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Do Multiple Tortoises Equal a Hare? The Utility of Nine Noncoding Plastid Regions for Species-Level Phylogenetics in Tribe Lycieae (Solanaceae)

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***Abstract*—**The identification of plastid DNA markers that provide sufficient phylogenetic resolution at the species and population levels is an important challenge for plant systematics. This is mainly due to the relatively slow rate of evolution of the plastid genome. In this study, we examine the utility of several plastid DNA regions for phylogenetic inference at low taxonomic levels in tribe Lycieae (Solanaceae). The regions employed here previously provided considerable numbers of potentially informative characters in investigations of sequence variation across the plastid genome, and one region (*trnH-psbA*) has been advocated for use in DNA barcoding. We sequenced nine noncoding plastid DNA regions (~8,400 bp) for a diverse sample of *Lycium* species and *Phrodus microphyllus* (tribe Lycieae), as well as the outgroup *Nolana,* and included published sequences from the distant outgroup *Atropa*. The nine regions varied with regard to their phylogenetic utility, as measured by the numbers of variable and parsimony informative characters and informative insertions/deletions. We advocate a combination of three regions, including *trnD*^{GUC}–*trnT*^{GGU}, *rpl32–trnL*^{UAG}, and *ndhF–rpl32*, for phylogenetic studies at the infrageneric or tribal level in Solanaceae. Collectively, these three plastid DNA regions had >100 variable characters across 24 taxa, with 72 parsimony informative characters, and 10 phylogenetically informative indels. Future studies should explore these three noncoding markers for low level phylogenetic questions in other taxonomic groups.

***Keywords*—**DNA barcoding, *Lycium*, molecular systematics, phylogeny, Solanaceae, species-level relationships.

The development of genetic markers to infer evolutionary relationships among closely related species and populations remains an important challenge in plant molecular systematics. In particular, knowledge of fine-scale evolutionary relationships is crucial for understanding character evolution among closely related taxa, and comparison of data from both organelle and nuclear genomes can help untangle complicated evolutionary histories (Cronn et al. 2002; Sang 2002; Small et al. 2004).

Data from the plastid genome have been influential in plant molecular systematics since the availability of universal primers (e.g. Taberlet et al. 1991) and the sequencing of coding regions *rbcL* (e.g. Chase et al. 1993) and *ndhF* (Olmstead and Sweere 1994). However, given the relatively slow rate of plastid evolution compared to widely used nuclear ribosomal ITS sequences (Baldwin et al. 1995; Álvarez and Wendel 2003; Nieto Feliner and Rosselló 2007) or low copy nuclear genes (Sang 2002; Small et al. 2004), the utility of plastid data has often been limited to relatively deep phylogenetic levels. Yet, the goal of identifying plastid regions that evolve at a rate useful for phylogenetic studies at lower taxonomic levels remains, given their many advantages including uniparental inheritance with lower effective population sizes, and their presence almost universally as a single copy. Comparison of topologies inferred from the maternally inherited plastid genome to those inferred from the biparentally inherited nuclear genome can facilitate identification of hybrid species histories (Sang et al. 1997; Lihová et al. 2006; but see Hansen et al. 2007).

In recent years, there have been considerable advances towards the identification and development of plastid noncoding intergenic spacer regions that are more amenable to species level studies (Shaw et al. 2005, 2007). Shaw et al. (2005) compared sequence variation across 21 plastid DNA regions in 29 species from 9 families (10 lineages) of seed plants. Because their objective was to identify plastid DNA regions that were useful at low taxonomic levels, only noncoding plastid DNA intergenic spacers or introns were included, as these regions are expected to evolve more rapidly than coding regions such as *rbcL* (Gielly and Taberlet 1994). The extent of phylogenetic information for these plastid regions was determined by assessing the number of potentially informative characters; sequence variation was considered both within families or lineages, as well as across all taxonomic groups (see Figs. 4 and 5 in Shaw et al. 2005). Although the magnitude of plastid DNA sequence variation varied dramatically across lineages, the authors reported predictable variation for particular regions of plastid DNA across diverse taxonomic groups. In a follow up paper, Shaw et al. (2007) used comparisons of entire plastid genomes (*Atropa* and *Nicotiana*, Solanaceae; *Lotus* and *Medicago*, Fabaceae; *Saccharum* and *Oryza*, Poaceae) to identify additional regions predicted to have high variability. The development and comparative analyses of noncoding plastid DNA sequence variation by Shaw et al. (2005, 2007) provides a tremendous resource for molecular systematic studies of plants. Importantly, the authors provide a framework for selecting plastid DNA markers potentially useful for low level phylogenetic reconstruction. However, given the broad scope of Shaw et al. (2005, 2007), these regions were not actually demonstrated as useful for fine-scale phylogenetic studies.

Here, we focus on the identification of noncoding plastid DNA regions that allow for the robust inference of species level relationships among closely related members of tribe Lycieae (Solanaceae), as well as the sister genus *Nolana*. Species in tribe Lycieae [~87 species in three genera: *Grabowskia* (three spp.), *Lycium* (ca. 83 spp.), and *Phrodus* (one spp.)] are distributed predominately in Argentina, Chile, and southwestern North America in the New World, and in southern Africa and eastern Asia in the Old World. A recent phylogenetic study (Levin et al. 2007) using the nuclear gene GBSSI (*waxy*) documented the monophyly of 32 species of Old World *Lycium*, implying a single dispersal event from the Americas to the Old World (clade C1 in Fig. 1). However, a previous study incorporating plastid sequence data (*trnL-trnF* and *trnT-trnL*; Levin and Miller 2005) found only weak support for the monophyly of Old World species. With additional sequence data, including more quickly evolving plastid DNA regions, the monophyly of Old World Lycieae using plastid data can be further examined.

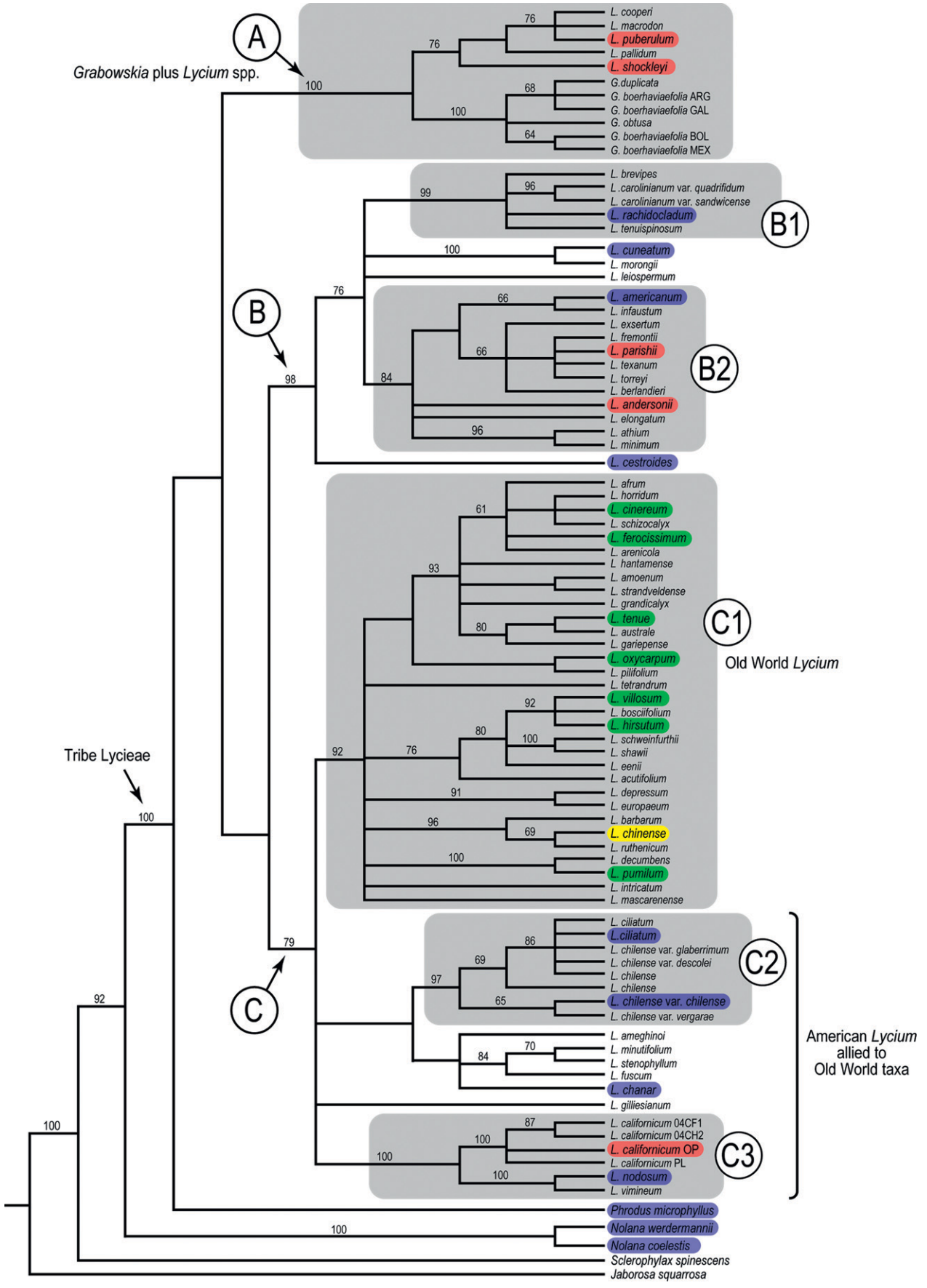

FIG. 1. Maximum likelihood topology of tribe Lycieae inferred from nuclear GBSSI data (Levin et al. 2007). Major clades are indicated with gray shading, and taxonomic sampling in the present study is indicated by color coding that corresponds to biogeographic region (blue, South America; red, North America; green, South Africa; yellow, China). Maximum likelihood bootstrap values >60 are indicated above the branches.

In this study, we compare the utility of nine plastid regions for phylogenetic inference at low taxonomic levels in the tribe Lycieae (Solanaceae). These plastid DNA regions were selected primarily on the basis of variability reported in Shaw et al. (2005, 2007). One of the regions included here (*trnH-psbA*) has also been suggested as useful for plant barcoding (Kress et al. 2005; Kress and Erickson 2007; Fazekas et al. 2008; Lahaye et al. 2008). We include six plastid DNA regions from 22 species in tribe Lycieae and two species of *Nolana*, plus an additional three regions for a subset of these taxa (12 Lycieae, two *Nolana*). As measures of utility, we compare the numbers of variable and parsimony informative characters, including their relationship with each other and to the aligned length of a region. Further, we evaluate levels of homoplasy, investigate the role of insertions and deletions (indels) in phylogenetic reconstruction, explore combinations of plastid DNA data that may yield the same result as the completely concatenated data set, and evaluate the barcoding utility of *trnH-psbA* for differentiating closely related species. Additionally, we compare topologies inferred using plastid DNA sequence data with existing hypotheses based on plastid (Fukuda et al. 2001; Levin and Miller 2005) and nuclear data (Fig. 1; Miller 2002; Levin and Miller 2005; Levin et al. 2007).

Materials and Methods

***Taxonomic Sampling*—**Members of tribe Lycieae (Solanaceae) are shrubby perennials most commonly found in arid or coastal (saline) environments. The present study contains 22 species, including 13 New World (American) *Lycium*, eight species from the Old World (seven from southern Africa and one from China), and the monotypic *Phrodus microphyllus* (collection and voucher information in Appendix 1). This sampling includes ~25% of species in tribe Lycieae, with a diverse sample of species from across a broad geographic area (Fig. 1), as well as both closely and distantly related species. Within the American clade B (see Fig. 1), we included members of all well supported clades (*L. rachidocladum*, *L. cuneatum*, *L. americanum*, *L. parishii*, and *L. andersonii*) as well as *L. cestroides*. Similarly, in the large Old World Clade (clade C1 in Fig. 1), we included both closely related species (*L. hirsutum* and *L. villosum*, *L. cinereum* and *L. ferocissimum*), as well as species from various clades across C1 (South African species *L. tenue*, *L. oxycarpum*, *L. pumilum*; Asian species *L. chinense*). Within the larger clade C we included five of the 11 American species (*L. ciliatum*, *L. chilense*, *L. chanar*, *L. californicum*, and *L. nodosum*) that were suggested previously as closely allied to the Old World species (Levin et al. 2007). Finally, we sampled two species in the strongly supported clade A (*L. puberulum* and *L. shockleyi*). Two species of *Nolana*, the sister group to Lycieae (Fig. 1; Levin and Miller 2005; Levin et al. 2007), were included as close outgroups, and corresponding sequences for all plastid DNA regions from the complete chloroplast genome of *Atropa belladonna* (Genbank AJ316582) were included as a distant outgroup.

***Plastid DNA Sampling*—**Nine plastid DNA regions were included in this study (Table 1), including the widely used *trnT^{UGU}-trnF^{GAA}* region, here amplified as two regions, *trnT^{UGU}-trnL^{UAA}* and *trnL^{UAA}-trnF^{GAA}* following Taberlet et al. (1991). We also included two regions, *trnD^{GUC}-trnT^{GGU}* and *trnH^{GUG}-psbA*, that were among the most variable regions in a comparison including three *Solanum* species (Shaw et al. 2005). The region *trnH-psbA* has also been suggested for plant barcoding given its relatively short length and high variability (Kress et al. 2005; Kress and Erickson 2007; Lahaye et al. 2008). Five other regions (*rpl32-trnL^{UAG}*, *ndhF- rpl32*, *3´trnV^{UAC}-ndhC*, *trnQ^{UUG}-5´rps16*, and *3´rps16–5´trnK^{UUU}*) are from Shaw et al. (2007) and were among those regions with the greatest variability in that study. All but two of the regions included here are located in the large single-copy region of the plastid genome, whereas two (*ndhF-rpl32*, *rpl32-trnL*) are located in the small single-copy region (Shaw et al. 2005, 2007). Sampling was stratified, such that fifteen taxa were first included for all nine plastid DNA regions (Appendix 1), and then an additional 10 taxa were added for those six regions with the highest variation (except for *trnL-trnF*, for which sequences were already in hand).

***DNA Extraction, Amplification, and Sequencing*—**Genomic DNA was isolated from silica dried leaf material using the DNeasy extraction protocol (Qiagen Inc., Valencia, California). For the *trnT-trnL* and *trnL-trnF* regions, amplification and sequencing followed Levin and Miller (2005). Specifically, we sequenced the intergenic spacer between *trnT* and 5´*trnL* and a few bases of 5´*trnL*, as well as the *trnL* intron, *3´trnL*, and the intergenic spacer between *3´trnL* and *trnF* (Table 1). Many of the sequences for the *trnT-trnL* and *trnL-trnF* regions were previously available (Levin and Miller 2005); however, 15 new sequences for eight taxa were generated in the present study (Appendix 1).

Amplification of the intergenic region between the *trnD* and *trnT* genes, including the *trnY* and *trnE* genes, used primers from Demesure et al. (1995; Table 1). Fifty microliter reactions contained 1 × buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.36 µM of each primer, 8.8 ng BSA, 1.25 units of *Taq* polymerase, and 1–2 µL DNA. Thermal cycler conditions included initial denaturation at 80°C for 5 min; 40 cycles at 94°C for 45 s, 50°C for 30 s, 72°C for 1 min, ending with an extension at 72°C for 7 min. Alternatively, a touchdown procedure was used with an initial denaturation at 94°C for 3 min; 20 cycles at 94°C for 45 s, 58°C-49°C (decreasing one degree every two cycles) for 30 s, 72°C for 1 min 30 s; 20 cycles at 94°C for 45 s, 48°C for 30 s, 72°C for 1 min 30 s; with a final extension at 72°C for 7 min.

For the other six regions (*trnH-psbA*, *rpl32-trnL*, *ndhF-rpl32*, *trnV-ndhC*, *trnQ-5´rps16*, and *3´rps16–5´trnK*), accessions were amplified in either 25 or 50 µl reactions containing 1 × buffer, 2.5–3.0 $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer, 4.4 ng BSA (per 25 µl), 0.625 units *Taq* polymerase (per 25 µl), and 1–2 µl DNA template. The thermal cycler conditions were the same as the touchdown procedure outlined above for *trnD-trnT*. Primer sequences (with references) are in Table 1.

All PCR amplification products were cleaned using either the Qiaquick PCR Purification Kit or the MinElute PCR Purification Kit (Qiagen Inc.). Sequencing in both directions using the same primers as in amplification was conducted at the Core Laboratories Center (Cornell University) or the Nucleic Acid Facility, University Park (Pennsylvania State University).

Table 1. The plastid DNA regions and primer sequences used for PCR amplification and sequencing.

Plastid DNA region	Primer sequence (5´–3´)	Reference
trnL^{UAA}–trnF^{GAA}	c: CGA AAT CGG TAG ACG CTA CG f: ATT TGA ACT GGT GAC ACG AG	Taberlet et al. 1991
trnT^{UGU}–trnL^{UAA}	a: CAT TAC AAA TGC GAT GCT CT b: TCT ACC GAT TTC GCC ATA TC	Taberlet et al. 1991
trnD^{GUC}–trnT^{GGU}	trnD^{GUC}: ACC AAT TGA ACT ACA ATC CC trnT^{GGU}: CTA CCA CTG AGT TAA AAG GG	Demesure et al. 1995
trnH^{GUG}–psbA	trnH^{GUG}: CGC GCA TGG TGG ATT CAC AAT CC psbA: GTT ATG CAT GAA CGT AAT GCT C	Tate and Simpson 2003 Sang et al. 1997
rpl32–trnL^{UAG}	trnL$^{(UAG)}$: CTG CTT CCT AAG AGC AGC GT rpL32-F: CAG TTC CAA AAA AAC GTA CTT C	Shaw et al. 2007
ndhF–rpl32	rpL32-R: CCA ATA TCC CTT YYT TTT CCA A ndhF: GAA AGG TAT KAT CCA YGM ATA TT	Shaw et al. 2007
3´trnV^{UAC}–ndhC	trnV$^{(UAC)}$x2: GTC TAC GGT TCG ART CCG TA ndhC: TAT TAT TAG AAA TGY CCA RAA AAT ATC ATA TTC	Shaw et al. 2007
trnQ^{UUG}–5´rps16	trnQ$^{(UUG)}$: GCG TGG CCA AGY GGT AAG GC rpS16x1: GTT GCT TTY TAC CAC ATC GTT T	Shaw et al. 2007
3´rps16–5´trnK^{UUU}	rpS16x2F2: AAA GTG GGT TTT TAT GAT CC trnK$^{(UUU)}$x1: TTA AAA GCC GAG TAC TCT ACC	Shaw et al. 2007

All sequences included in this study have been deposited in Genbank, with accession numbers in Appendix 1.

***Data Analyses*—**Individual sequences for all genomic accessions were checked manually, aligned using Sequencher v4.7 (Gene Codes Corp., Ann Arbor, Michigan), and combined into a single consensus sequence for each accession. Multispecies alignments for each plastid DNA region were constructed in Se-Al v2.0a11 (Rambaut 2002). As plastid regions are inherited as a single locus, we concatenated all of the regions used here into a single data set. Two concatenated data sets were created; one included sequence data for six regions across 25 taxa, and the other included sequence data for nine regions across 15 taxa (TreeBASE study number S2267).

We used maximum parsimony (MP) and maximum likelihood (ML) methods for inferring relationships among species. Parsimony analyses were conducted in PAUP* (Swofford 2002) using the branch and bound algorithm with the Multrees option in effect. Bootstrap support for the parsimony analyses was determined using 500 full heuristic searches, each with 10 random addition sequence replicates and Multrees in effect. To investigate the importance of indels, we conducted identical parsimony searches with and without indels coded as separate binary characters. Indels coded were all > 1 bp, nonautapomorphic, present in at least one species in tribe Lycieae, and did not occur in areas of mononucleotide repeats. For each of the two concatenated data sets, ML model parameters were determined using the Akaike information criterion in Modeltest v. 3.7 (Posada and Crandall 1998). The best-fit model for each data set was used in separate ML analyses in PAUP*. Support for ML topologies was assessed using 1,000 ML bootstrap searches conducted on the Amherst College computing cluster (Condor Project 2005).

For each of the nine plastid DNA regions, we calculated both the total number of variable sites and the percentage of variable sites out of the total number of aligned bases. We also determined the number of parsimony informative (PI) characters. All measures were calculated for species in tribe Lycieae (either 22 or 12 species depending on the plastid DNA region) and for tribe Lycieae plus their sister taxon *Nolana* (either 24 or 14 species depending on the plastid DNA region), but excluding the distant outgroup *Atropa*. To facilitate comparisons across all nine of the plastid DNA regions, we also calculated the number of variable sites and PI characters for an identical subset of taxa for the six regions with greater taxonomic sampling; this subset included the 12 species of Lycieae sampled for all nine regions and two species of *Nolana* (see Appendix 1). To determine whether the total number of variable sites was a reasonable indicator of phylogenetic information (Shaw et al. 2005; Mort et al. 2007), we used regression analyses to examine the relationship between the number of PI sites and the number of variable sites for each plastid DNA region. We also investigated whether the phylogenetic utility of a region, as measured by either variable sites (Shaw et al. 2005) or PI sites (Mort et al. 2007), increased with increasing sequence length.

Results

***Comparison of Plastid DNA Regions*—**Aligned lengths for the nine plastid DNA regions ranged from 524 bp in *trnH-psbA* to 1,224 bp in *trnQ-5´rps16*. There was no significant relationship between the aligned sequence length and the number of variable characters ($F_{1,8} = 0.126$, $p = 0.733$) or the number of PI characters ($F_{1,8} = 0.477$, $p = 0.512$) across the nine regions. There was, however, a significant positive association between the total number of variable sites and the number of PI characters ($F_{1,8} = 20.673$, $p < 0.003$; Fig. 2A). For all regions, the number of PI characters ranged from eight to 31 for comparisons of Lycieae and *Nolana* (Table 2), and from zero to 14 in comparisons of Lycieae alone (Table 2). Standardizing taxon sampling across all plastid DNA data sets resulted in a slight reduction of variable sites and PI characters, but did not change the relative rankings of the different regions (compare values in Table 2 and Fig. 2B). Three of the plastid DNA regions (*ndhF-rpl32*, *rpl32-trnL*, and *trnD-trnT*) had the highest number of PI characters. A fourth region, *trnH-psbA*, had among the highest percentage of variable sites, but, given its short length (Table 2),

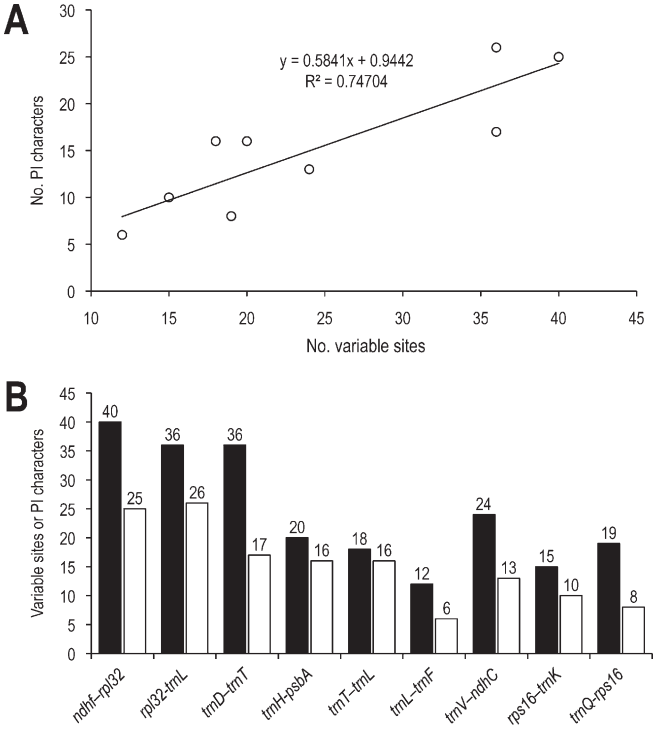

FIG. 2. The relationship between the number of variable characters and the number of parsimony informative characters (A) for nine plastid DNA data sets including indels. For each plastid DNA region, the total number of variable sites (B, closed bars) and parsimony informative characters (open bars) are compared for the identical subset of 14 species (12 Lycieae and two *Nolana*).

TABLE 2. Length and variability (nucleotide substitutions plus indels) for nine plastid regions sequenced in Lycieae and two *Nolana* species (either 24 or 14 species; outgroup *Atropa belladonna* excluded) and Lycieae alone (either 22 or 12 species).

	ndhF–rpl32	*rpl32–trnL*^{UAG}	*trnD*^{GUC}*–trnT*^{GGU}	*trnH*^{GUG}*–psbA*	*trnT*^{UGU}*–trnL*^{UAA}	*trnL*^{UAA}*–trnF*^{GAA}	*3´trnV*^{UAC}*–ndhC*	*3´rps16–5´trnK*^{UUU}	*trnQ*^{UUG}*–5´rps16*
Lycieae and *Nolana*									
No. Species included	24	24	24	24	24	24	14	14	14
Aligned length	860	932	1,160	524	714	904	1,125	796	1,221
No. Variable sites	44	39	42	26	21	15	24	15	19
No. PI characters	30	31	21	18	17	9	13	10	8
Indels	3	4	3	–	2	–	1	–	–
% Variable sites	0.051	0.042	0.036	0.050	0.029	0.017	0.021	0.019	0.016
Lycieae									
No. Species included	22	22	22	22	22	22	12	12	12
Aligned length	841	911	1,160	517	714	897	1,116	796	897
No. Variable sites	25	20	30	15	10	12	14	9	7
No. PI characters	14	14	12	7	7	6	3	3	0
Indels	3	4	3	–	2	–	1	–	–
% Variable sites	0.030	0.022	0.026	0.029	0.014	0.013	0.013	0.011	0.008

trnH-psbA was only fourth among the regions with the most PI characters (Table 2). In fact, comparison of *trnH-psbA* for Lycieae revealed four sets of species with identical *trnH-psbA* sequences: (1) *L. americanum*, *L. andersonii*, *L. californicum*, *L. chanar*, *L. nodosum*, *L. parishii*, *L. rachidocladum*, and *Phrodus microphyllus*, (2) *L. cinereum*, *L. ferocissimum*, *L. oxycarpum*, and *L. pumilum*, (3) *L. cestroides* and *L. cuneatum*, (4) *L. ciliatum* and *L. chilense*. Not surprisingly, both the number and percentage of variable sites decreased when only species in tribe Lycieae were considered; however, from 37–80% of the variable characters remained (compare the number of variable sites for Lycieae plus *Nolana* to the number for Lycieae alone, Table 2). For example, considering both Lycieae and *Nolana* there were 42 variable characters for *trnD-trnT*; 30 (71%) of these positions remain as variable characters when considering Lycieae alone.

Phylogenetic Relationships—Results of the maximum likelihood analyses for both the 15 and 25 taxa data sets are shown in Fig. 3. Likelihood bootstrap support values were high, and 80% of all resolved nodes had bootstrap values greater than 70%. All species in tribe Lycieae are supported as a monophyletic group in analyses of both data sets. In addition, *L. puberulum* and *L. shockleyi* are also strongly supported as monophyletic (Fig. 3A, BS = 99). There is limited support for the monophyly of Old World *Lycium* (BS = 56, Fig. 3A; BS = 51, Fig. 3B). However, there is strong support for two clades within the Old World. The first clade includes five South African species (*L. cinereum*, *L. ferocissimum*, *L. oxycarpum*, *L. pumilum*, *L. tenue*; Fig. 3A, BS = 99), and the second clade includes two South African species (*L. hirsutum* and *L. villosum*) and the Chinese *L. chinense* (Fig. 3A, BS = 100).

Maximum parsimony analyses with and without indels yielded similar topologies (trees not shown) as likelihood analyses, but with generally higher bootstrap support (Fig. 3). This increased support also resulted in one more resolved node within New World *Lycium*; a 5 bp indel in *rpl32-trnL* supports the monophyly of *L. andersonii* + *L. parishii* (BS = 87), rather than a polytomy with *L. californicum* (Fig. 3A). Inclusion of indels in maximum parsimony analyses also provided additional support for the monophyly of Old World *Lycium* (25 taxa, BS = 83; 15 taxa, BS = 84).

Discussion

Comparison of Plastid DNA Regions—As noted by Shaw et al. (2005), although the *trnT-trnL* and *trnL-trnF* regions are

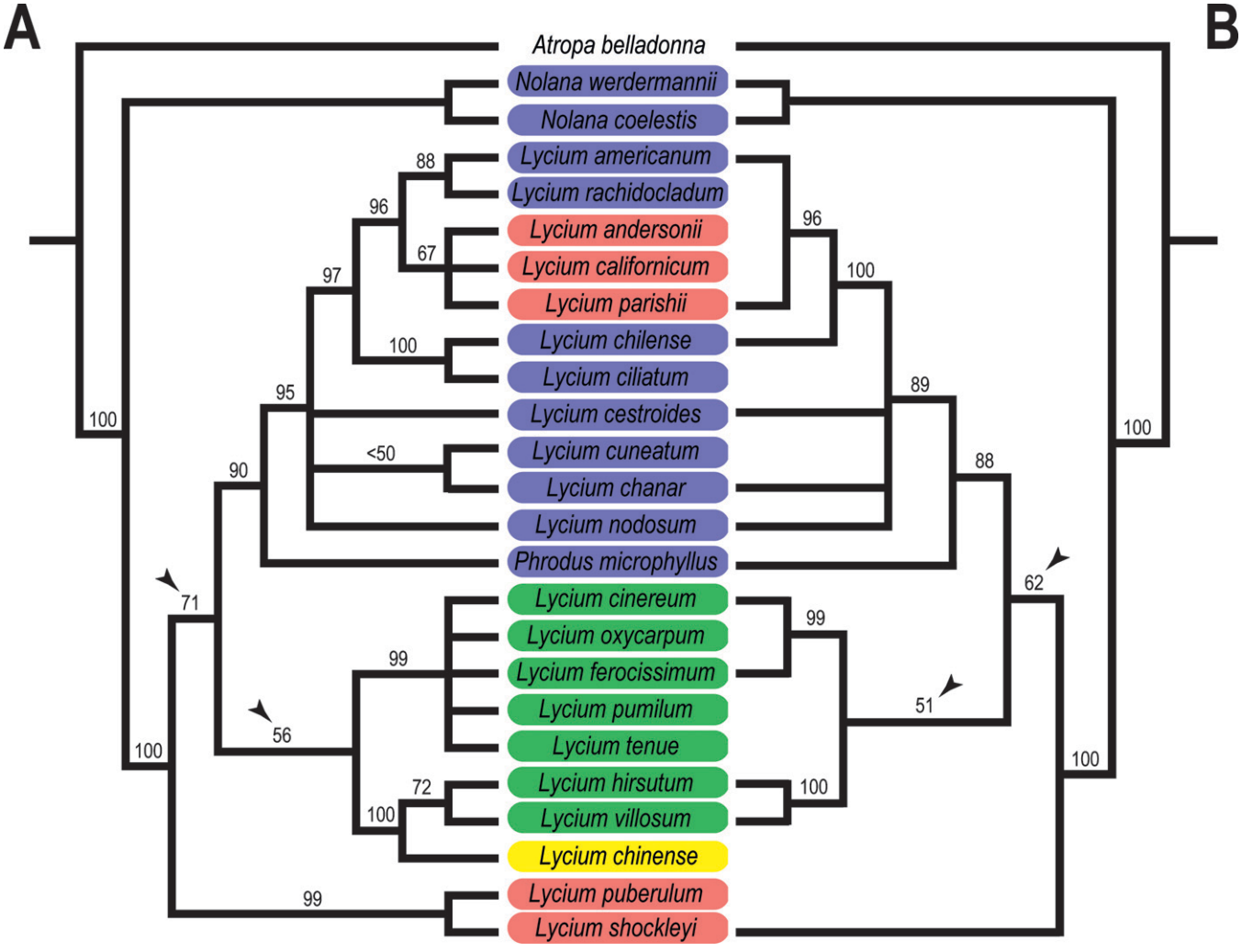

FIG. 3. Maximum likelihood topologies inferred for the 25 taxa, six region (A) and 15 taxa, nine region (B) data sets. Maximum likelihood bootstrap values are shown above the branches. Arrows indicate nodes for which inclusion of indels in parsimony analyses increases support to >75% (see results and discussion). Color coding as in Fig. 1.

widely used in molecular systematic studies, they are often not sufficiently variable to provide resolution at low taxonomic levels. In general our results agree with their assertion; in the twenty-five species, six region data set, *trnT-trnL* and *trnL-trnF* were among the least variable regions in terms of both PI and variable characters (Table 2). Likewise, in the standardized comparison of all nine regions (Fig. 2B), these regions (especially *trnL-trnF*) were among the least variable. Larger scale studies of tribe Lycieae, using only these regions (Levin and Miller 2005) or these regions in combination with the *matK* coding region (Fukuda et al. 2001), provide little resolution regarding species level relationships. Several authors have reported that *trnT-trnL* is more variable than *trnL-trnF* for equivalent comparisons (Böhle et al. 1994; Small et al. 1998). While this may be somewhat true for comparisons including Lycieae and *Nolana*, these two spacers provided roughly equal numbers of variable and PI characters within tribe Lycieae (Table 2).

Plastid noncoding DNA regions clearly vary with regard to their potential utility in phylogenetic studies (Shaw et al. 2005, 2007), and this may be particularly true for different taxonomic groups. Comparison of plastid DNA sequence variation across three *Solanum* species showed the lowest divergence alongside three taxon comparisons in other taxonomic groups (see Fig. 4 in Shaw et al. 2005); in that study, *trnD-trnT* and *trnH-psbA* were reported as most variable in *Solanum*. Shaw et al. (2005) defined potentially informative characters (PICs) as all nucleotide substitutions, indels, and inversions, which is comparable to the total number of variable characters in our study, including all nucleotide substitutions and indels. Levels of variation across Lycieae for these two regions (*trnD-trnT*: 30 variable characters; *trnH-psbA*: 15 variable characters) were higher than reported by Shaw et al. (2005) for *Solanum* (*trnD-trnT*: eight PICs; *trnH-psbA*: five PICs). Across all nine regions and taxa in the present study, *trnD-trnT* provided the second highest number of variable characters and the third highest number of PI characters (Table 2; Fig. 2). These results concur with a number of studies (Potter et al. 2002; Yang et al. 2002; Shaw et al. 2005) that have noted that *trnD-trnT* evolves more rapidly than either *trnT-trnL* or *trnL-trnF*.

Mort et al. (2007) included *trnD-trnT* in a comparison of nine plastid DNA regions in several taxonomic groups. Levels of variation in *trnD-trnT* varied considerably across lineages (see Fig. 3 in Mort et al. 2007), but for comparisons similar to those in the present study, *trnD-trnT* provided limited numbers of PI characters (i.e. fewer than 10 for *Aichryson*, *Crassula*, and *Zaluzianskya*; Mort et al. 2007). In contrast, there were 21 PI characters in a comparison of Lycieae and its closest outgroup *Nolana* (Table 2). On the other hand, *trnD-trnT* has proven even more useful in other lineages. Friesen et al. (2000) used *trnD-trnT* to investigate the phylogenetic position of monotypic *Milula spicata* in relation to 14 species in *Allium* (Alliaceae). Remarkably, the *trnD-trnT* data set (including two outgroups) contained 69 PI characters and parsimony analysis inferred a well resolved and supported tree, comparable to trees inferred using an equivalent data set for ITS (Friesen et al. 2000).

Recent efforts towards barcoding in plants suggest that a combination of coding and noncoding regions may prove useful in species identification and, by extension, in low level phylogenetic reconstruction. For example, Kress et al. (2005) have promoted the use of the *trnH-psbA* intergenic spacer, a result most recently supported by Lahaye et al. (2008). It should be noted that *trnH-psbA* was quite variable in the Shaw et al. (2005) study, but these authors did not recommend its use in phylogenetic studies given its short length (< 600 bp) and difficulties in alignment. We had no difficulty aligning *trnH-psbA* in our study, but this region was the shortest of all regions (467–506 bp in Lycieae) and provided intermediate numbers of characters in the plastid DNA comparisons (Table 2). Kress and Erickson (2007) suggest that the coding gene *rbcL*, in conjunction with the noncoding *trnH-psbA* spacer region, could correctly identify and discriminate among related species and serve as a universal bar code for plants. Sequences of *rbcL* for six species of *Lycium* are available on Genbank, including three taxa used in the present study (North American *L. andersonii*, South American *L. cestroides*, and South African *L. ferocissimum*). Analysis of combined *rbcL* and *trnH-psbA* sequence data for these taxa reveal either 19 or 20 differences between *L. ferocissimum* and the American taxa *L. andersonii* and *L. cestroides*, respectively, but only five differences between the two American taxa. However, given that these three taxa are all fairly distantly related (Figs. 1, 3), it is not surprising that the combination of these two regions should be able to clearly differentiate them. By contrast, the ability of *rbcL* plus *trnH-psbA* to act as a barcode and differentiate closely related species of *Lycium* has little support. In fact, among Lycieae there were four groups of species (comprising 16 total species) with identical *trnH-psbA* sequences; yet these species can all be distinguished based on morphology and other molecular data. Further, if taxon sampling within *Lycium* were increased (as in Fig. 1), then the number of species with identical *trnH-psbA* sequences could only increase. Sequence data for *rbcL* will be unlikely to improve this situation, as there is only one difference between Genbank retrieved *rbcL* sequences of *L. carolinianum* var. *sandwicense* and *L. andersonii*, which are resolved in different clades (B1 and B2) in Fig. 1. Of course, to fully evaluate the utility of the proposed two locus barcode in tribe Lycieae additional *rbcL* sequencing would need to be done, but these preliminary data suggest that it is not a promising approach for differentiating among closely related Solanaceae.

Recently, Shaw et al. (2007) identified over a dozen new plastid DNA regions predicted to show high levels of variation at the species level. Given differences among lineages in the utility of various plastid DNA regions, Shaw et al. (2005, 2007) suggested that a number of variable regions be screened for specific levels of variability within a particular group of interest before sequencing large numbers of taxa. Following such an approach, we screened nine regions for 15 species, broadening taxonomic sampling for those regions with the greatest numbers of PI characters in Lycieae. Three of the five regions included here (*trnV-ndhC*, *trnQ-5´rps16*, and *3´rps16–5´trnK*) were of limited utility for infrageneric phylogenetic inference, having very few variable (or PI) characters (Fig. 2B; Table 2). However, two of the more variable regions identified in Shaw et al. (2007; *rpl32-trnL* and *ndhF-rpl32*) stand out as particularly informative for phylogenetic studies in Lycieae (Fig. 2B; Table 2). Together with *trnD-trnT* (see above), these three plastid DNA regions account for two-thirds of all the variable sites in the six region data set (Table 2). In addition, a parsimony analysis including only these three regions and indels recovers a topology virtually identical to that in Fig. 3A. The only difference is the position of *L. californicum*, which moves from being allied with *L. andersonii* and *L. parishii* (in the ML analysis, see Fig. 3A) to a polytomy including *L. americanum* + *L. rachidocladum* and *L. andersonii* + *L. parishii*.

Thus, we advocate a combination of *ndhF-rpl32*, *rpl32-trnL*, and *trnD-trnT* for infrageneric studies in Solanaceae. These regions contained the most information in this study and are more useful for differentiating species. When sequence data for these three plastid regions are combined, all species have unique sequences except for four closely related African species (*L. cinereum*, *L. ferocissimum*, *L. oxycarpum*, and *L. pumilum*). *Lycium tenue* (also in this clade, Fig. 3), differs from the other four species by only a single nucleotide. In comparison, the average number of base pair differences is 13 or 12 among the American species or across the tribe, respectively. In addition to the high numbers of PI characters (Fig. 3; Table 2), these regions can all be sequenced easily in two reactions (once in each direction). Although *trnH-psbA* has a high percentage of variable sites (Table 2), given its short length it is more cost efficient to sequence *trnD-trnT*, which is over twice as long, allowing recovery of a greater number of informative characters for the same cost. Further, it is possible to coamplify *ndhF-rpl32* and *rpl32-trnL* in a single reaction (Shaw et al. 2007), thus increasing the efficiency and cost of obtaining sequences. It is clear from previous studies that *trnD-trnT* may also be useful for infrageneric studies in other taxonomic groups (Friesen et al. 2000; Hahn 2002; Potter et al. 2002; Cuenca and Asmussen-Lange 2007). To our knowledge, there have been no published studies of either *ndhF-rpl32* or *rpl32-trnL*, with the exception of their development (Shaw et al. 2007). Shaw et al. (2007) suggested that these regions should be useful for fine-scale phylogenetics and indeed they are, at least in the genus *Lycium*. Both of these plastid DNA regions had high numbers of potentially informative characters, with *rpl32-trnL* exhibiting the greatest number of potentially informative characters across all 34 plastid DNA regions examined in Shaw et al. (2005, 2007).

Relationships in Lycieae—Consistent with previous studies (Miller 2002; Levin and Miller 2005; Levin et al. 2007), tribe Lycieae is strongly supported as monophyletic using plastid DNA sequence data (Fig. 3). As in Levin and Miller (2005), the monophyly of Old World *Lycium* using plastid data remains weakly supported, despite the addition of considerable plastid DNA sequence data in the present study. This result, however, does not stem from the lack of variation in plastid DNA among the Old World taxa, but instead appears to be due to the level at which that variation occurs. For example, among the species sampled there are a total of 17 synapomorphies involving Old World *Lycium*; however, most of these (13 sites) cluster a subset of species within the Old World (Fig. 3), as opposed to all of the Old World species. In particular, there is support for two clades within the Old World species (*L. cinereum*, *L. oxycarpum*, *L. ferocissimum*, *L. pumilum*, and *L. tenue*; and *L. hirsutum*, *L. villosum*, and *L. chinense*) in the majority of plastid DNA data sets, and thus strong support in combined analyses. Inclusion of indels, rather than just nucleotide substitutions, provides strong support for Old World monophyly. In particular, there is one 12 bp indel in *rpl32-trnL* that is shared and unique to the Old World species sampled here. Within this Old World clade there is another large deletion (218 bp) in *ndhF-rpl32* that is shared among the Chinese species (*L. chinense* in this study; *L. barbarum*, *L. ruthenicum*, data not shown). Thus, inclusion of plastid DNA indels as separate characters is important for understanding relationships of Old World *Lycium*.

Previous analyses of relationships in tribe Lycieae using the nuclear GBSSI gene suggested that New World *Lycium* are paraphyletic (Fig. 1). In particular, Levin and Miller (2005) and Levin et al. (2007) identified eleven New World species that were supported as more closely related to Old World *Lycium* than to other New World species. Several of these (i.e. those species with affinities to Old World *Lycium*) were included in the present study (*L. californicum*, *L. chanar*, *L. chilense*, *L. ciliatum*, and *L. nodosum*). However, in contrast with previous work, the combined plastid tree (Fig. 3A) places all of these species and *Phrodus microphyllus* in a strongly supported (BS = 88) New World clade. Thus, there is conflict between the present plastid DNA data and previous analyses using the nuclear GBSSI gene (Levin et al. 2007). We tested the extent of the conflict by constraining these five taxa to be allied (i.e. included in a clade) with the Old World species; the constrained topology was significantly less likely than the unconstrained topology (one tailed nonparametric Shimodaira-Hasegawa test, $p < 0.05$). This placement of *Phrodus* is in agreement with Olmstead et al. (2008), who used two plastid regions (*trnL-trnF* and *ndhF*) in a large scale study of Solanaceae. Levin et al. (2009a) used both nuclear and plastid data for a larger taxon sample and found that the conflict in the placement of *Phrodus* is between nuclear and plastid data; three nuclear data sets (GBSSI, nrITS, and nitrate reductase) suggested that *Phrodus* is sister to the rest of tribe Lycieae, and only the plastid data placed *Phrodus* closely related to a clade of New World species (as shown in Fig. 3).

Relationships among the 12 species American clade (Fig. 3, *Lycium americanum* through *Phrodus microphyllus*) are not completely resolved with the present data. Lack of resolution, particularly at the base of this clade, could be due to a number of factors including homoplasy, lack of variation in plastid DNA, or incomplete taxon sampling. However, homoplasy is low, with rescaled consistency indices for the combined data sets (without indels) from 0.92 (25 taxa) to 0.91 (15 taxa). There are 58 variable (and 23 PI) characters among these twelve taxa; thus, variation at this level may not be limiting. Incomplete taxon sampling in this group may be a more likely cause of reduced resolution, as there are over 50 species of Lycieae in the New World, many of which are closely related to species included here. Thus, increased taxonomic sampling within American species could potentially help resolve relationships within this lineage by uniting closely related species and groups of species. By contrast, within the clade of Old World species that includes *L. ferocissimum*, this polytomy is undoubtedly due to insufficient variation (only four variable characters exist among these five species); thus, additional data from low-copy nuclear genes (Levin et al. 2009b) will be necessary to fully resolve relationships.

The primary goal of this study was to develop noncoding plastid DNA markers for phylogenetic analysis among closely related species in tribe Lycieae. We sequenced nine noncoding plastid regions for a diverse sample of Lycieae (either 22 or 12 species, mostly *Lycium*) and two near outgroups in *Nolana*. The nine plastid DNA regions varied with regard to their phylogenetic utility, and we advocate a combination of three regions (*trnD–trnT*, *rpl32–trnL*, and *ndhF–rpl32*) for future studies. Collectively, these three plastid DNA regions had 115 variable characters, 72 of which were parsimony informative, and 10 indels. In addition, two of the regions can be amplified in a single reaction, thus reducing the time and expense of amplification and sequencing. Although the *trnD–trnT* region has proven useful in other studies (Friesen et al. 2000; Hahn 2002; Potter et al. 2002), the other two regions (*rpl32–trnL* and

ndhF–rpl32) are virtually unexplored. It will be interesting to see if these regions, identified by Shaw et al. (2007) as potentially useful at low levels, continue to prove as promising in other groups as they do in this study.

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Appendix 1. Taxa, collection localities, voucher information, and Genbank accession numbers for all sequences included in this study. Genbank accession numbers are listed in the following order: *trnH*^{GUG}–*psbA*, *rpl32–trnL*^{UAG}, *ndhF–rpl32*, *trnL*^{UAA}–*trnF*^{GAA}, *trnT*^{UGU}–*trnL*^{UAA}, *trnD*^{GUC}–*trnT*^{GGU}, *3´trnV*^{UAC}–*ndhC*, *trnQ*^{UUG}–*5´rps16*, *3´rps16–5´trnK*^{UUU}. Voucher specimens are deposited in the following herbaria: ARIZ = University of Arizona; BLFU = University of the Free State; CORD = Universidad Nacional de Córdoba; MASS = University of Massachusetts.

Tribe Lycieae Hunz.: *Lycium americanum* Jacq.—Argentina, *Barboza 525* (CORD), FJ189606, FJ189735, FJ189668, DQ124561, DQ124438, FJ189630, FJ189654, FJ189692, FJ189706; *Lycium andersonii* A. Gray—Mexico, *Miller 97-12* (ARIZ), FJ189607, FJ189736, FJ189669, DQ124562, DQ124439, FJ189631, –, –, –; *Lycium californicum* Nutt. ex A. Gray—Pima Co., Arizona, USA, *Miller 01-2* (ARIZ), FJ189608, FJ189737, FJ189670, DQ124572, DQ124449, FJ189632, –, –, –; *Lycium cestroides* Schltdl.—Argentina, *Bernardello 878* (CORD), FJ189609, FJ189738, FJ189671, DQ124574, DQ124451, FJ189633, FJ189655, FJ189693, FJ189707; *Lycium chanar* Phil.—Argentina, *Miller et al. 04-71* (MASS), FJ189610, FJ189739, FJ189672, FJ189718, FJ189726, FJ189634, FJ189656, FJ189694, FJ189708; *Lycium chilense* Bertero—Argentina, *Miller et al. 04-101* (MASS), FJ189611, –, FJ189673, FJ189719, FJ189727, FJ189635, FJ189657, FJ189695, FJ189709, Argentina, *Miller et al. 04-109* (MASS), –, FJ189740, –, –, –, –, –, –, –; *Lycium chinense* Mill.—Eurasia, cult. Nijmegen #954750075, FJ189623, FJ189752, FJ189685, DQ124600, DQ124477, FJ189647, –, –, –; *Lycium ciliatum* Schltdt.—Argentina, *Bernardello 876* (CORD), FJ189612, FJ189741, FJ189674, DQ124577, DQ124454, FJ189636, –, –, –; *Lycium cinereum* Thunb.—South Africa, *Venter 649* (BLFU), FJ189613, FJ189742, FJ189675, DQ124578, DQ124455, FJ189637, FJ189658, FJ189696, FJ189710; *Lycium cuneatum* Dammer—Argentina, *Bernardello & Vesprini 897* (CORD), FJ189614, FJ189743, FJ189676, DQ124580, DQ124457, FJ189638, –, –, –; *Lycium ferocissimum* Miers—South Africa, *Miller et al. 05-199* (MASS), FJ189615, FJ189744, FJ189677, FJ189720, FJ189728, FJ189639, FJ189659, FJ189697, FJ189711; *Lycium hirsutum* Dunal—South Africa, *Venter 646* (BLFU), FJ189616, FJ189745, FJ189678, DQ124588, DQ124465, FJ189640, FJ189660, FJ189698, FJ189712; *Lycium nodosum* Miers—Argentina, *Barboza 515* (CORD), FJ189617, FJ189746, FJ189679, DQ124594, DQ124471, FJ189641, FJ189661, FJ189699, FJ189713; *Lycium oxycarpum* Dunal—South Africa, *Miller et al. 05-206* (MASS), FJ189618, FJ189747, FJ189680, FJ189721, FJ189729, FJ189642, –, –, –; *Lycium parishii* A. Gray—Arizona, USA, *Miller 97-22* (ARIZ), FJ189619, FJ189748, FJ189681, DQ124597, DQ124474, FJ189643, FJ189662, FJ189700, FJ189714; *Lycium puberulum* A. Gray—Texas, USA, *Levin 97-6* (ARIZ), FJ189620, FJ189749, FJ189682, DQ124599, DQ124476, FJ189644, –, –, –; *Lycium pumilum* Dammer—South Africa, *Miller and Levin 06-145* (MASS), FJ189621, FJ189750, FJ189683, FJ189722, FJ189730, FJ189645, –, –, –; *Lycium rachidocladum* Dunal—Chile, *Miller et al. 04-82* (MASS), FJ189622, FJ189751, FJ189684, FJ189723, FJ189731, FJ189646, –, –, –; *Lycium shockleyi* A. Gray—Nevada, USA, *Miller 98-1* (ARIZ), FJ189624, FJ189753, FJ189686, DQ124604, DQ124481, FJ189648, FJ189663, FJ189701, FJ189715; *Lycium tenue* Willd.—South Africa, *Miller et al. 05-220* (MASS), FJ189625, FJ189754, FJ189687, FJ189724, FJ189732, FJ189649, –, –, –; *Lycium villosum* Schinz—South Africa, *Venter 652* (BLFU), FJ189626, FJ189755, FJ189688, DQ124612, DQ124489, FJ189650, FJ189664, FJ189702, FJ189716; *Phrodus microphyllus* (Miers) Miers—Chile, *Miller et al. 04-92* (MASS), FJ189627, FJ189756, FJ189689, FJ189725, DQ124495, FJ189651, FJ189665, FJ189703, FJ189717.

Outgroups: *Nolana werdermannii* I. M. Johnst.—Chile, *Miller et al. 04-77* (MASS), FJ189604, FJ189733, FJ189666, DQ124616, DQ124493, FJ189628, FJ189652, FJ189690, FJ189704; *Nolana coelestis* Lindl.—Chile, *Miller et al. 04-98* (MASS), FJ189605, FJ189734, FJ189667, DQ124617, DQ124494, FJ189629, FJ189653, FJ189691, FJ189705.