LOCAL HIBRIDIZATION IN SUBTROPICAL MOUNTAIN HABITATS: CAN *CEDRELA* (MELIACEAE) MAINTAIN SPECIES' IDENTITY IN SYMPATRY?

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Abstract. Quiroga, M. P.; A. C. Premoli, A. Grau & L. Malizia. 2016. Local hibridization in subtropical mountain habitats: Can *Cedrela* (Meliaceae) maintain species' identity in sympatry? *Darwiniana*, nueva serie 4(2): 195-211.

Congener species with incomplete reproductive barriers that coexist along environmental gradients may be prone to ecological divergence, in spite of the potential for hybridization in sympatry. We analyzed distribution patterns of isozymes, plastid, and nuclear DNA sequences at regional and local scales in three timber *Cedrela* species of the subtropics in the northern Argentina (*Cedrela angustifolia*, *C. balansae*, and *C. saltensis*), to test whether populations of distinct species have diverged in montane habitats in relation to their ecological characteristics. *Cedrela balansae* and *C. angustifolia* can be identified by diagnostic isozyme alleles. Nuclear ITS sequences yielded intraindividual polymorphism; ambiguous bases were shared between *C. balansae* and *C. saltensis* while those of *C. angustifolia* were part of its intraspecific polymorphism. Chloroplast DNA consisted mainly of a low-elevation haplotype present in *C. balansae* and *C. saltensis* and other found in *C. angustifolia* which in turn was shared locally by all species in sympatry. Multivariate UPGMA analysis of isozymes and Bayesian phylogeny of haplotype ITS yielded concordant patterns. Populations of *C. angustifolia* clustered in one group and were separated from the rest whereas those of *C. saltensis* and *C. balansae* were grouped together in one cluster. This indicates that stronger reproductive barriers exist between *C. angustifolia* and the low-elevation taxa *C. saltensis* and *C. balansae* which seem to maintain continuous gene flow. Nonetheless, under particular environmental settings, i.e., the three species hybridized sometime in the past and later became differentiated through ecological divergence.

Keyword. Argentina; *Cedrela angustifolia*; *Cedrela balansae*; *Cedrela saltensis*; Yungas.

Resumen. Quiroga, M. P.; A. C. Premoli, A. Grau & L. Malizia. 2016. Hibridación local en hábitats de montaña subtropicales: ¿*Cedrela* (Meliaceae) puede mantener la identidad de las especies en simpatría? *Darwiniana*, nueva serie 4(2): 195-211.

Las especies congéneres con barreras reproductivas incompletas que coexisten a lo largo de un gradiente ambiental pueden ser proclives a la divergencia ecológica, a pesar del potencial de hibridación en simpatría. Estudiamos los patrones de distribución de isoenzimas y de secuencias de ADN nuclear y plastidial a escala local y regional en las tres especies de árboles del género *Cedrela* del subtrópico de noroeste de Argentina para analizar si las poblaciones de las distintas especies han divergido en sus hábitats montanos en relación a sus características ecológicas. *Cedrela balansae* y *C. angustifolia* pueden ser identificadas por alelos isoenzimáticos diagnósticos. Las secuencias nucleares del ITS mostraron polimorfismo intraindividual; bases ambiguas fueron compartidas entre *C. balansae* y *C. saltensis*, mientras que en *C. angustifolia* es parte de su polimorfismo intraespecífico. El ADN del cloroplasto consistió en un haplotipo compartido entre las especies de baja altitud *C. balansae* y *C. saltensis* y otro diferente para *C. angustifolia* el cual

comparte con las otras dos especies cuando están en simpatría. Los análisis de UPGMA de isoenzimas y filogenético bayesiano de haplotipos del ITS muestran patrones concordantes. Las poblaciones de *C. angustifolia* se agruparon dentro de un mismo cluster y se separaron de las de *C. saltensis* y *C. balansae* que se mantuvieron agrupadas. Esto indica que existen barreras reproductivas entre *C. angustifolia* y los otros taxa de baja elevación *C. saltensis* y *C. balansae*, las cuales mantienen un continuo flujo génico. No obstante, bajo los distintos ambientes que ocupan, las tres especies hibridaron en algún momento en el pasado y más tarde comenzaron a diferenciarse a través de divergencia ecológica.

Palabras clave. Argentina; *Cedrela angustifolia*; *Cedrela balansae*; *Cedrela saltensis*; Yungas.

INTRODUCTION

Classical debates on speciation focused on the geography of speciation in relation to reproductive isolation, and thus drift, causing divergence (Mayr, 1947). While allopatric speciation mostly takes place when physical barriers impede genetic exchange, and therefore prompt divergence, sympatric speciation occurs in the face of gene flow. Under the absence of reproductive isolating mechanisms, divergence may result from the action of ecologically-based divergent selection which arises as a consequence of individuals interacting with their environment, i.e. ecological speciation (Orr & Smith, 1998). Ecological selection is divergent when it acts in contrasting environmental settings, due to abiotic factors e.g. climate and distribution of resources as well as biotic effects such an individual's ability to obtain food and/or nutrients, attract pollinators, or avoid predators and various forms of interspecific interactions (Schluter, 2001). This differs from other models of speciation in which the evolution of reproductive isolation involves key processes other than ecological selection, including speciation by polyploidization, hybridization, genetic drift, and founder events/population bottlenecks (Rundle & Nosil, 2005).

Sympatric/parapatric congener species inhabiting mountain habitats provide the opportunity to study isolation mechanisms as a result of ecological segregation of niches in relation to distinct environmental factors that abruptly change with elevation. Nonetheless, if such taxa maintain incomplete reproductive barriers potential hybridization events may occur wherever they overlap. The occurrence of intermediate forms between sympatric species, i.e. potential hybrids, either result from the cross of taxa undergoing the process of splitting by sympatric speciation and thus show incipient reproductive isolation or alternatively, they are the product of secondary contact between taxa that have diverged

sometime in the past but have not developed complete reproductive barriers (Coyne & Orr, 2004). *Cedrela* species are taxonomically challenging because they can be easily confounded morphologically (see key to the species of *Cedrela* in Pennington & Muellner, 2010). Therefore, a concern arose about the possibility of hybridization between species with nearly similar ecological tolerances.

Recent studies in *Cedrela* suggest that interspecific reproductive isolation seems to be incomplete, particularly among some of the South American species (Köcke, 2015). Furthermore, neither species' monophyly of e.g. *C. fissilis*, nor the delimitation of species such as *C. odorata* and *C. fissilis* was supported by phylogenetic analyses (Garcia-Gonçalvez et al., 2011). In addition, cryptic speciation, i.e. the existence of genetically distinct but morphologically indistinguishable entities, was suggested for *C. odorata* (Muellner et al., 2009, 2010; Cavers et al., 2013) and *C. fissilis* (Garcia-Gonçalvez et al., 2011). Furthermore, *Cedrela* species can tolerate a wide array of environmental variables, i.e. precipitation and temperature (Koecke et al., 2013) which may favor range overlap and thus the potential formation of intermediate forms.

Three species of *Cedrela* inhabit subtropical forests of the southern Yungas in northwestern Argentina: *C. balansae*, *C. angustifolia* (following Pennington & Muellner, 2010)*,* and *C. saltensis* (Zapater et al., 2004). These are among the most valuable subtropical hardwoods of Argentina and therefore, they have been heavily logged (Malizia et al., 2006). They have a variable degree of geographic overlap along latitudinal and elevation gradients: *C. angustifolia* is found in Montane forests between 500 and 2300 m above sea level (a.s.l.) reaching its southern-most distributional range at 28º15' S; *C. balansae* occurs in Pedemont forests between 400 and 1100 m a.s.l. attaining its southern limit at 24º 30' S; and *C. saltensis*

occupies the transition of Montane and Pedemont forests between 700 and 1100 m a.s.l. with its southern limit at 24º 40' S (Fig. 1; Grau et al., 2006). The latter has a restricted range (<1000 km^2) most of which occurs in sympatry (600 km^2) with the other two species (Grau et al., 2006). In comparison with other *Cedrela* from South and Central America, the three species have similarly high variability in their climatic envelopes, e.g. temperature seasonality (Koecke et al., 2013). In a previous study, we analyzed the possible hybrid origin of *Cedrela saltensis* from *C. balansae* and *C. angustifolia*, based on a reduced number of populations and using isozymes. This preliminary analysis yielded the presence of diagnostic alleles in *C. saltensis* that suggested that this species is taxonomically distinct (Premoli et al., 2011). This result was in agreement with a phylogenetic analysis showing that *C. saltensis* probably had a separate evolutionary history (Muellner et al., 2009).

We analyzed distribution patterns of molecular diversity along the range of each of the three pure *Cedrela* species (i.e. at regional scale) and at a local scale where at least two such species coexist in sympatry. We test the hypothesis whether populations of *C. saltensis, C. balansae,* and *C. angustifolia* which in turn coexist with a variable degree of range overlap have diverged in montane habitats. We aim to solve the following questions: 1) Can the three *Cedrela* species be distinguished genetically by neutral markers in relation to the different habitats they occupy (i.e. elevation)? 2) Do they maintain gene flow particularly where their ranges overlap? 3) If so, does gene flow occur at present and/or has also occurred in the past? We used isozymes, plastid, and nuclear DNA sequences of samples collected within the three species' natural ranges to answer these questions.

MATERIALS AND METHODS

Sample collection

To study the distribution of genetic diversity of *Cedrela angustifolia*, *C. balansae*, and *C. saltensis,* we sampled what is considered putative pure species inhabiting seasonally dry forests of northern Argentina. They were identified by morphological characters and altitudinal distribution. During years 2008-2009 we collected fresh leaf tissue from several
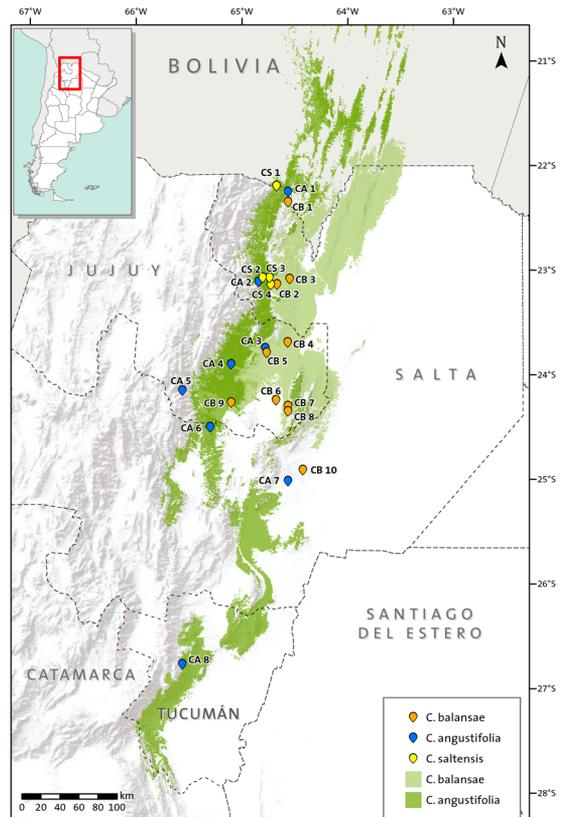

Fig 1. Distribution ranges of *Cedrela angustifolia*, *C. balansae*, and *C. saltensis*, and locations of the studied populations. The numbers correspond to populations indicated in Table 1. Color version at http://www.ojs.darwin.edu.ar/index.php/darwiniana/article/view/715/700

populations at randomly selected sites of each species (Table 1), collecting a total of 227 individuals. At each location all species present were sampled.

Isozymes

Protein extraction usually took place within a week of field work. However, because of the deciduous habit leaflets were released of the rachis before protein extraction, resulting in the loss of enzyme activity of some samples. Enzyme extracts were prepared by grinding ca. 1g of leaf tissue using 1–1.5 mL of extraction buffer (Mitton et al., 1979). Homogenates were stored at -80ºC until they were absorbed onto Whatman No. 3 paper wicks that were loaded into 12% starch gels (S-5651 starch potato; Sigma, St. Louis, MO, USA). Five enzymes were resolved in two combinations of buffer systems that yielded information on a

Table 1. Location of sampled populations of *Cedrela angustifolia* (CA), *C. balansae* (CB), and *C. saltensis* (CS) in northwestern Argentina. n: nuclear; H: haplotypes to ITS region; cp: chloroplast. Accession number H1 to H25: KX840898 to KX840922.

Species	Population	Name	Latitude (S)	Longitude (W)	Elevation	Samples isozymes	Samples DNA		
							n	H	cp
C. angustifolia	Los Toldos	CA 1	22° 15'	64° 42'	1299	10	4	H1, H2, H4	1
	Los Naranjos	CA 2	23° 05'	64° 51'	1710	5	1	H3	1
	Calilegua	CA 3	23° 41'	64° 52'	1450	15	5	H2, H4, H5, H18	3
	Particulares	CA 4	23° 54'	65° 06'	1000	-	6	H5, H15, H16	6
	Sur Jujuy	CA 5	24° 09'	65° 18'	1390	15	1	H1	1
	Provincia Salta	CA 6	24° 30'	65° 18'	1400	4	-	-	1
	Sur Salta	CA 7	25° 01'	64° 35'	800	11	2	H4, H5	2
	San Javier	CA 8	26° 46'	65° 19'	718	25	2	H5	1
C. balansae	Los Toldos	CB 1	22° 21'	64° 34'	770	10	3	H6, H7, H8	1
	Los Naranjos 1	CB 2	23° 07'	64º 40'	800	-	3	H10, H14	2
	Los Naranjos 2	CB 3	23° 07'	64º 27'	495	32	-	-	-
	Sauzalito	CB 4	23° 40'	64º 33'	380	22	-	-	-
	Calilegua	CB 5	23° 46'	64º 48'	535	5	2	H9, H12	-
	Ruta 34	CB 6	24° 17'	64º 54'	730	27	1	H11	1
	Sur Jujuy east	CB 7	24° 18'	64º 37'	770	9	1	H12	1
	Sur Jujuy west	CB 8	24° 21'	65º 01'	920	7	1	H13	2
	San Salvador de Jujuy	CB 9	24° 16'	65° 06'	990	-	2	H11	1
	Salta	CB 10	24° 54'	64° 25'	650	6	2	H12, H11	1
C. saltensis	Los Toldos	CS 1	22° 11'	64° 40'	1219	11	4	H20, H21, H22, H23, H24	2
	Acheral	CS 2	23° 05'	64° 47'	1200	5	1	H25	1
	Río Colorado	CS 3	23° 05'	64° 44'	1000	3	1	H25	-
	Los Naranjos	CS 4	23° 06'	64° 40'	830	5	3	H25	1

total of eight isozyme loci. These were Isocitrate dehydrogenase (Idh1, Idh2), Malic enzyme (Me1, Me2), and Shikimate dehydrogenase (Skdh) that were resolved in a buffer morpholine-citric system pH 7.5 (Ranker et al., 1989), which was run at a constant current of 25 mA for about 6 h. Peroxidase (Per1, Per2) and Phosphoglucoisomerase (Pgi) in buffer system pH 8.2 (Poulik, 1957) was run at a constant current of 20 mA for about 6 h.

Differences among populations and species were analyzed by heterogeneity of allelic frequencies by chi-square tests. Levels of within-population isozyme variation and at the species level were described by standard population genetic measures using Popgene v. 1.32 (Yeh & Boyle, 1997). These were the mean number of alleles (A), the effective number of alleles (N_E), the proportion of polymorphic loci using the *sensu stricto* criterion (P_{SS}), and the observed and expected heterozygosities under Hardy–Weinberg equilibrium conditions (H_O and H_E, respectively). Genetic diversity indices were compared statistically among species, which first calculates the average for each species over samples and loci calculated from the randomized data set with 9999 permutations using FSTAT v. 2.9.3.2 (Goudet, 1995).

The degree of population divergence (F_{ST}; Wright, 1965) between all population pairs was estimated using polymorphic loci by FSTAT v. 2.9.3.2 (Goudet, 1995). We compared average within- and between-species F_{ST} values by Kruskal Wallis non-parametric tests with pairwise multiple comparisons of mean rank sums. At the local level, we tested the degree of genetic divergence by paired between-species F_{ST} at the two localities were all species coexist in sympatry, i.e. Los Toldos (CA1, CB1, CS1) and Los Naranjos (CA2, CB3, CS4) populations (at 22 and 23º South latitude, respectively; Table 1).

Genetic relatedness among populations of the three species was analyzed with multivariate UPGMA cluster analysis using modified Rogers genetic distance (Wright, 1978).

DNA isolation, amplification and sequencing

We performed DNA extractions following the ATMAB method (Dumolin et al., 1995). Non-coding regions of chloroplast DNA (cpDNA) were amplified by polymerase chain reaction (PCR) using four intergenic spacer regions, namely: psbB-psbH (BH; Hamilton, 1999), psbA-trnH (AH; Hamilton, 1999),

trnD-trnT (DT; Demesure et al., 1995), and trnL-trnF (LF; Taberlet et al., 1991). Nuclear DNA (nDNA) was amplified for the internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence (ITS; Muellner et al., 2005). The PCR reaction mix contained 1 μL of DNA extract (~10 ng), 2.5 μl buffer (Invitrogen) 10x, 2.5 μl dNTPs 10x, 1.2 μl MgCl$_2$ 50 mmol, (0.75 μM of each primer 10μM), and 0.1 μl of Taq DNA polymerase (Invitrogen), as well as 2 μl dimethyl sulfoxide (DMSO) only to analyze the ITS region, on a total volume of 25 μL. The PCR amplification conditions were: 4 min at 95 ºC, 35X (1 min at 94 ºC; 1 min annealing at 53 ºC for BH, AH, DT, LF and 49 ºC for ITS; 1.5 min at 72 ºC), and 10 min at 72 ºC. The PCR products were purified by a reaction using 2.5 U of Exonuclease I (USB) and (0.25 U of Shrimp Alkaline Phosphatase (USB) for 10 μL of PCR product (conditions: 15 min at 37 ºC; 15 min at 85 ºC). The amplified DNA was sequenced with an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Laboratorio Ecotono, Universidad Nacional del Comahue, Bariloche) using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems). The cycle sequencing reactions were performed following the manufacturer's protocols. Sequences of each sampled haplotype were deposited in GenBank (Accession number KX840881 to KX840927).

Sequence data were aligned with Mega4 (Tamura et al., 2007) and concatenated manually into a single combined dataset for posterior analyses only for cpDNA regions for each species. Chloroplast and genomic DNA haplotypes were determined from both nucleotide substitutions and indels. We calculated different diversity parameters including variation in alignment size, number of haplotypes (H), number of variable sites (V) and parsimony informative sites (PI), and nucleotide (π) and haplotype (h) diversity with DNAsp 5.10 (Librado & Rozas, 2009) for each species.

Phylogenetic analysis

We inferred the phylogenetic relationships among haplotypes of *C. balansae*, *C. angustifolia*, and *C. saltensis* by Bayesian inference (BI) analysis using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). This analysis was carried out independently for the cpDNA and ITS datasets.

The alignment analyses of cpDNA yielded a total

of 3050 bp but only 4 parsimony-informative characters. The most appropriate models of nucleotide substitution identified by Modeltest v3.7 (Posada and Crandall, 1998) and MrModeltest v2.3 (Nylander, 2004) under the Akaike Information Criterion (Akaike, 1973) (AIC) was implemented in the BI analyses. The AIC indicated that the best-fit model was HKY+I among the 24 models of molecular evolution. The program MrBayes v3.1.2 was used to estimate an unrooted Bayesian phylogeny using the model selected. Bayesian analysis was performed using two simultaneous runs of 2 million generations each, with one cold and three heated chains in each run. Sampling was carried out once every 100 trees; the first 2500 trees were discarded as burn-in samples. A 50% majority-rule consensus tree of the two independent runs was obtained with posterior probabilities that were equal to bipartition frequencies.

We used sequences of the ITS region of the three species to perform BI at the regional level. The outgroup consisted of sequences of the closely related genus *Toona* (accession number: FJ518906, FJ462489, FJ462488), *C. salvadorensis*, and *C. dugesii* (accession numbers FJ462484 and FJ462483, respectively). The BI analysis was performed using four replicates with four chains (three heated and one cold) that were run for seven million generations sampling every 100 generations with a burn-in period of approximately 10% of the trees for the ITS region. Nodal support was determined using Bayesian posterior probabilities as implemented by MrBayes. The most appropriate models of nucleotide substitution were identified with the same methodology used for cpDNA. The best-fit model of AIC was HKY+I+G for data including the ambiguous bases and hybrid individuals and the model HKY+I without ambiguous bases and hybrid individual. A 50% majority-rule consensus tree of the two independent runs was obtained with posterior probabilities that were equal to bipartition frequencies. At the local scale, we preformed analysis of BI (using the same conditions as described above) including all individuals from the Los Toldos population which is the location where the three species coexist in sympatry and where ambiguities, i.e. intraindividual variation at single bases were detected. This ambiguity was solved following Garcia-Gonçalvez et al. (2011) so that each of the five sequences that harbored double peaks at a given location was separated into a pair of new sequences. These sequences were

identical to the respective parental sequence except that each sequence of the pair included one of the two possible nucleotide codes instead of the formerly ambiguous code (Garcia-Gonçalvez et al., 2011).

RESULTS

Isozyme variation

To obtain reliable isozyme profiles, several individuals per population and species were analyzed, although that enzyme activity depended on the freshness of samples upon arrival to the laboratory. Isozyme profiles yielded diagnostic alleles, i.e. those present exclusively in just one species, Idh1-1in three populations of *C. angustifolia* and Idh2-1, Me2-1, and Per1-2 which in turn were unique to just one population of *C. balansae*. Neither diagnostic (i.e. species-specific) nor unique (i.e. population-specific for a given population within a species) alleles were present in the range-restricted *C. saltensis* [see Supporting Information]. Allelic frequencies at isozyme loci differed among populations of the three analyzed *Cedrela* species. Seven of eight tests of heterogeneity in allelic frequencies yielded significant differences among populations by means of chi-square tests [see Appendix].

All three species were genetically diverse by isozymes (Table 2). Relatively reduced among-population divergence was measured within each species: *C. angustifolia* F_{ST} = 0.170 [CI 0.057-0.292], *C. balansae* F_{ST} = 0.115 [CI 0.040-0.232], and *C. saltensis* F_{ST} = 0.255 [CI 0.059-0.444] (Fig. 2).
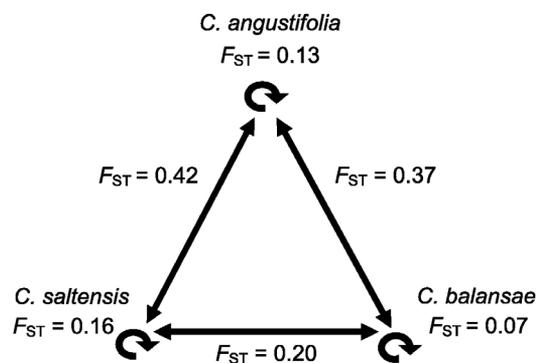

Fig. 2. Diagram of population divergence by (F_{ST}) within- (circular arrows) and between-species (linear arrows) of *Cedrela*.

Table 2. Parameters of genetic variation within populations and species of *Cedrela* from the southern Yungas, Argentina. These are: mean number of alleles (*A*), effective number of alleles (N_E), percentage of polymorphic loci sensus tricto (*P*ss), and observed and expected heterozygosity (H_O and H_E, respectively). Population averages and species values are shown at the bottom of each column. Standard errors are shown in parentheses. *Cedrella angustifolia* (CA), *C. balansae* (CB) and *C. saltensis* (CS). Location of the populations are shown in Table 1.

Locus	A		N_E		P_{SS}	H_O		H_E	
C. angustifolia									
CA1	1.50	(0.18)	1.24	(0.11)	37.5	0.163	(0.098)	0.135	(0.077)
CA2	2.38	(0.32)	1.74	(0.14)	62.5	0.265	(0.105)	0.326	(0.106)
CA3	1.88	(0.36)	1.42	(0.21)	50.0	0.225	(0.088)	0.228	(0.096)
CA5	2.63	(0.17)	1.54	(0.09)	87.5	0.287	(0.075)	0.310	(0.072)
CA6	1.50	(0.19)	1.23	(0.10)	50.0	0.198	(0.080)	0.180	(0.071)
CA7	1.25	(0.15)	1.11	(0.07)	12.5	0.034	(0.034)	0.062	(0.062)
CA8	1.63	(0.11)	1.22	(0.05)	50.0	0.101	(0.048)	0.133	(0.067)
Total species CA	3.13	(0.12)	1.39	(0.03)	87.5	0.175	(0.013)	0.231	(0.016)
Pop Mean CA	1.82	(0.04)	1.36	(0.02)	50.0	0.182	(0.007)	0.196	(0.008)
C. balansae									
CB1	2.13	(0.39)	1.53	(0.27)	62.5	0.215	(0.106)	0.197	(0.095)
CB3	2.25	(0.20)	1.63	(0.09)	75.0	0.336	(0.100)	0.321	(0.087)
CB4	1.50	(0.24)	1.26	(0.15)	37.5	0.125	(0.065)	0.150	(0.085)
CB5	2.25	(0.13)	1.20	(0.04)	75.0	0.099	(0.048)	0.133	(0.061)
CB6	2.13	(0.17)	1.39	(0.08)	75.0	0.149	(0.063)	0.215	(0.075)
CB7	1.88	(0.20)	1.21	(0.04)	62.5	0.085	(0.035)	0.164	(0.050)
CB8	1.50	(0.29)	1.09	(0.05)	25.0	0.071	(0.054)	0.067	(0.050)
CB10	1.25	(0.22)	1.06	(0.06)	12.5	0.021	(0.021)	0.040	(0.040)
Total species CB	3.50	(0.10)	1.36	(0.03)	100.0	0.159	(0.010)	0.297	(0.012)
Pop Mean CB	1.86	(0.03)	1.30	(0.01)	53.1	0.138	(0.007)	0.161	(0.006)
C. saltensis									
CS1	1.88	(0.37)	1.56	(0.32)	37.5	0.145	(0.109)	0.140	(0.105)
CS2	1.38	(0.18)	1.36	(0.17)	37.5	0.225	(0.149)	0.205	(0.100)
CS3	1.50	(0.22)	1.32	(0.16)	50.0	0.292	(0.133)	0.225	(0.091)
CS4	1.50	(0.17)	1.24	(0.11)	50.0	0.225	(0.122)	0.164	(0.073)
Total species CS	2.38	(0.28)	1.57	(0.18)	75.0	0.197	(0.046)	0.204	(0.039)
Pop Mean CS	1.56	(0.03)	1.37	(0.02)	43.8	0.222	(0.009)	0.184	(0.006)

Statistically lower between-species divergence (p<0.001, Kruskal-Wallis test: $H_{(2, N=113)}=33.66$) was obtained for *C. balansae* and *C. saltensis* population pairs ($F_{ST} = 0.20$) than between *C. angustifolia* and each of the two former species (*C. angustifolia* v *C. balansae* $F_{ST} = 0.37$ and *C. angustifolia* v *C. saltensis* $F_{ST} = 0.42$). A similar pattern was detected at the local level with greater divergence between *C. angustifolia* and any of the other species growing in sympatry (*C. angustifolia* v *C. balansae* $F_{ST} = 0.32$ and 0.46, *C. angustifolia* v *C. saltensis* $F_{ST} = 0.45$ and 0.40) than between *C. balansae* and *C. saltensis* $F_{ST} = -0.1$ and 0.11 at Los Toldos and Los Naranjos, respectively.

Multivariate cluster analysis (UPGMA) using modified Rogers genetic distance showed that populations of *C. angustifolia* cluster together in one group which resulted isoenzimatically distinct from populations of *C. saltensis* and *C. balansae* (Fig. 3).

DNA variation

Sequences of cpDNA yielded low polymorphism and reduced intra- and inter-specific variation (Table 3, Fig. 4). The final cpDNA dataset that resulted from the concatenation of the four chloroplast regions was 3045 bases long and included four haplotypes (Table 3). Nonetheless, chloroplast haplotypes were geographically structured two of them were widespread (H1 and H2) and the other two were unique to population CB2 of *C. balansae* and CA3 of *C. angustifolia* (H3 and H4, respectively). Wide ranging H1 was mostly exclusive to populations of *C. angustifolia*
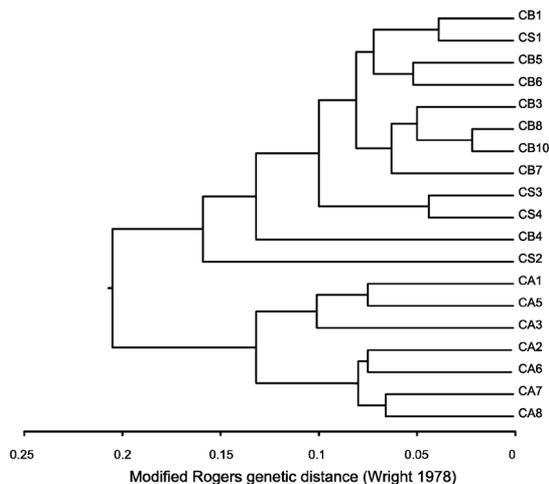

Fig. 3. Multivariate cluster analysis (UPGMA) by means of modified Rogers distance (Wright, 1978) of *C. angustifolia* (CA), *C. balansae* (CB), and *C. saltensis* (CS) populations. Numbers correspond to location of sampled populations as described in Table 1.

Table 3. Genetic diversity of cpDNA for three species of *Cedrela*. N: Number of haplotypes; # VS/ PI: # Variable sites / Parsimony informative sites; h: Haplotype diversity; π: Nucleotide diversity. Standard errors are shown in parenthesis.

Region	Samples	Size bp	N	# VS/ PI	Accession number of each haplotype	h	π
psbA-trnH	23	382	2	1/1	AH1: KX840881, KX840882, KX840884 AH2: KX840883, KX840885	0.498 (0.037)	0.0013 (0.0001)
psbB-psbH	20	609	4	1/0	BH1: KX840886, KX840887, KX840889, KX840890 BH2: KX840888 BH3: KX840891 BH4: KX840892	0.100 (0.062)	0.0001 (0.0001)
trnD-trnT	18	1370	2	2/2	DT1: KX840893, KX840894, KX840896 DT2: KX840895, KX840897	0.515 (0.037)	0.0007 (0.0001)
trnL-trnF	18	728	2	1/1	LF1: KX840923, KX840924, KX840926 LF2: KX840925, KX840927	0.523 (0.034)	0.0007 (0.0001)
Concatenated* cpDNA	11	3045	4	4/4	H1= AH1, BH1, DT1, LF1 H2= AH2, BH2, DT2, LF2 H3= AH2, BH3, DT2, LF2 H4= AH1, BH4, DT1, LF1	0.709 (0.049)	0.0007 (0.0001)

* As depicted in fig. 4

except at the high-elevation northern-most studied populations at Los Toldos where the three species coexist in sympatry/parapatry and share this chloroplast haplotype. The other H2 widespread haplotype was exclusively present in *C. balansae* and *C. saltensis* wherever they occur as pure populations (Fig. 4).

The aligned nITS region matrix consisted of 578 characters. Forty-seven sites were variable, 26 of which were potentially parsimony informative. In total, 25 haplotypes were identified (Table 4). Out of the total variable sites, 43% were shared between the three species, 27% between *C. balansae* and *C. saltensis*, 15% between *C. angustifolia* and *C. balansae*, 10% between *C. angustifolia* and *C. saltensis*, whereas 5% differed among the three species. Bayesian consensus trees using the nITS dataset showed that haplotypes of *C. angustifolia* constitute a monophyletic group while haplotypes of *C. balansae* and *C. saltensis* constitute a shared group (Fig. 5).

Seven of the 47 variable sites showed at least one ambiguity, that is, they presented overlapping double peaks at the same site. The pattern of double peaks in the electropherograms suggested intraindividual polymorphism. We detected double peaks (ambiguities) in different individuals of the three species. Ambiguities of *C. saltensis* and *C. balansae* consist of double peaks present in each of
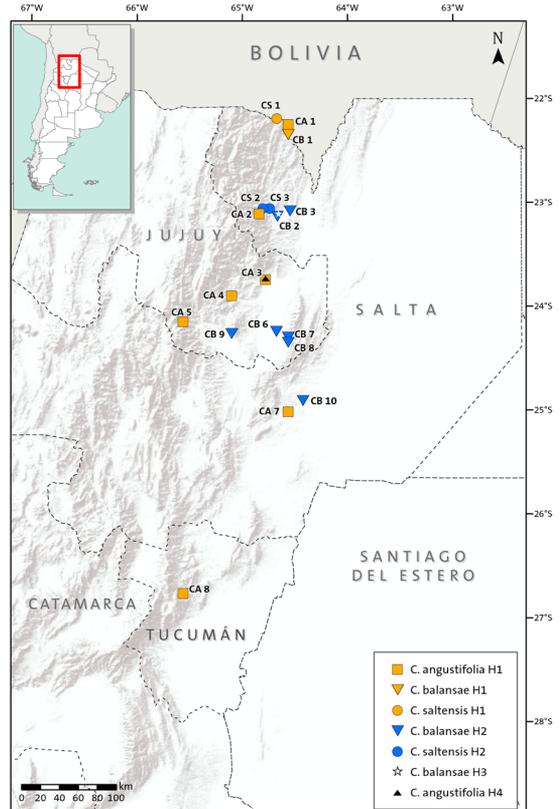
Fig. 4. Geographical distribution of chloroplast DNA haplotypes. The numbers correspond to populations indicated in Table 1. Haplotypes (H) correspond to Table 3. Color version at http://www.ojs.darwin.edu.ar/index.php/darwiniana/article/view/715/700

Table 4. Genetic diversity of the ITS1-2 region for three species of *Cedrela*. N: number of haplotypes; # VS/ PI: Number of variable sites / Parsimony informative sites; h: Haplotype diversity; π: Nucleotide diversity. Standard errors are shown in parenthesis.

	Samples	Size bp	N	# VS/ PI	h	π
C. angustifolia	21*	562	9	8 / 5	0.843 (0.019)	0.0039 (0.0001)
C. balansae	13*	577-578	9	22 /4	0.923 (0.004)	0.0077 (0.0004)
C. saltensis	11*	577-578	7	13 / 9	0.818 (0.012)	0.0077 (0.0001)
Total	45*	562-578	25	40 / 26	0.951 (0.005)	0.0177 (0.0004)

* includes ambiguous sequences.

Fig. 5. Bayesian phylogeny (consensus tree) of nITS data of *C. angustifolia*, *C. balansae*, and *C. saltensis*, including *Toona ciliata* (3 samples), *C. salvadorensis*, and *C. dugesii* as outgroup. Numbers correspond to locations of the sampled po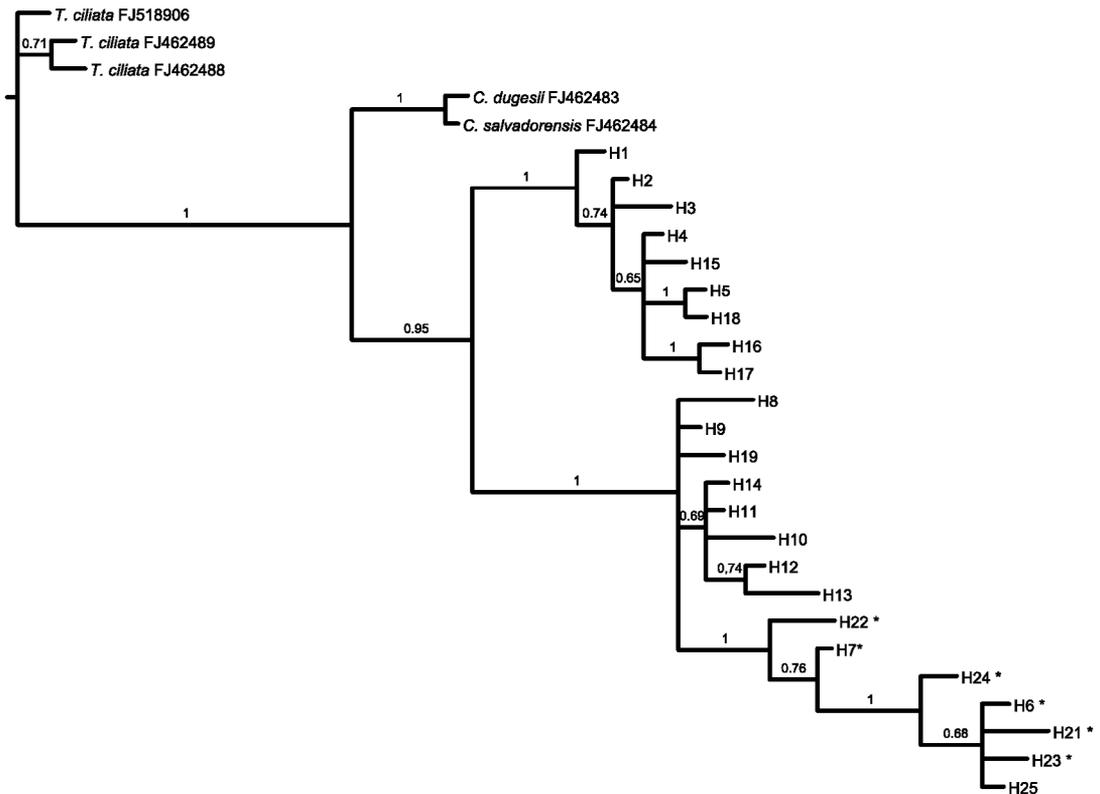pulations following Table 1. Numbers above branches indicate posterior probabilities values of Bayesian analyses. * correspond to individuals with ambiguities.

the pure species (interspecific variation), whereas those of *C. angustifolia* corresponded to the within-species variation (intraspecific variation).

At Los Toldos, out of a total of 578bp of the ITS region, 25bp were variable (Table 5). In 10 of those sites one individual of *C. balansae* presented ambiguities, i.e. double peaks that were always shared with bases of *C. saltensis*. Also, two individuals of *C. saltensis* presented ambiguities at four sites which in turn were shared with *C. balansae*. In contrast, one individual of *C. angustifolia* from Los Toldos present ambiguities that corresponded to intraspecific variation. Nonetheless, the phylogenetic relationships among individuals of the three species at Los Toldos by ITS showed that *C. angustifolia* individuals are different from those of the other two species (Fig. 6).

DISCUSSION AND CONCLUSIONS

Concordant genetic relationships were depicted by ITS and isozymes and show that high-elevation *C. angustifolia* has diverged from the low-elevation *C. balansae* and *C. saltensis*, which in turn seem to be genetically more similar. This result is supported by phylogenetic analysis of ITS haplotypes and clustering schedule of populations using isozyme data (Fig. 2, 3 and 5). At the local scale in Los Toldos and Los Naranjos, where the three taxa coexist in sympatry the same pattern was obtained; i.e. a greater divergence measured by F_{ST} values between *C. angustifolia* and each of the other two species than between *C. balansae* and *C. saltensis*. These results suggest the relevance of

Table 5. Samples of nuclear ITS sequences with nonambiguous and ambiguous bases of three *Cedrela* species' populations. Sequences with ambiguities labeled as A or B are indicated in boxes. The location of the populations are shown in Table 1. The number after the hyphen corresponds to distinct individuals.

| Variable sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	gap
Parsimony informative	1	2	3	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24							25
Sequence Position	009	014	034	079	085	112	126	178	182	204	224	248	256	271	280	308	314	318	327	336	345	354	329	341	348	355	355	358	360	369	361	53-68
C. angustifolia H1	T	T	G	C	A	T	T	T	G	C	G	T	T	A	A	C	G	T	G	G	C	C	A	C	T	C	C	A	G	T	G	-----------
C. angustifolia H2	T	T	G	G	A	T	T	T	G	C	G	T	T	A	A	C	G	T	G	G	C	C	A	C	T	C	C	A	G	T	G	-----------
C. angustifolia H3	T	T	C	G	A	T	T	T	G	C	G	T	T	A	A	C	G	T	G	G	C	C	A	C	T	C	C	A	G	T	G	-----------
C. angustifolia H5	T	T	C	G	A	T	T	T	G	C	G	T	T	A	A	C	G	T	G	G	C	C	A	C	T	C	C	A	G	T	G	-----------
C. balansae H6	[T]	G	G	C	G	A	T	[T]	[T]	C	[C]	C	[T]	A	C	[T]	A	C	G	[A]	C	C	[A]	C	C	G	C	G	T	?	?	GCGAGGCGTCCGGCGC
C. balansae H7	[C]	G	G	C	G	A	T	[C]	[G]	C	[G]	C	[C]	A	C	[C]	A	C	G	[G]	G	C	[G]	C	C	G	C	G	T	?	?	-AAAGGCGTCCGGCGC
C. balansae H8	T	C	G	C	G	A	T	T	T	C	G	C	T	A	C	C	A	C	G	G	C	C	A	C	T	C	C	G	T	C	A	-AAAGGCGTCCGGCGC
C. balansae H13	T	C	G	C	G	A	T	T	T	C	C	C	C	G	C	C	A	C	C	A	C	C	A	C	T	C	C	G	T	C	A	-AAAGGGCGTCCGGCGC
C. balansae H20	T	C	G	C	G	A	T	T	T	C	C	C	C	A	C	T	A	C	C	A	C	C	A	C	T	C	C	G	T	C	A	-AAAGGGCGTCCGGCGC
C. saltensis H21	[C]	G	G	C	G	A	T	[C]	[C]	C	[G]	C	T	A	C	C	A	C	C	G	C	C	G	C	C	G	C	G	T	C	A	GCGAGGCGTCCGGCGC
C. saltensis H22	[T]	G	G	C	G	A	T	[T]	[T]	C	[C]	C	T	A	C	C	A	C	C	G	C	C	G	C	C	G	C	G	T	C	A	-AAAGGCGTCCGGCGC
C. saltensis H23	[T]	G	G	C	G	A	C	C	G	C	G	C	T	A	C	C	A	C	C	G	C	C	G	C	C	G	C	G	T	C	A	GCGAGGCGTCCGGCGC
C. saltensis H24	[C]	G	G	C	G	A	C	C	G	C	G	C	T	A	C	C	A	C	C	G	C	C	G	C	C	G	C	G	T	C	A	-AAAGGGCGTCCGGCGC
C. saltensis H25	C	G	G	C	G	A	T	C	G	G	G	C	T	A	C	C	A	C	G	G	C	C	G	C	C	G	C	G	T	C	A	GCGAGGCGTCCGGCGC

205
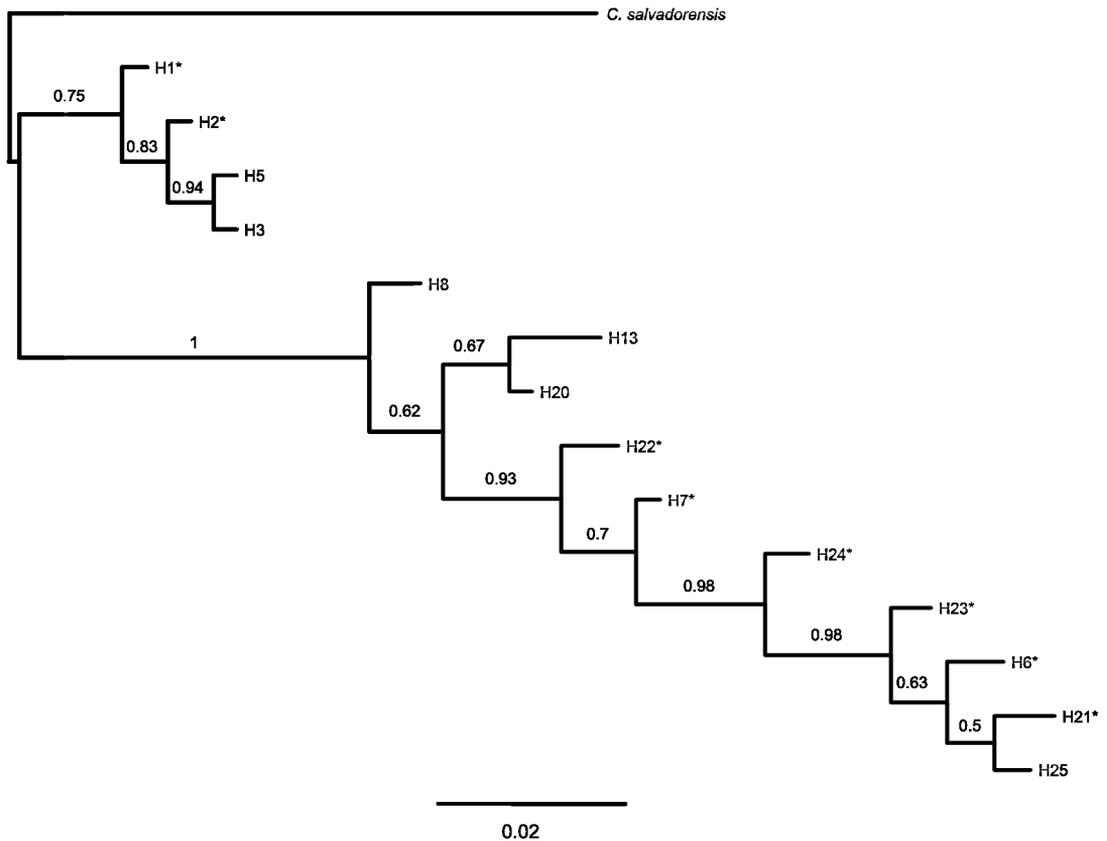
Fig. 6. Bayesian phylogeny (consensus tree) of nITS data of sympatric populations of *C. angustifolia*, *C. balansae*, and *C. saltensis* from Los Toldos population, allopatric populations of each species, and *C. salvadorensis* as outgroup. Numbers correspond to location of sampled populations following Table 1. * correspond haplotypes with ambiguous bases, i.e. alternative possible nucleotide sequences. Numbers above branches indicate posterior probabilities values of Bayesian analyses.

distinct environmental settings, i.e. elevation, as promotor of ecological divergence in *Cedrela*. The distribution of cpDNA haplotypes indicated a shared evolutionary history among the species. In particular, concordant patterns of the widely distributed H2 haplotype in *C. balansae* and *C. saltensis* provide evidence of potential interspecific gene flow at the regional level. In addition, while the widespread H1 haplotype is mostly restricted to *C. angustifolia*, it was shared by all species in the northern-most population suggesting that hybridization has probably also occurred locally among the three species (see below).

High levels of genetic diversity by means of isozymes and nuclear DNA correlate with the elevated variability suggested for the three studied species in relation to tolerance to a wide range of temperatures, e.g. temperature seasonality as the difference between temperature maximum of the warmest month and temperature minimum of the coldest month (Koecke et al., 2013). Nonetheless, they generally occupy spatially segregated microhabitats at three elevation strata of the southern Yungas: *C. angustifolia* at higher elevations while *C. saltensis* are more common at middle and lower elevations and *C. balansae* at the lowest end. Quantitative traits measured under common gardens on seedlings of *C. odorata* from distinct provenances, mesic and dry, suggest adaptive genetically-based differences which were interpreted as potential incipient speciation in relation to contrasting habitats (Navarro et al., 2002).

The nuclear ITS region yielded intraindividual polymorphism, e.g. ambiguous bases, as previously reported in *Cedrela* (Garcia-Gonçalvez et al., 2011; Zelener et al., 2016). Interestingly, the three species show ambiguous bases at Los Toldos where they occur in sympatry. While such intraindividual polymorphism was shared between *C. balansae* and *C. saltensis* and thus suggesting local interspecific gene flow, that of *C. angustifolia* was part of intraspecific polymorphism within this species' gene pool. The intraspecific ambiguities detected in *C. angustifolia* are similar to those found in other *Cedrela* species such as *C. fissilis* (Garcia-Gonçalvez et al., 2011). These authors argue that hybridization, polyploidy, and long generation times in plants are factors that contribute to incomplete concerted evolution that has probably contributed to the intra-individual polymorphism in this species. Other *Cedrela* species analyzed by Muellner et al. (2009) presented ambiguities (*C. angustifolia* FJ462478, *C. balansae* FJ462474, *C. odorata* FJ462464), reflecting high variation in ITS sequences within the genus. Hence, both interspecific and intraspecific hybridization bring together different alleles from the parental sources into a single genome and therefore can give rise to intraindividual polymorphism (Koch et al., 2003; Muir et al., 2001), as in our case.

The three species at Los Toldos shared the same chloroplast haplotype at this location which indicates that either hybridization and thus chloroplast capture probably occurred sometime in the past among the three species and/or that they derived relatively recent from a common ancestor and thus they presents incomplete lineage sorting. The genetic similarity by isozymes and shared interspecific polymorphism in ITS sequences between *C. balansae* and *C. saltensis* are in accordance with Koenen et al. (2015) and support the hypothesis of their relatively recent origin product of a contemporaneous expansion possibly during the Holocene. On the other hand, the greater genetic distinctiveness of *C. angustifolia*, even when growing in sympatry with the other two species, is in accordance with ancestral lineage divergence during the late Miocene (following Koenen et al., 2015). As a consequence, interspecific reproductive isolation seems to be incomplete between *C. balansae* and *C. saltensis* as shown by the common widespread H2 cpDNA haplotype shared by the two species. In a recent study, where *C. balansae* and *C. saltensis*

occur in sympatry, genetically pure samples of either species were found together with hybrids and introgressant forms in several populations (Zelener et al., 2016). Nonetheless, shared cpDNA sequences at the northern-most studied location where the three species grow in sympatry highlights the potential for hybridization among the three species. While it is unknown if pollen from one species of *Cedrela* can pollinate other species, the lack of chloroplast DNA differentiation and thus chloroplast sharing may be due to deficient reproductive barriers by relatively recent speciation or secondary contact that occurred under particular ecological and climatic conditions along altitudinal gradients. Previous phylogenetic studies of the Meliaceae based on cpDNA resulted in polytomies (Muellner et al., 2009) suggesting that hybridization may be common.

Chloroplast sharing, i.e. the lack of interspecific polymorphism at cpDNA, may be due to convergent evolution, incomplete lineage sorting, and/or reticulate evolution (Acosta & Premoli, 2010). Sequence convergence would be highly unlikely. The high potential for interspecific gene flow in plants (Rieseberg & Soltis, 1991) may result in chloroplast capture. This is where the cytoplasm of one species is replaced by that of another species through hybridization/introgression (Fehrer et al., 2007). Chloroplast capture occurs frequently in species with sympatric distributions and reproductive compatibility (Acosta & Premoli, 2010). Thus, in areas with range overlap and under similar ecological settings, *Cedrela* species may produce hybrids whereas under contrasting environmental conditions, e.g. elevation, they may diverge by ecological selection even under potential interspecific gene flow. Hence, varying conditions along elevation gradients have probably driven ecological divergence in *Cedrela*.

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Appendix. Allele frequencies of three *Cedrela* species at eight isozyme loci. CA: *C. angustifolia*, CB: *C. balansae*, CS: *C. saltensis*.

Locus	CA1	CA2	CA3	CA5	CA6	CA7	CA8	CB1	CB3	CB4	CB5	CB6	CB7	CB8	CB10	CS1	CS2	CS3	CS4
Idh1*																			
1	0.556						0.467												
2	0.333	1.000	0.5	0.9	0.875	1.000	0.4	0.15					0.167						
3	0.111	0.000	0.286	0.1	0.125		0.033	0.85	0.984	1.000	1.000	0.926	0.833	1.000	1.000	1.000	1.000	0.833	0.9
4			0.214				0.1		0.016			0.074						0.167	0.1
Idh2																			
1													0.056						
2	1.000	1.000	1.000	0.976	0.833	1.000	0.964	0.875	0.984	0.909	1.000	1.000	0.889	1.000	1.000	0.909	1.000	1.000	1.000
3				0.024	0.167		0.036	0.125	0.016	0.091			0.056			0.091			
Me1*																			
1							0.133						0.188						
2	1.000	0.8	1.000	1.000	1.000	1.000	0.833	1.000	1.000	0.69	1.000	0.907	0.813	1.000	1.000	1.000	1.000	0.667	0.9
3		0.2					0.033			0.31		0.093						0.333	0.1
Me2*																			
1													0.056						
2	0.85	0.8	0.867	1.000	0.75	1.000	0.8	0.917	0.953	0.818	1.000	0.788	0.889	1.000	1.000	1.000	1.000	1.000	1.000
3	0.15	0.2	0.133		0.25		0.2	0.083	0.047	0.182		0.212	0.056						

Table (allele frequencies along populations). Values read across sampled populations for each locus/allele.

Locus / allele																
Per1																
1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.929	1.000	1.000	1.000	1.000	1.000	1.000
2										0.071						
Per2*																
1		0.333		0.1			0.167	0.182	0.2	0.016				0.6		
2	1.000	0.667	1.000	0.8	1.000	1.000	0.833	0.659	0.8	0.919	1.000	1.000	1.000	0.4	1.000	1.000
3				0.1				0.159								
Pgi*																
1					0.05		0.159	0.074		0.083	0.056					
2	0.1	0.033		0.091	0.25		0.048	0.25	0.241	0.143	0.071	0.083	0.222			
3	0.5	0.567	0.78	0.75	0.682	0.667	0.3	0.645	0.523	0.857	0.786	0.833	0.537	0.5	0.222	
4	0.2	0.033	0.22	0.227	0.3		0.306	0.045	0.111	0.111						
5	0.2	0.2	0.25	0.033	0.1		0.023	0.037	0.167	0.4						
6		0.067	0.25	0.222												
7		0.1	0.05	0.05	0.6	0.6	0.5									
Skdh*																
1		0.367	0.325	0.143	0.05	0.031	0.1	0.1								
2	1.000	0.5	0.65	1.000	0.821	0.95	1.000	0.859	0.389	0.9	0.926	1.000	1.000	0.955	0.5	0.833	0.8
3	0.1	0.133	0.025	0.036	0.063	0.611	0.074	0.045	0.5	0.167	0.2					
4	0.1	0.047														

*p<0.05 chi-square test of heterogeneity of allele frequencies along populations.