

Evolutionary Relationships in Tribe Lycieae (Solanaceae)

Rachel A. Levin, Joshua R. Shak and
Jill S. Miller
Department of Biology, Amherst
College
Amherst, MA 01002
USA

Gabriel Bernardello
Instituto Multidisciplinario de Biología Vegetal
Universidad Nacional de Córdoba-CONICET
C.C. 495, 5000 Córdoba
Argentina

Andor M. Venter
Department of Plant Sciences
University of the Free State
Bloemfontein 9300
South Africa

Keywords: *Lycium*, *Grabowskia*, *Phrodus*, phylogeny, GBSSI

Abstract

We examine evolutionary relationships among the three genera in tribe Lycieae using DNA sequence data from the nuclear granule-bound starch synthase gene (GBSSI, *waxy*). Tribe Lycieae is comprised of the large cosmopolitan genus *Lycium* and the predominately South American genera *Grabowskia* and *Phrodus*. Phylogenetic results strongly suggest that *Lycium* contains *Grabowskia* and may also include *Phrodus*. Further, we examine relationships among several clades of American *Lycium* and within the monophyletic Old World lineage. This study has the largest taxon sampling to date for tribe Lycieae, with 85% of the named species. Increased sampling within eastern Asia and South America, with the world's highest species-richness of *Lycium*, as well as the addition of more rapidly evolving genetic markers, are the areas on which to focus future work.

INTRODUCTION

Tribe Lycieae Hunz. is one of the largest tribes within Solanaceae (ca. 87 species) and includes ca. 83 species in the large genus *Lycium*, *Grabowskia* (3–4 species), and monotypic *Phrodus microphyllus* (Hunziker, 1977; D'Arcy, 1991; Hunziker, 2001). *Phrodus microphyllus* and the *Grabowskia* species have limited geographic distributions. *Phrodus microphyllus* is endemic to the Atacama and Coquimbo regions of Chile, and *Grabowskia* species are restricted to South America including the Galapagos Islands, although one wide-ranging species (*G. boerhaviaefolia*) occurs in both South America and Mexico. In contrast, *Lycium* is one of only three cosmopolitan genera in Solanaceae, along with *Solanum* and *Physalis* (Hunziker, 2001). *Lycium* is most species-rich in southern South America, southern Africa and southwestern North America. In addition, a number of species are distributed in the Mediterranean and across Asia, and one species is apparently native to Australia. Sister to tribe Lycieae is tribe Nolaneae (Olmstead et al., 1999; Levin and Miller, 2005), which includes only the genus *Nolana* (ca. 83 species; D'Arcy, 1991; Tago-Nakazawa and Dillon, 1999), endemic to Chile and Peru, with one species on the Galapagos Islands.

Species in tribe Lycieae are uniformly woody shrubs that generally inhabit arid to semi-arid environments. Hunziker (2001) characterized members of Lycieae by developing buds with imbricate corolla aestivation, and fruits that are usually berries or, alternatively, drupaceous having 2–4 seeds. Fruit type has served as the distinguishing character among the three genera, varying from fleshy fruits with two “pyrenes” of 1–2 seeds each (i.e., each seed or pair of seeds is surrounded by a stony layer) in *Grabowskia* to mucilaginous, multi-seeded berries with two apical sclerifications in *Phrodus microphyllus* (Bernardello and Hunziker, 1987; Hunziker and Bernardello, 1995; Bernardello and Chiang-Cabrera, 1998; Hunziker, 2001) to true berries in most *Lycium*.

The majority of *Lycium* tend to produce red, yellow, or black, fleshy, multi-seeded (>10 seeds) berries, although four American species (*L. athium*, *L. minimum*, *L. ameghinoi* and *L. californicum*) have drupaceous fruits containing two seeds with each seed surrounded by a stony layer (Bernardello, 1986; Miller, 2002). Five other North American *Lycium* species (*L. cooperi*, *L. macrodon*, *L. puberulum*, *L. shockleyi* and *L. schaffneri*) have modified berries that have partial sclerification and a reduced seed number (Chiang-Cabrera, 1981; Miller, 2002).

The worldwide distribution of *Lycium* poses questions regarding its biogeographical history. Recently, Levin and Miller (2005) have shown that the Old World *Lycium* species are monophyletic and nested within an American group of *Lycium* species. Among the New World *Lycium*, the North and South American species are not in distinct clades; thus, geography is not a good predictor of phylogenetic relationships. Given that *Grabowskia*, *Phrodus* and *Nolana* are all endemic to the Americas, it is likely that *Lycium* arose in the Americas and then dispersed to the Old World. Further, *Lycium* probably arose in South America, given that its closest relatives, *Nolana*, *Sclerophylax* and *Jaborosa*, are all endemic to South America (Di Fulvio, 1961; Tago-Nakazawa and Dillon, 1999; Hunziker, 2001).

Within Lycieae, previous work (Miller, 2002; Levin and Miller, 2005) suggests that generic definitions require reconsideration. There is clear evidence that a small group of North American *Lycium* is more closely related to a monophyletic *Grabowskia* than to the other species of *Lycium*. Interestingly, most of the species in this small group of *Lycium* species share a hardened fruit and glaucous leaves, characteristics that are more similar to *Grabowskia* species than to other species of *Lycium*. Further, the placement of *Phrodus* is equivocal in Levin and Miller (2005); it may be nested within *Lycium* or sister to the rest of the tribe.

In the present study our goals center around the addition of sequence data for the nuclear GBSSI region and increased taxon sampling, mainly from South America and southern Africa, in order to 1) determine the placement of *Phrodus microphyllus* relative to other members of Lycieae, 2) evaluate species circumscriptions within *Grabowskia*, 3) better understand relationships among the various clades of New World *Lycium* species, and 4) examine relationships within Old World *Lycium*, especially the placement of *L. australe*, the only species of *Lycium* native to Australia.

MATERIALS AND METHODS

Taxon Sampling

Included in this study are 68 species of *Lycium* (85% of the genus), including 16 North American species (one species ranges into the Pacific islands), 20 South American species, all 27 African species (including a species from the Canary Islands), four Asian and one Australian species. Multiple accessions including named varieties of the closely related South American *L. chilense* and *L. ciliatum* were included, as were several accessions of the North American species *L. californicum*, which has both monomorphic and dimorphic populations (Yeung et al., 2005; Miller and Levin, unpubl. data). We have also sampled the other genera in tribe Lycieae, including three species of *Grabowskia* (six total accessions sampled across the wide geographic range of *G. boerhaviaefolia*) and monotypic *Phrodus microphyllus*. Also included are two *Nolana* species, the sister group to Lycieae (Levin and Miller, 2005), as well as representatives of other genera closely related to Lycieae, including *Sclerophylax* and *Jaborosa* (Olmstead et al., 1999; R. Olmstead and L. Bohs, pers. commun.). All 89 taxa with voucher information and GenBank accession numbers are in Table 1.

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted from fresh or silica gel-dried leaf material following Miller (2002) and Levin et al. (2004).

We amplified exons 2 through 10 (ca. 1800 bp) of the nuclear GBSSI (*waxy*) gene

using forward primer waxyF (Levin et al., 2006) or occasionally 181F (Walsh and Hoot, 2001) and the reverse primer 2R [Miller et al., 1999; but also see Levin et al. (2005)] using the protocols of Levin et al. (2006). For a number of accessions, this 1800 bp region was amplified in two separate pieces, rather than one long piece, using the primer pair waxyF and 1171R (5'-TCA TAC CCA TCA ATG AAA TC-3'; Walsh and Hoot, 2001), the primer pair IwaxyF [5'-ATT CCC TGC TAC CTG AAG TC-3'; a *Lycium*-specific version of primer 1058F, Levin et al. (2006)], and 2R (see protocol in Levin et al., 2006). Alternatively, amplifications were done for a shorter (ca. 900 bp) GBSSI region, from the 3' end of exon 3 through the 5' end of exon 8 using primers 622-B (5'-CAC TGC TAT AAA CGT GGG GTT GA-3'; Peralta and Spooner, 2001) and CR [5'-GGC ATA GTA TGG GCT CAC AGT AA-3'; modified from primer 1555-CR of Peralta and Spooner (2001)], following conditions in Levin and Miller (2005).

PCR products were cleaned either by PEG precipitation and ethanol cleanup (Morgan and Soltis, 1993) or the QIAquick PCR purification kit (Qiagen, Inc., Valencia, California) and sequenced on an ABI automated sequencer 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; when amplification was done with waxyF (or 181F) and 2R, sequencing was done with the primers used for amplification as well as internal primers 1171R and IwaxyF; occasionally 3F (5'-GAT ACC CAA GAG TGG AAC CC-3'; Miller et al., 1999) was also used.

For the polyploid taxa, previously published direct sequences for seven species were used (DQ124504, DQ124509, DQ124510, DQ124521, DQ124525, DQ124528, DQ124544, DQ124547). The two additional polyploid taxa, *Lycium strandveldense* and *L. garipeense*, were cloned before sequencing as part of a project to study possible hybrid origins of African *Lycium* species (Miller et al., unpubl. data). For these two taxa, a single representative sequence was used in the present study.

Sequence Alignment

Sequences were edited and aligned using Autoassembler DNA Sequence Assembly Software v. 1.4.0 (Applied Biosystems, 1989–95) or Sequencher v. 4.6 (Gene Codes Corp., 1991–2006). All sequences (usually four) for each genomic accession were combined into a single consensus sequence. Disagreements between these sequences were very rare, but if present that site was coded with the appropriate ambiguity code. In some species there was evidence for multiple alleles (i.e., double peaks in the chromatogram) in the direct sequences (up to 10 of the nucleotide sites), and such sites were also coded with the appropriate ambiguity code. All of the consensus sequences were then aligned manually in SeAl (Rambaut, 2002) and MacClade 4.0 (Maddison and Maddison, 2000) for use in phylogenetic analysis.

Maximum Likelihood Analysis

We used a maximum likelihood (ML) algorithm to analyze the complete data set, which included 89 taxa. ML model parameters were determined using the Akaike information criterion in Modeltest v. 3.7 (Posada and Crandall, 1998). The best-fit model TVM + I (nucleotide frequencies of A = 0.2725, C = 0.1896, G = 0.2041 and T = 0.3338; a substitution rate matrix of A to C: 1.1451, A to G: 2.837, A to T: 0.7266, C to G: 1.3132, C to T: 2.837 and G to T: 1; assumed proportion of invariable sites = 0.3837; and equal rates of change across variable sites) was used in an ML analysis in PAUP* (Swofford, 2002). ML settings in PAUP* included the heuristic search option, all most-parsimonious trees from a parsimony analysis of the data set (1000 random addition sequence replicates, Multrees disabled) as the starting trees (note that only one of the most-parsimonious starting trees was actually used by PAUP*, as all others had higher ML scores), tree bisection reconnection (TBR) branch-swapping, and the MulTrees option in effect. *Jaborosa squarrosa* was defined as the outgroup, as it is likely the most distant taxon to Lyceae included in this analysis (Olmstead et al., unpubl.). All most-likely trees were combined in a strict consensus tree. An ML nonparametric bootstrap

(BS) analysis was also conducted, using the same model parameters as above, and 200 full heuristic bootstrap replicates, each with five random-addition sequence replicates and TBR branch-swapping; the MulTrees option was not in effect. Due to computer intensity this ML bootstrap analysis was conducted using PAUP* version 4.0b10 for UNIX (Swofford, 2002) run on the Condor (Condor Project, 2005) computer cluster at Amherst College. Bootstrap replicates were parsed for processing using RepMaker (Wilgenbusch, 2003).

RESULTS

Sequences across all 89 taxa have an aligned length of 1968 bp. The ML analysis yielded two trees with $-\ln L = 6589.81093$. Both ML trees (see one of the ML trees, Fig. 1) show strong support for a monophyletic tribe Lycieae (BS = 100), with *Nolana* (BS = 100) strongly supported as the sister group to tribe Lycieae (BS = 92). Within Lycieae, there is limited support (BS = 54; not indicated in Fig. 1) for *Phrodus microphyllus* as sister to all other species in tribe Lycieae, which are in two clades [clade A (BS = 100) and a group that includes clades B and C (BS = 79)]. Clade A is a well supported lineage of five species of *Lycium* (*L. cooperi*, *L. macrodon*, *L. puberulum*, *L. pallidum* and *L. shockleyi*) plus *Grabowskia* (BS = 100). All other species of *Lycium* are in two strongly supported lineages, clade B (BS = 98) and clade C (BS = 92).

Clade B is a strictly American clade comprised of both North and South American taxa. In this clade there is support for the South American *Lycium cestroides* as sister to the other species (BS = 76). Among these species there is a strongly supported group (clade B1; BS = 99) composed of the North American *L. brevipes* and *L. carolinianum* (var. *sandwicense* occurs on Pacific Islands) and the South American *L. rachidocladum* and *L. tenuispinosum*. This clade is also supported by a large (59–62 bp) insertion. Also within clade B is a well supported (clade B2; BS = 84) lineage of North and South American taxa, including the sister species *L. minimum* + *L. athium* (BS = 96) and a North American clade (BS < 60) including *L. exsertum* + *L. fremontii* + *L. parishii* + *L. texanum* + *L. torreyi* + *L. berlandieri* that is nested among South American species and the North American *L. andersonii*.

Clade C includes a strongly supported clade of Old World *Lycium* species (clade C1; BS = 92) as well as several species of South American *Lycium* (except for North American *L. californicum*). There is a strongly supported relationship (BS = 97, clade C2 in Fig. 1) between the South American species *L. chilense* and *L. ciliatum*. There is also support for a group of American species (BS = 100, clade C3 in Fig. 1), including the South American species *L. nodosum* and *L. vimineum* (BS = 100) that are sister to all accessions of the North American *L. californicum* (BS = 100). Further, a close relationship between *L. minutifolium*, *L. stenophyllum* and *L. fuscum* has moderate support (BS = 84). Beyond these relationships, however, there is only weak support for relationships among these groups and the remaining three species of South American *Lycium* (*L. ameghinoi*, *L. chanar* and *L. gilliesianum*) in clade C.

In the Old World clade of *Lycium* (clade C1, Fig. 1), the East Asian species *L. barbarum*, *L. chinense* and *L. ruthenicum* comprise a monophyletic group (BS = 96), but their relationship to other Old World *Lycium* species is equivocal. *Lycium australe* is in a clade with the southern African species *L. tenue* and *L. gariepense*, which is nested within a well supported clade of twelve southern African species (BS = 93). There is also support for a clade (BS = 80) comprised of both southern and northern African *Lycium* species, including the southern African clade of *L. villosum*, *L. hirsutum* and *L. bosciifolium* (BS = 92) and the sister species relationship of *L. schweinfurthii* plus *L. shawii*. Finally, in this group there is support for relationships between *L. decumbens* and *L. pumilum* (BS = 100), as well as for *L. europaeum* and *L. depressum* (BS = 91).

DISCUSSION

Relationships within Lycieae

Consistent with previous studies, tribe Lycieae is strongly supported as a natural group, confirming Hunziker's (1977) tribal circumscription. Further, the present study suggests that *Phrodus microphyllus* is sister to all other taxa in tribe Lycieae. Interestingly, the fruits of *Phrodus* are intermediate between the mostly fleshy berries found in *Lycium* and the uniformly hardened fruits of *Grabowskia*. *Phrodus* fruits have a hardened distal region composed of two large sclerifications, but their middle and lower sections are fleshy. They also differ from the fruits of all other members of Lycieae in being mucilaginous (Bernardello and Hunziker, 1987).

In agreement with previous work (Levin and Miller, 2005), the earliest diverging clade within the rest of Lycieae includes all species of *Grabowskia* plus a small lineage of North American *Lycium* species. *Grabowskia* has been described as having four species, *G. boerhaviaefolia*, *G. obtusa*, *G. duplicata* and *G. megalosperma* (Hunziker, 1997, 2001), distinguished by corolla size, number of flowers per inflorescence, and whether flowers are produced on old versus young branches (Hunziker, 1997). In reality, it is exceedingly difficult to distinguish *Grabowskia* species morphologically, and species circumscriptions may need to be re-evaluated. In the present study we included *G. boerhaviaefolia* accessions from across its geographic range, including accessions from the Galapagos Islands, the interior of Mexico, as well as Argentina and Bolivia. Results suggest that *G. duplicata* and *G. obtusa* are nested within a diverse *G. boerhaviaefolia*. The branch lengths shown in the phylogram of one of the two ML trees (Fig. 1) indicate that the branch leading to *Grabowskia* is actually quite long, especially compared to its sister group of *Lycium*, but within *Grabowskia* the branches are short to non-existent. Given that there is limited resolution within *Grabowskia* (Fig. 1), it is possible that all three species [*G. megalosperma*, a questionable species that has only been collected once (Hunziker, 2001), was not included] are monophyletic species. The nomenclatural circumscription of *Grabowskia* as a genus is of course also in question, given that *Lycium* is not monophyletic unless it includes *Grabowskia*.

Relationships among American *Lycium*

Compared with previous studies (Fukuda et al., 2001; Miller, 2002; Levin and Miller, 2005), considerably more South American species were included in this study. This increased sampling strengthens the finding that the North and South American species are not reciprocally monophyletic. Further, a number of South American species (plus one North American species) are clearly more closely related to Old World *Lycium* species than they are to other American *Lycium*.

Interestingly, within clade B (Fig. 1), *Lycium cestroides* appears sister to all other American *Lycium* in this clade, albeit with only moderate BS support (BS = 76). *Lycium cestroides* has unique, long, dark, purple corollas that are hummingbird-pollinated, a floral morphology and pollinator affinity not found among any other American *Lycium* species (Galletto et al., 1998). Also within clade B, *Lycium athium* and *Lycium minimum* are strongly supported as sister taxa. This finding is further supported by the shared presence of a two-seeded drupaceous fruit, a morphology similar to that shared by *L. californicum* and *L. ameghinoi* (both species in clade C). But in *L. athium* and *L. minimum* the pyrenes are incompletely sclerified, whereas the pyrenes in *L. californicum* and *L. ameghinoi* are completely sclerified (Bernardello, 1986). Bernardello (1986) noted an affinity between *L. athium* and *L. minimum* based on morphological characteristics, as both species have caducous corollas of similar size and shape and exerted stamens with short anther thecae. Geographically, it is remarkable that these two species are so closely related given that *L. athium* is known only from thorn scrub forests in the northern Argentinean province of Formosa, whereas *L. minimum* is endemic to the Galapagos Islands. Bernardello (1986) also noted from examination of herbarium material that flowers of *L. minimum* appear to be dimorphic; if confirmed, this would be the only South American *Lycium* species described as dimorphic.

Previous studies (Bernardello and Chiang-Cabrera, 1998; Fukuda et al., 2001; Miller, 2002; Levin and Miller, 2005) have offered little support for infrageneric sections as previously circumscribed (Hitchcock, 1932; Chiang-Cabrera, 1981; Bernardello, 1986). However, the one section that remains supported is the South American section *Schistocalyx*, including the sister species *Lycium chilense* and *L. ciliatum*, which share an enlarged ciliate base on the filaments (Bernardello, 1986, 1987). *Lycium chilense* has eight named varieties (var. *chilense*, var. *vergarae*, var. *minutifolium*, var. *filifolium*, var. *comberi*, var. *glaberrimum*, var. *descolei*, and var. *confertifolium*) from across Argentina and central Chile (Bernardello, 1986) and from near sea level to at least 3470m elevation in the Andes. Although *L. ciliatum* has a more limited geographic range, occurring in central to northern Argentina and into Bolivia, some populations are difficult to distinguish from *L. chilense* (Bernardello, 1986).

Given that these two species are observed to be rather heterogeneous and overlapping in their morphological characteristics, it is reasonable to ask whether these are indeed two species. In this study we included GBSSI sequences from a total of six accessions (five named varieties) of *Lycium chilense* and two accessions of *L. ciliatum*. Although all eight taxa are strongly supported as a monophyletic group, the *L. ciliatum* accessions appear nested within *L. chilense*, with some varieties of *L. chilense* more closely related to *L. ciliatum* than to other varieties of *L. chilense*. These two species are thought to hybridize (Bernardello, 1986), and both taxa have diploid and tetraploid populations (Stiefkens and Bernardello, 2000). Given that present data intermingle the two species, consideration of a single widespread species may be warranted. Unfortunately, despite greatly increased taxon sampling within South America, the closest relatives of *L. chilense* and *L. ciliatum* remain equivocal. However, both ML topologies suggest that the Argentinean and Chilean species *L. ameghinoi*, *L. chanar*, *L. fuscum*, *L. stenophyllum*, and *L. minutifolium* are sister to *L. chilense* and *L. ciliatum*. Further, it is clear that all of these taxa are more closely related to the Old World clade than they are to the American clade B (Fig. 1). As there are still 10 South American taxa outstanding from our analyses, additional taxon sampling in this part of the world is needed.

Among these South American taxa, the close relationship between *Lycium stenophyllum* and *L. minutifolium* is also supported by morphology. Hitchcock (1932) considered *L. stenophyllum* to be included within *L. minutifolium*, and Bernardello (1986) also suggested a close relationship, while still maintaining them as distinct species based partly on the absence of revolute leaves in *L. minutifolium*. It is surprising that *L. fuscum* is not sister to *L. gilliesianum*, as suggested by both Hitchcock (1932) and Bernardello (1986), as both species share relatively long (typically > 1cm) tubular-obconic, four-merous, yellow-green corollas. However, the affinities of *L. gilliesianum* within clade C remain unclear.

Relationships among Old World *Lycium*

Within the Old World clade, *Lycium australe* is in a well supported clade comprised of twelve southern African *Lycium* species. This finding concurs with that of Levin and Miller (2005) using chloroplast *trnT-trnF* data. Fukuda et al. (2001) also used chloroplast sequence data, and found that *L. australe* was within the Old World clade, but relationships within that clade were equivocal. Haegi (1976) reports one native species of *Lycium* (*L. australe*) from Australia and three naturalized species, including the South African species *L. afrum* and *L. ferocissimum* and the Asian species *L. barbarum*. Results of the present study show that *L. australe* is closely related to the South African *L. tenue*, suggesting that a dispersal event from southern Africa to Australia best explains the position of *L. australe* in our analyses. Given that two South African species are known to have been introduced to Australia, it is possible that *L. tenue* was also introduced. Further study including additional accessions of *L. tenue* and *L. australe* may provide more information regarding the origin of *L. australe*. For example, if *L. australe* were nested within *L. tenue*, then there would be support for the introduction of *L. tenue* into Australia

and the synonymy of the two species.

The present study is the first to include *Lycium mascarenense* (Venter and Scott, 1999) from the Mascarene Islands, Madagascar, and the northeast coast of southern Africa. *Lycium mascarenense* was originally thought conspecific with *L. acutifolium*, but with morphology adapted for a coastal habitat (Venter and Scott, 1999). These two species might also be thought closely related given geography, as they are the only species that occur in northeastern South Africa (Venter, 2000). However, the current study clearly shows these two species as distinct and not particularly closely related. It is likely that the current occurrence of *L. mascarenense* in northeastern South Africa is due to dispersal (the fruits are fleshy and likely bird-dispersed as in most *Lycium*) from Madagascar or the Mascarene Islands.

Also notable within the Old World clade is the sister relationship between *Lycium decumbens* and *L. pumilum*. *Lycium decumbens* is a small prostrate species that occurs in coastal northern Namibia into southern Angola, whereas *L. pumilum* occurs throughout much of central and western South Africa into central Namibia. Floral morphology concurs with the DNA data in the present study, as both species have short campanulate corollas, with the stamens inserted just below the mouth of the tube, and a calyx that is equal or slightly shorter than the corolla tube (Venter, 2000).

As in previous studies (Miller, 2002; Levin and Miller, 2005), the East Asian species comprise a clearly monophyletic lineage. Although results of the present study do not resolve whether the East Asian species are sister to the African species or nested within the other Old World taxa, results from chloroplast data (Miller et al., unpubl. data) suggest that the East Asian species are nested within the African species.

Several species included here (*Lycium intricatum*, *L. schweinfurthii*, *L. europaeum*, and *L. shawii*) have ranges in the Mediterranean regions of Eurasia and many also make it into northern Africa, the Middle East or Asia. Our results suggest that some of these species are more closely related to African species, whereas others have affinities with Asian *Lycium*. Among the species with affinities to *Lycium* in Africa, *Lycium schweinfurthii* is closely related to *L. shawii*; *L. shawii* is the only *Lycium* species that occurs in both southern and northern Africa, with its range extending into Mediterranean Europe, the Middle East, and Asia (Venter, 2000). Given the presence of *L. shawii* in southern Africa, it is perhaps not surprising that *L. schweinfurthii* and *L. shawii* are in a clade with species restricted to southern Africa. On the other hand, *L. europaeum* is sister to *L. depressum*, an Asian species that makes it as far west as Israel. This may suggest that the presence of *L. europaeum* in northern Africa is due to dispersal from Asia. The current placement of *L. intricatum* in our analyses leaves ambiguous its affinities towards Africa or Asia. It is certainly possible that the accession that we used of this species, from the Canary Islands, has actually diverged considerably from mainland populations of *L. intricatum* (perhaps indicated by its long branch length, Fig. 1).

Given that East Asian *Lycium barbarum* has been naturalized around the world, there has been considerable confusion over species circumscriptions (see Feinbrun, 1963), with some local varieties of *L. barbarum* currently synonymized with *L. europaeum*, a species native to Mediterranean Eurasia, the Middle East, and northern Africa (Feinbrun, 1968). Further, varieties of *L. europaeum* have been synonymized with both *L. schweinfurthii* and *L. intricatum* (Feinbrun, 1968; Stearn, 1972). Greater sampling of taxa from across Mediterranean Eurasia and northern Africa, as well as increased sequence data are needed to elucidate relationships among these geographically close species and between these species and their east Asian or southern African relatives.

Gender Dimorphism in *Lycium*

Gender dimorphism (the presence of two sexual mating types that coexist within populations) is rare in Solanaceae, known only in six of 94 genera and only ca. 0.8% of species (Sawyer and Anderson, 2000). Accordingly, most *Lycium* species have perfect flowers on all plants and are hermaphroditic in gender expression. However, studies of

North American *Lycium* document functional dioecy in three species (Miller and Venable, 2002). In addition, there are seven species of *Lycium* in Africa with separate male and female plants (Minne et al., 1994; Venter, 2000, 2007; Venter and Venter, 2003a, b). Levin and Miller (2005) demonstrated recently that gender dimorphism evolved twice among North American *Lycium* and perhaps as many as three times in Africa. Further, gender dimorphism is more developed in African compared to American *Lycium* species, both in terms of its frequency and morphological expression. Gender dimorphism is much more common among African *Lycium*, occurring in seven of the 27 African species (26%), but in only three of 50 American species (6%). In addition, morphologically the African *Lycium* have more advanced dimorphism, with the polleniferous morph completely female sterile (Venter, 2000), whereas the functional males are morphologically hermaphroditic in American *Lycium* (Miller and Venable, 2003).

The evolution of gender dimorphism in *Lycium* is particularly interesting because it has occurred on a background of self-incompatibility (SI), which is well documented in *Lycium* (Richman, 2000; Aguilar and Bernardello, 2001; Miller and Venable, 2002; Savage and Miller, 2006). Miller and Venable (2000) have proposed that gender dimorphism evolved in *Lycium* following polyploidy, which acts as a trigger for this transition because it disrupts the incompatibility system. Indeed, there is much empirical work demonstrating the effect of polyploidy on SI in Solanaceae and other groups in which SI is gametophytically controlled (Stone, 2002; Mable, 2004). In accordance with this hypothesis, polyploidy is associated with gender dimorphism in both American and African species, whereas diploid species are hermaphroditic (Miller and Venable, 2000). Studies of American *Lycium* species have further shown that the dimorphic species are self compatible (SC), whereas the hermaphroditic species are SI (Miller and Venable, 2002; Savage and Miller, 2006). Remarkably, the association of ploidy level and breeding system is also present within *L. californicum*. Phylogenetic analyses including eleven diploid and seven polyploid accessions indicate that this taxon is monophyletic; however, all diploid populations are hermaphroditic, whereas all polyploid populations are dimorphic (Yeung et al., 2005; Miller and Levin, unpubl. data).

CONCLUSIONS

Considerable progress in understanding evolutionary relationships among tribe Lycieae and its relatives has been made in recent years. This study has included all three genera of tribe Lycieae and 85% of its largest genus *Lycium*. Our current understanding of evolutionary relationships suggests that the taxonomy of Lycieae requires revision. There is strong support for the combination of *Grabowskia* with *Lycium*. The placement of *Phrodus* relative to *Lycium* is less clear, although it is well supported within tribe Lycieae. In addition, chloroplast DNA data (Olmstead et al., unpubl.) suggest that *Phrodus* is nested within *Lycium*, and a conservative classification could place both *Grabowskia* and *Phrodus* within *Lycium*. Efforts must now turn to increased sampling of East Asian *Lycium*, as we have to date included only three of the seven described species from this area (Zhang et al., 1994), and of South American *Lycium* (we have included ca. two-thirds of the South American species in the present study). In addition, more rapidly evolving genetic markers may yet help resolve relationships among the very closely related southern African taxa; however, given the recent age of many of these species (as suggested by the short branch lengths, Fig. 1) and evidence for considerable hybridization (Venter, 2000; Miller et al., unpubl. data), deciphering relationships among the African species may pose a considerable challenge.

ACKNOWLEDGEMENTS

The authors thank the National Science Foundation for grant DEB-0343735 to J.S.M and R.A.L.; the Amherst College Department of Biology; M. Dillon (Field Museum, Chicago), and A. Tye and the Charles Darwin Research Station, Galapagos Islands, for providing *Lycium minimum* and *Grabowskia. boerhaviaefolia*; A. Danin,

D. Symon, R. Olmstead, A. Forcone, G. Barboza, G. van der Weerden, G. Anderson, L. Bohs, B. Tan, A. Hempel and D. Lorence for providing leaf material and/or DNA; Amherst College undergraduate students A. Savage, A. Neymark, K. Gamson, J. Eastman and P. Murphy for laboratory assistance; and the Namibian Ministry of Environment and Tourism, the Northern Cape Nature Conservation Service, the Western Cape Nature Conservation Board, the Free State Department of Tourism, Environmental and Economic Affairs, and Ezemvelo Kwa-Zulu Natal Wildlife for plant collection permits. The authors also sincerely thank the organizers of the VI International Solanaceae Conference, two anonymous reviewers, and especially Richard Olmstead for his helpful comments on the manuscript and his great contribution to editing this conference volume.

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Tables

Table 1. Taxa, vouchers, localities, and GenBank accession numbers for all GBSSI sequences included in this study. BIRM samples have the seed accession number of the Solanaceae collection at the University of Birmingham, UK; Nijmegen accession numbers refer to the Solanaceae collection at the University of Nijmegen, The Netherlands. Notation in parentheses is used in Fig. 1 for species with multiple accessions. Voucher specimens are deposited in the following herbaria: AD = Plant Biodiversity Centre, Adelaide, Australia; ARIZ = University of Arizona; BLFU = University of the Free State; CONN = University of Connecticut; CORD = Universidad Nacional de Córdoba; F = Field Museum; 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.

Tribe Lycieae Hunz.

***Grabowskia* Schltld.**

- G. boerhaviaefolia* Schltld.—Argentina, *Bernardello* 894 (CORD); EF137751. (ARG)
G. boerhaviaefolia Schltld.—Bolivia, *Nee* 51864 (NY); DQ124499. (BOL)
G. boerhaviaefolia Schltld.—Ecuador, Galapagos Islands, *Dillon and Tye* 8502 (F); EF137752. (GAL)
G. boerhaviaefolia Schltld.—Mexico, *Chiang et al.* F-2206 (ARIZ); EF137750. (MEX)
G. duplicata Arnott—Argentina, *Bernardello & Vesprini* 898 (CORD); DQ124497.
G. obtusa Arnott—Argentina, *Bernardello* 891 (CORD); DQ124498.

***Lycium* L.**

- L. acutifolium* E. Mey ex Dunal.—South Africa, *Miller et al.* 05-229 (MASS); EF137753.
L. afrum L.—South Africa, *Miller et al.* 05-200 (MASS); EF137754.
L. ameghinoi Speg.—Argentina, *Forcone* 790 (CORD); DQ124501.
L. americanum Jacq.—Argentina, *Barboza* 525 (CORD); DQ124502.
L. amoenum Dammer—South Africa, *Miller et al.* 05-177 (MASS); EF137755.
L. andersonii A. Gray—Mexico, *Miller* 97-12 (ARIZ); DQ124503.
L. arenicola Miers—South Africa, *Venter* 647 (BLFU); DQ124504.
L. athium Bernardello—Argentina, *Miller et al.* 05-25 (MASS); EF137756.
L. australe F. Muell.—Australia, *Philpott s.n.* (AD); EF137757.
L. barbarum L.—Cult. Michigan, USA, *Olmstead* S-35 (WTU); EF137758.
L. berlandieri Dunal—Arizona, USA, *Miller* 01-1 (ARIZ); DQ124506.
L. bosciifolium Schinz—South Africa, *Venter* 654 (BLFU); EF137759.
L. brevipes Benth.—Mexico, *Miller* 97-19 (ARIZ); DQ124508.
L. californicum Nutt. ex A. Gray—Pima Co., Arizona, USA, *Miller* 01-2 (ARIZ); DQ124511. (OP)
L. californicum Nutt. ex A. Gray—Pinal Co., Arizona, USA, *Miller & Levin* 04-12 (MASS); DQ124510. (04CH2)
L. californicum Nutt. ex A. Gray—Pinal Co., Arizona, USA, *Miller & Levin* 04-15 (MASS); DQ124509. (04CF1)
L. californicum Nutt. ex A. Gray—Mexico, *Miller and Levin* 05-81 (MASS); EF137760. (PL)
L. carolinianum Walt. **var. quadrifidum** (Moc. & Sessé ex Dun.) C. L. Hitchcock—Texas, USA, *Hempel* 843 (TAIC); DQ124512.

- L. carolinianum* Walt. var. *sandwicense* (Gray) C. L. Hitchcock—Hawaii, USA, Lorence 9367 (US); DQ124538.
- L. cestroides* Schltl.—Argentina, *Bernardello* 878 (CORD); DQ124513.
- L. chanar* Phil.—Argentina, *Miller et al.* 04-71 (MASS); EF137761.
- L. chilense* Bertero—Argentina, *Bernardello* 877 (CORD); DQ124514.
- L. chilense* Bertero var. *chilense*—Argentina, *Miller et al.* 04-101 (MASS); EF137765.
- L. chilense* var. *confertifolium* (Miers) F. A. Barkley—Argentina, *Miller et al.* 04-67 (MASS); EF137762.
- L. chilense* var. *descolei* F. A. Barkley—Argentina, *Forcone* 792 (CORD); EF137766.
- L. chilense* var. *glaberrimum* Phil.—Argentina, *Miller et al.* 04-78 (MASS); EF137763.
- L. chilense* var. *vergarae* (Phil.) Bernardello—Argentina, *Miller et al.* 04-109 (MASS); EF137764.
- L. chinense* Mill.—China, cult. Waimea Bot. Garden, Hawaii, USA, *Olmstead* 92-212 (WTU); EF137767.
- L. ciliatum* Schltl.—Argentina, *Bernardello* 876 (CORD); DQ124516.
- L. ciliatum* Schltl.—Argentina, *Miller et al.* 05-05 (MASS); EF137768.
- L. cinereum* Thunb.—South Africa, *Venter* 649 (BLFU); EF137769.
- L. cooperi* A. Gray—Arizona, USA, *Miller* 97-1 (ARIZ); DQ124518.
- L. cuneatum* Dammer—Argentina, *Bernardello & Vesprini* 897 (CORD); DQ124519.
- L. decumbens* Welw. ex Hiern—Namibia, *Miller et al.* 05-145 (MASS); EF137770.
- L. depressum* Stocks—Israel, *Danin* 01-01-2006 (MASS); EF137771.
- L. eenii* S. Moore—Namibia, *Miller et al.* 05-139 (MASS); EF137772.
- L. elongatum* Miers—Argentina, *Bohs* 2940 (UT); DQ124520.
- L. europaeum* L.—Israel, *Danin* 01-05-2005 (MASS); EF137773.
- L. exsertum* A. Gray—Pima Co., Arizona, USA, *Miller* 01-3 (ARIZ); DQ124521.
- L. ferocissimum* Miers—South Africa, cult. Strybing Arboretum and Botanical Gardens 98-0143; DQ124523.
- L. fremontii* A. Gray—Mexico, *Miller* 97-9 (ARIZ); DQ124525.
- L. fuscum* Miers—Argentina, *Miller et al.* 04-110 (MASS); EF137774.
- L. garipeense* A.M. Venter—Namibia, *Miller et al.* 05-164 (MASS); EF137775.
- L. gilliesianum* Miers—Argentina, *Forcone* 789 (CORD); DQ124526.
- L. grandicalyx* Joubert & Venter—Namibia, *Miller et al.* 05-160 (MASS); EF137776.
- L. hirsutum* Dunal—South Africa, *Venter* 646 (BLFU); EF137777.
- L. horridum* Thunb.—South Africa, *Venter* 655 (BLFU); DQ124528.
- L. infaustum* Miers—Argentina, *Bernardello* 893 (CORD); DQ124529.
- L. intricatum* Boiss.—Canary Islands, *Anderson* 5027 (CONN); EF137778.
- L. leiospermum* I. M. Johnst.—Mexico, *Miller and Levin* 05-53 (MASS); EF137779.
- L. macrodon* A. Gray—Arizona, USA, *Miller* 97-21 (ARIZ); DQ124530.
- L. mascarenense* A.M. Venter & A.J. Scott—South Africa, *Miller et al.* 05-232 (MASS); EF137780.
- L. minimum* C.L. Hitchcock—Ecuador, Galapagos Islands, *Dillon and Tye* 8501 (F); EF137781.
- L. minutifolium* Remy—Chile, *Miller et al.* 04-105 (MASS); EF137782.
- L. morongii* Britton—Bolivia, *Nee* 46749 (NY); DQ124531.
- L. nodosum* Miers—Argentina, *Barboza* 515 (CORD); EF137783.
- L. oxycarpum* Dunal—South Africa, *Miller et al.* 05-206 (MASS); EF137784.
- L. pallidum* Miers—Arizona, USA, *Miller* 97-20 (ARIZ); DQ124534.

- L. parishii* A. Gray—Arizona, USA, *Miller* 97-22 (ARIZ); DQ124535.
L. pilifolium C.H. Wright—South Africa, *Venter* 466 (BLFU); EF137785.
L. puberulum A. Gray—Texas, USA, *Levin* 97-6 (ARIZ); DQ124537.
L. pumilum Dammer—South Africa, *Miller et al.* 05-125 (MASS); EF137786.
L. rachidocladum Dunal—Chile, *Miller et al.* 04-82 (MASS); EF137787.
L. ruthenicum Murray—Eurasia, cult. Nijmegen #954750075; EF137788.
L. schizocalyx C.H. Wright—South Africa, *Venter* 658 (BLFU); EF137789.
L. schweinfurthii Dammer—Israel, *Danin* 01-01-2005 (MASS); EF137790.
L. shawii Roem. & Schult.—Israel, *Danin* 01-02-2006 (MASS); EF137791.
L. shockleyi A. Gray—Nevada, USA, *Miller* 98-1 (ARIZ); DQ124540.
L. sp. nov.—South Africa, *Nänni* 309 (BLFU); DQ124541.
L. stenophyllum Remy—Chile, *Miller et al.* 04-86 (MASS); EF137792.
L. strandveldense A.M. Venter—South Africa, *Miller et al.* 05-193 (MASS); EF137793.
L. tenue Willd.—South Africa, *Miller et al.* 05-220 (MASS); EF137794.
L. tenuispinosum Miers—Argentina, *Bernardello* 892 (CORD); EF137795.
L. tetrandrum L. f.—South Africa, *Olmstead* 99-24 (WTU); DQ124544.
L. texanum Correll—Texas, USA, no voucher; DQ124545.
L. torreyi A. Gray—Arizona, USA, *Miller* 01-5 (ARIZ); DQ124546.
L. villosum Schinz—South Africa, *Venter* 652 (BLFU); DQ124547.
L. vimineum Miers—Argentina, *Bernardello & Vesprini* 896 (CORD); EF137796.
- Phrodus** Miers
Phrodus microphyllus (Miers) Miers—Chile, *Miller et al.* 04-92 (MASS); EF137801.
- Taxa outside of Lycieae**
Jaborosa squarrosa (Miers) Hunz. & Barboza—Bolivia, *Nee* 51819 (NY); EF137798.
Nolana werdermannii I.M. Johnst.—Chile, *Miller et al.* 04-77 (MASS); EF137799.
Nolana coelestics Lindl.—Chile, *Miller et al.* 04-98 (MASS); EF137800.
Sclerophylax spinescens Miers—Argentina, *Miller et al.* 05-11 (MASS); EF137797.
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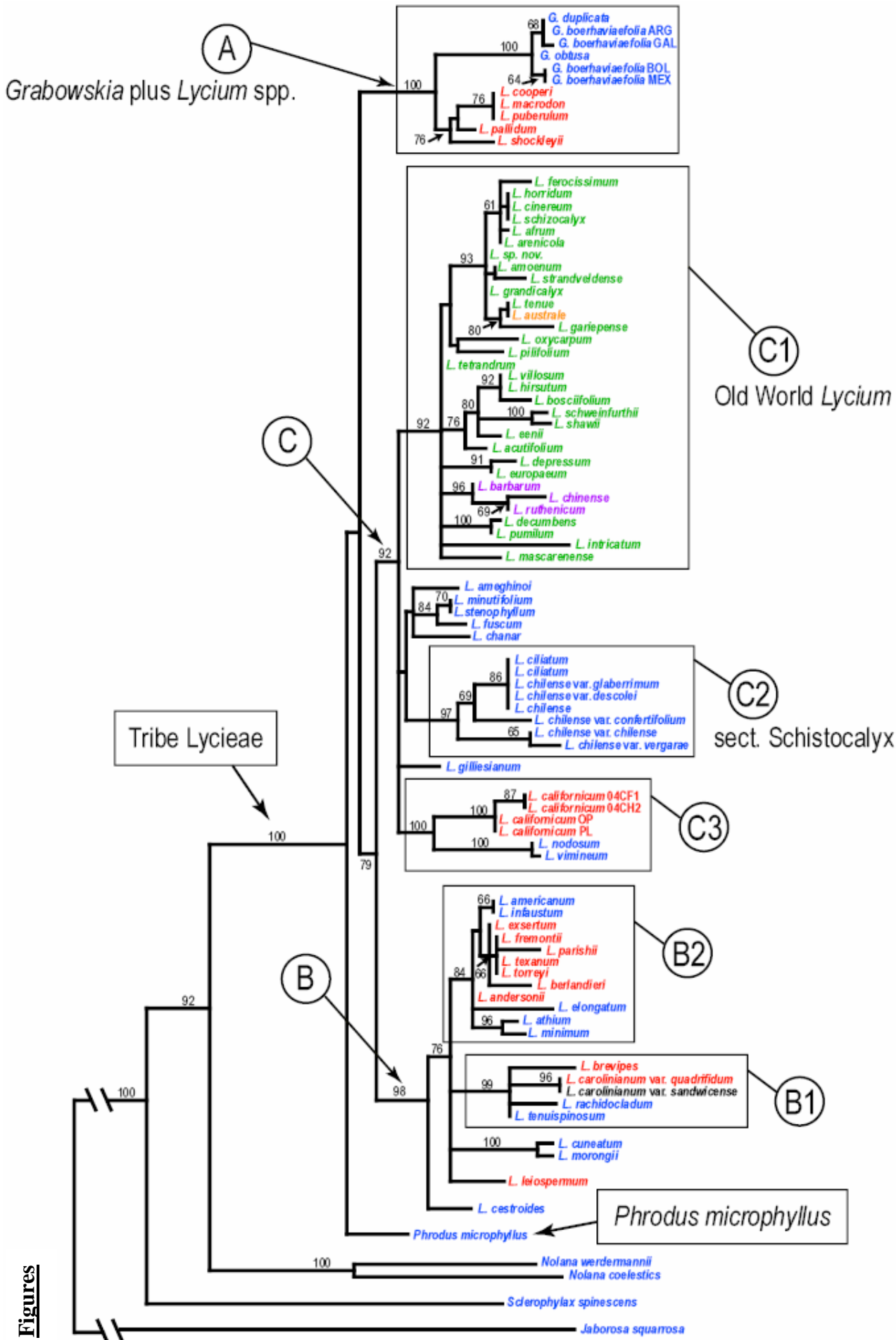


Fig. 1b. A phylogram of one of the two most likely trees from a maximum likelihood analysis of the data. Bootstrap values (> 60) are indicated at the nodes. Taxa are color-coded according to geography, with South American taxa in blue, North American taxa in red, African, and Mediterranean taxa in green, east Asian taxa in purple, the Australian species in orange and the Pacific Islands taxon in black.

