic DNA was extracted from fresh or silica gel-dried leaf material using the protocols described in Miller (2002) and Levin et al. (2004).

**waxy**—Amplification of the 3′ end of exon 3 through the 5′ end of exon 8 of the nuclear GBSSI gene (Fig. 1) was done using primers 622 B (GBSSI 5′-CAC TGC TAT AAA CGT GGG GTT GA-3′) and CRmod 5′-GGC ATA TGA TGG GCT AAC GAT AA-3′; modified from primer GBSSI CR of Peralta and Spooner (2001). Twenty-five microliter reactions contained 1X buffer, 2.5 mM MgCl2, 0.20 mM dNTPs, 0.40 µM of each primer, 1X Qiagen Q-solution (Qiagen, Valencia, California, USA), 0.625 units of *Taq* polymerase, and 1 µL DNA. The thermal cycler program used was a touchdown procedure with an initial denaturing at 94°C
for 4 min; 14 cycles at 94°C for 30 s, 57°C–51°C (decreasing one degree every two cycles) for 1 min; 72°C for 1 min 30 s; 26 cycles at 94°C for 30 s, 50°C for 1 min, 72°C for 1 min 30 s; ending with an extension at 72°C for 10 min. Occasionally amplifications were done with forward primers 181F (Walsh and Hoot, 2001) or waxyF (Levin et al., 2006) and 2R (Miller et al., 1999); but note that one base is missing in the primer sequence given in this reference, see Levin et al. (2006). PCR products were cleaned using either polyethylene glycol precipitation and ethanol cleanup (Morgan and Soltis, 1993) or the QIAquick PCR purification kit (Qiagen, Valencia, California, USA). Sequencing was done on an ABI automated sequencer (Applied Biosystems, Foster, California, USA) by the DNA Sequencing Facility of the Biotechnology Resource Center at Cornell University, Ithaca, New York, USA. Cycle sequencing was done with both amplification primers 622B and CRMod; when amplification was done with 181F or waxyF and 2R, sequencing was done with the primers used for amplification as well as internal primers 1171R (5’-TAC CTG AAG TC-3’; Walsh and Hoot, 2001) and IwaxyF (5’-ATT CCC TGC TAC CTG AAG TC-3’; a Lycium-specific version of primer 1058F; Levin et al., 2006); occasionally 3F (5’-GAT ACC CAA GAG TGG AAC CC-3’; Miller et al., 1999) was also used (Fig. 1).

**Table 1.** Comparison of the 58 taxa data sets for the waxy and trnT-trnF regions.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>waxy</th>
<th>trnT-trnF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range of raw length</td>
<td>851–1866 bp</td>
<td>1563–1610 bp</td>
</tr>
<tr>
<td>Aligned length</td>
<td>1963 bp</td>
<td>1691 bp</td>
</tr>
<tr>
<td>Variable sites (proportion)</td>
<td>0.15</td>
<td>0.048</td>
</tr>
<tr>
<td>Intron sites (proportion)</td>
<td>140 (0.071); Introns only 94 (0.114)</td>
<td>30 (0.018)</td>
</tr>
<tr>
<td>Range of pairwise distances</td>
<td>0–0.113</td>
<td>0–0.028</td>
</tr>
<tr>
<td>CI (RC); RI</td>
<td>0.93 (0.89); 0.95</td>
<td>0.98 (0.97); 0.99</td>
</tr>
</tbody>
</table>

*Note: PI = parsimony-informative, CI (RC) = rescaled CD = consistency index, RI = retention index.

*The large range for raw length is due to the use of different primers (Fig. 1), rather than true differences in length.

**trnT-trnF**—We amplified the chloroplast region between the trnT and trnF genes, including the intergenic spacer between trnT and trnD, the trnL intron, the trnL 3’ exon (we sequenced only a few bases of the trnL 5’ exon), and the intergenic spacer between the trnL 3’ exon and trnF. For ease of amplification, this piece was amplified with two separate PCRs; one reaction used primers a and b, and the other reaction used primers c and f (Taberlet et al., 1991). This set of primers resulted in a sequence gap of 24 bp in the trnL 5’ exon, and 26 bp at the 3’ end of the trnL intron. For both sets of primers, 50 μL reactions were done using 1× buffer, 2.0 mM MgCl₂, 0.20 mM dNTPs, 0.36 μM of each primer, 8.8 ng BSA, 1.25 units of Taq polymerase, and 1–2 μL DNA. The thermal cycler program used with primers a and b was a touchdown procedure with an initial denaturing at 94°C for 4 min; 12 cycles at 94°C for 1 min, 54°C–49°C (decreasing one degree every two cycles) for 1 min, 72°C for 1 min 30 s; 28 cycles at 94°C for 1 min, 48°C for 1 min, 72°C for 1 min 30 s; ending with an extension at 72°C for 7 min. For PCR amplifications using primers c and f, two different thermal cycler programs were used; 94°C for 4 min; 30 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 1 min 30 s; 72°C for 7 min; or 94°C for 4 min; 40 cycles at 94°C for 45 s, 52°C for 1 min, 72°C for 1 min; 72°C for 7 min. PCR products were cleaned and sequenced as before using the same primers as for amplification.

**Sequence alignment**—Sequences of all primers were edited and aligned using Autassembler DNA Sequence Assembly Software, version 1.4.0 (Applied Biosystems, 1989–1995) to construct a consensus sequence for each taxon. Taxon sequences were aligned manually in SeAl (Rambaut, 2002) and MacClade 4.0 (Maddison and Maddison, 2000).

**Parsimony analyses**—The two data sets were analyzed separately (Table 1), combined, and both with and without indels coded as additional binary characters. Parsimony analyses were conducted in PAUP* version 4.0b10 (Swofford, 2002) using heuristic searches with 100 random addition sequence replicates and tree-bisection-reconnection (TBR) branch swapping. Constant characters were excluded, and gaps were treated as missing data. For the waxy only and the combined data sets, due to large numbers of equal length trees, each addition replicate was limited to 200 trees following the analysis protocol in Levin et al. (2004). The strength of support for individual tree branches was estimated using bootstrap values (BS) (Felsenstein, 1985) and decay indices (DI) (Bremer, 1988; Donoghue et al., 1992). Bootstrap values were from 500 full heuristic bootstrap replicates, each with 10 random addition sequence replicates. The MulTrees option was not in effect. Decay values for each branch were determined using the PAUP decay index command file in MacClade to prepare a set of trees each with a single branch resolved. To find the shortest trees consistent with each constraint, this file was executed in PAUP* using the heuristic search option with 100 random addition sequence replicates and the MulTrees option disabled. The decay index for each branch is the difference in length between the shortest trees consistent with each constraint and the globally shortest trees.

In the trnT-trnF only analysis, all 65 taxa in the Appendix were included. For the waxy only and the waxy + trnT-trnF combined analyses, 58 taxa were included, because waxy data were either missing or considerably incomplete for seven species. The trnT-trnF only analysis was rooted with Jaborosa integrifolia and J. squarrosa; the other analyses were rooted with J. squarrosa, because waxy data were incomplete for J. integrifolia.

Congruence of the 58 taxa data sets was tested using the incongruence length difference test (ILD; Farris et al., 1994, 1995) as implemented by the partition homogeneity test in PAUP*. One thousand heuristic partition homogeneity replicates were completed, each with 10 random addition sequence replicates, TBR branch swapping, MulTrees off, gaps treated as missing data, and constant characters excluded.

**Maximum likelihood analysis**—An analysis using a maximum likelihood (ML) model was conducted with the 58 taxa combined data set. ML model parameters were determined using Modeltest version 3.5 (Posada and Crandall, 1998). This program tests the fit of 56 substitution models to the data; based on a hierarchical likelihood ratio test, a model that best fits the data is identified. The best model was used in an ML analysis in PAUP* using the heuristic search option, all 94 most parsimonious trees from a parsimony analysis of the combined data set (100 random addition sequence replicates, MulTrees disabled) as the starting trees, TBR branch swapping, and the MulTrees option in effect. As with the parsimony analysis of the combined data set, Jaborosa squarrosa was defined as the outgroup. An ML bootstrap analysis was also conducted using 100 full heuristic bootstrap replicates, each with three nearest neighbor interchange (NNI) swapping replicates; the MulTrees option was not in effect.

**RESULTS**

**waxy**—Sequences across all 58 taxa had an aligned length of 1963 bp (because of primer choice, 16 taxa had sequences that started in the 3’ end of exon 2 and extended through most of exon 10; the rest began in the 3’ end of exon 3 or the 5’ end of intron 3 and extended through the 5’ end of exon 8) (Fig. 1, Table 1). Of these 1963 characters, 140 were parsimony informative (PI), and phylogenetic analysis yielded the maximum number (20,000) of most parsimonious trees (MPTs).
allowed under the search method, with a tree length of 360 steps.

The waxy data show strong support for many relationships within tribe Lycieae (Fig. 2A). A clade comprising Nolana spp. sister to a monophyletic Lycieae (Lycium + Grabowskia + Phrodus; BS = 100; DI = 6) is well-supported (BS = 99; DI = 5). Within this clade, Grabowskia is monophyletic (BS = 100; DI = 8), and is part of a well-supported group (clade A; BS = 96; DI = 3) in which it is sister to a group of Lycium comprised of L. cooperi, L. macrodon, L. pallidum, L. puberulum, and L. shockleyi (clade B; BS = 89; DI = 2). There is a basal trichotomy within tribe Lycieae that includes Phrodus microphyllus, clade A (i.e., Grabowskia + clade B, Fig. 2A), and a weakly supported clade comprised of the majority of Lycium species (BS = 69; DI = 1). Within this latter clade of Lycium, there are two well-supported lineages: clade C (BS = 98; DI = 4) and a clade (BS = 86; DI = 2) that includes the strongly supported clade D (BS = 95; DI = 3), L. nodosum + L. viniforum + L. californicum (BS = 96; DI = 3), L. chilense + L. ciliatum (BS = 98; DI = 4), and L. ameghinoi + L. gilbertianum (BS = 69; DI = 1) (Fig. 2A). Within clade C there is not much resolution, although L. brevipes + L. carolinianum var. quadrifidum + L. carolinianum var. sandwicense + L. tenuispinosum are strongly supported (BS = 98; DI = 4), as is the sister relationship of L. cuneatum + L. morongii (BS = 99; DI = 6) (Fig. 2A). The clade comprised of L. brevipes + L. carolinianum var. quadrifidum + L. carolinianum var. sandwicense + L. tenuispinosum is further supported by a large (59–62 bp) insertion. There is also an 11-bp deletion supporting the sister relationship of L. cuneatum + L. morongii. Inclusion of indels as additional binary characters in a phylogenetic analysis (topology not shown) did not affect either the topology or support values for the waxy only analysis.

trnT-trnF—Sequences across 65 taxa had an aligned length of 1691 bp. Of these 1691 characters, 42 were PI, and phylogenetic analysis yielded five MPTs of 98 steps. Although
there was strong signal in the data [consistency index (CI) = 0.97], there were too few characters to yield a well-supported topology (Fig. 2B). Unlike the waxy only analysis, Nolana + Sclerophylax (BS = 80; DI = 2) are sister to a monophyletic tribe Lycieae (BS = 100; DI = 9). However, within the well-supported clade containing Lycium + Grabowskia + Phrodus (BS = 98; DI = 3), there is generally limited resolution of relationships. Grabowskia is well supported as monophyletic (BS = 98; DI = 4), as is the sister species relationship between L. chilense and L. ciliatum (BS = 95; DI = 1). Notably, seven taxa including L. ameghinoi, L. californicum, L. chilense, L. ciliatum, L. gilliesianum, L. nodosum, and L. viminalis are included within the moderately supported clade E (BS = 78; DI = 1; Fig. 2B) in the chloroplast only analysis, whereas these taxa are all placed as sister to clade D (BS = 86; DI = 2; Fig. 2A) in the waxy only analysis.

In Lycieae there are four indels >1 bp within trnT-trnF. A 6-bp deletion is shared by all taxa in a clade (BS = 64; DI = 1) that is nested within clade E (Fig. 2B), and a separate 6-bp deletion is shared by L. barbarum + L. chinense + L. ruthenicum (BS = 62; DI = 1; Fig. 2B). There is also a 12-bp deletion shared by six taxa (L. americanum, L. carolinianum var. quadrifidum, L. carolinianum var. sandwicense, L. elongatum, L. infaustum, and L. tenuispinosum). In addition, there is a 6-bp deletion shared by L. cooperi, L. pallidum, and L. puberulum. Not surprisingly, the strict consensus topology inferred from a trnT-trnF analysis including indels as additional characters (not shown) is the same as Fig. 2B, except that the six taxa with the 12-bp deletion have weak support (BS = 44) as a monophyletic group. Similarly, a clade of L. cooperi + L. pallidum + L. puberulum also has weak support (BS = 62) in this analysis.

**Data sets combined**—Results of an ILD test comparing the waxy and trnT-trnF data sets suggest that they are not congruent (P = 0.001). Visual examination of the topologies suggests that the source of the conflict between the topologies inferred from chloroplast trnT-trnF and the nuclear waxy data is primarily the placement of seven taxa (bolded in Fig. 2), although there is limited support for the placement of these taxa in the cp only analysis. To investigate the effects of these seven taxa, an ILD test was conducted with these taxa excluded; however, the data sets remained incongruent (P = 0.001). Because Sclerophylax sp. was placed somewhat differently by the cp and waxy data, this taxon was excluded from an additional ILD analysis, with no effect on data set incongruence. Visual examination of the topologies inferred from these two regions suggests few differences in relationships (except those previously outlined), with the topologies differing mainly at the level of resolution (Fig. 2). Contributing to the significant incongruence may be the large disparity in the number of PI characters (Table 1), as well as a difference in the substitution rates between the relatively slowly evolving trnT-trnF region and the faster evolving waxy region (see also Dolphin et al., 2000; Barker and Lutzoni, 2002; Dowton and Austin, 2002).

Thus, despite the apparent conflict between data sets, 58 taxa were included in a combined analysis of waxy and trnT-trnF data. This analysis included 170 PI characters, resulting in 20,000 MPTs (the maximum saved under the search method, see Materials and Methods) of 459 steps, with CI = 0.91, retention index (RI) = 0.94. Despite the large number of MPTs, the bootstrap consensus tree shows considerable resolution among taxa (Fig. 3). Tribe Lycieae is strongly supported as a monophyletic group (BS = 100; DI = 10) and contains two supported clades (clades A and F in Fig. 3), as well as Phrodus microphyllus, which cannot be placed within either clade A or clade F. Clade A (BS = 92; DI = 3) is comprised of the monophyletic Grabowskia (BS = 100; DI = 13) that is sister to a group of North American Lycium species (clade B; BS = 89; DI = 2). The large clade F (BS = 86; DI = 2) includes a single Old World Lycium clade with high support (clade D, BS = 100; DI = 5) that is sister to a weakly supported group of North American Lycium species (clade B; BS = 99; DI = 5). L. californicum + L. nodosum + L. viminalis (BS = 94; DI = 3), and the strongly supported L. chilense + L. ciliatum (BS = 100; DI = 7). As in the separate analyses, inclusion of indels as binary characters had little effect on either the topology or the support values for the combined analysis of the trnT-trnF and waxy data sets (topology not shown). However, bootstrap support for the 16 taxa cladestested within clade C in Fig. 3 (BS = 73; DI = 1) did increase to 92%, likely due to the 6-bp deletion in trnT-trnF
shared by all taxa in the clade (BS = 64; Fig. 2B) nested within clade E (Fig. 2B).

**Maximum likelihood**—Maximum likelihood (ML) analysis of the 58 taxa combined data set was conducted with parameters estimated using Modeltest. The AIC procedure indicated that the GTR + G model best fit the data. The ML model parameters included nucleotide frequencies of A = 0.3156, C = 0.1787, G = 0.1828, and T = 0.3229; a substitution rate matrix of A to C: 1.0034, A to G: 1.9793, A to T: 0.5, C to G: 1.3265, C to T: 2.4417, and G to T: 1; assumed proportion matrix of A to C: 1.0034, A to G: 1.9793, A to T: 0.5, C to G: 0.1787, G to T: 1.3229, T to C: 0.3156, and T to G: 0.3229; a gamma rate distribution at 0.2477. Using this model, the number in intron 5.

**DISCUSSION**

**Comparison of waxy vs. trnT-trnF**—The waxy data provide greater resolution of relationships compared to the trnT-trnF data (Fig. 2). Although both the cp trnT-trnF and nuclear waxy regions have strong phylogenetic signal, with similarly high consistency and retention indices, waxy is more phylogenetically useful, with a considerably higher percentage of PI sites (Table 1). This high level of information is likely due to the mix of noncoding introns and coding exons (Fig. 1). In fact, introns accounted for 67% of the total PI characters observed for waxy (Table 1). The distribution of PI characters across the waxy region is similar to that observed by Levin et al. (2005) for Solanum. For those introns with complete sampling among Lycieae (Fig. 1, introns 3–7), both Lycieae and Solanum have the highest number of PI characters in intron 3 and the lowest number in intron 5.

There is an apparent conflict (Fig. 2) in the phylogenetic placement of the seven previously mentioned American taxa (bold, Fig. 2) that group with the Old World species (clade D; Fig. 2A) in the waxy only topology (BS = 86; DI = 2; Fig. 2A), whereas these same taxa are part of a larger American set of species in the trnT-trnF topology (clade E; BS = 78; DI = 1; Fig. 2B). To test whether there are true topological differences between the data sets, each data set was constrained to find the MPTs consistent with the placement of these seven taxa in the position suggested by the other data set. When a parsimony analysis of the waxy data is constrained to place the seven taxa in clade E (Fig. 2B), there is a cost of two steps; such constrained topologies are not significantly less likely than unconstrained topologies (one-tailed nonparametric Shimodaira-Hasegawa [S-H] test; P > 0.05). However, when a parsimony analysis of the trnT-trnF data is constrained to place the seven taxa in a clade with all taxa in clade D (Fig. 2A), there is a cost of five steps; these constrained topologies are significantly less likely than unconstrained topologies (one-tailed S-H test; P < 0.05). Thus, there is stronger evidence that these seven taxa belong within the American clade E (Fig. 2B), a finding not surprising given that these taxa are also placed in this position in the combined analysis (Fig. 3), albeit with weak support. It is likely that increased taxon sampling within American Lycium will help determine the true affinities of these seven species.

**Monophyly of Lycieae and Lycium**—Tribe Lycieae, including 48 Lycium species, three Grabowskia species, and the monotypic Phrodus, is strongly supported as monophyletic in all analyses (Figs. 2–4). This result concurs with Olmstead and Bohs (University of Washington and University of Utah, personal communication), in which a monophyletic Lycieae (4 Lycium, 2 Grabowskia, Phrodus microphyllus) was recovered using cp ndhF and trnL-trnF data. Regarding the monophyly of Lycium, results from the present study corroborate previous work (Olmstead et al., 1999; Miller, 2002) and confirm that
Grabowskia is nested within Lycium (clade A) and sister to a small North American group of Lycium (clade B), most of which possess berries with sclerifications and a reduced seed number (see Discussion). Grabowskia species share several morphological characters with this group of North American Lycium in addition to their sclerified fruits, including relatively large (compared to other Lycium species), typically white, pendulous flowers, calyx lobes that are longer than the calyx tube, and flattened, often glaucous leaves. The ML topology (Fig. 4) suggests that this clade of Grabowskia and Lycium species diverged first within Lycieae, with Phrodus microphyllus sister to all Lycium species except those species in clade A. However, as these relationships are not well supported, more data are needed to clarify basal relationships within Lycieae.

Relationships within Lycium—Lycium sandwicense occurs on islands across the Pacific (Easter Island, Hawaiian Islands, and Ogasawara Islands and Daitou Island in Japan), and is the only Lycium species found in both the northern and southern hemispheres (Hitchcock, 1932; Chiang-Cabrera, 1981; Yamasaki, 1991). It has been thought to be closely related or conspecific with L. carolinianum, a species found from Florida to Texas in the United States and in Mexico (Hitchcock, 1932; Chiang-Cabrera, 1981). Fukuda et al. (2001) and Miller (2002) confirmed this close relationship, supporting the nomenclatural combination of this taxon as L. carolinianum var. sandwicense (Gray) C.L. Hitchcock (Hitchcock, 1932). However, their sampling did not allow determination of the affinities of these taxa beyond being related to various American species. In agreement with previous studies, L. carolinianum var. sandwicense is sister to L. carolinianum var. quadridium, and results of the present study strongly support the close relationship of L. carolinianum var. quadridium and L. carolinianum var. sandwicense with L. brevipes and L. tenuispinosum (BS = 97; DI = 4; Fig. 3). These four species are also supported by a large indel in waxy intron 7. This clade is one of two well-supported lineages within the strongly supported American clade C (Fig. 3).

Sister to the aforementioned L. carolinianum lineage is a clade of 10 species (BS = 80; DI = 1; Fig. 3), among which there is no resolution. Understanding phylogenetic relationships within this clade is especially important, because it contains North and South American species and both hermaphroditic and gender dimorphic species. Gender expression has been studied by Miller and Venable (2000). Despite variation in sexual systems among populations, accessions of L. carolinianum as distinct from the other North American morphic species (Fig. 3), but the presence of both monomorphic (i.e., hermaphroditic) and dimorphic populations of L. carolinianum suggests a separate origin of gender dimorphism in this species (Yeung et al., 2005; J. S. Miller and R. A. Levin, unpublished data). Population sexual expression (monomorphism vs. dimorphism) and the ploidy level (diploidy vs. tetraploidy) of individuals in this species are clearly associated (Yeung et al., 2005). Specifically, diploid populations are always hermaphroditic, whereas tetraploid populations have separate hermaphroditic and female plants; a result consistent with the ploidy-driven hypothesis of gender dimorphism proposed by Miller and Venable (2000). Despite variation in sexual systems among populations, accessions of L. carolinianum from a dimorphic, tetraploid population (HRF and HRH) and an accession from a monomorphic, diploid population (OP) are clearly supported as monophyletic in our analyses (Fig. 3). In addition, a more extensive study including 15 accessions from 10 populations of L. carolinianum also confirms the monophyly of this species (Yeung et al., 2005).

Results from the present study cannot definitively place L. exsertum as sister to L. fremontii, such that dimorphism could have evolved separately in each of these three taxa. However, L. exsertum and L. fremontii share a number of morphological characters and are likely each other’s closest relative. Thus, it seems that dimorphism has evolved at least twice within North America.

Previous analyses have not included sufficient taxon sampling among African Lycium to determine the number of times that dimorphism evolved on this continent. Here we have included five African dimorphic species (L. arenicola, L. horridum, L. tetrandrum, L. villosum, and an undescribed Lycium species), but lack of resolution among the African species limits inference of the number of times dimorphism has arisen.
Given current topologies and levels of support (Fig. 3), it is likely that dimorphism evolved a minimum of twice in southern Africa, but perhaps more, because there are two additional dimorphic species in southern Africa (Venter, 2003a, b) that have not been included.

Venter (2000, 2003a, b) suggested that hybridization has been important in the evolution of African Lycium. The presence of dioecy and tetraploidy in *L. villosum* distinguishes this taxon from the closely related *L. hirsutum* (Venter, 2000); thus, Venter (2000) proposed that *L. villosum* is of hybrid origin, with *L. hirsutum* as one of the parents. Our data are consistent with this hypothesis and support a close relationship between these two species (Figs. 2A, 3). Additionally, she hypothesizes that hexaploid *L. arenicola* is a hybrid, with either *L. horridum* or *L. tetrandum* as a likely parent. Our data place *L. arenicola* closer to *L. horridum* (than to *L. tetrandum*) with moderate support (Figs. 2A, 3), which is consistent with the cytological observations of Venter (2000). However, at present the chloroplast marker is uninformative with regard to the African *Lycium*. Venter (2000, 2003a, b) also suggests hybrid origins for two additional polyploid African *Lycium* species, *L. strandveldense* and *L. gariepense*, which are not included in this study. Inclusion of these taxa in future studies will allow evaluation of Venter’s ideas, as well as exploration of the more general phenomenon of introgression following hybridization (e.g., Okuyama et al., 2005).

Finally, Bernardello (1986) has suggested that gender dimorphism may be present in *L. minimum*, a Galapagos endemic not included in the present study. However, field observations of *L. minimum* are needed to confirm gender dimorphism in this species.

**Biogeography**—The present study finds a clearly monophyletic group of Old World taxa (Fig. 3, BS = 100; DI = 5) in agreement with Fukuda et al. (2001), including all Old World taxa except for the Pacific island taxon *L. carolinianum* var. *sandwicense*, which is derived from a dispersal event of an American ancestor onto islands in the Pacific. The Old World taxa appear as a well-supported clade nested within the American species, which comprise the rest of Lycieae. Among the New World species, neither the North or South American species are monophyletic, a finding consistent with Fukuda et al. (2001) and Miller (2002). This is not surprising, since most *Lycium* have red, fleshy, bird-dispersed fruits that could easily be moved between North and South America. Current data support one dispersal event from America to the Old World, but our data are silent on dispersal patterns within the Old World. All of the *Lycium* species in the Old World have fleshy berries, a pattern consistent with past bird dispersal of the seeds from a berry-fruited American *Lycium*. No subsequent shift to sclerified fruits has occurred in the African and Eurasian lineage.

**Fruit evolution within Lycium**—A red, fleshy, bird-dispersed berry is the dominant fruit type in *Lycium* (Bernardello, 1983; Bernardello and Chiang-Cabrera, 1998). Bernardello (1983) suggests that fruit evolution in *Lycium* has been from a berry (ancestral condition) to a berry with sclerifications (initially at the berry apex) to drupaceous with a reduced seed number. Knapp (2002) examined fruit evolution across the entire family and found that berries likely evolved three times within Solanaceae, including along the branch leading to subfamily Solanoideae, which includes tribe Lycieae. Berries containing stone cells (i.e., sclerified areas) are found across the subfamily (Knapp, 2002), suggesting either that berries with stone cells are the symplesiomorphic condition for Solanoideae or that such modification to the berry fruit in Solanaceae is easily accommodated evolutionarily.

Berries with stone cells have been reported from across tribe Lycieae (Bernardello and Chiang-Cabrera, 1998), and in some species the amount of sclerification is considerable (Fig. 4). For example, fruits of *L. cooperi*, *L. macrodon*, and *L. puberulum* have a hardened endocarp that partially encloses the seeds, a transverse split separating the upper and lower halves of the fruit, and a reduced number of seeds (Miller, 2002). Although *L. shockleyi* and *L. pallidum* lack the hardened endocarp, *L. shockleyi* and *L. pallidum* var. *oligospermum* share a reduced seed number with the other species (Miller, 2002). Further, fruits of *L. pallidum* have a sclerified apex, and fruits of *L. shockleyi* have a transverse suture. Several other taxa (*L. ameghinoi*, *L. californicum*, Fig. 4; *L. athium* and *L. minimum*, not included here) have distinctive, two-seeded drupaceous fruits, which may be inferred to have evolved multiple times. Additionally, the genus *Grabowskia* has been defined by its unique fruits that are fleshy with two sclerified pyrenes. In all of the ML topologies (Fig. 4), the earliest diverging lineages within Lycieae are clade A and *Phrodus microphyllus*, both of which have fruits with some type of sclerification, suggesting that fruits with stone cells may be ancestral in the tribe.

The closest relatives of Lycieae have fairly distinctive fruits within Solanaceae, offering little insight as to the fruit type of the common ancestor of all Lycieae. Tago-Nakazawa and Dillon (1999) suggested that the unique mericarp fruits of *Nolana* are derived from a berry (Tago-Nakazawa and Dillon, 1999, as cited in Knapp, 2002), likely with structures similar to stone cells playing a role in development (Knapp, 2002). Among the other relatives, *Sclerophylax* have dry, two-seeded indehiscent fruits (Di Fulvio, 1961), and *Jaborosa* have berries (Hunziker, 2001). Understanding the development of these fruits is necessary to more confidently infer the likely ancestral fruit type for Lycieae.

**Conclusions**—Tribe Lycieae is strongly supported as monophyletic, and it is likely that both *Grabowskia* and *Phrodus microphyllus* are nested within the largest genus *Lycium*. *Lycium* is not monophyletic; a small group of North American *Lycium* species are more closely related to *Grabowskia* than they are to other *Lycium* species. Nomenclatural revision of the tribe will be forthcoming, particularly with regard to this group of *Lycium* plus *Grabowskia*. Further analyses (in progress) including greater taxon sampling and additional sequence data will strengthen understanding of relationships among the rest of Lycieae.

Contrary to Miller (2002), gender dimorphism appears to have evolved at least twice in North America. In southern Africa, dimorphism has evolved at least twice, but perhaps three or more times. Old World *Lycium* comprise a monophyletic group nested within a group of North and South American lineages. The diversity of fruit morphologies within Lycieae offers the opportunity to better understand fruit evolution. Berries with stone cells may be ancestral within Lycieae; however, developmental studies are necessary to assess homologies among fruit types.


Appendix: Taxa, localities, vouchers, and GenBank accession numbers for all sequences included in this study. A dash indicates that the region was not sampled for that accession. BIRM samples have the seed accession number of the Solanaceae collection at the University of Nijmegen, Netherlands. Notation in parentheses is used in Figs. 2–4 for species with multiple accessions. Voucher specimens are deposited in the following herbaria: AD = Plant Biodiversity Centre, Adelaide, Australia; ARIZ = Arizona University; BLFU = University of the Free State; CORD = Universidad Nacional de Córdoba; MASS = University of Massachusetts; NY = New York Botanical Garden; TAIC = Texas A&M University, Kingsville; US = Smithsonian Institution; UT = University of Utah; WTU = University of Washington.

Taxon, Locality, Voucher information; GenBank accession numbers: waxy, trnT-trnL, trnL-trnF.