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Molecular evidence of hybrid zones of *Cedrela* (Meliaceae) in the Yungas of Northwestern Argentina

Noga Zelener [a,*], Daniela Tosto [b], Luiz Orlando de Oliveira [c], María Cristina Soldati [a], María Virginia Inza [a], Luis Fernando Fornes [d]

[a] Instituto de Recursos Biológicos, INTA Castelar-CIRN-CNIA, De los Reseros y N. Repetto (ex Las Cabañas) s.n., Hurlingham 1686, Buenos Aires, Argentina
[b] Instituto de Biotecnología, INTA Castelar-CICVyA-CNIA, De los Reseros y N. Repetto (ex Las Cabañas) s.n., Hurlingham 1686, Buenos Aires, Argentina
[c] Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, 36570-000 Viçosa, MG, Brazil
[d] INTA-EEAFamaillá, Ruta 301 – km 32, 4132 Famaillá, Tucumán, Argentina

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ABSTRACT

In the Yungas of Northwestern Argentina, three endangered species of *Cedrela* (*C. angustifolia*, *C. saltensis*, and *C. balansae*) follow altitudinal gradients of distribution with contact zones between them. We sampled 210 individuals from 20 populations that spanned most of *Cedrela*'s geographical range in the Yungas, and used Amplified Fragment Length Polymorphism (AFLP) markers and DNA sequences of the nuclear Internal Transcribed Spacer (ITS) to investigate hybrid zones. Data analyses employed an array of complementary methods, including principal coordinate analyses, Bayesian clustering analyses, maximum likelihood tree-building, and network techniques. Both nuclear molecular systems – AFLP and ITS – provided insights into the evolutionary history of *Cedrela* in the Yungas in a congruent manner. We uncovered strong support for the occurrence of natural hybridization between *C. balansae* and *C. saltensis*. Additionally, we identified hybrid zones in areas of sympatry (at both the Calilegua National Park and the San Andrés farm) and in transition zones from 820 to 1100 meters above sea level (localities of Pintascayo and Acambuco). There was no evidence for hybridization of either *C. balansae* or *C. saltensis* with *C. angustifolia*. The role of hybrid populations in conservation and use of genetic resources in the Yungas were discussed.

1. Introduction

The understanding of natural zones of hybridization may provide insights to address key evolutionary and ecological processes (e.g., Barton and Hewitt, 1985; Rieseberg and Wendel, 1993; Carney et al., 2000; Pelser et al., 2012). Natural hybridization between closely related forest tree species is a common event in nature and has been documented in several economically important genera, such as *Quercus* (Whittemore and Schaal, 1991), *Eucalyptus* (Potts and Wiltshire, 1997), *Nothofagus* (Stecconi et al., 2004), *Prosopis* (Vega and Hernández, 2005), and *Populus* (DiFazio et al., 2011). After successful hybridization events took place, first-generation hybrids may mate with one or both parental species and give rise to gene introgressions, which result in the transfer of genetic material across species boundaries (Heiser, 1949; Anderson, 1953; Arnold, 2004). Furthermore, ongoing hybridization or introgression may lead to the establishment of hybrid zones (Harrison, 1993). Environmental changes may favor this process and lead to distinct evolutionary consequences (e.g., Aitken et al., 2008; Hoffmann and Agro, 2011).

The genus *Cedrela* (Meliaceae) comprises 18 currently described hardwood species (and a few additional yet unnamed entities), some of which are highly valued in the domestic and international forestry sector (Smith, 1960; Pennington et al., 1981; Pennington and Muellner, 2010). This genus is distributed in Central and South America; it reaches its southernmost edge in the Yungas, a subtropical montane rainforest located in Northwestern Argentina (NWA). This ecosystem is an invaluable biogeographic relict, a reserve of biodiversity, genetic resources, and endemic species. In addition, it provides important environmental services such as the restoration of soil fertility and the stability of river basins. The Yungas extends from 22°S (on the border with Bolivia) to 28°15′S through a narrow and discontinuous strip that crosses the Argentinean provinces of Salta, Jujuy, and Tucumán (North–South direction). A wide altitudinal gradient ranging from 300 to

* Corresponding author.
E-mail addresses: noga.zelener@gmail.com (N. Zelener), tosto.daniela@inta.gob.ar (D. Tosto), luiz.ufv@hotmail.com (L.O. de Oliveira), soldati.maria@inta.gob.ar (M.C. Soldati), inza.maria@inta.gob.ar (M.V. Inza), fornes.luis@inta.gob.ar (L.F. Fornes).

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3000 meters above sea level (masl) also characterizes the Yungas, which produces changes in the species composition of woody vegetation (Brown et al., 2001).

Three species of *Cedrela* follow latitudinal and altitudinal patterns of distribution in the Yungas, with elevation transition zones related to an environmental gradient (Brown et al., 2001) where the species co-occur. The distribution of *C. angustifolia* (syn = *C. lilloi*; Pennington and Muellner, 2010) is in the upper level of the Montane Rainforest and the Temperate Montane Forest (from 900 to 2500 masl), between 22° to 28°15′S at the southernmost edge of *Cedrela*'s range. *Cedrela balansae* typically grows in the Piedmont Rainforest (from 300 to 700 masl) within a small latitudinal range (22° to 24°30′S). *Cedrela saltensis* occurs in the Montane Rainforest (from 700 to 1100 masl) within restricted areas of sympatry between the other two species; its southern limit is 24°40′S (Brown et al., 2001; Zapater et al., 2004; Malizia et al., 2006).

Cedrela is among the most important forest resources in Argentina and consequently has been subjected to intense processes of logging, which impact genetic diversity and structure of populations (Inza et al., 2012; Soldati et al., 2013). Currently, *C. angustifolia* is included in the IUCN Red List 'endangered' category (IUCN, 2016). Conservation policies to counteract anthropogenic pressure on native forests have been implemented in Argentina (SAyDS, 2007; Forestry Act No. 26,331). Nonetheless, effective conservation strategies depend on the ability to unambiguously identify species and hybrids (Heinze, 1998; Rajora and Mosseler, 2001; Muellner et al., 2009). Very little is known about the occurrence of interspecific hybridization in *Cedrela*; some early literature proposed that interfertility may take place among species of *Cedrela* (Smith, 1960; Lamb, 1968).

The delimitation of species and their populations based exclusively on traditional morphological characters is not always conclusive. It is frequently inadequate to document introgression and differentiate interspecific natural hybrids from parental species because backcross hybrids often closely resemble the parental species (Anderson, 1948; Rieseberg and Wendel, 1993; Allendorf et al., 2001). Species delimitation in *Cedrela* is difficult because of the lack of unique qualitative morphological diagnostic characters. In *Cedrela*, species can be defined only by reference to six widely variable and overlapping characters (Pennington and Muellner, 2010).

Molecular genetics enables more rigorous tests for hybridization or introgression (Mallet, 2005). Multi-locus DNA fingerprinting by AFLP (Amplified Fragment Length Polymorphism) technique generates a large number of available genetic markers from genomic DNA far exceeding the number of morphological characters. Thus, AFLP has proven to be a powerful and sensitive tool to detect hybrids, dissect hybrid genomes, and investigate hybridization that occurred as a result of current admixture and historical introgressions (e.g., Beismann et al., 1997; Ishida et al., 2003; Chauhan et al., 2004; Fossati et al., 2004; Zha et al., 2008; Smulders et al., 2008; Gaskin et al., 2009; Koerber et al., 2013; Sahu et al., 2015). Additionally, the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA (Baldwin et al., 1995) have played a significant role in uncovering evidence of hybridization processes among related taxa in a wide range of plant groups. In hybrids, ITS sequences often reveal additive patterns from both parental donors (e.g., Whittall et al., 2000; Rauscher et al., 2002; Kaplan and Fehrer, 2007; Du et al., 2010; Zha et al., 2010; Zhao and Woeste, 2011) and provide additional evidence of evolutionary states (Baldwin et al., 1995; Campbell et al., 1997; Nieto Feliner and Rosselló, 2007; Poczai and Hyvönen, 2010).

In previous studies, we used molecular markers to investigate patterns of genetic diversity and structure in *C. angustifolia* (Inza et al., 2012) and *C. balansae* (Soldati et al., 2013) in the Yungas. These two studies were carried out for each species independently and did not take into consideration *C. saltensis*, the third species of *Cedrela* in the Yungas. Thus, the extent of which interspecific gene flow, gene introgression, and hybrid formation among *Cedrela* took place in the Yungas remained an unexplored issue. In the study we report herein, we explored patterns of genetic diversity and structure in *C. angustifolia*, *C. balansae*, and *C. saltensis* using a combined dataset of AFLP markers and ITS sequence data to: (i) investigate whether natural hybridization took place among *C. angustifolia*, *C. balansae*, and *C. saltensis*, thus supporting the existence of hybrid zones for *Cedrela* in NWA; (ii) characterize genetic patterns of variation in the hybrid zones; (iii) define geographic areas of the hybrid zones and delimit the extension of their altitudinal ranges; (iv) and discuss conservation implications and use of gene resources of *Cedrela* in the Yungas. To the best of our knowledge, the existence of interspecific hybrids among species of *Cedrela* has not been investigated. In this sense, our work will contribute to the specific delimitation of the three species of *Cedrela* in the Yungas, as a prerequisite to the application of proper conservation strategies, to guide germplasm collection and to design suitable seed orchards for breeding programs.

2. Materials and methods

2.1. Sampling strategy and DNA isolation

The geographic area of study covered most of the distribution range of *Cedrela* in the Yungas of NWA. For this study, we had available: (i) 104 samples from 10 populations of *C. angustifolia* from the work of Inza et al. (2012); (ii) 73 samples from seven populations of *C. balansae* (Soldati et al., 2013); (iii) 11 samples from one additional population of *C. balansae*; and (iv) 22 samples from two populations of *C. saltensis* located in zones of sympatry between *C. saltensis* and *C. angustifolia* and between *C. saltensis* and *C. balansae*, in Calilegua National Park and San Andrés farm, respectively (Table 1; Fig. 1). Total genomic DNA from 22 samples of *C. saltensis* and 11 samples of *C. balansae* was isolated from silica-dried leaves according to Hoisington et al. (1994) with minor modifications. For information about sampling and DNA extraction of *C. angustifolia* and *C. balansae* see Inza et al. (2012) and Soldati et al. (2013).

2.2. AFLP procedure and data analyses

We used 104 samples of *C. angustifolia*, 11 samples of *C. balansae*, and 22 samples of *C. saltensis*. We followed the procedures described by Vos et al. (1995) and used two primer pair combinations (EcoRI + AT/MseI + ACCA and EcoRI + AC/MseI + AATA) for selective amplification. These same primer pair combinations were used by Soldati et al. (2013) to genotype the 73 samples of *C. balansae*. Thus, the raw electrophoresis data we obtained for the current study (137 samples) could be combined with those of Soldati et al. (2013) to give rise to a single AFLP matrix, which contained data from 210 samples and included the three *Cedrela* species that occur in the Argentinean Yungas.

The 210 electropherograms were then analyzed using GeneMapper 3.7 software (Applied Biosystems). We considered AFLP fragments of different samples that migrated at the same position in the electrophoretic profiles to be homologous. Fragments were treated as dominant genetic markers and scored as 1 (present) or 0 (absent) binary characters. For further analyses, we included only polymorphic fragments that we scored across all samples unambiguously. We used two complementary methods to analyze the AFLP data matrix. Firstly, we employed a multivariate approach as Principal Coordinates Analysis (PCoA) using GenAlEx 6.3 (Peakall and Smouse, 2006). The PCoA was carried out at the

Table 1
Study populations of *Cedrela* in the Yungas of Northwestern Argentina, with locality, population number and code, sample sizes (*N*, number of samples in AFLP analyses; *N′*, number of samples in ITS sequence analyses), geographic coordinates, and mean altitude of the population (meters above sea level).

Taxa	Locality/province	Number/code	*N*/*N′*	Latitude (S)/longitude (W)	Altitude (masl)
C. angustifolia	Los Pizarros/Tucumán	1/ClLPi	10/3	27°45′/65°42′	1113
	La Florida-Provincial Park/Tucumán	2/ClFL	10/3	27°07′/65°47′	1328
	El Siambón/Tucumán	3/ClLS	14/0	26°42′/65°27′	1389
	Sunchal/Tucumán	4/ClLSu	8/3	26°31′/65°06′	1468
	Metán/Salta	5/ClMT	8/0	25°23′/65°01′	1102
	El Arenal/Jujuy	6/ClAL	7/0	24°20′/64°21′	1111
	La Ramada/Jujuy	7/ClLLR	9/0	23°58′/65°10′	1656
	Calilegua-National Park/Jujuy	8/ClPNC	8/2	23°41′/64°54′	1642
	San Andrés/Salta	9/ClSA	15/0	23°05′/64°51′	1744
	Baritú-NP/Salta	10/ClD	15/2	22°30′/64°45′	1704
C. balansae	Apolinario Saravia/Salta	11/CbAS	6/2	24°20′/64°12′	589
	Calilegua-National Park/Jujuy	12/CbPNC	10/6	23°44′/64°51′	707
	Yuto/Jujuy	13/CbYuto	12/6	23°40′/64°33′	416
	San Andrés/Salta	14/CbSA	10/9	23°06′/64°27′	471
	Pintascayo/Salta	15/CbPin	11/8	22°51′/64°36′	926
	Río Seco-FSB/Salta	16/CbRS	10/6	22°31′/63°55′	699
	Río Seco-FF/Salta	17/CbRSFF	9/4	22°27′/63°59′	665
	Acambuco/Salta	18/CbACAM	16/11	22°05′/63°56′	911
C. saltensis	Calilegua-National Park/Jujuy	19/CsPNC	9/6	23°41′/64°51′	1069
	San Andrés/Salta	20/CsSA	13/10	23°06′/64°42′	837

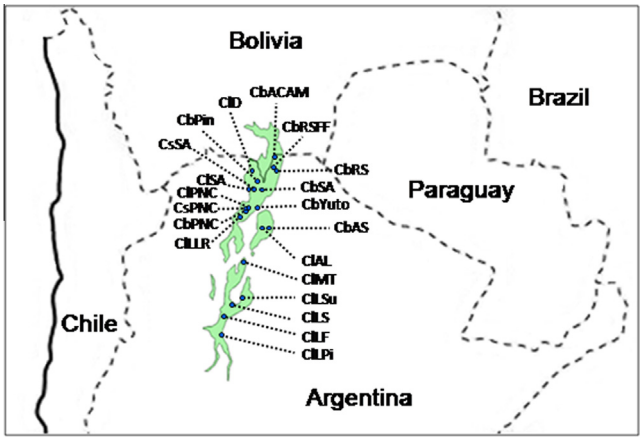

Fig. 1. Location of study populations of *Cedrela* in the Yungas of Northwestern Argentina. Labels indicate populations (codes are as in Table 1).

sample level, using Nei's genetic distance (Nei, 1978). Secondly, we used statistical inferences based on the Bayesian clustering method of Structure 2.3.4 (Pritchard et al., 2000; Falush et al., 2007). In Structure, we set a first run using data from the three species jointly (20 populations). We used admixture model with a burn-in period of 250.000 iterations and a Monte Carlo Markov Chain (MCMC) of 750.000 iterations, with 8 independent replications for $K = 1$ to 21. Structure Harvester (Earl and vonHoldt, 2012) indicated $K = 2$ as the best K, according to the ΔK method (Evanno et al., 2005). This preliminary analysis showed that samples of *C. balansae* together with samples of *C. saltensis* (*Cbal-Csal* hereafter) were set apart from samples of *C. angustifolia*. PCoA analysis had indicated analogous results. Therefore, we set two subsequent runs in which data of *Cbal-Csal* and data of *C. angustifolia* were analyzed independently. For each run, we used admixture model and applied 250.000 and 750.000 iterations, for the burn-in period and the MCMC length, respectively; we used 20 independent replications for $K = 1$ to 12. We found the best K value for each independent run using Structure Harvester and the ΔK method.

2.3. ITS amplification, cloning and sequencing

We used standard protocols for the polymerase chain reaction (PCR) to amplify the entire ITS region of the 18S–26S nuclear ribosomal genes (which included the 5.8S gene) of 81 samples, as follows: 13 samples from five populations of *C. angustifolia*, 52 samples from the eight populations of *C. balansae*, and 16 samples from the two populations of *C. saltensis*. PCR amplifications were carried out with primer pairs F1-ITS and R1-ITS (Muellner et al., 2005) following the thermal-cycling conditions published elsewhere (Muellner et al., 2003). Amplification products were resolved on 2% agarose gels and then gel purified using the commercial kit Nucleo Spin Extract II (Macherey–Nagel) according to the manufactures' instructions. The purified PCR products were directly sequenced in both directions using primers F1-ITS and R1-ITS. Dye terminator sequencing reactions were assayed with Big Dye Terminator v3.1 kit (Applied Biosystems) and analyzed in Genetic Analyzers 3130XL and 3500XL (Applied Biosystems).

Visual inspections of the electropherograms in direct and reverse strands distinguished two categories of sequences. Firstly, 70 sequences exhibited ambiguities owing to the presence of overlapping double peaks at few sites (usually less than 12 sites). However, some sequences deposited in GenBank (FJ462473 and FJ462474, for *C. balansae*; and FJ462478, for *C. angustifolia*) also displayed some of these ambiguities. Secondly, seven sequences displayed multiple overlapping double peaks. Moreover, four sequences contained displacement between peaks, which suggested that these four samples contained more than one ribosomal sequence differing in length. To further characterize the intraindividual polymorphisms in these 11 sequences, we cloned the PCR products into DH5α competent cells using the pGEM-T Vector System II cloning kits (Promega, Madison, Wisconsin). Additionally, we cloned the PCR products from two samples of *C. saltensis* (CsSA3 and CsPNC14). Our PCoA and Bayesian assignment methods based on AFLP markers had suggested a hybrid origin for these two samples of *C. saltensis*. After cloning, we used the QIAprep (Mini-Prep) DNA isolation protocol (Qiagen AG, Basel, Switzerland) to isolate plasmids that contained the inserts and we submitted them to sequencing using universal primers T7 and SP6. From each of the 13 samples subjected to bacterial cloning analysis, we obtained sequences from 4 to 10 clones.

2.4. Sequence analysis

The ITS dataset contained a total of 128 sequences. Direct sequencing of PCR amplicons produced ITS sequences of *C. angustifolia* (13 samples), *C. balansae* (43), and *C. saltensis* (12), while sequencing of bacterially-cloned amplicons yielded 60 clones from a total of nine samples of *C. balansae* and four samples of *C. saltensis* (Table 1). Additionally, we included four ITS sequences that had been deposited in GenBank as belonging to *C. saltensis* (FJ462462), *C. balansae* (FJ462473), *C. angustifolia* (FJ462478), and *Toona ciliata* (FJ462488, used as outgroup in subsequent analysis). Visual inspection revealed that a set of 25 sequences obtained through bacterial cloning displayed recombination events at 1 to 4 positions. The *Phi* test (Bruen et al., 2006) implemented in Splits Tree 4.13.1 software (Huson and Bryant, 2006) provided further evidence for recombination (unpublished data). These 25 sequences were excluded from further analyses.

For editing, the sequences were imported into Sequencher 4.8 (Gene Codes); introduction of gaps were necessary to account for the presence of insertion or deletion events (indels). The boundaries of the coding and spacer regions were established in accordance with Garcia et al. (2011). Double peaks were scored as proposed by Fuertes Aguilar and Nieto Feliner (2003), and International Union of Pure and Applied Chemistry (IUPAC) nucleotide ambiguity codes were used for coding polymorphic positions. Indels were coded by applying the approach of Simmons and Ochoterena (2000) implemented in SeqState 1.4.1 software (Müller, 2005). However, indels that were bordered by mononucleotide repeats were discarded for subsequent statistical analyses (Garcia et al., 2011). We refrained from using information from singletons (i.e., a sequence that occurred only once in the dataset) that had been obtained through bacterial cloning. These point mutations are very likely to be PCR artefacts that can arise from DNA polymerase misincorporation during PCR or bacterial cloning (see Queiroz et al., 2011; Freire et al., 2012). In order to exclude putative pseudogenes among functional paralogs of ITS, we carried out structural analyses using the approach of Garcia et al. (2011), with the programs MFOLD 3.2 – RNA folding (Mathews et al., 1999; Zuker, 2003) to estimate secondary structure within the 5.8S and ITS2 regions and 4SALE (Seibel et al., 2008) to draw secondary structures for the 5.8S and ITS2 regions.

2.5. Genetic relationships among Cedrela

Nucleotide substitution model was selected using the Akaike Information Criterion (AIC) as implemented in jModelTest 0.1.1 (Posada, 2008). A maximum likelihood (ML) analysis was then performed to construct a phylogenetic tree using PhyML3.0 software (Guindon and Gascuel, 2003). The tree topology space was searched using the Best of Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) algorithms from BioNJ starting tree. The robustness of the ML tree was tested with 1000 bootstrap replicates. The ML tree was visualized and edited by FigTree1.3.1 (http://tree.bio.ed.ac.uk/). In addition, we constructed a network using the Median Joining network method (Bandelt et al., 1999) as implemented in NETWORK 4.6.1.1 software (Fluxus Technology Ltd. http://www.fluxus-engineering.com). For the network analysis, we resolved the ambiguities up to two polymorphic sites into a pair (one ambiguity) or quartet (two ambiguities) of sequences.

2.6. Geographical patterns of population structure and delimitation of hybrid zones

We constructed two maps displaying the geographic distribution of the AFLP and ITS markers. The first map shows the latitudinal distribution of the genetic clusters that were estimated by Bayesian method from AFLP markers. In this map we represent each of the 20 populations (Table 1) with the proportion of assignment to each genetic cluster. The second map shows the latitudinal distribution of the ITS ribotypes that were identified in each of the 15 populations (Table 1). The geographic limits of the putative hybrid zones were drawn into the first map to include all populations with ancestry in more than one species. In the second map, we included the populations that showed ITS ribotypes from distinct species. In addition, we addressed the altitudinal distribution of genetic clusters from AFLPs at sample level including 178 out of the 210 samples (first map). Similar analysis was carried out from ITS ribotypes comprising 64 samples (second map).

3. Results

3.1. Population structure

Fingerprinting of the 210 samples of *Cedrela* from NWA resulted in 577 well-scorable polymorphic AFLP markers. The PCoA (Fig. 2) showed that the first two principal coordinates accounted together for 87.9% of the total variation. Most of the samples grouped together according to species; there was a main cluster for each of the three species (*C. angustifolia*, *C. balansae*, and *C. saltensis*). The first principal coordinate (73.1%) clearly distinguished samples of *C. angustifolia* from samples of the other two species. The second principal coordinate accounted for 14.8% of the total variation. This coordinate placed most of the samples of either *C. saltensis* or *C. balansae* in the extremes of the distribution, while the remaining samples of these two species were scattered along intermediate positions. The scattering was most noticeable for samples from four populations (CbPin, CbSA, CbACAM, and CbPNC) of *C. balansae*. Remarkably, most samples of CbPin rested near the *C. saltensis* cluster (Fig. 2).

An analysis carried out on Structure revealed that the 210 samples of *Cedrela* split into two groups (best $K = 2$) (Fig. 3A). The first group was congruent with a taxonomical-delimited species (*C. angustifolia*, depicted in violet;[1] Fig. 3B). The second group brought together samples of *C. balansae* and *C. saltensis* (*Cbal-Csal*, depicted in red; Fig. 3B). Increasing the number of groups beyond the best K ($K > 2$) did not lead to mixing of the two previously identified groups. Instead, additional sub-groups were formed within each of the existing group at $K = 2$ (Appendix, online supplementary material). Interestingly, at $K = 3$, samples of *C. saltensis* formed a separate sub-group (depicted in green), which included most of the samples of population CbPin of *C. balansae*. The remaining samples of *C. balansae* grouped together within the second group (depicted in red), which showed varying levels of admixture. From $K = 4$ to $K = 20$, samples of *C. angustifolia* were kept split into two sub-groups, both of which remained nearly unchanged throughout the analyses (Appendix, online supplementary material).

Next, we carried out analyses on Structure for *C. angustifolia* and *Cbal-Csal*, independently. Both analyses showed best $K = 2$ (*C. angustifolia*, Fig. 3C; *Cbal-Csal*, Fig. 3D). The two groups of *C. angustifolia* (group I depicted in violet and group II depicted in lilac; Fig. 3C) were broadly represented amongst the populations, which suggests that populations behaved as metapopulations associated with latitudinal and altitudinal distributions across the Yungas (Table 1). There was a tendency of finding populations located at higher latitudes and lower altitudes (ClLPi, ClFl, ClLS, ClLSu, ClMT, and ClAL) with a higher percentage of membership in group I (74%–100%). Populations located towards lower latitudes and

[1] For interpretation of color in Fig. 2, the reader is referred to the web version of this article.

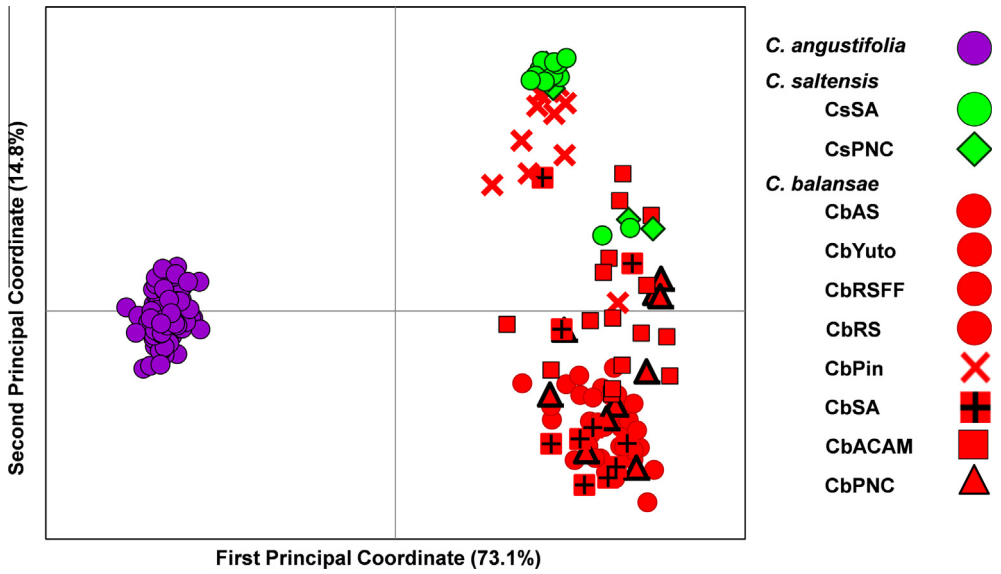

Fig. 2. Plot of the first two principal coordinates for each of the 210 samples of *Cedrela* in the Yungas. The analysis was carried out using data from 577 polymorphic AFLP markers.

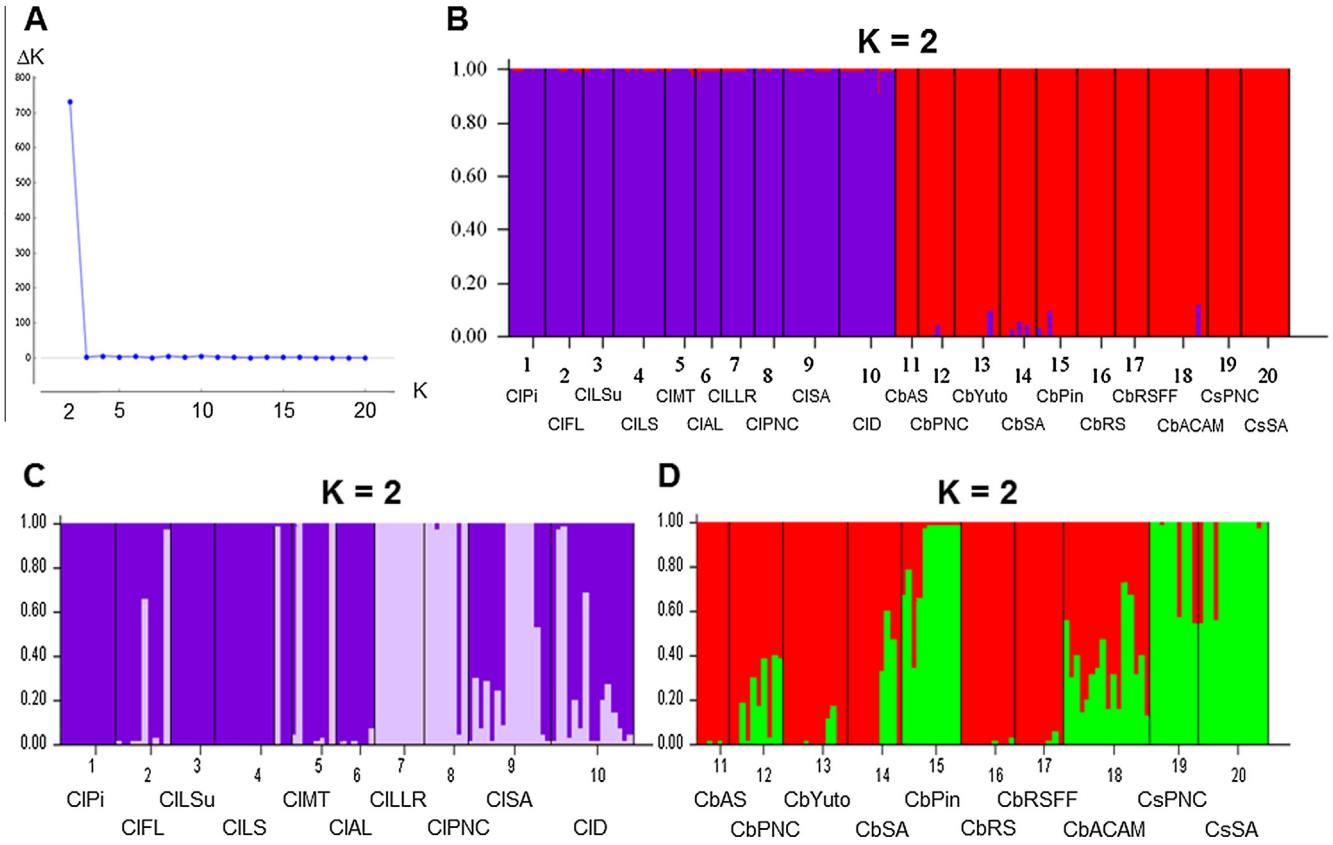

Fig. 3. Bayesian clustering analyses in STRUCTURE inferring the ancestry of 210 samples of *Cedrela* in the Yungas based on AFLP dataset. The taxonomic identity of each population is as follows: *C. angustifolia*, 1 to 10; *C. balansae*, 11 to 18; and *C. saltensis*, 19 and 20. Each sample is represented as a vertical colored bar; the extension of the color in each bar indicates the probability of belonging to the inferred genetic group in that sample. (A) The best *K* (*K* = 2) was calculated according to the Delta *K* method (Evanno et al., 2005). (B) Clustering analysis (*K* = 2) of the 20 populations of *Cedrela*. (C) Clustering analysis (*K* = 2) of the 10 populations of *C. angustifolia* (1–10). (D) Clustering analysis (*K* = 2) of eight populations of *C. balansae* (11–18) together with two populations of *C. saltensis* (19–20). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

higher altitudes (ClSA, ClPNC, ClLLR, and ClD) showed substantial increase in the percentage of membership in group II (43%–100%), while exhibiting varying levels of admixture between groups I and II (Table 1; Fig. 3C).

To a certain extent, the two groups of *Cbal-Csal* were congruent with taxonomical-delimited species: group I (depicted in red; Fig. 3D) brought together samples of *C. balansae*, whilst group II (depicted in green; Fig. 3D) brought together samples of *C. saltensis*.

Accordingly, four populations of *C. balansae* (CbAS, CbYuto, CbRSFF, and CbRS) showed percentage of membership >97% only in group I; therefore, they may constitute genetically pure populations of *C. balansae*. In contrast, four populations that were defined *a priori* as *C. balansae* (CbACAM, CbPNC, CbPin, and CbSA) contained varying levels of admixture (Fig. 3D). The admixture levels were much higher than the 5% threshold which might be attributed to stochastic noise (Meudt et al., 2009). These results suggest a hybrid or introgressed origin for these four populations. In fact, CbACAM displayed no genetically pure samples of *C. balansae*, as all samples of this population displayed percentage of membership around 50% in both groups (Fig. 3D). The two populations of *C. saltensis* showed percentage of assignment in group II >97% for most of the samples, except for four of them (CsPNC10, CsPNC14, CsSA3, and CsSA6). In congruence with their clearly intermediate positions in PCoA, these four samples of *C. saltensis* exhibited levels of admixture of groups I and II >45% (Fig. 3D). CbPin— a population sampled as *C. balansae* according to morphological characteristics — showed percentage of membership >83% in group II, the *C. saltensis* group. Actually, four out of 11 samples of CbPin showed percentage of membership $\geqslant$99% in the *C. saltensis* group (Fig. 3D), which suggests that these four samples detained a large contribution of the *C. saltensis* genome.

3.2. ITS dataset

The aligned ITS dataset consisted of a total of 131 sequences; it contained 68 sequences obtained from direct sequencing, 60 sequences obtained through bacterial clones, and three sequences from GenBank (accessions FJ462462, FJ462473, and FJ462478). The sequences ranged from 588 to 640 bases owing to the presence of indels. Taking into account the three sequences deposited at GenBank as reference sequences during alignments, the ITS1 subset comprised 251 bases. ITS1 displayed 18 variable sites and three indels: a 1-bp indel in *C. balansae*; a 16-bp indel in *C. angustifolia*; and a 52-bp indel (present in 7 out of 12 samples of *C. saltensis*). The 5.8S subset spanned 156 bases and lacked polymorphic sites. The ITS2 subset contained 232 bases, including nine variable sites and two indels: a 1-bp indel (present in both *C. saltensis* and *C. angustifolia*) and a 1-bp indel (present in 30 out of 43 samples of *C. balansae* and in 5 out of 13 samples of *C. angustifolia*). Intraindividual polymorphisms (double peaks) were detected in some samples of *C. angustifolia*; the number of double peaks ranged from one to six positions. In *C. balansae*, the presence of intraindividual polymorphisms varied from 1 to 13 positions. Some of these ambiguities were present in GenBank accessions. These ambiguities suggested the presence of intraspecific variation in ITS region and were resolved in a number of sequences of the ITS dataset.

The approach of Garcia et al. (2011) indicated no evidence for putative pseudogenes among the sequences of ITS. In *C. angustifolia* and *C. saltensis*, the conserved ITS1 angiosperm motif 5′-GGCRY(4–7n)GYGYCAAGGAA-3′ of Liu and Schardl (1994) appeared as 5′-GGCGC(GAGCT)GCGCCAAGGAA-3′; in *C. balansae*, it appeared as 5′-GGCGC(GAGCY)GCGCCAAGGAA-3′. The sequence of the 5.8S gene exhibited the predicted secondary structure for a functional 5.8S ribosomal gene. In the three species, the ITS2 region displayed highly similar secondary structures, including the conserved four helices (Coleman, 2007; Koetschan et al., 2010).

3.3. Phylogenetic and network analyses

A ML analysis was carried out using the TrN + G best-fit model of nucleotide substitution. This analysis returned a tree that grouped the sequences into clades with strong bootstrap supports (Fig. 4A). Sequences of *C. angustifolia* from this study and a sequence of *C. angustifolia* from GenBank (FJ462478) formed in the first clade, with 90% support. Sequences of *C. balansae* and *C. saltensis* occupied the second clade, with 95% support; within this second clade, there were two sub-clades. In agreement with the results of the AFLP analyses, the placement of some sequences within each of the sub-clades did not take place according to species; both sub-clades contained sequences of both *C. balansae* and *C. saltensis*. With 91% bootstrap support, the first sub-clade grouped together 38 out of 43 sequences of *C. balansae* obtained through direct sequencing, 47 sequences obtained through bacterial cloning from samples that belong to either *C. balansae* or *C. saltensis*, and a sequence of *C. balansae* from GenBank (FJ462473). The second sub-clade (100% bootstrap support) included 17 sequences from direct sequencing (12 sequences from samples of *C. saltensis* and five sequences from samples of *C. balansae*), 13 sequences from samples classified as either *C. balansae* or *C. saltensis* that had been obtained through bacterial cloning, and a sequence of *C. saltensis* from GenBank (FJ462462). Network analysis further assessed the genealogical relationships among the sequences (Fig. 4B), which were mostly congruent with the ML analysis. Four ribotypes were found among the 128 sequences (Fig. 4B). Ribotype 1 was present exclusively in *C. angustifolia*; ribotype 2 was a singleton, which appeared in a sample of *C. balansae* (CbAcBA11); ribotypes 3 and 4 occurred in sequences from samples of *C. balansae* and *C. saltensis*, respectively. Interestingly, ribotypes 3 and 4 appeared in bacterially-cloned sequences that had been obtained from both a sample of *C. balansae* (CbPin253) and a sample of *C. saltensis* (CsSA6) (drawn as slices in Fig. 4B).

4. Discussion

4.1. Evidence for natural hybridization among Cedrela in the Yungas

For hybridization to occur among closely related species, several events should take place simultaneously. Overlapping distribution ranges of the target species represents the first of such requirements (Rajora and Mosseler, 2001; Lepais et al., 2009), shared pollinators and synchronicity of flowering are also essential factors (Carney et al., 2000).

In the Yungas, the distribution range of *C. angustifolia* partly overlaps with the range of *C. saltensis* at lower elevation of the Montane Rainforest, while there is no overlap of ranges of *C. angustifolia* and *C. balansae*. The two genetic groups of *C. angustifolia*, derived from Bayesian analysis, did not occur in any sample of *C. saltensis*. Therefore, range overlapping does not seem to be enough to trigger hybridization between *C. angustifolia* and *C. saltensis* or between *C. angustifolia* and putative *C. saltensis–C. balansae* hybrids. According to phylogenetic studies, the clade that contains *C. angustifolia* is sister to the clade that contains *C. saltensis* and *C. balansae* (Muellner et al., 2009; Garcia et al., 2011; Koecke et al., 2013). This phylogenetic distance may account for the absence of hybridization involving either *C. angustifolia* and *C. saltensis* or *C. angustifolia* and *C. balansae*.

Studies on cross-species transferability of SSR markers provide further insights about the phylogenetic relationships among species of *Cedrela* in the Yungas (Soldati et al., 2014). Accordingly, a greater transferability was observed towards *C. saltensis* and *C. balansae*, while the transferability efficiency of the same set of markers decreased significantly when *C. angustifolia* was involved. This is consistent with the greater phylogenetic and morphological distance that exists between *C. angustifolia* and each of the other two species. In this sense, our results show inconsistencies with those reported by Premoli et al. (2011) regarding studies based on isozyme markers. Based on genetic distance indices and multivariate cluster analysis, these authors showed that *C. saltensis* is more closely related to *C. angustifolia* than to *C. balansae*. However, the

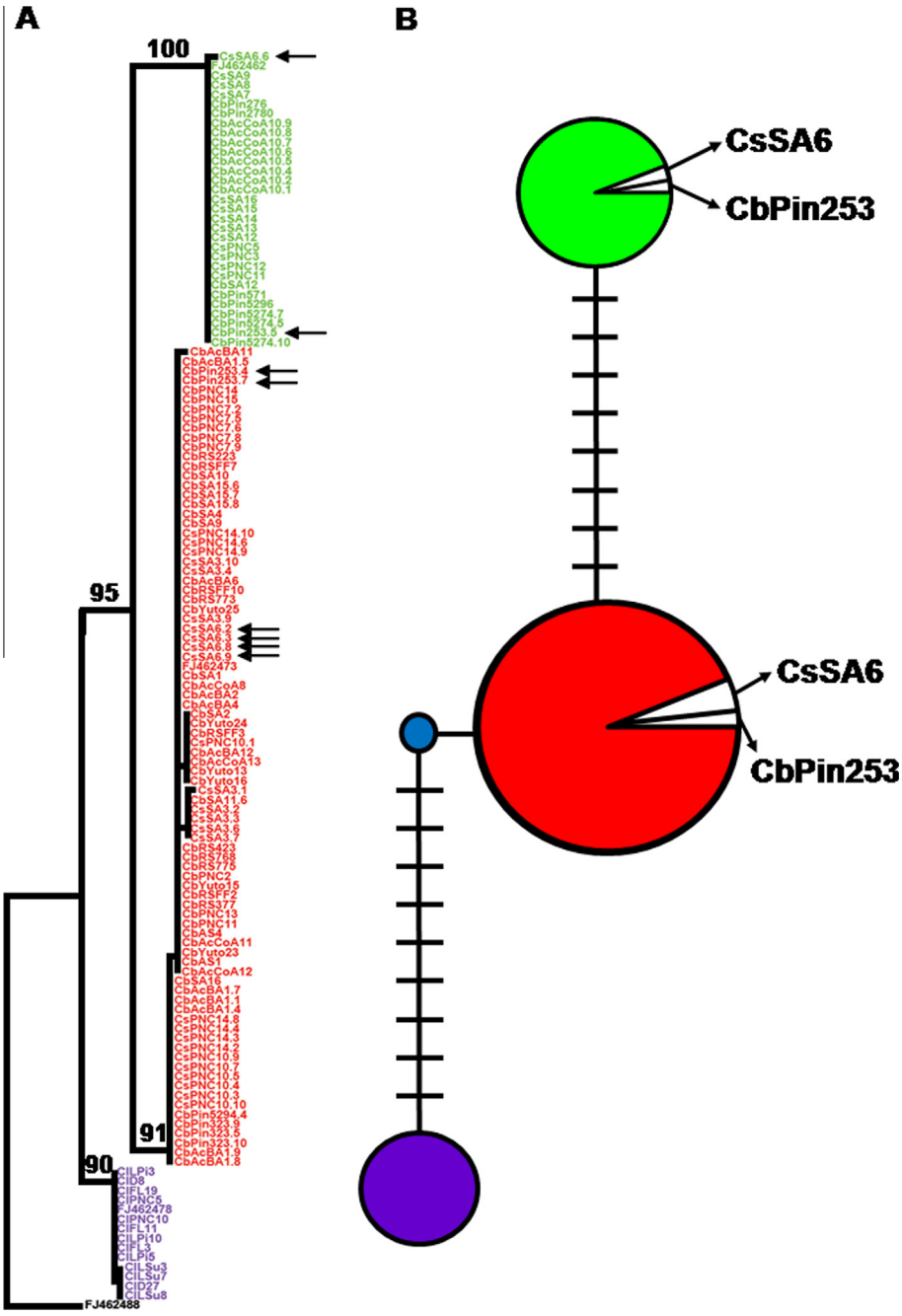

Fig. 4. Phylogenetic relationships among *Cedrela* in the Yungas. (A) Maximum likelihood tree resulting from the ITS dataset of 132 sequences of *Cedrela* with *Toona ciliata* (GenBank accession FJ462488) as outgroup. Nodal support values are given as bootstrap supports (%) above the branches. Tip labels indicate samples and were color-coded for reference purposes: *C. angustifolia*, violet; *C. balansae*, red; *C. saltensis*, green. Black arrows denote unexpected placements of sequences obtained though bacterially-derived clones from samples CsSA6 and CbPin253. (B) Median-joining network for the four ribotypes of ITS in *Cedrela* of the Yungas. Each circle represents a ribotype and was color-coded for reference purposes: *C. angustifolia*, violet; *C. balansae*, red; *C. balansae* sample CbAcBA11, blue; *C. saltensis*, green. Circle size is proportional to the relative frequencies of each ribotype. Numbers of substitutions are indicated with bars when there is more than one. Slices in circles represent unexpected placements of sequences obtained though bacterially-derived clones from samples CsSA6 and CbPin253. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

study we reported herein rejects this inference. Our results support a previous hypothesis (Premoli et al., 2011), which postulated that *C. saltensis* is not a hybrid species between *C. balansae* and *C. angustifolia*.

The overlapping distribution ranges of *C. saltensis* and *C. balansae* follows a downstream transect in the same altitudinal stratum. Studies of reproductive biology revealed that species of *Cedrela* may share pollinators (Bawa et al., 1985; Kageyama et al., 2004; Ward et al., 2005; Aschero, 2006; Pennington and Muellner, 2010). In the Yungas, the three species of *Cedrela* exhibit synchronicity of the flowering period (Zapater et al., 2004). If *C. saltensis* and *C. balansae* share pollinators, as postulated previously, and there is some degree of interfertility between these two species, hybridization is likely to take place in sites where they co-occur.

Hybridization can lead to an increase in genetic diversity at population level (Donoso et al., 2004; Aitken et al., 2008; Broughton et al., 2011; Hoffmann and Agro, 2011). The detection of hybrids by means of morphological characters generally assumes that trees of hybrid origin will be phenotypically interme-

diate between parental species. However, this assumption is often not valid (Allendorf et al., 2001). For *Cedrela*, morphometric characters did not discriminate between introgressant hybrids and trees harboring the expected morphology of *C. balansae* (Soldati et al., 2013). These findings suggest that an extensive range of phenotypic plasticity exists and that it may obscure morphological differences between *C. balansae* and *C. saltensis*.

The simultaneous application of different nuclear molecular systems provided an integrated approach to understand the genetic patterns of *Cedrela* in the Yungas. Both markers, AFLP and ITS, provided reliable insights about the genetic relatedness of samples at species level; the markers were mostly congruent to detect natural hybridization between *C. balansae* and *C. saltensis*.

In recent hybrids, nucleotide additive patterns of parental ribotypes into the hybrid genome are expected (Campbell et al., 1997; Nieto Feliner and Rosselló, 2007; Coleman, 2009). Direct sequencing of ITS fragments revealed that intragenomic variation was widespread. Intraspecific variation alone would not account for the high level of intragenomic variation we uncovered. Some samples of *C. balansae* (CbPin253) and *C. saltensis* (CsSA6), for example, retained ITS paralogs that had originated in either *C. balansae* or *C. saltensis*. The existence of samples that possess paralogs with dual origins provides a strong support for the occurrence of natural hybridization between *C. balansae* and *C. saltensis* in the Yungas. Additionally, AFLP analyses clearly showed that the genotype of some samples were intermediate between *C. balansae* and *C. saltensis*; some of these samples (CsPNC14, CsSA3, CbAcBA1, and CbAcCoA10) contained tandem copies of a single ribotype. Most likely, unidirectional concerted evolution took place in distinct samples (Hillis and Dixon, 1991; Dover, 1994), which led to ITS homogenization towards one of the parental species (Álvarez and Wendel, 2003).

4.2. Hybrid zones of *C. balansae* and *C. saltensis* in the Yungas

The abundance of samples with mixed ancestry in both *C. balansae* and *C. saltensis* corroborated the existence of hybrid contact zones in the Yungas. These hybrid zones exist in areas of sympatry as well as at intermediate elevations where *C. balansae* and *C. saltensis* co-occur (Figs. 5 and 6).

The changes in the frequencies of AFLP markers and ITS ribotypes were strongly congruent along the Yungas and strata elevation. Intermediate genotypes displayed ancestry in both species at sympatry areas that occurred in the Calilegua National Park (CbPNC and CsPNC) and the San Andrés farm (CbSA and CsSA) (Figs. 5A and 6A). Moreover, increasing proportions of ancestry admixture occurs towards altitude ranges approximately from 820 to 1100 masl (CbPin and CbACAM) (Figs. 5B and 6B). Almost genetically pure populations of *C. saltensis* were identified towards increasing altitudes of the Montane Rainforest. Meanwhile, pure populations of *C. balansae* were widely represented in the Piedmont Rainforest, in accordance with vegetation strata described for the Yungas (Figs. 5B and 6B) (Brown et al., 2001; Zapater et al., 2004; Malizia et al., 2006). In addition, genetically pure samples of either *C. balansae* or *C. saltensis* were found together with hybrids and introgressant forms between these species in several populations (CbPNC, CbSA, CbPin, CsPNC, and CsSA) (Figs. 5 and 6).

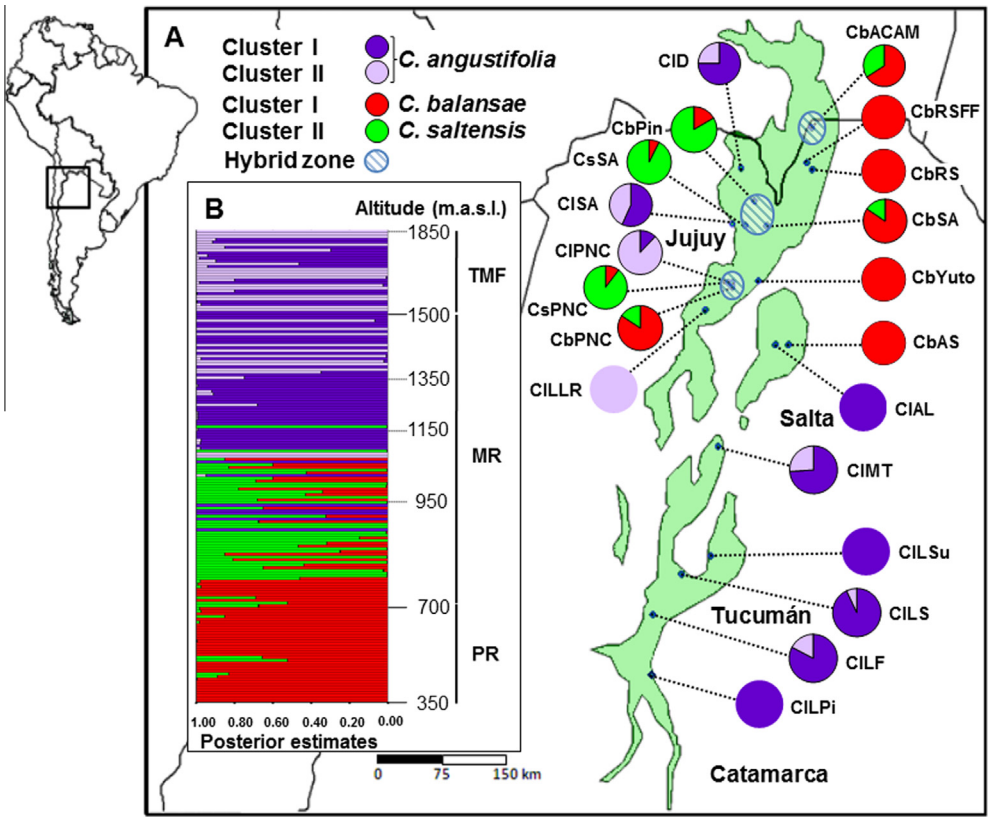

Fig. 5. Hybrid zones. Latitudinal and altitudinal distribution of the genetic groups inferred from Structure for *Cedrela* in the Yungas. (A) Latitudinal distribution. Pie charts at sampled locations show the proportion of membership of populations to *C. angustifolia*, *C. balansae*, and *C. saltensis*. Labels at right of pie charts indicate populations (codes are as in Table 1). Hatched areas in the map depict locations where *C. balansae* and *C. saltensis* form hybrid zones. (B) Altitudinal distribution. Each sample is represented as a horizontal colored bar; the extension of the color in each bar indicates the probability of belonging to the inferred genetic cluster in that sample. Altitudes of each sample (in meters above sea level) are shown at right and altitudinal strata are indicated as TMF (Temperate Montane Forest), MR (Montane Rainforest), and PR (Piedmont Rainforest). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

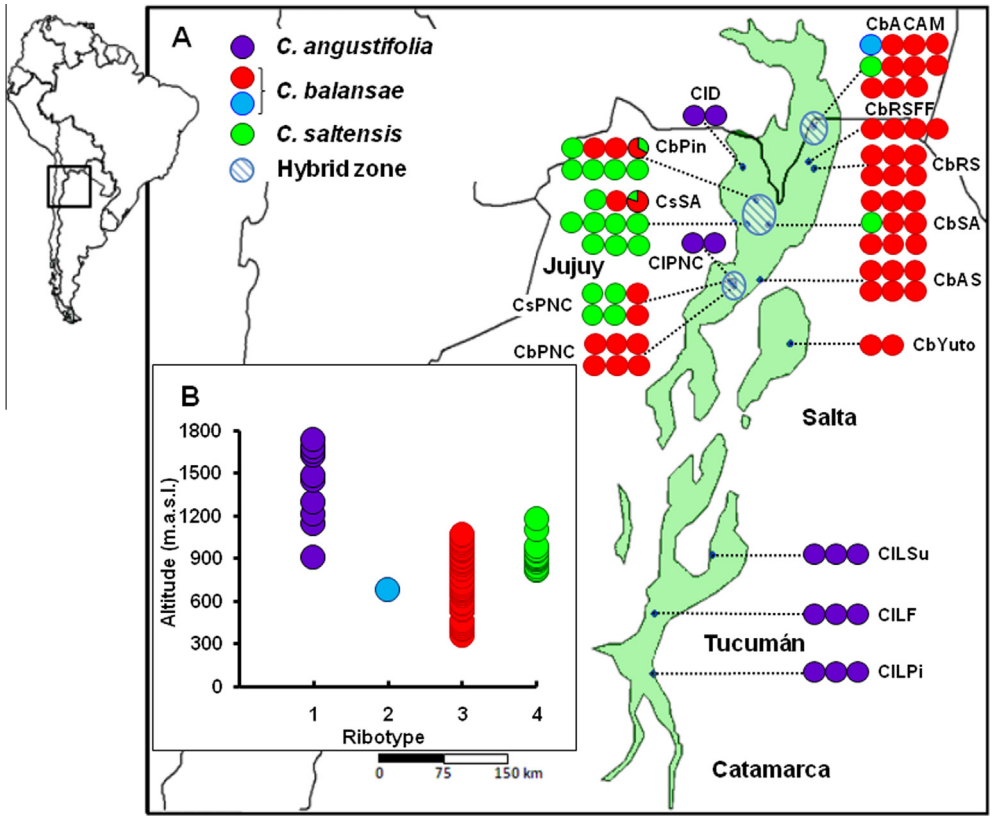

Fig. 6. Hybrid zones. Latitudinal and altitudinal distribution of the four ribotypes of ITS of *Cedrela* in the Yungas. (A) Spatial distribution. Each circle represents the ribotype of a sample; ribotypes have been color-coded for reference purposes: *C. angustifolia*, violet; *C. balansae*, light blue and red; *C. saltensis*, green. Labels near circles indicate populations (codes are as given in Table 1). Populations of hybrid origin display circles of two or more colors; multicolored circles represent samples harboring intragenomic polymorphism for ITS. Hatched areas in the map depict locations where *C. balansae* and *C. saltensis* form hybrid zones. (B) Altitudinal distribution, showing the correspondence between ribotype assignment and the altitude (in meters above sea level) where the sample was obtained. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We did not find any genetically pure sample of *C. balansae* in the CbACAM population (locality of Acambuco); it is worth mentioning that an accession of *C. saltensis* was reported in Tarija (South of Bolivia) at 22°16′S, 64°30′W, and 1100 masl (James C. Solomon collector, No.10066, herbaria LPB and MO; http://www.tropicos.org), not far from locality of Acambuco. The existence of *C. saltensis* nearby may explain the presence of introgressant forms of *C. balansae* in CbACAM population.

4.3. Implications for conservation and use of genetic resources

Habitat disturbance has long been known to promote hybridization (e. g. Anderson, 1948; Rieseberg and Carney, 1998; Lamont et al., 2003; vanHengstum et al., 2012). Since the second half of the last century, the Yungas has faced depletion processes. Most Piedmont Rainforest surface has been modified by anthropogenic intervention (e.g., Brown et al., 2006; SAyDS, 2007, 2008; Seghezzo et al., 2011). Gene flow that likely occurred within *C. balansae* and *C. saltensis* contact zones suggests that barriers to the gene exchange remained weak for an extended period of time in the Yungas. In addition, climate changes affect the colonization and evolutionary dynamics of the species (e.g., Aitken et al., 2008; Linder et al., 2010; Hoffmann and Agro, 2011; Koecke et al., 2013; Köcke et al., 2015). Genetic field trials conducted in the Yungas provided evidence of better adaptive behavior (survival and height in the third year) in hybrids between *C. balansae* and *C. saltensis* (e.g., population of Pintascayo), than in provenances of genetically pure populations of both species, in transition zones between them (Grignola, personal communication). This evidence suggests that combination of introgression and selection may further contribute to novel allelic associations enhancing adaptation of introgressed forms. Thus, habitat forest degradation assembled with effects of climate changes may be potent evolutionary forces that contribute to structuring hybrid zones in the Yungas.

The genetic identity of *Cedrela* germplasm of the Yungas has implications for restoration planning and also provides valuable baseline information for commercial forestry-related activities, such as genetic improvement programs that currently implement purifying seedling and clonal seed orchards to supply the demand of proper reproductive genetic material. In addition, most of the geographical area we sampled belongs to private land, where forestry productive activities are developed in the bounded areas according to the Forestry Act No. 26,331. The domestic market of forest products applies differential pricing to different *Cedrela* wood, punishing the round logs that do not adjust to the expected timber features. The molecular characterization of populations in areas where *C. balansae* and *C. saltensis* showed overlapping distribution can ensure the genetic provenance of the wood offered to the forestry sector.

5. Conclusions

The extensive searching of the Yungas for *Cedrela* in addition to comparative genomic assessments (Inza et al., 2012; Soldati et al., 2013; and the present work) allowed us to identify both genetically pure populations of *C. balansae* and hybrid populations containing a significant proportion of trees that harbor genetic contributions of both *C. balansae* and *C. saltensis* parental species.

This knowledge contributes to their retrieval by conservation activities and highlights the role that hybrid populations could play in the evolutionary dynamics of species. Also, the knowledge of hybridization between *C. balansae* and *C. saltensis* promotes research concerning the geographical expansion of the hybrid zone, the intensity and directionality of the hybridization between both species, as well as, the hybrid fitness for new environmental adaptation in the Yungas, and its implications for genetic improvement.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.05.020.

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