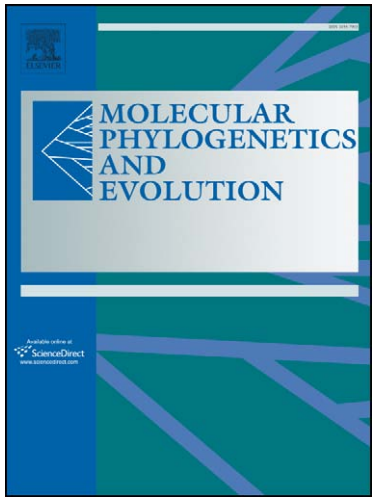

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PII: S1055-7903(08)00374-6
DOI: 10.1016/j.ympev.2008.07.018
Reference: YMPEV 2970

To appear in: *Molecular Phylogenetics and Evolution*

Received Date: 18 March 2008
Revised Date: 1 July 2008
Accepted Date: 26 July 2008

Please cite this article as: Tu, T., Dillon, M.O., Sun, H., Wen, J., Phylogeny of *Nolana* (Solanaceae) of the Atacama and Peruvian Deserts inferred from sequences of four plastid markers and the nuclear *LEAFY* second intron, *Molecular Phylogenetics and Evolution* (2008), doi: 10.1016/j.ympev.2008.07.018

Phylogeny of *Nolana* (Solanaceae) of the Atacama and Peruvian Deserts inferred from sequences of four plastid markers and the nuclear *LEAFY* second intron

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Running title: Phylogeny of *Nolana*

Abstract

The phylogeny of *Nolana* (Solanaceae), a genus primarily distributed in the coastal Atacama and Peruvian deserts with a few species in the Andes and one species endemic to the Galápagos Islands, was reconstructed using sequences of four plastid regions (*ndhF*, *psbA-trnH*, *rps16-trnK*, and *trnC-psbM*) and the nuclear *LEAFY* second intron. The monophyly of *Nolana* was strongly supported by all molecular data. The *LEAFY* data suggested that the Chilean species, including *Nolana sessiliflora*, the *N. acuminata* group and at least some members of the *Alona* group, are basally diverged, supporting the Chilean origin of the genus. Three well supported clades in the *LEAFY* tree were corroborated by the SINE (short interspersed elements) or SINE-like insertions. Taxa from Peru are grouped roughly into two clades. *Nolana galapagensis* from the Galápagos Island is most likely to have derived from a Peruvian ancestor. The monophyly of the morphologically well diagnosed *Nolana acuminata* group (*N. acuminata, N. baccata, N. paradoxa, N. parviflora, N. pterocarpa, N. rupicola* and *N. elegans*) was supported by both plastid and *LEAFY* data. Incongruence between the plastid and the *LEAFY* data was detected concerning primarily the positions of *N. sessiliflora*, *N. galapagensis*, taxa of the *Alona* group, and the two Peruvian clades. Such incongruence may be due to reticulate evolution or in some cases lineage sorting of plastid DNA. Incongruence between our previous GBSSI trees and the plastid -*LEAFY* trees was also detected concerning two well-supported major clades in the GBSSI tree. Duplication of the GBSSI gene may have contributed to this incongruence.

Keywords: Gene duplication; Lineage sorting; *ndhF*; *psbA-trnH*; Reticulate evolution; *rps16-trnK*; *trnC-psbM*.

1. Introduction

The genus *Nolana* L. f. consists of 89 species primarily distributed in the Atacama and Peruvian deserts, with 43 species in Peru, 49 species in Chile, a few species in the inland regions of the Andes (e.g., *N. chapiensis, N. lezamae, N. sessiliflora*, *N. urubambae, N. tarapacana*) and one species endemic to the Galápagos Islands. It is the fourth largest genus in the family Solanaceae after *Solanum* (ca. 1500 species), *Cestrum* (ca. 160 species) and *Physalis* (ca. 120 species). Members of this genus are annuals, or perennial herbs or woody shrubs. Adapting to the unusual arid *lomas* environment in coastal Peru and Chile (see Dillon et al. 2007), species of *Nolana* have developed somewhat succulent leaves arranged in rosettes and shoots with short internodes. When the water conditions are favorable for growing, the rosettes may increase in size and the flowering period may prolong. This suite of adaptive characters may confound phylogenetic analysis using morphology. Flowers of *Nolana* are hermaphroditic with corollas varying greatly in size, actinomorphic to weakly zygomorphic, tubular-salverform to campanulate, and white to blue in color with variable colored spots and veins in the throat. The most significant character separating *Nolana* from other Solanaceae taxa is the presence of the unusual sclerified fruits called mericarps in this genus (Knapp, 2002). The mericarp number can be reduced to as few as two or as many as 30, often with several seeded mericarps arising through incomplete radial fission of the fertile carpels (Bondeson, 1896; Saunders, 1936; Tago-Nakawaza and Dillon, 1999).

Because of its unique fruit type, *Nolana* has been widely accepted as a highly distinct group since its description by Linnaeus f. in 1762 (Don, 1838; Hunziker, 2001; Johnston, 1936; Mesa, 1981). The monophyly of *Nolana* is also strongly supported by sequence data from the plastid *matK* gene, the nuclear ribosomal internal transcribed spacer (ITS) (Tago-Nakawaza and Dillon, 1999) and partial sequences of the nuclear granule-bound starch synthase I (GBSSI) gene (Dillon et al., 2007). Some workers recognize *Nolana* at the familial rank (Cronquist, 1981; Hunziker, 2001), or as a subfamily, i.e., Nolanoideae of Solanaceae (D'Arcy, 1979; D'Arcy, 1991; Dahlgren, 1980; Takhtajan, 1997; Thorne, 1983). Data from plastid DNA restriction site mapping and plastid *ndhF* gene sequences, with most Solanaceous genera sampled, have strongly supported the

placement of *Nolana* within the Solanaceae, and suggested its sister relationship with the tribe Lycieae (Olmstead and Palmer, 1992; Olmstead and Sweere, 1994; Olmstead et al., 1999).

In our previous efforts, the plastid *matK*, ITS (Tago-Nakawaza and Dillon, 1999) and the nuclear GBSSI sequences (Dillon et al., 2007) were employed to elucidate interspecific relationships of *Nolana*. The initial phylogenetic study using ITS and *matK* data sampling 37 species produced poorly resolved phylogenies. The sequence data from the third to the eighth exon of the GBSSI gene produced a much better resolved phylogeny of the genus. The GBSSI tree suggested the sister relationship between *Nolana sessiliflora* and the remainder of the genus, two strongly supported major clades for the remaining species, and eight strongly to moderately supported subclades within the two major clades. The subclade (the *Nolana acuminata* group) comprised of *Nolana paradoxa, N. acuminata, N. reichei, N. elegans, N. rupicola, N. pterocarpa, N. baccata, and N. parviflora* was supported by the GBSSI data. This subclade in the GBSSI tree is also supported by morphology and distribution, as all taxa in the subclade share the characters of basal rosettes, large showy flowers and 10-20 mericarps, and are generally distributed in coastal Chile. The other seven subclades did not contradict relationships inferred from morphology and geographic distribution. However, the interspecific relationships within most subclades were largely unresolved. Furthermore, each of the two major clades includes species with diverse morphological characters and each has species from both Chile and Peru. Several mechanisms including adaptive radiation, reticulate evolution, or gene duplication may lead to a clade of taxa with diverse morphology based on molecules. If the last scenario is true, the phylogeny based on these sequences may be misleading. Special caution should be made when using low-copy nuclear genes because they are prone to gene duplications through polyploidy or retrotransposition even losing gene copies (Sang, 2002). Duplication of the GBSSI gene has been reported in some groups of flowering plants, including Rosaceae (Evans et al., 2000), *Viburnum* (Carprifoliaceae) (Winkworth and Donoghue, 2004) and *Spartina* (Poaceae) (Fortune et al., 2007). In Solanaceae, initial phylogenetic studies detected only one copy of the GBSSI gene, such as in the diploid *Solanum* (Levin et al., 2006; Levin et al., 2005; Peralta and Spooner, 2001), the Iochrominae group of the tribe

Physaleae (Smith and Baum, 2006), *Schizanthus* (Perez et al., 2006), and *Nolana*'s close relative Lycieae (Levin and Miller, 2005). In a recent study, more than two copies of this gene were found in Hyoscyameae, a polyploid group from the northern hemisphere closely related to *Nolana* (Yuan et al., 2006). Evolution of the GBSSI gene in Solanaceae thus may be more complex than previously thought, and the GBSSI phylogeny of Solanaceae taxa needs to be tested using additional markers.

In this study, we employed four plastid markers to test the phylogeny of *Nolana*. We chose *ndhF* because the gene has been used for a broad range of taxa in Solanaceae (Olmstead and Sweere, 1994). Partial sequences between the *trnC* and the *psbM* genes were sequenced because of its relatively high rate of nucleotide substitution in *Panax* (Araliaceae) (Lee and Wen, 2004). The intergenic region *trnH-psbA* (Shaw et al., 2005) has been demonstrated to be highly variable at infraspecific and interspecific levels in the Solanaceous genus *Petunia* (Lorenz-Lemke et al., 2006). Considerable variation of the *rps16-trnK* spacer (Shaw et al., 2007) was detected from the alignment among sequences of *Solanum*, *Nicotiana*, and *Atropa* (GenBank accession nos. NC 007500, NC 001879, NC 008096, NC 007943, NC 004561).

We also used the nuclear *LEAFY* gene in our analysis. The *LEAFY* gene is a homeotic gene which regulates the floral meristem induction during the early stages of reproductive ontogeny (Blazquez, 1997; Blazquez et al., 1997; Schultz and Haughn, 1991; Wada et al., 2002; Weigel, 1995). In some cases, it affects the vegetative morphogenesis (Hofer et al., 1997; Kelly et al., 1995; Pouteau et al., 1997). It was first described as *FLORICAULA* in *Antirrhinum majus* (Coen et al., 1990) and then as *LEAFY* in *Arabidopsis thaliana* (Schultz and Haughn, 1991). Some other names have been used to designate the orthologues of *LEAFY* in plants, such as *NFL* in *Nicotiana tabacum* (Kelly et al., 1995), *Imp-flo* in *Impatiens* (Pouteau et al., 1997), and *alf* in *Petunia* (Souer et al., 1998). More than one copy has been reported for the *LEAFY* orthologs in the gymnosperms, and some basal or polyploid angiosperms (Bomblies et al., 2003; Cronk, 2001; Frohlich and Meyerowitz, 1997; Frohlich and Parker, 2000; Theissen, 2000). Whereas this gene has been generally suggested to be single-copy in most diploid angiosperm species studied so far, exceptions include two or more possible copies in the diploid *Eucalyptus* L. (Southerton et al., 1998) and at least two clear copies in certain

taxa of the Lamiales (Aagaard et al., 2005; Aagaard et al., 2006), Leguminosae (Archambault and Bruneau, 2004) and Brassicaceae (Baum et al., 2005). In Solanaceae, only one copy of *alf*, the ortholog of *LEAFY*, was presumed for *Petunia* Juss. based on the southern blot and the inflorescence cDNA library screening experiment (Souer et al., 1998). Two copies of *NFL*, the homolog of *FLORICAULA* and *LEAFY,* were detected in the cultivated allotetraploid *Nicotiana tabacum* L. As expected, a single copy of this gene was observed from both paternal (*N. sylvestris* Speg.) and maternal (*N. tomentosiformis* Goodspeed) parents of the allotetraploid *N. tabacum* (Kelly et al., 1995). The generally low copy number of *LEAFY* in angiosperms and the relatively high level of variation within the introns make it an excellent candidate as a phylogenetic marker for resolving interspecific even intraspecific relationships or for testing hypothesis of hybridization (Grob et al., 2004; Hoot and Taylor, 2001; Howarth and Baum, 2005; Oh and Potter, 2003, , 2005). The first use of *LEAFY* for phylogenetic study of Solanaceae (Smith and Baum, 2006) demonstrated that the second intron of *LEAFY* contains more informative characters than those from ITS and GBSSI together. The *LEAFY* sequences were also shown to be useful in detecting hybridization in the Iochrominae group of Solanaceae.

Objectives of this study are to: (1) elucidate the interspecific relationahips within *Nolana* using multiple molecular markers; (2) test the GBSSI phylogeny of the genus; and (3) evaluate the phylogenetic utility of the nuclear *LEAFY* second intron.

2. Materials and Methods

2.1 Taxon Sampling, DNA Extraction and Amplification, and Sequencing

All 63 species analyzed in our previous GBSSI study (Dillon et al. 2007) as well as two additional species, *Nolana tocopilensis* and *N. ivaniana*, were sequenced for four plastid markers in the present study. However, only 55 species were sequenced for the nuclear *LEAFY* gene because of difficulties in amplifying this gene from some degenerated leaf tissue samples. DNA extractions followed Dillon et al. (2007) and voucher information was presented in Table 1.

Target regions were amplified in 25 µl reaction-mixture volumes using the Bioline Taq polymerase and associated reagents at 2.0 mM $MgCl_2$ concentration except for *trnC-psbM*, which used 4.0 mM $MgCl_2$. Primers for *ndhF*, *trnH-psbA* and *rps16-trnK* followed Olmstead and Sweere (1994), Shaw et al. (2005) and Shaw et al. (2007), respectively. The primers trnC (5'-CCAGTTCAAATCCGGGTGTC-3') and 2039R (5'-TTTTCTACTTATCATTTACG-3') were used to amplify the *trnC-psbM* region and one internal primer 690F (5'-TTTATATTTATAGAGATAGGGGAC-3') was designed for sequencing.

The second intron of the *LEAFY* gene was initially amplified and sequenced from a subset of taxa using degenerate primers F2 and R1 (Howarth and Baum, 2005). These sequences were used to design *Nolana* specific primers (LFYNol3F: 5'-TATTGCCAAGGAACGAGGTG-3'; and LFYNol3R: 5'-CGTACCTGAACACTTGATTTG-3'). Two internal sequencing primers were also designed (LFYNol5F: 5'-TACGGACTGATGGGCTGAAC-3', and LFYNol5R: 5'-GACAAGGTTACAGGTGGAGATAC-3'). Most amplified products contained one band and were sequenced directly. Cloning was conducted using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, U.S.A.) when ambiguous sequences were obtained by direct sequencing or when more than one band was detected during the amplification. At least five clones representing each band of the PCR products were sequenced. To capture potential hidden copies, we selected four samples which showed multiple bands, and used low annealing temperature (45℃) in the PCR reactions and sequenced 20 clones for each of the four samples.

The PCR reactions for *LEAFY* differed from the *ndhF* reactions in that 10 μM BSA was used in the *LEAFY* amplification. The PCR program for the *LEAFY* amplification was 95℃ for 3 min, then 35 cycles of 94℃ for 40 s, 50℃ for 40 s, 72℃ for 2 min, followed by a final extension of 72℃ for 10 min. The amplified products were then purified using the polyethylene glycol (PEG) precipitation.

Cycle sequencing was conducted using the BigDye 3.1 reagents with an ABI 3700 automated sequencer (Applied Biosystems, Foster City, California, U.S.A.). The program Sequencher 4.5 (Gene Codes Corporation, 2005) was used to evaluate chromatograms for base confirmation and to edit contiguous sequences. Sequences were initially aligned with ClustalX version 1.83 (Thompson et al., 1997), followed by manual adjustments on Se-Al v2.0a11 (Rambaut, 2007).

2.2 Phylogenetic Analyses

Parsimony analysis was performed using a heuristic search with 100 random sequences addition replicates, tree bisection-reconnection (TBR) swapping, collapse of zero-length branches, multiple tree option in effect and character state changes equally weighted in the analysis. Because too many trees were found for the *LEAFY* data, trees were limited to 10,000 during each of 10 random sequences addition replicate. Gaps were treated either as missing data or coded as simple indels using the program Gapcoder (Young and Healy, 2003). Bootstrap values (BP) (Felsenstein, 1985) of the internal nodes were obtained with 500 replicates. In each replicate, we performed 10 random sequences addition replicates following by tree bisection-reconnection (TBR) swapping algorithm and keeping no more than 1000 trees per replicate.

Bayesian inference (Rannala and Yang, 1996) was conducted using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) with the model estimated by Modeltest version 3.7 (Posada and Buckley, 2004; Posada and Crandall, 1998). The Markov chain Monte Carlo algorithm was run for 2,000,000 generations with four incrementally heated chains, starting from random trees and sampling one out of every 100 generations. The first 2,000 to 5,000 trees were discarded, depending on when chains appeared to have become stationary, and the remaining trees were used to construct the Bayesian consensus tree.

Internodes with posterior probabilities (PP) ≥ 95% were considered statistically significant.

3. Results

3.1 Phylogenetic analyses of plastid DNA data

The four plastid markers had 5172 aligned positions, of which 220 were variable (4.2%) and 150 (2.9%) were parsimony-informative (PI). The aligned length of each marker was 1998 from the *ndhF* gene, 815 from *rps16-trnK*, 501 from *psbA-trnH* and 1858 from partial sequences of *trnC-psbM*. Treating gaps as missing data, the parsimony analysis generated 7717 equally most parsimonious trees (MPTs) with a tree length of 268 steps, a consistency index (CI) of 0.84, a consistency index excluding uninformative characters of 0.78, and a retention index (RI) of 0.95 (Table 2). The strict consensus tree is presented in Fig.1. In the Bayesian analysis, 5000 trees were eliminated before generating the 50% majority-rule tree. The topology of the tree is similar to that of the MPTs. All nodes with high bootstrap value (>90%) had high PP (1.0) values as well. Some nodes with moderate to low bootstrap values also had good PP support, such as the cp-I clade, the cp-G calde, the cp-C clade, and the cp-FH clade (Fig. 1).

Except for *ndhF*, all other three plastid DNA regions contained gaps. There were 25 new indel characters in the plastid DNA data set, of which ten were parsimony-informative, including eight repeats, one deletion and one insertion. All the unambiguous indels supported the topology of the base substitution tree with two exceptions. One was the 7-base repeat in the outgroup and the cp-D clade, and the other was the 42-base repeat detected in the outgroup taxa *Grabowskia glauca* and *Phrodus microphylla*. The analysis of the plastid DNA with indels as new characters produced 15448 MPTs with a tree length of 296 steps, a CI of 0.85, a CI excluding uninformative characters of 0.78, and an RI of 0.95. The topology of the strict consensus tree from gaps as new characters was identical to that of the tree with gaps as missing data. The bootstrap values of clades were similar in both analyses.

3.2 Phylogenetic analysis of LEAFY

Amplification of the *LEAFY* second intron yielded one or two bands. All sequences from the larger bands can be aligned with *LEAFY* sequences of Solanaceae from

GenBank. The sequences of the smaller bands did not match any *LEAFY* sequences or other genes. The nature of the smaller fragments remained unknown and these sequences were not included in the phylogenetic analysis.

Variation in the second intron of the *LEAFY* gene is higher than that in the four plastid markers (Table 2). Sequences across 113 accessions ranged from 843 bp to 1771 bp and had an aligned length of 4175 bp. Of these 4175 characters, 900 were variable (21%) and 564 were parsimony-informative (13.5%) (Table 2). Treating gaps as missing data, the parsimony analysis yielded 10,920 MPTs with a tree length of 1493 steps, a CI of 0.75, a CI excluding uninformative characters of 0.65, and an RI of 0.90. The strict consensus tree is presented in Fig. 2.

The indels in the second intron of the *LEAFY* gene composed of simple deletions, insertions, mononucleotide repeats, or tandemly arranged multibase repeats. After the ambiguous blocks in the alignment were deleted, there were 325 indel characters, which ranged from 1 bp to 789 bp in size. The analysis treating indels as new characters had a tree length of 1898 steps, a CI of 0.74, a CI excluding uninformative characters of 0.65, and an RI of 0.90. The topology with indels as new characters was generally congruent with that of the tree when indels were treated as missing data. Nevertheless, the indel characters increased the bootstrap values of many clades (Fig. 2).

3.2 Phylogenetic results

The monophyly of *Nolana* has been recovered by both the plastid regions and sequences of the *LEAFY* second intron. Two large clades for *Nolana* (cp-I and cp-II) were detected in the plastid DNA tree, one containing taxa from Chile and the other with taxa from Chile and Peru. *Nolana acuminata*, *N. baccata*, *N. paradoxa*, *N. parviflora*, *N. pterocarpa*, *N. rupicola*, *N. elegans*, *N. balsamiflua*, *N. linearifolia* and *N. sessiliflora* formed the cp-I clade, which was sister to the well-supported cp-II clade composed of the remaining species of the genus (Fig. 1). Within the cp-I clade, subclade cp-A consisting of *Nolana acuminata*, *N. baccata*, *N. paradoxa*, *N. parviflora*, *N. pterocarpa*, *N. rupicola* and *N. elegans* was strongly supported, whereas the other clade (cp-D, generally corresponding to clade D in the previous GBSSI tree (Dillon et al., 2007)) including species of *N. balsamiflua*, *N. linearifolia* and *N. sessiliflora* is only weakly supported. In the cp-II clade, five subclades (cp-B, cp-C, cp-E, cp-G, cp-FH, corresponding to clade

B, C, E, G and F and H in the previous GBSSI tree (Dillon et al., 2007)) were recovered.

In the *LEAFY* tree, *Nolana sessiliflora* is sister to a clade composed of the remainder species of *Nolana* like in our previous GBSSI tree (Dillon et al., 2007). Six clades (LFY-A, LFY-BE, LFY-C, LFY-D, LFY-F, LFY-GB, see Fig. 2) has been recovered with strong to moderate bootstrap support. Generally, the components of each clade in the *LEAFY* tree are comparable with those of species in the plastid tree except LFY-BE and LFY-GB, which are distributed from Peru to northern Chile.

4. Discussion

4.1 Monophyly of Nolana

Nolana was strongly supported to be closely related to the tribe Lycieae based on the plastid *ndhF* and *rbcL* sequences as well as the restriction site mapping data (Olmstead and Palmer, 1992; Olmstead and Sweere, 1994). When taxa of the tribe Lycieae were used as outgroups, the monophyly of *Nolana* was strongly supported by the plastid DNA, *LEAFY* and GBSSI data with bootstrap support of 100. Taxa of *Nolana* share the unique morphological synapomorphy of having one-seeded mericarps in the fruits.

4.2 Plastidt phylogeny

Members from the cp-I clade in the plastid tree have largely overlapping distributions, with the majority confined to northern Chile (18ºS to 30ºS) and, one species, *N. paradoxa*, extending from central to southern Chile (29º15'S to 42º30'S). The cp-A clade can be easily diagnosed by a set of characters including herbs with a basal rosette of leaves, and >10 mericarps. The monophyly of the cp-A clade was also suggested in previous molecular studies of ITS and *matK* (Tago-Nakawaza and Dillon, 1999) as well as GBSSI (Dillon et al., 2007). *Nolana balsamiflua* shared a strongly supported sister relationship with *N. linearifolia*. However, this relationship is not congruent with their morphology. The non-apical style, mericarp morphology and number (~10), and weakly lignified perennial herbaceous habit of *N. linearifolia* make it easily distinguishable from *N. balsamiflua*, which is more similar to other Chilean species, e.g., *N. rostrata, N. filifolia*, and *N. stenophylla*. The sister relationship of the *N. balsamiflua - N. linearifolia* clade to *N. sessiliflora* should be re-examined due to the low bootstrap support value.

The species that make up the cp-B clade are generally similar as they are herbs with showy blue to purple corollas, and ~5 mericarps in the fruits. Most species in this subclade are restricted to the Peruvian coast, 7ºS - 16ºS. The only exceptions to the coastal distribution are *N. urubambae*, *N. lezamae*, and *N. chapiensis* which occur above 2000 m and 50-500 km from the coast. However, this clade lacks internal resolution and forms a large polytomy, with the only sister relationship between *Nolana gayana - N. humifusa* is detected (BP=81, PP=100). These two species have overlapping distributions between 8 ºS - 15ºS.

The cp-E clade contains *Nolana galapagensis* from the Galápagos Island and *N. adansonii* from southern Peru and northern Chile. Nevertheless, support for this clade is low and their relationships should be viewed with caution.

The cp-G clade has moderate support and contains species restricted to northern Chile (i.e., *N. intonsa* and *N. tarapacana*) or southern Peru (the remaining species) except *N. lycioides*, which occurs in both countries. In this clade, *Nolana inflata*, *N. weissiana* and *N. plicata* form a subclade with high bootstrap support sister to the remaining taxa that form a strongly supported subclade. In the latter subclade, the two Chilean species, *N. intonsa* and *N. tarapacana* are nested in the clades of taxa from Peru. The grouping of *N. tarapacana* with *N. arequipensis* and *N. tomentella* is weakly supported, whereas the sister relationship of *N. intonsa* with the remaining Peruvian species is strongly supported.

The cp-C clade is moderately supported and is a morphologically well-diagnosed group with woody or shrubby habit, linear leaves, large showy flowers, and a Chilean distribution. These species share the synapomorphy of fused mericarps with apical stigmas. Johnston (1936) recognized this group as the segregate genus *Alona*. It has been accepted at the subgeneric level by modern workers (e.g., Tago-Nakawaza & Dillon, 1999). Two additional species, *N. balsamiflua* and *N. stenophylla* also share the fruit morphology and were included in *Alona* by Johnston (1936). However neither species is grouped with the cp-C clade in the plastid phylogeny (Fig. 1). Rather, they are sister to the cp-A clade.

Sister to the cp-C clade is the cp-FH clade with moderate support. Within this clade, *Nolana aplocaryoides* and *N. clivicola* diverged first with the remaining taxa forming a large polytomy. Taxa of the cp-FH clade are restricted to northern Chile (22º-30ºS), either inhabiting highly saline beach dunes (e.g., *N. aplocaryoides, N. crassulifolia*, *N. salsoloides*, *N. peruviana*, and *N. divaricata*) or occurring in inland/upland habitats (e.g., *N. leptophylla*, *N. flaccida, N. mollis, N. glauca,* and *N. werdermannii*). Many species within this clade, including *N. villosa* and *N. incana*, grow in the habitats known as "aguadas" which are moist areas fed by the underground water in an otherwise dry and saline quebradas (Tago-Nakawaza and Dillon, 1999). Lack of resolution within this subclade makes it unsuitable to explain the relationships between species based on the

maternally inherited plastid DNA data in this study.

4.3 LEAFY phylogeny

Different clones of *Nolana sessiliflora* form a clade (BP=100/100) sister to the large clade consisting of the remaining *Nolana* species. The LFY-A clade contains *N. parviflora*, *N. pterocarpa*, *N. baccata*, *N. rupicola* and *N. paradoxa* with strong support. This clade is also supported by the GBSSI and plastid DNA data, and the morphology.

The LFY-D clade consists of *Nolana balsamiflua* and *N. stenophylla* with weak support and is sister to a strongly supported the LFY-C clade, which contains *N. rostrata*, *N. filifolia*, *N. coelestis* and *N. carnosa* (i.e., subgenus *Alona*). These two clades were within the same large clade in the previous GBSSI tree (Dillon et al., 2007), but formed a polytomy. In the plastid DNA tree, *Nolana balsamiflua* groups with the basal *N. sessiliflora* whereas *N. stenophylla* is nested within the cp-FH clade, which is sister to the cp-C clade. However, both clades in the plastid DNA tree are only weakly supported. The relationship between LFY-C and LFY-D are consistent with distribtutional patterns and morphological characters of this group, since species in both clades are restricted to northern Chile and can be well diagnosed morphologically by woody or shrubby habit, large showy flowers, and highly fused mericarps with apical stigmas.

The LFY-BE clade (BP=99) includes species from southern Peru and the Galápagos Island (*Nolana galapagensis*). *Nolana galapagensis* grouped with *N. arenicola* in the GBSSI tree with weak support (Dillon et al., 2007), yet these two morphologically highly distinct species do not form a clade in the *LEAFY* tree. *Nolana adansonii* was closely related to *N. galapagensis* in the GBSSI tree (BP=83) and in the plastid DNA tree (BP=56, PP<95), but it is sister to *N. thinophila* in the *LEAFY* tree. *Nolana galapagensis* and *N. adansonii* differ significantly in habit, leaves, floral structure, and mericarp number. All of these taxa are southern Peruvian in distribution, only with the exception of *N. galapagensis*.

For the remaining species, the Chilean *Nolana clivicola* is sister to a clade (BP<50) containing species from Chile as well as Peru. Considering the low bootstrap support, the position of *Nolana clivicola* needs to be further tested. The sister relationship between LFY-GB and LFY-FH also needs further study due to low support values. The

LFY-FH clade is moderately supported and the taxa recovered are all distributed from northern to north-central Chile and share morphological characters including erect shrubby habit, small tubular and often white corollas, and generally 5-7 mericarps. However, the bootstrap support of the internal node is generally low and the relationship among species within this clade remains unresolved.

Species in the LFY-GB clade range from central Peru to northern Chile. A subclade consisting of *N. thinophila* and different clones of *N. adansonii* is sister to the remaining species in the LFY-GB clade. The morphology and habitats of these two species are quite different. The former species forms large (> 1m in diameter) prostrate mats on near-ocean beaches and have cylindrical or terete leaves, whereas the latter species occurs at greater distances from the ocean and has distinctly petiolate leaves. Their relationship has moderate support from the base-substitution data set, and has a high PP value and high bootstrap value when the indels are included in the analysis. Sequences from additional nuclear genes may test this relationship. Although there is strong support for the remainder clade, the internal nodes lack strong support, except for some terminal clades, such as, *N. humifusa, N. gayana,* and *N. urubambae*, and *N. cerrateana, N. pallida, N. arequipensis* and *N. tomentella* and a weakly supported clade of *N. confinis* and one clone of *N. pallida* (5c).

4.4. Reticulate evolution, lineage sorting or gene duplication

The congruence of topologies from plastid DNA and *LEAFY* data has been detected in several clades of *Nolana*. Two clades had similar or identical component taxa on both plastid DNA and *LEAFY* trees. One clade comprised of *Nolana elegans*, *N. acuminata*, *N. baccata*, *N. paradoxa*, *N. parviflora*, *N. pterocarpa* and *N. rupicola*; and the other contained *N. carnosa*, *N. coelestis*, *N. rostrata*, and *N. filifolia*, although the position of the two clades was not the same in the plastid DNA and the *LEAFY* trees. Morphologically species in each of these two clades are similar overall and form cohesive, well-diagnosed species groups.

Nevertheless, some relationships are more complex and not congruent among different gene trees. Strong incongruence among gene trees may be the result of processes such as reticulate evolution (especially hybridization and introgression), recombination,

or lineage sorting (Wendel and Doyle, 1998). A striking case is *Nolana sessiliflora*. It groups with the Chilean species of the clade consisting of the cp-A clade and the cp-D clade in the plastid DNA tree, whereas it has been suggested to be the first diverged species in *Nolana* in the nuclear data (GBSSI and *LEAFY*). Morphologically, it is quite distinct from taxa in the cp-D clade and very different from those of the cp-A clade. The incongruence may suggest reticulate evolution of *N. sessiliflora* with perhaps the common ancestor of the cp-D clade or the ancestor of the cp-D and the cp-A clades.

Another major incongruence concerns the subgenus *Alona* (Johnston, 1936). We sampled seven of the 13 species of this subgenus. In the *LEAFY* tree, the monophyly of *Alona* was strongly supported. The *Alona* group is morphologically unique with fruits having fused mericarps and apical styles. In the plastid DNA tree (Fig. 1), species of *Alona* are in three different clades: the cp-C clade, the cp-D clade, and *Nolana stenophylla* within the cp-FH clade. Given that species from both the cp-A and the cp-FH clades (see Fig. 1) overlap in distribution with those of *Alona*, reticulate evolution among taxa of this subgenus and the other Chilean species in the cp-A and the cp-FH clades may have occurred.

The last major incongruence between plastid DNA and *LEAFY* trees is the relationships among the Peruvian species. Both plastid DNA and *LEAFY* sequences suggest that the Peruvian species are derived and they are nested within the Chilean species (Fig. 1 and Fig. 2). *Nolana gayana* and *N. humifusa* share similar distributional ranges from central to northern Peru whereas *N. aticoana* is confined to southern Peru and *N. urubambae* is found nearly 500 km from the coast at the elevation of 3000 m. These taxa are all annual to perennial herbs with blue to lavender corollas. Of these taxa, only *N. gayana* has stellate pubescence and a different calyx form. In the plastid tree, *Nolana humifusa* and *N. gayana* group together (BP=81) and are nested within the cp-B clade, which comprises *N. scaposa*, *N. lezamae*, *N. laxa*, *N. chapiensis*, *N. chancoana* and *N. aticoana*. In the *LEAFY* tree, they group with species mostly from the cp-G clade instead of cp-B clade in the plastid DNA tree. *Nolana inflata*, *N. plicata* and *N. weissiana* group together in the plastid DNA tree and are sister to the cp-G clade. They are however sister to the LFY-BE clade in the *LEAFY* tree, which are generally corresponding to the cp-B clade. Reticulate evolution is perhaps the most likely reason

for this incongruence.

The artificial hybrids of *Nolana* (Freyre et al., 2005; Saunders, 1934) demonstrated that cross were successful between species such as far related *N. paradoxa* and *N. aplocaryoides*. These results may indirectly suggest the probability of reticulate evolution in the diversification of *Nolana*. Nevertheless, lineage sorting (especially in the plastid DNA phylogeny) can not be ruled out because the branches in the plastid DNA tree are comparatively short and some of the conflicting clades are only weakly or moderately supported. But this interpretation of lineage sorting is hampered by the general lack of informative sites in the plastid genome at the species level.

We detected major incongruence between the phylogeny of GBSSI and the other two markers (plastid and *LEAFY*) concerning the two large clades in the GBSSI tree, each containing elements from both Chile and Peru. The two major clades in the GBSSI tree each also exhibit a high level of morphological diversity, yet they are strongly supported with high bootstrap values and each has a branch length much longer than most other terminal branches (ML tree not shown). The results from the parsimony and likelihood analyses of GBSSI data are congruent, suggesting that long branch attraction is unlikely (Sanderson et al., 2000) for most branches with perhaps the exception of the *Nolana adansonii-N. galapagensis* clade. Lineage sorting due to ancient polymorphisms of the same orthologous gene copy may also be ruled out because of the long internal branches of these two major clades. An alternative hypothesis of gene duplication of the GBSSI gene may be reasonable for explaining this incongruence. However, this hypothesis is not consistent with (1) the absence of direct evidence that two or more copies from the same sample, and (2) some morphologically cohesive species (e.g., species of the cp-A or LFY-A clade and of the subgenus *Alona*) grouping together instead of randomly resolving into both major clades. Because only five samples were cloned and no more than 20 clones were sequenced in our study, inadequate sampling of clones may have not recovered all copies. The second situation may be refuted if PCR selection occurs, i.e., the reaction favored certain paralogues of a multi-copy gene because of differences in primer affinity related to differences in primary or secondary structure of DNA at the potential target sites (Wagner et al., 1994). Moreover, Lynch and Conery (2000) estimated an average half-life of duplicate gene copies to be about 4-million years.

GBSSI was recognized as a single-copy gene in many plant families, but duplicated GBSSI copies may be undetected in some previous studies due to insufficient sampling of species and genomes. A particularly compelling example of this situation is the studies of GBSSI for *Spartina* (Poaceae) (Baumel et al., 2002; Fortune et al., 2007). Baumel et al (2002) initially detected only one copy of the GBSSI gene for most species of the *Spartina*. A further study with more clones sampled revealed repeated gene duplication followed by deletion or sometimes without deletion (Fortune et al., 2007). In the case of *Nolana*, duplication of the GBSSI gene may have occurred in the early history of the genus, and we perhaps have two main copies of the gene in *Nolana* corresponding to the two major clades (clade I and clade II in Dillon et al. 2007).

4.5. *Implications on biogeographic diversification*

The *LEAFY* data suggested the basal-most position of the Chilean *Nolana sessiliflora*. The Chilean *Nolana acuminata* group (LFY-A) and the *Alona* group (LFY-C and LFY-D) then diverged next. Even though the basal-most position of *Nolana sessiliflora* was not detected in the plastid DNA phylogeny, it is nested within the clade of the genus consisting of the *N. acuminata* group and the *Alona* group from Chile. Reticulate evolution may have complicated the construction of the early diversification history of the basally branching taxa or their ancestors. Nevertheless, our *LEAFY* data suggest the basal position occupied by taxa from Chile and all Peruvian species are supported to be nested within groups of Chilean taxa.

There are at least two cases of secondary dispersal/migration from Peru to Chile on the species level. The northern Chilean species *Nolana intonsa* is nested within a clade of Peruvian species in both plastid DNA and the nuclear trees (*LEAFY* and GBSSI), suggesting its dispersal/migration from Peru to northern Chile. *Nolana intonsa* is also morphologically similar to *N. lycioides*, *N. cerrateana* and *N. pallida* from Peru. Another case is the northern Chilean species *Nolana tarapacana*, which is nested in a clade of Peruvian species in the plastid DNA tree. However, the *LEAFY* sequences of *N. tarapacana* were not available and it formed a polytomy with other species from Peru in the GBSSI tree (Dillon et al., 2007).

The GBSSI data suggested a close relationship between *Nolana galapagensis* from

the Galápagos Islands and the Peruvian *N. adansonii* and *N. arenicola* (Dillon et al., 2007). Morphologically, *Nolana galapagensis* is similar to the Chilean *N. sedifolia* in a set of characters including the robust shrub habit, succulent leaves, and small white tubular corollas. In the plastid DNA tree, *N. galapagensis* is sister to *N. adansonii* from Peru with weak support and the Peruvian *N. arenicola* groups other Peruvian species (BP=100). In the *LEAFY* tree *N. galapagensis* is nested in a group of Peruvian species including *N. arenicola* along with a few other Peruvian species (clade LFY-BE in Fig. 2). Although the position of N. galapagensis needs to be further resolved, our results support the evolution of *Nolana galapagensis* of its Peruvian relatives. Its morphological similarities with the Chilean *N. sedifolia* may be due to convergence or adaptive evolution after it reached the Galápagos Islands.

4.6 SINE or SINE-like insertions in Nolana

In recent years, a new source of phylogenetic characters, transposable elements, especially SINE (short interspersed repetitive element) families, have been employed as a unique tool for phylogenetic study (Ray, 2007; Shedlock and Okada, 2000). The utility of SINE has been basically restricted to animal phylogenetic reconstruction (Lum et al., 2000; Murata et al., 1993; Nikaido et al., 2006; Nikaido et al., 2007; Shimamura et al., 1997) and has not attracted much attention among plant phylogenetists. Only a few SINEs have been employed as phylogenetic markers in plants, including the SINE detected in GBSSI exclusively in the monophyletic tribe of Hyoscyameae (Yuan et al., 2006), a putative relative of *Nolana*. At least one of these insertions from the *LEAFY* second intron may be identified as a SINE. This insertion (labeled as SINE1 in Fig. 2.) is about 789 bp in size between the position 3119 and the position 3908 in the alignment and is flanked by a repeat of AATCCAAAAT. The SINE1 occurs exclusively in a strongly supported clade of species from Chile and can be aligned with the TS (Tobaccco SINE) sequences. The TTG repeat of variable length at the 3' end of the SINE1 sequence was considered to be characteristic of the TS family (Yoshioka et al., 1993). The second SINE-like insertion was detected exclusively for a clade (BP=99/100) of species from Peru and Chile (labeled SINE2 in Fig. 2). This SINE-like insertion is ca. 472 bp in size and is flanked by a repeat of GGWGT. The third SINE-like insertion was

detected exclusively for *Nolana paradoxa-N. rupicola* clade. It is about 263 bp in size and is flanked by a sequence repeat of ACTAGRAAT. Two additional SINE-like insertions were found in *N. werdermannii* (512 bp flanked by TTTAGTT) and *N. aplocaryoides* (218 bp flanked by ASCCCTS) respectively. All these five insertions are longer than 200 bases and are flanked by a short direct repeat of sequences, which have been considered a hallmark of transposition and retroposition (Li, 1997). The three SINEs possessed by the three clades (SINE1, SINE2 and SINE3) corroborate the monophyly of these clades, supporting the significance of the SINEs in phylogeny reconstruction. The later two SINE-like insertions (SINE4 and SINE5) are only autapomorphies for each of the two species (*Nolana aplocaryoides* and *N. werdermannii*). The functions of the SINEs or SINE-like insertions in *Nolana* need to be explored and may be helpful for understanding the molecular evolution of the *LEAFY* gene in the genus.

Acknowledgments

Support of the study was provided by National 973 Project of China (2007CB411601), National Science Foundation of China (30625004), the National Science Foundation (DEB-0415573 to Michael Dillon and Jun Wen), and the Laboratory of Analytical Biology at the National Museum of Natural History of the Smithsonian Institution. We thank the many colleagues who have helped with field and lab work, discussed the study, and/or supplied plant material for this study, including: Federico Luebert, Jeff Hunt, Lee Weigt, Zelong Nie, Kin Han, Nicolás García, Marucha Benavides, Magda Chanco, Diane Dillon, Miguel and Erica Finger, Jorge Guerra, Oswaldo Ibicache, Mauricio Ibicache, Paulina Ossa, Manuel and Gabriela Ossa, José Miguel Fariña, Segundo Leiva, Pedro Lezama, Victor Quipuscoa, Eduardo and Tina Martell, Rachel Pinto, Adriana Hoffmann, Clodomiro Marticorena, Max Quezada, Eric Rodriguez, Abundio Sagástegui, Sebastián Teillier, Carmen Trujillo Cadiz, Alfredo Tupuachi, Maximino (Nino) Villarroel, Mario Zapata, Miyuki Nakazawa, Mélica Muñoz-Schick, Sergio Moreira, Aldo Mesa, Mikio Ono, Rosanna Freyre, Edgardo Ortiz, Massiel Corrales, Gina Castillo, Juan Gonzales, and Gina Arancio.

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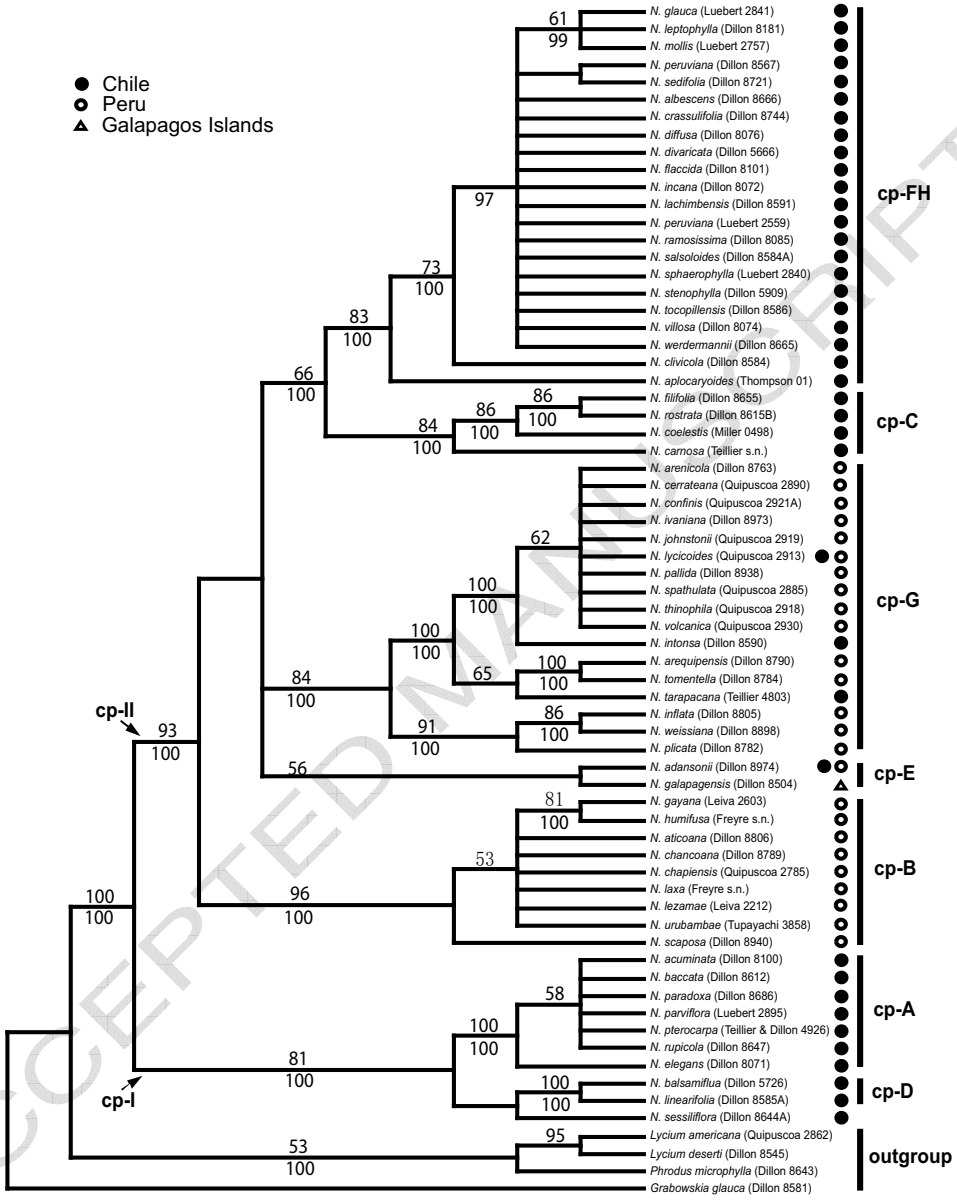
Legend

Fig. 1. Strict consensus tree of the most parsimonious trees of *Nolana* based on combined sequences of four chloroplast markers. Bootstrap values are provided above the branches leading to the nodes and Bayesian posterior probabilities are below the branches, bootstrap values below 50% and Bayesian values below 95% are not shown.

Fig. 2. Strict consensus tree of the most parsimonious tree of *Nolana* based on sequences of the *LEAFY* second intron. Numbers next to the nodes indicate the bootstrap value based on the base-substitution data/ the bootstrap values based on the analysis treating indels as new characters; The "-" means the bootstrap values are not changed. Bold branches indicate the nodes have Bayesian values > 95%. Clades are annotated as LFY-A to LFY-FH. SINE1-SINE5 indicate the SINE or SINE-like insertions detected for the clades or species.


Chile
Peru
Galapagos Islands
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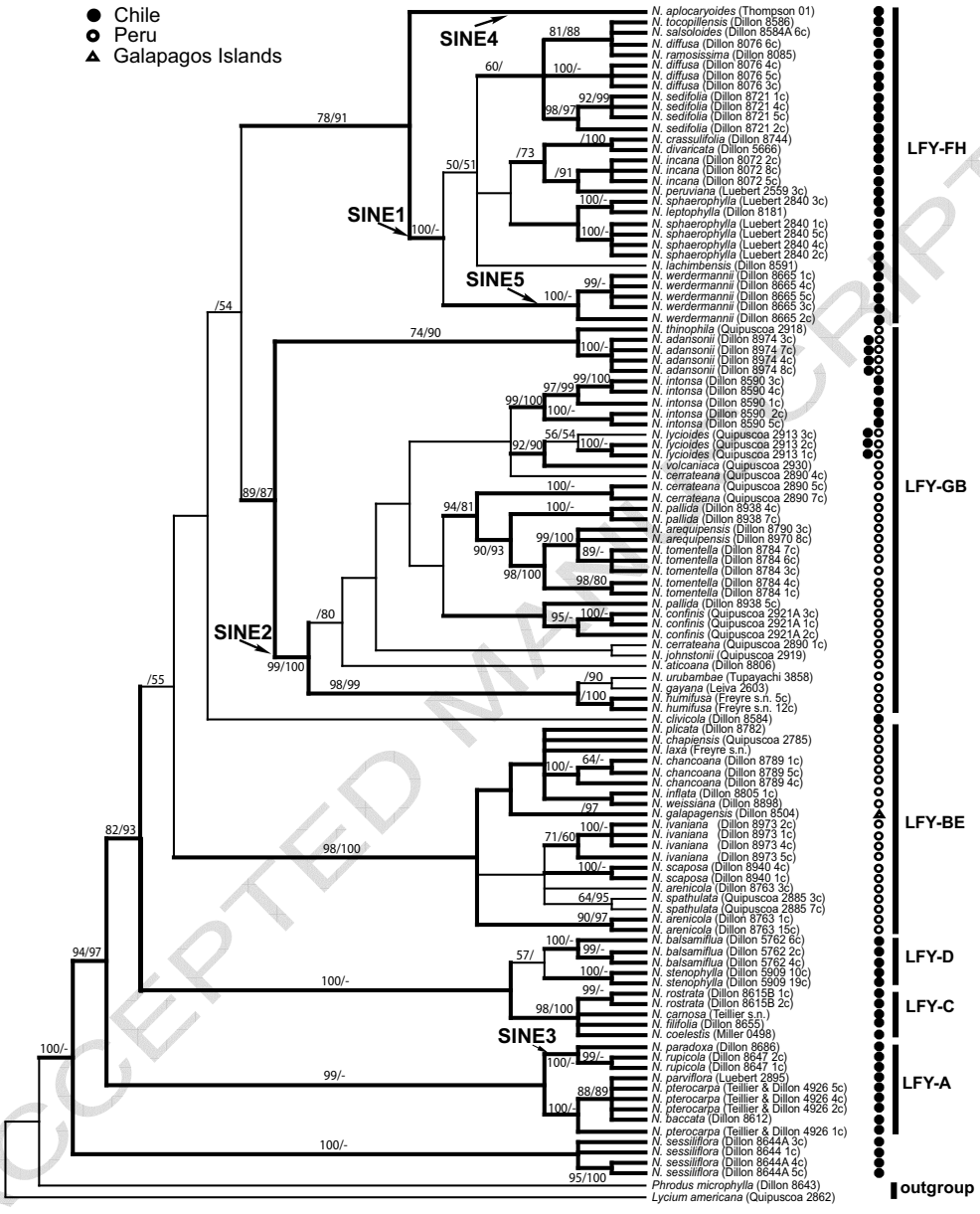
N. leptophylla (Dillon 8181)
N. mollis (Luebert 2757)
N. peruviana (Dillon 8567)
N. sedifolia (Dillon 8721)
N. albescens (Dillon 8666)
N. crassulifolia (Dillon 8744)
N. diffusa (Dillon 8076)
N. divaricata (Dillon 5666)
N. flaccida (Dillon 8101)
N. incana (Dillon 8072)
N. lachimbensis (Dillon 8591)
N. peruviana (Luebert 2559)
N. ramosissima (Dillon 8085)
N. salsoloides (Dillon 8584A)
N. sphaerophylla (Luebert 2840)
N. stenophylla (Dillon 5909)
N. tocopillensis (Dillon 8586)
N. villosa (Dillon 8074)
N. werdermannii (Dillon 8665)
N. clivicola (Dillon 8584)
N. aplocaryoides (Thompson 01)
N. filifolia (Dillon 8655)
N. rostrata (Dillon 8615B)
N. coelestis (Miller 0498)
N. carnosa (Teillier s.n.)
N. arenicola (Dillon 8763)
N. cerrateana (Quipuscoa 2890)
N. confinis (Quipuscoa 2921A)
N. ivaniana (Dillon 8973)
N. johnstonii (Quipuscoa 2919)
N. lycicoides (Quipuscoa 2913)
N. pallida (Dillon 8938)
N. spathulata (Quipuscoa 2885)
N. thinophila (Quipuscoa 2918)
N. volcanica (Quipuscoa 2930)
N. intonsa (Dillon 8590)
N. arequipensis (Dillon 8790)
N. tomentella (Dillon 8784)
N. tarapacana (Teillier 4803)
N. inflata (Dillon 8805)
N. weissiana (Dillon 8898)
N. plicata (Dillon 8782)
N. adansonii (Dillon 8974)
N. galapagensis (Dillon 8504)
N. gayana (Leiva 2603)
N. humifusa (Freyre s.n.)
N. aticoana (Dillon 8806)
N. chancoana (Dillon 8789)
N. chapiensis (Quipuscoa 2785)
N. laxa (Freyre s.n.)
N. lezamae (Leiva 2212)
N. urubambae (Tupayachi 3858)
N. scaposa (Dillon 8940)
N. acuminata (Dillon 8100)
N. baccata (Dillon 8612)
N. paradoxa (Dillon 8686)
N. parviflora (Luebert 2895)
N. pterocarpa (Teillier & Dillon 4926)
N. rupicola (Dillon 8647)
N. elegans (Dillon 8071)
N. balsamiflua (Dillon 5726)
N. linearifolia (Dillon 8585A)
N. sessiliflora (Dillon 8644A)
Lycium americana (Quipuscoa 2862)
Lycium deserti (Dillon 8545)
Phrodus microphylla (Dillon 8643)
Grabowskia glauca (Dillon 8581)
cp-FH
cp-C
cp-G
cp-E
cp-B
cp-A
cp-D
outgroup
cp-II
cp-I

1


Chile
Peru
Galapagos Islands
SINE4
SINE1
SINE5
SINE2
SINE3
LFY-FH
LFY-GB
LFY-BE
LFY-D
LFY-C
LFY-A
outgroup
N. aplocaryoides (Thompson 01)
N. tocopillensis (Dillon 8586)
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N. sedifolia (Dillon 8721 2c)
N. crassulifolia (Dillon 8744)
N. divaricata (Dillon 5666)
N. incana (Dillon 8072 2c)
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N. incana (Dillon 8072 5c)
N. peruviana (Luebert 2559 3c)
N. sphaerophylla (Luebert 2840 3c)
N. leptophylla (Dillon 8181)
N. sphaerophylla (Luebert 2840 1c)
N. sphaerophylla (Luebert 2840 5c)
N. sphaerophylla (Luebert 2840 4c)
N. sphaerophylla (Luebert 2840 2c)
N. lachimbensis (Dillon 8591)
N. werdermannii (Dillon 8665 1c)
N. werdermannii (Dillon 8665 4c)
N. werdermannii (Dillon 8665 5c)
N. werdermannii (Dillon 8665 3c)
N. werdermannii (Dillon 8665 2c)
N. thinophila (Quipuscoa 2918)
N. adansonii (Dillon 8974 3c)
N. adansonii (Dillon 8974 7c)
N. adansonii (Dillon 8974 4c)
N. adansonii (Dillon 8974 8c)
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N. intonsa (Dillon 8590 4c)
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N. intonsa (Dillon 8590 2c)
N. intonsa (Dillon 8590 5c)
N. lycioides (Quipuscoa 2913 3c)
N. lycioides (Quipuscoa 2913 2c)
N. lycioides (Quipuscoa 2913 1c)
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N. cerrateana (Quipuscoa 2890 4c)
N. cerrateana (Quipuscoa 2890 5c)
N. cerrateana (Quipuscoa 2890 7c)
N. pallida (Dillon 8938 4c)
N. pallida (Dillon 8938 7c)
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N. arequipensis (Dillon 8970 8c)
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N. tomentella (Dillon 8784 4c)
N. tomentella (Dillon 8784 1c)
N. pallida (Dillon 8938 5c)
N. confinis (Quipuscoa 2921A 3c)
N. confinis (Quipuscoa 2921A 1c)
N. confinis (Quipuscoa 2921A 2c)
N. cerrateana (Quipuscoa 2890 1c)
N. johnstonii (Quipuscoa 2919)
N. aticoana (Dillon 8806)
N. urubambae (Tupayachi 3858)
N. gayana (Leiva 2603)
N. humifusa (Freyre s.n. 5c)
N. humifusa (Freyre s.n. 12c)
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N. plicata (Dillon 8782)
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N. chancoana (Dillon 8789 1c)
N. chancoana (Dillon 8789 5c)
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N. ivaniana (Dillon 8973 5c)
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N. scaposa (Dillon 8940 1c)
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N. spathulata (Quipuscoa 2885 3c)
N. spathulata (Quipuscoa 2885 7c)
N. arenicola (Dillon 8763 1c)
N. arenicola (Dillon 8763 15c)
N. balsamiflua (Dillon 5762 6c)
N. balsamiflua (Dillon 5762 2c)
N. balsamiflua (Dillon 5762 4c)
N. stenophylla (Dillon 5909 10c)
N. stenophylla (Dillon 5909 19c)
N. rostrata (Dillon 8615B 1c)
N. rostrata (Dillon 8615B 2c)
N. carnosa (Teillier s.n.)
N. filifolia (Dillon 8655)
N. coelestis (Miller 0498)
N. paradoxa (Dillon 8686)
N. rupicola (Dillon 8647 2c)
N. rupicola (Dillon 8647 1c)
N. parviflora (Luebert 2895)
N. pterocarpa (Teillier & Dillon 4926 5c)
N. pterocarpa (Teillier & Dillon 4926 4c)
N. pterocarpa (Teillier & Dillon 4926 2c)
N. baccata (Dillon 8612)
N. pterocarpa (Teillier & Dillon 4926 1c)
N. sessiliflora (Dillon 8644A 3c)
N. sessiliflora (Dillon 8644 1c)
N. sessiliflora (Dillon 8644A 4c)
N. sessiliflora (Dillon 8644A 5c)
Phrodus microphylla (Dillon 8643)
Lycium americana (Quipuscoa 2862)

1

1 Table 1

2 List of the taxa sampled with geographic origins, voucher numbers, and GenBank Accession numbers.

Species	Location	Voucher	GenBank accession number					
			ndhF	*psbA-trnH*	*rps16-trnK*	*trnC-psbM*	*LEAFY*	
Grabowskia glauca (Phil.) I.M.Johnst.	Chile (Antofagasta)	Dillon 8581 (F)	EU742303	EU742439	EU742371	EU742507	-	
Lycium americana Jacq.	Peru (Arequipa)	Quipuscoa 2862 (F)	EU742304	EU742440	EU742372	EU742508	EU742190	
L. deserti Phil.	Chile (Antofagasta)	Dillon 8545 (F)	EU742305	EU742441	EU742373	EU742509	-	
Nolana acuminata (Miers) Miers ex Dunal	Chile (Región II)	Dillon 8100 (F)	EU742307	EU742443	EU742375	EU742511	-	
N. adansonii (Roem. & Schult.) I.M. Johnst.	Peru (Arequipa)	Dillon 8984 (F)	EU742308	EU742444	EU742376	EU742512	EU742223 EU742224 EU742225 EU742226	clone3 clone7 clone4 clone8
N. albescens (Phil.) I.M. Johnst.	Chile (Región III)	Dillon 8666 (F)	EU742309	EU742445	EU742377	EU742513	-	
N. aplocaryoides (Guadich.) I.M. Johnst.	Chile (Región II)	Thompson 01 (F)	EU742310	EU742446	EU742378	EU742514	EU742192	
N. arenicola I.M. Johnst.	Peru (Arequipa)	Dillon 8763 (F)	EU742311	EU742447	EU742379	EU742515	EU742275 EU742276 EU742279	clone3 clone1 clone15
N. arequipensis M.O. Dillon & Quipuscoa	Peru (Arequipa)	Dillon 8790 (F)	EU742312	EU742448	EU742380	EU742516	EU742241 EU742242	
N. aticoana Ferreyra	Peru (Arequipa)	Dillon 8806 (F)	EU742313	EU742449	EU742381	EU742517	EU742254	
N. baccata (Lindl.) Dunal	Chile (Región III)	Dillon 8612 (F)	EU742314	EU742450	EU742382	EU742518	EU742297	
N. balsamiflua (Gaudich.) Mesa	Chile (Región II)	Dillon 5726 (F)	EU742315	EU742451	EU742383	EU742519	EU742280 EU742281 EU742282	clone6 clone2 clone4
N. carnosa (Lindl.) Miers ex Dunal	Chile (Caldera)	Teillier& Dillon s.n.	EU742316	EU742452	EU742384	EU742520	EU742287	
*N.*cerrateana Ferreyra	Peru (Arequipa)	Quipuscoa 2890 (F)	EU742317	EU742453	EU742385	EU742521	EU742236 EU742237 EU742238 EU742252	clone4 clone5 clone7 clone1
N. chancoana M.O. Dillon &	Peru (Arequipa)	Dillon 8789 (F)	EU742318	EU742454	EU742386	EU742522	EU742263	clone1

Quipuscoa							EU742264	clone5
							EU742265	clone4
N. chapiensis M.O. Dillon & Quipuscoa	Peru (Arequipa)	Quipuscoa 2785 (F)	EU742319	EU742455	EU742387	EU742523	EU742261	
N. clivicola (I.M. Johnst.) I.M. Johnst.	Chile (Región II)	Dillon 8584 (F)	EU742320	EU742456	EU742388	EU742524	EU742259	
N. coelestis (Lindl.) Miers ex Dunal	Chile (Región IV)	Miller 0498 (US)	EU742321	EU742457	EU742389	EU742525	EU742289	
N. confinis I.M. Johnst.	Peru (Moquegua)	Quipuscoa 2921A (F)	EU742322	EU742458	EU742390	EU742526	EU742249	clone3
							EU742250	clone1
							EU742251	clone2
N. crassulifolia Poepp.	Chile (Región III)	Dillon 8744 (F)	EU742323	EU742459	EU742391	EU742527	EU742204	
N. diffusa I.M. Johnst.	Chile (Región II)	Dillon 8076 (F)	EU742324	EU742460	EU742392	EU742528	EU742195	clone6
							EU742197	clone4
							EU742198	clone5
							EU742199	clone3
N. divaricata (Lindl.) I.M. Johnst.	Chile (Región II)	Dillon 5666 (F)	EU742325	EU742461	EU742393	EU742529	EU742205	
N. elegans (Phil.) Reiche	Chile (Región II)	Dillon 8071 (F)	EU742326	EU742462	EU742394	EU742530	-	
N. filifolia (Hook. & Arn.) I.M. Johnst.	Chile (Región III)	Dillon 8655 (F)	EU742327	EU742463	EU742395	EU742531	EU742288	
N. flaccida (Phil.) I.M. Johnst.	Chile (Región II)	Dillon 8101 (F)	EU742328	EU742464	EU742396	EU742532	-	
N. galapagensis (Christoph.) I.M. Johnst.	Ecuador(Galápagos Islands)	Dillon 8504 (F)	EU742329	EU742465	EU742387	EU742533	EU742268	
N. gayana (Gaudich.) Koch	Peru (Ancash)	Leiva 2603 (F)	EU742330	EU742466	EU742398	EU742534	EU742256	
N. glauca (I.M.Johnst.) I.M. Johnst.	Chile (Chañaral)	Luebert & Becker 2841 (F)	EU742331	EU742467	EU742399	EU742535	-	
N. humifusa (Gouan) I.M. Johnst.	Peru	Freyre s.n. (F)	EU742332	EU742468	EU742400	EU742536	EU742257	clone5
							EU742258	clone12
N. incana (Phil.) I.M. Johnst.	Chile (Región II)	Dillon 8072 (F)	EU742333	EU742469	EU742401	EU742537	EU742206	clone2
							EU742207	clone8
							EU742208	clone5
N. inflata Ruiz & Pav.	Peru (Arequipa)	Dillon 8805 (F)	EU742334	EU742470	EU742402	EU742538	EU742266	
N. intonsa I.M. Johnst.	Chile (Región I)	Dillon & Finger 8590 (F)	EU742335	EU742471	EU742403	EU742539	EU742227	clone3
							EU742228	clone4
							EU742229	clone1
							EU742230	clone2
							EU742231	clone5
N. ivaniana Ferreyra	Peru (Arequipa)	Dillon 8973 (F)	EU742336	EU742472	EU742404	EU742540	EU742269	clone2

							EU742270	clone1
							EU742271	clone4
							EU742272	clone5
N. johnstonii Vargas	Peru (Moquega)	Quipuscoa 2919 (F)	EU742337	EU742473	EU742405	EU742541	EU742253	
N. lachimbensis M.O. Dillon & Luebert	Chile (Región II)	Dillon 8591 (F)	EU742338	EU742474	EU742406	EU742542	EU742216	
N. laxa (Miers) I.M. Johnst.	Peru (Lima)	Freyre s.n.	EU742339	EU742475	EU742407	EU742543	EU742262	
N. leptophylla (Miers) I.M. Johnst.	Chile (Región II)	Dillon 8181 (F)	EU742340	EU742476	EU742408	EU742544	EU742211	
N. lezamae M.O. Dillon, S. Leiva & Quipuscoa	Peru (Ancash)	Leiva 2212 (F),	EU742341	EU742477	EU742409	EU742545	-	
N. linearifolia Phil.	Chile (Región II)	Dillon 8585A (F)	EU742342	EU742478	EU742410	EU742546	-	
N. lycioides I.M. Johnst.	Peru (Arequipa)	Quipuscoa 2913 (F)	EU742343	EU742479	EU742411	EU742547	EU742232	clone3
							EU742233	clone2
							EU742234	clone1
N. mollis Phil.	Chile (Antofagasta)	Luebert & Gracía 2757 (F)	EU742344	EU742480	EU742412	EU742548	-	
N. pallida I.M. Johnst.,	Peru (Arequipa)	Dillon 8938 (F)	EU742345	EU742481	EU742413	EU742549	EU742239	clone4
							EU742240	clone7
							EU742248	clone5
N. paradoxa Lindl.	Chile (IV)	Dillon 8686 (F)	EU742346	EU742482	EU742414	EU742550	EU742290	
N. parviflora (Phil.) Phil.	*N. parviflora* (Phil.) Phil.	Luebert 2895 (F)	EU742347	EU742483	EU742415	EU742551	EU742293	
N. peruviana (Gaudich.) I.M. Johnst.	Chile (Región II)	Luebert 2559 (F)	EU742348	EU742484	EU742416	EU742552	EU742209	
N. peruviana (Gaudich.) I.M. Johnst.	Chile (Región II)	Dillon 8567 (F)	Eu742349	EU742485	EU742417	EU742553	-	
N. plicata I.M. Johnst.	Peru (Arequipa)	Dillon 8782 (F)	EU742350	EU742486	EU742418	EU742554	EU742260	
N. pterocarpa Phil.	Chile (Región III)	Teillier & Dillon 4926 (F)	EU742351	EU742487	EU742419	EU742555	EU742294	clone5
							EU742295	clone4
							EU742296	clone2
							EU742298	clone1
N. ramosissima I.M. Johnst.	Chile (Región II)	Dillon 8085 (F)	EU742352	EU742488	EU742920	EU742556	EU742196	
N. rostrata (Lindl.) Miers ex Dunal	Chile (Región III)	Dillon 8615B (F)	EU742353	EU742489	EU742921	EU742557	EU742285	clone1
							EU742286	clone2
N. rupicola Gaudich.	Chile (Región II)	Dillon 8647 (F)	EU742354	EU742490	EU742922	EU742558	EU742291	clone2
							EU742292	clone1

N. salsoloides (Lindl.) I.M. Johnst.	Chile (Región II)	Dillon 8584A (F)	EU742355	EU742491	EU742923	EU742559	EU742194	
N. scaposa Ferreyre	Peru (Arequipa)	Dillon 8940 (F)	EU742356	EU742492	EU742924	EU742560	EU742273	clone4
							EU742274	clone1
N. sedifolia Poepp.	Chile (Región II)	Dillon 8721 (F)	EU742357	EU742493	EU742925	EU742561	EU742200	clone1
							EU742201	clone4
							EU742202	clone5
							EU742203	clone2
N. sessiliflora Phil.	Chile (Región II)	Dillon 8644A (F)	EU742358	EU742494	EU742926	EU742562	EU742299	clone3
							EU742300	clone1
							EU742301	clone4
							EU742302	clone5
N. spathulata Ruiz & Pav.	Peru (Arequipa)	Quipuscoa 2885 (F)	EU742359	EU742495	EU742927	EU742563	EU742277	clone3
							EU742278	clone7
N. sphaerophylla (Phil.) Mesa ex Dillon	Chile (Chañaral)	Luebert & Becker 2840 (F)	EU742360	EU742496	EU742928	EU742564	EU742210	clone3
							EU742212	clone1
							EU742213	clone5
							EU742214	clone4
							EU742215	clone2
N. stenophylla I.M. Johnst.	Chile (Región II)	Dillon 5909 (F)	EU742361	EU742497	EU742929	EU742565	EU742283	clone10
							EU742284	clone19
N. tarapacana (Phil.) I.M. Johnst.	Chile (Región I)	Teillier 4803 (F)	EU742362	EU742498	EU742930	EU742566	-	
N. thinophila I.M. Johnst.	Peru (Arequipa)	Quipuscoa 2918 (F)	EU742363	EU742499	EU742931	EU742567	EU742222	
N. tocopillensis (Phil.) I.M.Johnst.	?	Dillon 8586 (F)	EU742364	EU742500	EU742932	EU742568	EU742193	
N. tomentella Ferreyre	Peru (Arequipa)	Dillon 8784 (F)	EU742365	EU742501	EU742433	EU742569	EU742243	clone7
							EU742244	clone6
							EU742245	clone3
							EU742246	clone4
							EU742247	clone1
N. urubambae Vargas	Peru (Cusco)	Tupayachi 3858 (F)	EU742366	EU742502	EU742434	EU742570	EU742255	
N. villosa (Phil.) I.M. Johnst.	Chile (Región II)	Dillon 8074 (F)	EU742367	EU742503	EU742435	EU742571	-	
N. volcanica Ferreyra	Peru (Arequipa)	Quipuscoa 2930 (F)	EU742368	EU742504	EU742436	EU742572	EU742235	
N. weissiana Ferreyra	Peru (Arequipa)	Dillon 8898 (F)	EU742369	EU742505	EU742437	EU742573	EU742267	
N. werdermannii I.M. Johnst.	Chile (Región IV)	Dillon 8665 (F)	EU742370	EU742506	EU742438	EU742574	EU742217	clone1
							EU742218	clone4
							EU742219	clone5
							EU742220	clone2
							EU742221	clone3

Phrodus microphylla (Miers) Miers	Chile (Región II)	Dillon 8643 (F)	EU742306	EU742442	EU742374	EU742510	EU742191	

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Table 2.

Comparison of four plastid cpDNA markers and the nuclear *LEAFY* second intron. Note that the four plastidcpDNA markers were sequenced for 68 OTUs and the *LEAFY* second intron had 113 OTUs.

Region	*ndhF*	*rps16-trnK*	*psbA-trnH*	*trnC-psbM*	Combined cpDNA plastid DNA without gaps	Combined plastid cpDNA with gaps	*LEAFY*	*LEAFY* with gaps
Aligned length	1998	815	501	1858	5172	5196	4175	4500
Variable sites/proportion	76/3.8%	40/4.9%	46/9.1%	58/3.1%	220/4.2%	245/4.7%	900/21.0%	1204/26.7%
PI sites/proportion	52/2.6%	25/3.1%	28/5.6%	45/2.4%	150/2.9%	162/3.1%	564/13.5%	752//16.7%
CI/ RI.	0.94/0.98	0.95/0.99	0.75/0.91	0.89/0.97	0.84/0.95	0.85/0.95	0.75/0.90	0.74/0.90
Tree length	84	43	65	65	268	296	1493	1898

Note: PI = parsimony-informative; CI = cons istency index; RI = retention index.