American Journal of Botany 96(8): 1571–1580. 2009.

POPULATION STRUCTURE AND PHYLOGEOGRAPHY OF THE MISTLETOES *TRISTERIX CORYMBOSUS* AND *T. APHYLLUS* (LORANTHACEAE) USING CHLOROPLAST DNA SEQUENCE VARIATION[1]

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The mistletoe *Tristerix corymbosus* (Loranthaceae) is present in the temperate forest and Chilean matorral biomes of Chile and northwest Patagonia. The closely related cactus-specific species, *T. aphyllus*, occurs only in the matorral biome. The population structure of these mistletoes was examined to determine whether the distribution of haplotypes corresponds mostly to geographic zone, biome, or other biotic factors. Samples from 108 individuals in 26 localities of *T. corymbosus* and 13 individuals in four localities of *T. aphyllus* were collected. Sequences were obtained from two chloroplast genome regions: the *atpB-rbcL* spacer and the *trnL-F* region. Haplotypes were analyzed using parsimony and Bayesian trees as well as parsimony networks. All methods placed the haplotypes in four clades, one of which corresponded to *T. aphyllus* and the others to *T. corymbosus*. Within *T. corymbosus*, the different clades did not correlate with biome, geographical region, host, or any apparent morphological feature of the mistletoe. The morphologically distinct cactus parasite *T. aphyllus* likely arose in sympatry from an unspecialized tree parasite, *T. corymbosus,* after a host switch. The present day haplotype distribution is complex and resulted from post-glaciation migrations from multiple Pleistocene refugia.

Key words: *atpB-rbcL* spacer; cpDNA; glacial refugia; historical biogeography; host; Loranthaceae; parasitic plants; seed dispersal; South America; *Tristerix*.

Mistletoes are aerial parasitic plants found in the order Santalales (families Loranthaceae, Misodendraceae, Santalaceae, and Viscaceae) that are intimately dependent upon their hosts for water and nutrients (Kuijt, 1969; Norton and Carpenter, 1998; Mathiasen et al., 2008). These plants have also evolved complex associations with animals that pollinate their flowers and disperse their seeds (Kuijt, 1969; Reid, 1991). Indeed, mistletoes can be keystone species that determine community structure and diversity (Watson, 2001). Therefore, understanding mistletoe phylogeography will help illuminate more global historic processes of the community, particularly among those organisms closely associated with mistletoes. Although intraspecific genetic diversity has been examined in dwarf mistletoes (*Arceuthobium*, Viscaceae) using isozymes (Nickrent and Butler, 1990, 1991; Nickrent and Stell, 1990; Linhart et al., 2003) and AFLPs (Jerome and Ford, 2002a, b), to date no population genetic or phylogeographic study has been conducted using DNA sequence data, nor on Loranthaceae, the largest mistletoe family.

South America harbors several mistletoes considered relictual in Loranthaceae (Barlow, 1983; Vidal-Russell and Nickrent, 2008a), including the genus *Tristerix*, which has 11 species distributed along the Andes from Colombia to Chile. The only *Tristerix* present in the temperate forest biome is the austral species *T. corymbosus*, whereas other *Tristerix* species are found in wet or dry and/or high elevation areas (Kuijt, 1988; Amico et al., 2007). *Tristerix corymbosus* (Fig. 1) is distributed from 30° to 42°S in Chile and between 40° to 41°S in Argentina. This distribution spans two distinct habitats: the temperate forest of southern South America and the Chilean matorral. In a previous phylogenetic study that examined all species in the genus, *T. corymbosus* emerged as paraphyletic (Amico et al., 2007). Specifically, the Chilean matorral populations were sister to a clade composed of the cactus mistletoe *T. aphyllus* (Fig. 1), and all were sister to the temperate forest *T. corymbosus* populations. There are several morphological autapomorphies found in *T. aphyllus* that justify its recognition as a distinct species including the absence of leaves, fused red cotyledons, spherical white fruits, red inflorescences, extensive endophytic growth, and erect flowers. The phylogenetic results prompted the current investigation of the phylogeography of these species.

Tristerix corymbosus has geographical variation in fruit color associated with the two biomes it occupies (Fig. 1). All temperate forest mistletoe populations produce a fruit that is green at maturity, whereas in the Chilean matorral populations the fruits are yellow (Kuijt, 1988; Amico, 2007; Amico et al., 2007). In addition, the only seed disperser in the temperate forest is the nocturnal arboreal marsupial *Dromiciops gliroides* (Microbiotheriidae). This mode of seed dispersal differs from the situation in the Chilean matorral populations of *T. corymbosus* (and most mistletoes) where birds serve as dispersers (Hoffmann et al., 1986;

[1] Manuscript received 4 September 2008; revision accepted 31 March 2009.

The authors thank L. Amico, M. Nuñez, M. Rodríguez-Cabal, L. Suarez, C. Smith-Ramirez, and N. Tercero Bucardo for help obtaining specimens. They especially thank R. Vidal-Russell for her help in the field and laboratory and for discussions that improved the manuscript. M. Aizen, K. Ibrahim, O. Moya, A. Premoli, and two anonymous reviewers greatly improved an earlier draft of the manuscript with their useful comments. Corporación Nacional Forestal (Chile), Universidad Austral, and Parques Nacionales (Argentina) are thanked for granting permits to collect these mistletoes. The authors thank S. Sipes for generously allowing use of her automated DNA sequencer. Financial support (to G.C.A.) was provided by a Ph.D. fellowship from Consejo Nacional de investigacion Científicas y Técnicas (CONICET) and the National Geographic Society, and grants from the National Science Foundation (to D.L.N.).

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doi:10.3732/ajb.0800302

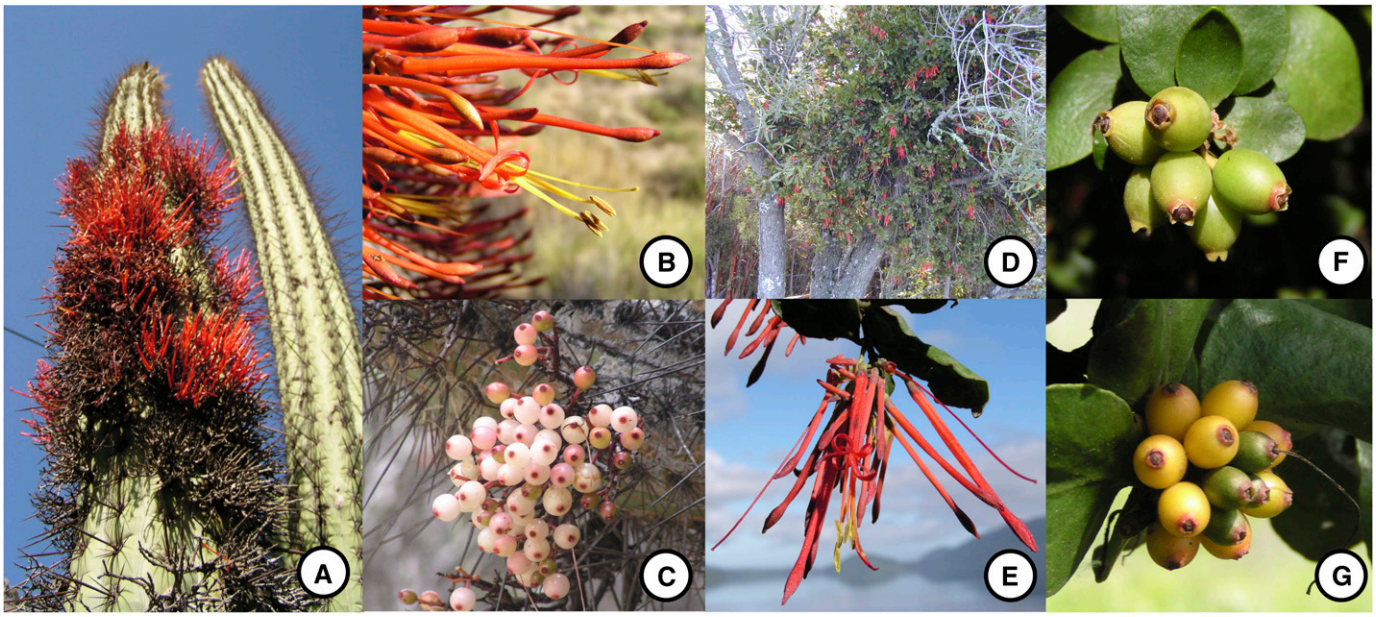

Fig. 1. *Tristerix aphyllus* and *T. corymbosum* morphological features. (A) Flowering individuals of *T. aphyllus* parasitizing the cactus host *Echinopsis chilensis*. Flowers (B) and fruits (C) of *T. aphyllus*. (D) *T. corymbosus* parasitic on *Maytenus boaria*. (E) Flowers of *T. corymbosus*. (F) Mature fruits of *T. corymbosus* from plants growing in the Chilean matorral, and (G) from temperate forest.

Amico and Aizen, 2000; Amico, 2007). Although different seed dispersers occur in each biome, the major pollinator across the entire geographic range is the hummingbird *Sephanoides sephaniodes*.

The distribution and composition of the temperate forest and Chilean matorral floras and faunas have been strongly influenced by geological and climatic events during the Tertiary (Hinojosa and Villagrán, 1997, 2005; Villagrán and Hinojosa, 1997; Gayo et al., 2005; Hinojosa et al., 2006). The fundamental features of these habitats were determined by the separation of South America from Antarctica and the uplift of the Andes. Between 15 and 8 million years ago (Ma) (Reynolds et al., 1990), the uplift of the Andes completely blocked the easterly flow of air masses originating in the tropics, leading to the establishment of what we now know as the mediterranean climate in central Chile with only one rainy season in winter and a dry summer (Hinojosa and Villagrán, 1997, 2005; Villagrán and Hinojosa, 1997). During the Quaternary, volcanism and glaciations have also affected this area, mainly the temperate forest, and have determined the distribution and composition of the extant flora. Glaciations were apparently patchy (Markgraf et al., 1995; McCulloch et al., 2000), thus leaving many possible refugia for plants and animals.

In this study, we obtained chloroplast DNA (cpDNA) sequences and used these haplotypes to examine phylogeographic patterns across the entire range of *T. corymbosus* and *T. aphyllus*. A phylogeny of the cpDNA haplotypes was reconstructed, and an analysis of molecular variance (AMOVA) was employed to evaluate any possible geographic structure. Given the history of the flora in southern South America and the close associations between the mistletoes and their seed dispersers, we predicted finding unique haplotypes in the temperate forest and Chilean matorral biomes. Thus, this study addresses four major questions: (1) what is the genetic structure of these mistletoes, (2) does the distribution of haplotypes correspond mostly to biome or geographic zone (North, Central and South), (3) did the two major geographical barriers, the Andean Cordillera and the waterway separating the Chilean mainland and Chiloé Island, structure the populations in the southern part of the geographic range of *T. corymbosus*, and (4) what role did host and seed dispersers play in determining the genetic structure of these mistletoes?

MATERIALS AND METHODS

Sampling—Samples from 26 populations of *Tristerix corymbosus* and four populations of *T. aphyllus* were collected across the entire geographic range of these species (Fig. 2, Table 1, Appendix 1). At each population, up to six plants were randomly sampled, and the host trees were recorded. Because sample sizes were low, we do not know whether overall genetic variability in the population was represented. Thus, the term locality instead of population will be used throughout this paper. Fresh leaves or flowers from all sampled individuals were dried in silica gel for later DNA isolation. Vouchers of each individual were deposited in the Department of Botany Herbarium, Universidad Nacional del Comahue, Bariloche, Argentina (BCRU). For the 26 localities of *T. corymbosus*, 18 were from the temperate forest and eight from the Chilean matorral. Six of the localities within the temperate forest were located in Argentina east of the Andes range. All the *T. aphyllus* localities were located in the Chilean matorral, the biome where this species is endemic. The latitude and longitude of each locality was recorded using a global positioning device, and elevation was determined with an altimeter.

DNA extraction, amplification, and sequencing—DNA was extracted from dried leaf or flower tissue using a modified CTAB protocol for high carbohydrate plants (Tel-Zur et al., 1999). A one-tenth dilution of genomic DNA was used for all PCR amplifications. Typical PCR amplification reactions included 1× Promega (Madison, Wisconsin, USA) buffer (10 mM Tris HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 50 μM dNTPs, 1 unit *Taq* polymerase, 0.4 μM of each primer, and ca. 30 ng of genomic DNA.

The *atpB-rbcL* spacer and the *trnL-trnF* region were amplified and sequenced using the primers described in Amico et al. (2007) and Taberlet et al. (1991). The *atpB-rbcL* spacer sequences were obtained from 121 individuals from 30 localities (Table 1). For the *trnL-trnF* region, which was less variable than *atpB-rbcL*, only one individual per locality was amplified (26 for *T. corymbosus* and four for *T. aphyllus*).

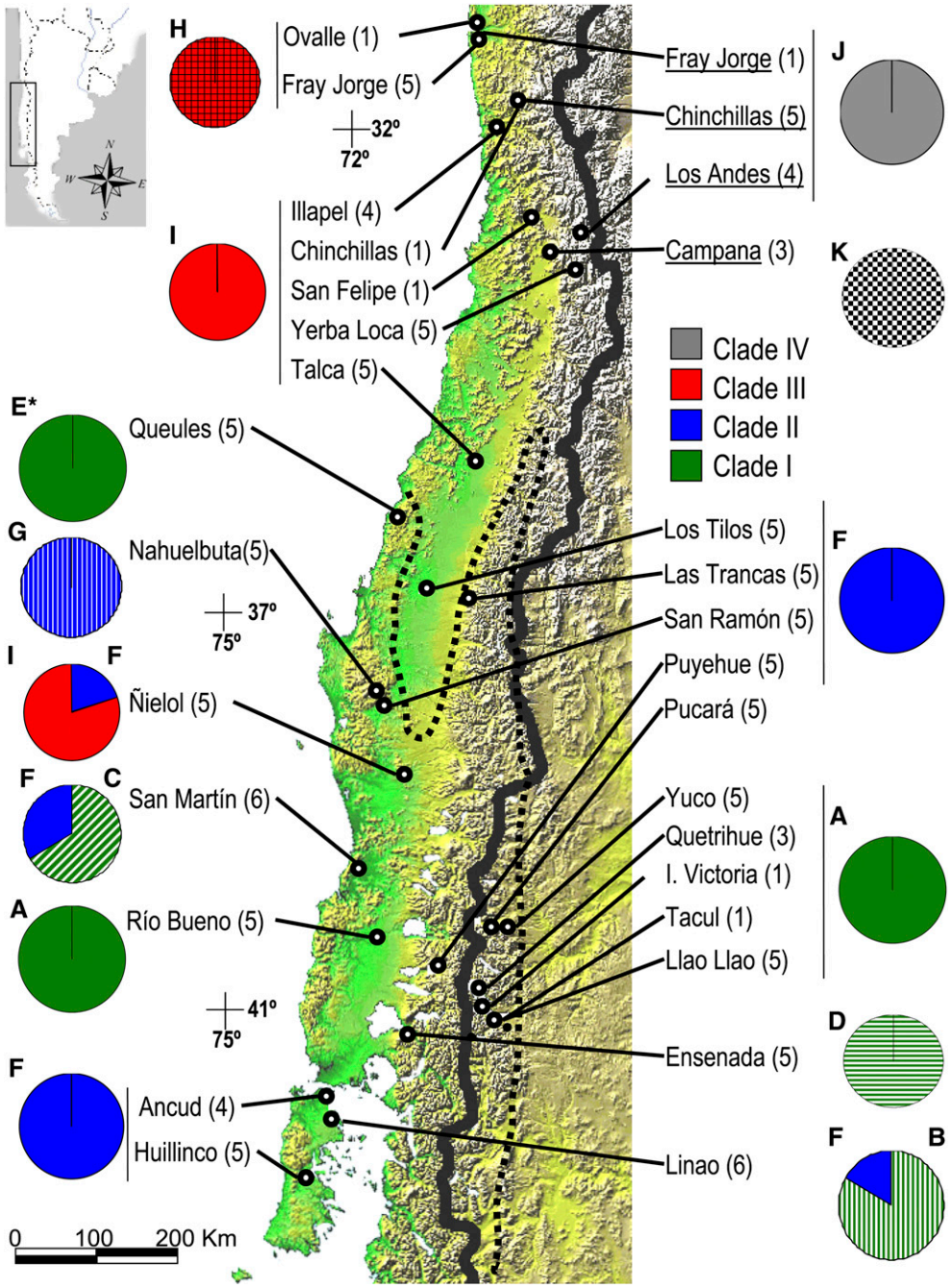

Fig. 2. The distribution of cpDNA haplotypes within and among localities of *Tristerix corymbosus* and *T. aphyllus*. Underlined locality names are for *T. aphyllus*. Capital letters correspond to the different haplotypes (see Table 2 and Fig. 3). The solid line is the border between Argentina and Chile, and the dotted line delimits the boundary between the Chilean matorral (to the North) and the temperate forest (to the South). The numbers within brackets are the sample sizes for each locality for the *atpB-rbcL* spacer.

A touch-down PCR thermal cycle profile was used consisting of 5 min at 95°C; 5 cycles of 30 s at 94°C, 30 s at 52°C, and 1 min 72°C; followed by 33 cycles of 30 s at 94°C, 30 s at 48°C, and 1 min at 72°C; with a final extension of 10 min at 72°C. In all reactions, negative controls that lacked genomic DNA were run to check for DNA contamination. Cycle sequencing reactions (following standard protocols) were performed directly on the purified PCR products using the BigDye terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, California, USA) with Better Buffer (Gel Company, San Francisco, California, USA). Sequences were determined with an ABI 377 automated sequencer (Applied Biosystems). Sequences of each haplotype generated in this study have been deposited with NCBI GenBank under the following accession numbers: DQ442919–DQ442923, DQ442943–DQ442948, and EF050531–EF050535.

Alignment and phylogenetic analyses—Sequences were aligned manually using the program BioEdit (Hall, 1999). The alignment generated several gaps that were unambiguously alignable. We used maximum parsimony (MP) and Bayesian inference (BI) analyses to estimate evolutionary relationships among *atpB-rbc*L spacer and *trnL-trnF* haplotypes. Gaps were considered homologous only when they shared identical boundaries and length and were manually

TABLE 1. Collection information for 26 localities of *Tristerix corymbosus* and four localities of *T. aphyllus* sampled in this study. Only one individual per locality was obtained for the *trnL-trnF* region.

Localities	Latitude (S)	Longitude (W)	Altitude	Country	Geog.	Biomes	Samples	Clades	Haplotypes
Tristerix corymbosus									
Ovalle	30°39′30″	71°40′53″	450	CL	NO	CM	1	III	H
Fray Jorge	30°38′27″	71°22′57″	550	CL	NO	CM	5	III	H
Chinchillas	31°30′15″	71°07′37″	365	CL	NO	CM	1	III	I
Illapel	31°46′13″	71°19′07″	460	CL	NO	CM	4	III	I
San Felipe	32°47′05″	70°51′35″	460	CL	NO	CM	1	III	I
Yerba Loca	33°20′22″	70°19′56″	1800	CL	NO	CM	5	III	I
Talca	35°24′32″	71°37′33″	160	CL	CE	CM	5	III	I
Queules	35°58′58″	71°41′42″	250	CL	CE	TF	5	I	E [a]
Los Tilos	36°46′35″	72°18′48″	100	CL	CE	CM	5	II	F
Chillán	36°49′44″	71°43′01″	985	CL	CE	TF	5	III	F
Nahuelbuta	37°49′26″	71°57′54″	1205	CL	CE	TF	5	II	G
San Ramón	37°51′01″	72°57′50″	1023	CL	CE	TF	5	II	F
Ñielol	38°44′40″	72°35′17″	105	CL	CE	TF	5	II and III	F (1), I (4)
San Martín	39°38′57″	73°11′23″	40	CL	SW	TF	6	I and II	C (4), F (2)
Yuco	40°09′41″	71°31′46″	660	ARG	SE	TF	5	I	A
Pucará	40°09′55″	71°37′35″	640	ARG	SE	TF	5	II	F
Rió Bueno	40°20′58″	72°55′31″	450	CL	SW	TF	5	I	A
Puyehue	40°39′51″	71°12′58″	809	CL	SW	TF	5	II	F
Quetrihue	40°47′59″	71°32′40″	785	ARG	SE	TF	3	I	A
Isla Victoria	40°57′48″	71°31′58″	790	ARG	SE	TF	1	I	A
Llao Llao	41°03′00″	71°32′40″	785	ARG	SE	TF	5	I	A
Tacul	41°04′06″	71°32′67″	780	ARG	SE	TF	1	I	A
Ensenada	41°10′44″	72°32′41″	70	CL	SW	TF	5	I	D
Ancud	41°58′50 ″	73°30′40″	20	CL	SW	TF	4	II	F
Linao	41°59′01″	73°30′38″	15	CL	SW	TF	6	I	B (5), F(1)
Huillinco	42°40′45”	73°54′21″	25	CL	SW	TF	5	II	F
Tristerix aphyllus									
Fray Jorge	30°38′27”	71°22′57”	550	CL	NO	CM	1	IV	J
Chinchillas	31°30′15”	71°07′37”	365	CL	NO	CM	5	IV	J
Los Andes	32°50′39”	70°31′21”	860	CL	NO	CM	4	IV	J
Campana	32°55′31”	71°05′08”	350	CL	NO	CM	3	IV	K

Notes: Country: East and West of Andes range, CL = Chile, ARG = Argentina. Geographical region (Geog.): NO = North, CE = Central, SW = Southwest, SE = Southeast. Biomes: CM = Chilean matorral, TF = temperate forest.

[a] from *trnL-trnF* region

coded as “A” or “T” for MP and as the states “0” or “1” for BI. For BI, gaps were treated as restriction data in a mixed matrix input file. Only one individual representing each haplotype was used to conduct MP analysis in the program PAUP* (Swofford, 2002) and BI analysis with the program MrBayes (Ronquist and Huelsenbeck, 2003). *Tristerix verticillatus* and *T. penduliflorus* were used as outgroups because they are the closest relatives of *T. corymbosus* (Amico et al., 2007). We executed MP using the branch and bound search option for the combined chloroplast regions. Nodal support was assessed using the nonparametric bootstrap (BS) (Felsenstein, 1985) with 1000 pseudoreplicates using a branch and bound search.

For BI, a model of sequence evolution was determined using the program MrModeltest (Nylander et al., 2004). The hierarchical likelihood ratio test selected the HKY85 (Hasegawa et al., 1985) model, which was then used for BI analysis. We executed BI in two independent analyses, each with four chains, for five million generations. Trees and parameters were saved every 100 generations, producing 50 000 trees. Starting model parameters were assigned a uniform prior probability distribution except for the base frequencies where a Dirichlet distribution was assigned. Parameters were estimated as part of the analysis, but in cases where both partitions were analyzed, the estimates between them were unlinked, thus allowing each to vary independently. The burn-in was determined by stationary in the –ln likelihood score, but was ca. 12 500 in every analysis. The split frequency (variance between the two independent runs) in all cases was below 0.001, thus confirming that sampling was from the posterior probability distribution.

Parsimony network—The haplotype network was constructed using the program TCS version 1.3 (Clement et al., 2000). Gap size was disregarded; thus, each one was coded as one substitution. The network with probabilities above the parsimony limit (0.95) was selected. Nested clade analysis was not performed because of the small sample sizes, which introduce statistical inference problems.

Analysis of molecular variance—Analysis of molecular variance, performed only with the *atpB-rbcL* spacer data, was used to examine genetic relationships between the two mistletoe species as well as among localities of *T. corymbosus*. The matrix of haplotype data was analyzed with the program Arlequin (Excoffier et al., 2005) using the Kimura (1980) two-parameter distance. The significance of the fixation indices was tested using a nonparametric approach with 1000 permutations.

Haplotypes from each species were grouped to test the species designation. To assess the significance of various factors that could affect partitioning of genetic variability in *T. corymbosus*, localities were grouped according to two criteria: biome type (temperate forest and Chilean matorral) and geographic zone (North, Central, and South) (Table 1, Fig. 2). Further grouping was done only within the southern region, where localities were grouped based on two biogeographic barriers: the Andes (Southwest and Southeast) and the waterway separating the Chilean mainland and Chiloé Island (Fig. 2).

RESULTS

Molecular features and phylogenetic reconstruction—The *atpB-rbcL* spacer, including the outgroup, had 752 aligned positions with 35 variable sites, of which 19 were parsimony informative. Including nucleotide substitutions and coded gaps, 20 informative sites were found for *T. corymbosus* and *T. aphyllus* (Table 2). Gap sizes in this chloroplast partition ranged from 5–42 nucleotides long (Table 2). The *trnL-trnF* region that included the outgroup had 612 aligned positions with 28 variable sites, of which 10 were parsimony informative (Table 2). Considering just *T. corymbosus* and *T. aphyllus*, this chloroplast

region contained only six informative sites, which correspond to three coded gaps and three nucleotide substitutions (Table 2).

On the basis of nucleotide substitutions and coded gaps, 10 haplotypes were recognized for *T. corymbosus* and *T. aphyllus* for the *atpB-rbcL* spacer. In addition to these, the *trnL-trnF* region (sampled for 30 individuals) added only one new haplotype, corresponding to the individual from the Queules locality (Table 2). All individuals sampled from this locality (five) had the same haplotype (A) for the *atpB-rbcL* spacer. When considering both chloroplast regions, the total number of haplotypes increases to 11, nine for *T. corymbosus* and two for *T. aphyllus*.

Analyses of the combined chloroplast regions using both MP and BI resulted in trees with congruent topologies. Parsimony analysis generated one tree (length = 97, CI = 0.99, RI = 0.98) that contained four strongly supported clades (I–IV), the first three of which pertain to *T. corymbosus* and the fourth to *T. aphyllus* (Fig. 3). All clades on the BI tree had posterior probabilities higher than 0.98. Clade I contained haplotypes A–E (all arising from a polytomy) that showed several unique changes with respect to the other clades (Table 2). Using both MP and BI analyses, strong support is obtained for the paraphyly of *T. corymbosus* as revealed by the sister relationship between clade III (*T. corymbosus*) and clade IV (*Tristerix aphyllus*).

Haplotype network—The haplotype network generated by TCS (Fig. 4) reflected the same topological features as seen on the MP and BI trees (Fig. 3). Clade I, with haplotypes A to E, is separated from the rest of the clades by several changes that included duplications, deletions, and substitutions (Table 2). Within clade I, haplotype A is central, and haplotypes B and C differed from it by one substitution. Haplotype E differed from haplotype A by three changes (substitution, duplication, and deletion) found in the *trnL-trnF* region (Table 2). Haplotype D differed from the others in clade I by the absence of duplications and deletions. Clade II comprised haplotypes F and G characterized by one substitution and one deletion. Haplotype G differed from F by one deletion and by the absence of a duplication. Within clade III, haplotype H differed from I by two unique substitutional changes and a unique duplication. Clade IV, representing *T. aphyllus*, contained two haplotypes (J and K) that together shared a substitution with clade III. Three changes were synapomorphies for this clade: one substitution and two duplications. The insertion of two bases distinguished haplotypes J and K.

Geographical distribution of clades and haplotypes—After plotting the 11 haplotypes on the map of Chile and Argentina (Fig. 2), the geographical distribution pattern that emerges is complex and does not appear to correspond to relationships among the phylogenetic clades. Localities that are geographically proximal do not necessarily share the same haplotype or haplotypes from the same clade. This is especially the case in central Chile where overlap occurs among different haplotypes (Fig. 2).

Clade I (haplotypes A to E) and clade II (haplotypes F and G) were distributed from the central to southern part of the *T. corymbosus* range, whereas clade III (haplotypes H and I) was present mainly in the northern part. Clade IV (haplotypes J and K) represents the other species, *T. aphyllus*, which is endemic to the Chilean matorral and occurs in sympatry with clade III of *T. corymbosus*.

Seven haplotypes (B, C, D, E, G, H, and K) are confined to one specific locality, while haplotypes A, F, I, and J are found in several (Table 1, Fig. 2). Haplotypes A and F are found mainly in the temperate forest on both sides of the Andes, and haplotypes I and J in the Chilean matorral. The *atpB-rbcL* spacer data indicate that only three localities have haplotype diversity: haplotype F is present with B in Linao, with C in San Martín, and with H in Ñielol.

Analysis of molecular variance—The AMOVA (Table 3) for the *atpB-rbcL* spacer, showed no statistical differences between the two species, *T. corymbosus* and *T. aphyllus* (Table 3). In this analysis, the greatest percentage of the variation was attributed to within group (i.e., within each species). In the grouping of *T. corymbosus* by biome type, the AMOVA showed about the same proportion (46%) of variation among and within each biome. When grouping by geographical region, the major variation was found within each region (50%), with lesser amounts (40%) among groups. The AMOVA examining localities from the southern region across two potential geographical barriers (the Andean Cordillera and the waterway between the Chilean mainland and Chiloé Island) did not reveal significant variation between the regions. Thus, the Andes and the water separation did not influence differentiation of cpDNA haplotypes in *T. corymbosus.*

DISCUSSION

Different historical and ecological events have shaped the genetic structure of *Tristerix corymbosus* and *T. aphyllus* such that today four well-differentiated haplotype clades exist: three for *T. corymbosus* and one for *T. aphyllus*. The phylogeographic history of these two species in southern South America appears more complex than suggested by a simple extrapolation based on region or biome. The phylogenetic relationships among the various haplotypes are not reflected in their geographical pattern. The four clades are not completely geographically segregated and overlap was observed between them (Fig. 2). Clade III of *T. corymbosus* occurs in the same area as *T. aphyllus* in clade IV. It has been postulated that *T. aphyllus* speciated from a population of *T. corymbosus* in sympatry (Amico et al., 2007). The genetic and morphological differentiation between these two sister species is discussed in more detail later.

The geographical distribution of the haplotypes of *T. corymbosus* is complex, especially in the central region of Chile. In this area, haplotypes from each clade are present in proximal localities, thereby resulting in little geographical structure for the species. When *T. corymbosus* is grouped by geographical location, AMOVA shows that this factor contributes less than half the among-group variance. If one focuses on the northern and southern portions of the range for the species, geographical location may play some role in shaping the genetic structure of *T. corymbosus*. Clades I and II are found mainly in the south and Clade III in the north; however, the observed pattern has likely been masked by recent migration of some haplotypes discussed later. A similar result is found when *T. corymbosus* is grouped by biome, where the proportion of variation among the temperate forest and the Chilean matorral is similar to within each biome, indicating that this ecological feature is not a major factor explaining the population genetic structure of *T. corymbosus*.

The other two geographical barriers examined in the southern part of the *T. corymbosus* distribution, the Andean Cordillera and mainland Chile–Chiloé Island waterway, also do not

TABLE 2. Variable sites from the *atpB-rbcL* spacer ($N = 121$) and *trnL-trnF* region ($N = 30$) alignments for the 11 haplotypes of *Tristerix corymbosus* and *T. aphyllus*. Numbers 1 to 13 correspond to indels (Del. = deletion; Dup. = duplication). The indels and autapomorphies for the outgroup are not shown. Dots = nucleotide same as haplotype A, dashes = nucleotide(s) not present.

		atpB-rbcL aligned positions																						*trnL-trnF* aligned positions											
		3	6	7	1	1	2	2	2	2	3	3	3	3	4	4	4	5	5	5	5	5	6	6	6	9	9	1	1	1	1	1	1	1	1
		9	4	8	1	5	0	5	8	8	9	0	1	7	8	1	5	7	0	0	2	2	5	3		3	4	1	0	2	3				
					6	0	0	0	2	3	8	7	2	8	2	2	7	3	1	4	5	0	9	6	1	7	8	3	8	6	4				
Clade	Haplotype																															0	7	2	2
I	A	G		C	C	2	A	3	T			G	G	6		A			A	–	–	–		C	T				A	C			T	
I	B	C			·	2	·	3	T			·	·	6		·			·	–	–	–		·	·				·	·			·	
I	C	·			·	A	2	·	3	T			·	6		·			·	–	–	–		·	·				·	·			·	
I	D	·			·		2	·	3	T		5	·	6		·			·	–	–	–		·	·				·	·			·	
I	E	·			·		2	·	3	T			·	6		·			·	–	–	–		·	·			12	·	·		13	·	
II	F	·			·			C		–	4		T	T		·	8		·	–	–	–		·	C				G	·			C	
II	G	·		1	?	·		C		–	4		T	T		·			·	–	–	–		·	C				G	·			C	
III	H	·			·	·		·		–			T	T		7	G		·	–	–	A		T	C		11		G	·			C	
III	I	·			·	·		·		–			T	T			G		T	A	–	A		T	C		11		G	·			C	
IV	J	·			T	·		·		–			T	T		·		9	·	–	AA	–	10	T	C				G	·			C	
IV	K	·			T	·		·		–			T	T		·		9	·	–	–	–	10	T	C				G	·			C	
T. verticillatus		·			·	·		·		–			·	T		·			·	–	–	–		·	C				G	T			C	
T. penduliflorus		·			·	·		·		–			·	T		·			·	–	–	–		·	C				G	T			C	

Notes: 1. Deletion = CAATCTCGCCGAATCCAATTCAATTGTTCTATTGTTTACTTA; 2. duplication = TTTAAAACA; 3. duplication = CCCTTGAAA; 4. deletion = ATCCATTTTTTT; 5. deletion = TTTTATATT; 6. deletion = ATAGG; 7. duplication = ATTATAG; 8. duplication = ATTATTT; 9. duplication = AGAGTTT; 10. duplication = AAGGGGT; 11. duplication =ATGGAG; 12. duplication = TTGG; 13. deletion = TTGGTCA

contribute to the genetic structure in the species. This result provides evidence for gene flow in *T. corymbosus* between the two sides of the mountain range for haplotype A (clade I) and haplotype F (clade II). Migration between both sides of the Andes has been suggested for other southern taxa, where the mountains are not very high and the forest is continuous (Marchelli and Gallo, 2004; Palma et al., 2005; Himes, et al., 2008). As with the Andean Cordillera, migration between the mainland and Chiloé Island has occurred in *T. corymbosus*. During the Pleistocene, lower sea levels associated with glacial oscillations most likely provided opportunities for exchange between insular and mainland biota (Heusser et al., 1999).

Clade and haplotype differentiation—The molecular phylogeny of the genus *Tristerix* indicates that *T. corymbosus* and *T. aphyllus* are the most austral species of a southern clade that also contains *T. verticillatus* and *T. penduliflorus* (Amico et al., 2007). A chronogram for Santalales calibrated with fossil pollen from *Anacolosides* indicates that the genus *Tristerix* diverged in the Oligocene ~25 Ma (Vidal-Russell and Nickrent, 2008b). The time of divergence of *T. corymbosus* cannot be determined with current data but is likely contemporaneous with the emergence of the Andes during the Miocene. At this time, the vegetation in the southern part of South America was dominated by a mixed and subtropical paleoflora more similar to what is now seen in the southern part of the Chilean matorral. With the cooling of South America, the southern flora experienced greater change than the northern flora, likely affecting the mistletoe populations by reducing population sizes and isolating them from others. These two factors could be responsible for the differentiation of clade I from the other three clades. Clade I is found mainly in the south and has a large number of autapomorphies (Table 2), possibly indicating long periods of isolation. Although causation is lacking, the differentiation of clade II from clades III/IV appears to follow a geographic pattern, given that clade II is mainly southern and the others mainly northern.

Interestingly, each clade has haplotypes that are widely distributed (A for clade I, F for clade II, I for clade III, and J for clade IV), while other haplotypes in each clade are unique to one locality or to a limited area. For the widely distributed haplotypes, only A and J are internal in the network, suggesting that these are older and had more time to disperse. Such is not the case for haplotypes of clades II and III. In the south, the higher number of rare haplotypes belonging to clade I (B, C, and D) suggest a complex history that can be associated with the last glaciations, which formed several refugia in that area. The southern part of the distribution of *T. corymbosus* suffered ice cover during the last glaciation period (Heusser, 1984; Villagrán, 1991). These haplotypes (B, C, and D) are in localities previously identified as refugia for canopy tree species (Premoli et al., 2000, 2002; Silla et al., 2002; Allnutt et al., 2003; Marchelli and Gallo, 2004).

The other haplotypes found in unique localities in the central and northern regions, haplotype G (clade II) and haplotype H (clade III), are found in areas that have long been recognized as forest remnants. Nahuelbuta (haplotype G, clade II) and Queules (haplotype E, clade I) localities occur in an isolated mountain range, which contains relictual floristic elements (Hinojosa et al., 2006). The Fray Jorge region, characterized by haplotype H (clade III), is considered to have a relictual flora derived from the mixed and subtropical flora of coastal Chile that was reduced and isolated by the end of the Tertiary (Troncoso, et al.,

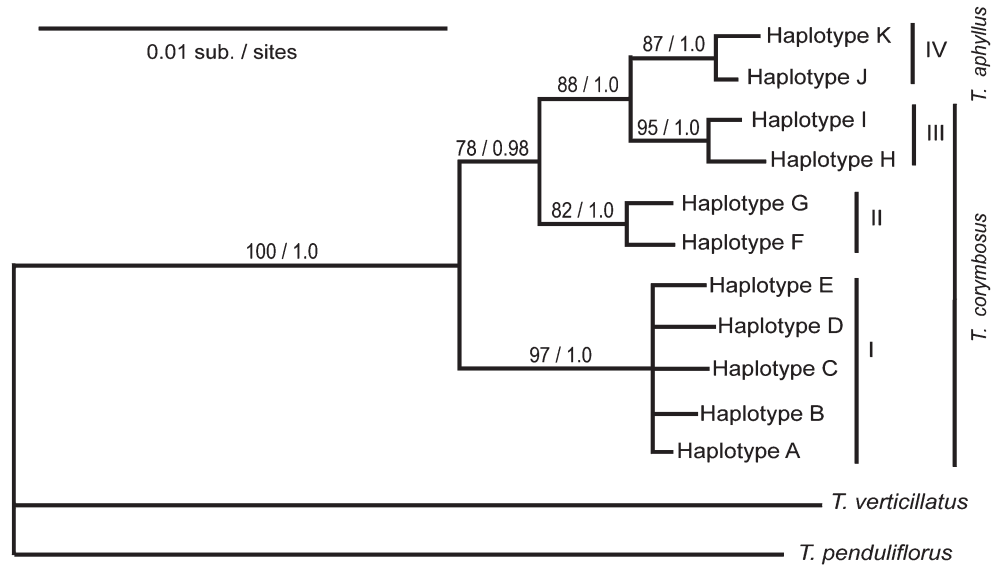

Fig. 3. Phylogram obtained from maximum parsimony (MP) and Bayesian inference (BI) analyses of the 11 cpDNA *atpB-rbcL* spacer and *trnL-trnF* region haplotypes obtained from *Tristerix corymbosus* and *T. aphyllus*. Roman numerals represent the clades. Numbers above branches represent MP bootstrap values (1000 pseudoreplicates) followed by BI posterior probabilities.

1980; Villagrán and Armesto, 1980; Hinojosa and Villagrán, 1997; Villagrán and Hinojosa, 1997). In *T. aphyllus*, haplotype K was located on La Campana mountain, an area that harbors unique flora and fauna in the central region of Chile (San Martín et al., 1988; Villagrán, 1995; Armesto et al., 2008).

Association of ecological factors with mistletoe genetic structure—Despite its morphological distinctiveness (Kuijt, 1988), *Tristerix aphyllus* has relatively few molecular differences when compared with *T. corymbosus* (Table 2). The high degree of genetic similarity between these two species is also seen in nuclear ribosomal ITS sequences (Amico et al., 2007). For the *atptB-rbcL* region, no statistically significant differences were found in the AMOVA between these species. One possible explanation is that the *T. aphyllus* speciation event is quite recent; thus, insufficient time has elapsed for the accumulation of genetic differences. In addition, the greatest proportion of variability resides within the populations of *T. corymbosus* and not as much within populations of *T. aphyllus*. Although morphological differentiation between *T. corymbosus* and *T. aphyllus* is apparent, no obvious differences have been identified among the three *T. corymbosus* clades. The polymorphism seen in mature fruit color is not associated with the clades but with biome. For example, the Los Tilos locality of the Chilean matorral has haplotype F (clade II), and mistletoes here produce yellow fruits, whereas the nearby temperate forest locality, Las Trancas, has the same haplotype (F), but the fruits are green at maturity. Furthermore, locality Ñielol in the temperate forest contains haplotypes F and I (clades II and III), and all individuals produce green fruits regardless of the haplotype.

Fruiting time differences are apparent between the two biome types: spring in the Chilean matorral and late summer in the temperate forest. This timing as well as fruit color differences could be the phenotypic expressions of environmentally controlled characters. Although some of the Chilean bird species that disperse *Tristerix* fruits are present in the temperate forest, they do not function as seed dispersers there because they do not recognize the fruits (G. Amico, unpublished data). The endemic marsupial *Dromiciops gliroides* does recognize the fruits and effectively serves as the sole disperser of *Tristerix* seeds in the temperate forest (Amico and Aizen, 2000; Amico, 2007). Although selection for fruit color appears to involve seed disperser type, the expectation that mistletoe genetic structure was shaped by this selection was not supported by the results of this study. If genetic differentiation ever existed, it is possible that it has since been erased by later migrations. A recent phylogeographic study of *D. gliroides* (Himes et al., 2008) showed a different haplotype distributional pattern as compared with the present mistletoe study. That study reported three major geographical groups within the temperate forest, but these are not concordant with the *T. corymbosus* groups. The current study did not reveal a common pattern as might be expected given a "comparative phylogeographic" approach (Arbogast and Kenagy, 2001). This result reflects the fact that *Dromiciops* does

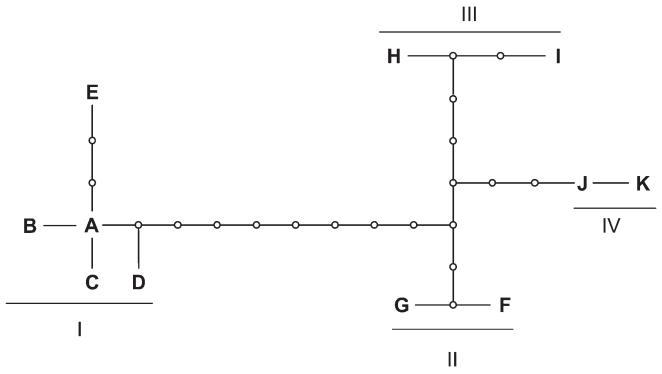

Fig. 4. Parsimony haplotype network of *atpB-rbcL* spacer and *trnL-trnF* region for *Tristerix corymbosus* and *T. aphyllus.* Identified haplotypes are represented in capital letters, nodal circles represents haplotypes not sampled (or extinct). Roman numerals represent the clades shown in Fig. 3.

TABLE 3. Analyses of molecular variance (AMOVA) of *atpB-rbcL* spacer sequence data given various groupings of *Tristerix* localities.

Grouping	Source of variation	df	Sum of squares	Variance components	% of total variation	*P*
Taxa[a]	Among groups	1	26.0	0.27	12.4	0.144
	Within groups	8	172.6	1.96	87.6	<0.001
	Total	9	198.6			
Biome[b]	Among groups	1	47.1	1.03	45.8	<0.001
	Within groups	24	108.2	1.05	46.5	<0.001
	Within locality	82	14.4	0.17	7.7	<0.001
	Total	107	169.7			
Region[b]	Among groups	2	59.9	0.78	40.5	<0.001
	Within groups	23	95.4	0.97	50.4	<0.001
	Within locality	82	14.3	0.17	9.1	<0.001
	Total	107	169.6			
Andes[b]	Among groups	1	2.9	–0.07	–6.2	0.438
	Within groups	11	48.3	0.97	84.4	<0.001
	Within locality	43	10.8	0.25	21.8	<0.001
	Total	55	62.0			
Chiloé[b]	Among groups	1	7.5	0.12	10.2	0.292
	Within groups	11	43.6	0.89	69.9	<0.001
	Within locality	43	10.9	0.25	19.9	<0.001
	Total	55	62.0			

[a] *T. corymbosus* (haplotypes A–H), *T. aphyllus* (haplotypes I, J)

[b] For groupings according to biome, geographical region, Andes, and the waterway separating the Chilean mainland and Chiloé Island (Table 1 and Fig. 2).

not depend exclusively on *Tristerix* fruits (Amico et al., 2009) as the mistletoe depends on the marsupial (Rodríguez-Cabal et al., 2007). So other factors such as breeding habits or foraging for other resources could be shaping the genetic structure of the marsupial.

Host–parasite interactions can result in the formation of host races, and these have been reported in other mistletoe species. In *Arceuthobium americanum* (dwarf mistletoe, Viscaceae), genetic structure (measured with AFLPs) was shaped mainly by the host, resulting in three distinct host races (Jerome and Ford, 2002a, b). *Tristerix corymbosus* is a generalist species, parasitizing at least 27 different hosts in 13 families, and these typically do not occur in large monospecific stands (Amico, 2007). For the 108 individuals of *T. corymbosus* sampled in this study, 16 species of shrubs or vines from 12 families were recorded as hosts. The common host was *Aristotelia chilensis* (Elaeocarpaceae), an endemic species of the temperate forest. Individuals that parasitized *A. chilensis* contained six of the nine recorded haplotypes. No haplotype was observed to be solely associated with any of the 16 recorded hosts. Haplotypes F and I were found in mistletoes parasitizing at least six host species. Moreover, haplotypes that occurred only in specific localities were found in mistletoes using more than one host or the common host (*A. chilensis*); thus, host races are not apparent in *T. corymbosus*. Conversely, *T. aphyllus* is host specific, parasitizing only the cactus genera *Echinopsis* and *Eulychnia* (Mauseth et al., 1984; Kuijt, 1988; Medel et al., 2002). If *T. aphyllus* is viewed as conspecific with *T. corymbosus*, it could be considered a host race; however, we propose that *T. aphyllus* is a distinct species formed via recent sympatric speciation following a host switch promoted by the behavior of the main seed disperser (Amico et al., 2007). Medel et al. (2002) postulated that *T. aphyllus* originated during the Pliocene because during this epoch central Chile changed to a drier climate, thus allowing the development of the xeric-adapted flora (including the cactus host of this mistletoe).

Conclusions—In contrast to *Tristerix aphyllus*, where factors underlying its genetic differentiation are apparent, the major factors associated with *T. corymbosus*, such as geographic region, biome, seed disperser, and host, were discounted. These factors may have played a role in the past, but the current distribution of haplotypes seems to reflect modifications after dispersal. Movement of the seed dispersers after the retreat of glacial ice could explain the current distribution of *Tristerix* haplotypes. In Europe post-glacial plant movement is well documented (Petit et al., 2002a, b, 2005; Grivet and Petit, 2003; Hampe et al., 2003), but for southern South America, there is little or no information of this type. The mistletoe and its dispersers (bird and marsupial) may have migrated from multiple refugia (localized in the temperate forest) or from the north (Chilean matorral) to areas in the south that were covered with ice during glaciations. Current gene flow via the seed disperser is mixing the haplotypes found in the temperate forest, thus obscuring the genetic structure created before and during the glacial period. The major seed disperser in the central region, where the most haplotype diversity for the mistletoe was found, is the tyrant flycatcher *Elaenia albiceps* (Amico, 2007). The migrational route of this neotropical bird overlaps the distribution of *T. corymbosus*, and the timing of its arrival coincides with the mistletoe fruiting season. Bird migration could produce a strong seed rain in a north to south direction, thus generating the dispersal of haplotypes from the center to the south. This strong directional gene flow could have erased past phylogeographic pattern and generated the complex genetic structure found in *T. corymbosus* today.

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APPENDIX 1. Collection information for 26 localities of *Tristerix corymbosus* and four localities of *T. aphyllus* sampled in this study. Vouchers of each individual were deposited in the Department of Botany Herbarium, Universidad Nacional del Comahue, Bariloche Argentina (BCRU). All collections from the same locality were given a number followed by a letter for each individual (letter indicated in sample column). DNA accession number (DNA acc.) from the collection maintained by D. L. Nickrent at SIUC.

Locality name	Location	Date of collection	Collector	DNA acc.	Samples
Tristerix corymbosus					
Ovalle	Near Fray Jorge National Park, IV Región, Chile	20-Jan-03	G Amico 85	4595	A
Fray Jorge	Fray Jorge National Park, IV Región, Chile	7-Sep-02	G Amico 81	4572	H, J, K, L, M
Chinchillas	Chincillas National Park, IV Región, Chile	8-Sep-02	G Amico 80	4571	A
Illapel	On the way to Illapel, IV Región, Chile	11-Sep-02	G Amico 78	4569	A, B, C, D
San Felipe	San Felipe, Santiago, V Región, Chile	13-Sep-02	G Amico 82	4573	A
Yerba Loca	Yerba Loca National Park, Región Metropolitana, Chile	14-Sep-02	G Amico 77	4568	E, D, K, N, O
Talca	Route 5 near Talca, VII Región, Chile	11-Sep-02	G Amico 83	4574	A, B, C, D, F
Queules	Los Queules National Park,VII Región, Chile	4-Feb-03	G Amico 86	4596	D, G, J, H, K
Los Tilos	Route 5 near Los Tilos, VIII Región, Chile	23-Jan-03	G Amico 87	4597	A, B, C, D, E
Chillán	On the way to Las Trancas from Chillán, VIII Región, Chile	23-Jan-03	G Amico 88	4598	A, B, C, D, E
Nahuelbuta	Nahuelbuta National Park, VIII Región, Chile	24-Jan-03	G Amico 89	4599	A, B, C, D, E
San Ramón	On the way to Nahuelbuta National Park, VIII Región, Chile	25-Jan-02	G Amico 74	4508	A, B, C, D, E
Ñielol	Ñielol National Park, IX Región, Chile	25-Jan-03	G Amico 90	4600	A, B, C, D, E
San Martín	Fundo San Martín, X Región, Chile	15-Jan-02	G Amico 75	4509	A, B, C, D, E, F
Yuco	Yuco, Lanin National Park, Neuquén, Argentina	18-Feb-03	G Amico 92	4602	A, B, C, D, E
Pucará	Pucará, Lanin National Park, Neuquén, Argentina	18-Feb-03	G Amico 91	4601	A, B, C, D, E
Rió Bueno	Rio Bueno, X Región, Chile	26-Jan-02	G Amico 72	4506	B, C, D, E, F
Puyehue	Puyehue National Park. X Región, Chile	14-Feb-03	G Amico 93	4603	A, B, C, D, E
Quetrihue	Quetrihue Peninsula, Nahuel Huapi National Park, Argentina	18-Feb-02	G Amico 71	4505	A, D, E
Isla Victoria	Victoria Island, Nahuel Huapi National Pak, Neuquén, Argentina	6-Oct-02	M Nuñez s.n.	4570	A
Llao Llao	Llao Llao, Bariloche, Río Negro, Argentina	30-Aug-02	G Amico 84	4575	F, I, N, O, T
Tacul	Villa Tacul, Bariloche, Río Negro Argentina	4-Feb-03	G Amico 94	4604	A
Ensenada	Ensenada, Perez Rosales National Park, X Región, Chile	14-Feb-03	G Amico 95	4605	A, B, C, D, E
Ancud	Near Ancud, Chiloé Island, X Región, Chile	1-Feb-03	G Amico 98	5023	A, B, C, D
Linao	Near Linao, Chiloé Island, X Región, Chile	13-Jan-03	G Amico 96	4606	A, B, C, D, E, F
Huillinco	On the way to Chiloé National Park, X Región, Chile	1-Mar-02	G Amico 73	4507	A, B, C, D, F
Tristerix aphyllus					
Fray Jorge	Fray Jorge National Park, IV Región, Chile.	20-Jan-03	G. Amico 97	4585	A
Chinchillas	Chinchillas National Park, IV Región, Chile.	3-Aug-05	L Suarez s.n.	4895	A, B, D, E., F
Los Andes	Near Los Andes, V Región, Chile.	17-Feb-05	G. Amico 166	4917	A, B, C, E
Campana	La Campana National Park, V Región, Chile.	17-Feb-05	G. Amico 162	4918	A, B, C