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Understanding the extensive hybridization in South American *Nothofagus* through karyotype analysis

M. CRISTINA ACOSTA[1,*] and ANDREA C. PREMOLI[2]

[1]*Instituto Multidisciplinario de Biología Vegetal (IMBIV), CONICET-Universidad Nacional de Córdoba; Facultad de Ciencias Exactas, Físicas y Naturales-Universidad Nacional de Córdoba, Casilla de Correo 495, 5000 Córdoba, Argentina*
[2]*INIBIOMA CONICET - CRUB Universidad Nacional del Comahue, Quintral 1250, 8400 Bariloche, Río Negro, Argentina*

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Extensive phylogeographic and population studies in *Nothofagus* revealed that hybridization, introgression and plastid capture are common phenomena that have occurred throughout the evolutionary history of the genus. Here, detailed karyotypes of ten South American *Nothofagus* spp. were constructed using chromosome fluorescent banding with the aim of (1) investigating if karyotype features were compatible with the formation of fertile interspecific hybrids, particularly when growing in sympatry and (2) identifying species-specific chromosomal markers to enable further studies of hybridization in *Nothofagus*. Similar karyotype formulas and heterochromatin patterns among species of the same clade (i.e. subgenus) revealed a low rate of chromosomal change. This finding reinforces the idea that hybrids between *Nothofagus* spp. can be fertile and that chromosome pairing in meioses could be successful. Genome conservation and extensive hybridization that resulted in plastid capture has been observed in other woody genera. Hybridization in tree species could be a survival strategy to enable the successful colonization of sites after disturbance and the introgression of genes from their congeners (adaptive introgression) may play an important role in adapting to climate change. Finally, *N. antarctica* has one more nucleolus organizing region (NOR) than its congeners that is easily identifiable and therefore could be used in future studies of hybrids.

ADDITIONAL KEYWORDS: chromosome banding – chromosome character mapping – evolution – karyotype phylogeny.

INTRODUCTION

Hybridization is a common and widespread phenomenon in vascular plants and can lead to genetic exchange between related species and the introgression of selectively favoured alleles from one population into another (Abbott *et al.*, 2013). Extensive hybridization is enabled by weak barriers to genetic exchange between closely related species, which in plants can be distinguished as pre- or post-pollination barriers (Baack *et al.*, 2015). Adaptive divergence in response to ecological factors such as pollinators and distinct habitats commonly drives the evolution of pre-pollination barriers, which contribute to total reproductive isolation in plants more often than post-pollination barriers do (Lowry *et al.*, 2008). In contrast, the evolutionary forces responsible for the development of intrinsic post-pollination barriers (i.e. hybrid inviability, sterility and the failure or reduction in successful reproduction in subsequent generations) are less-well known, but can frequently result in within-species polymorphism of incompatibility factors (Stacy *et al.*, 2017). Fixation of chromosomal arrangements in different lineages can also generate reproductive barriers and speciation, especially via a reduction in gene flow through the suppression of recombination (Rieseberg & Willis, 2007; Fuller *et al.*, 2017). Differences in chromosome number or structural differences between homologous chromosomes in hybridizing taxa tend to disrupt chromosome pairing and assortment during meiosis, yielding defective gametes; this process results in chromosomal sterility or partial sterility of hybrid progeny (Rieseberg *et al.*, 1995; Pikaard, 2001). Thus, the conservation

*Corresponding author. E-mail: mcacosta@imbiv.unc.edu.ar

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of chromosome characteristics may favour gene exchange, limiting among-species divergences.

Nothofagus Blume has been the focus of many genetic and morphological studies showing the occurrence of extensive hybridization, especially between South American species (Acosta & Premoli, 2010 and references herein). *Nothofagus* is the most important component of the temperate forest in southern South America, which lies on both sides of the Andes Mountains covering a broad latitudinal range (33° to 55°) (Donoso, 2006). Ten species belonging to three *Nothofagus* clades are present in South America: *N. glauca* (Phil.) Krasser., *N. macrocarpa* (A.DC.) F.M.Vázquez & R.A.Rodríguez, *N. nervosa* (Phil.) Dim. & Mil. [=*N. alpina* (Poepp. & Endl.) Oerst.] and *N. obliqua* (Mirb.) Oerst. in subgenus *Lophozonia* (Turcz.) Krasser; *N. antarctica* (G.Forst.) Oerst., *N. betuloides* (Mirb.) Oerst., *N. dombeyi* (Mirb.) Oerst., *N. nitida* (Phil.) Krasser and *N. pumilio* (Poepp. & Endl.) Krasser) in subgenus *Nothofagus* and *N. alessandrii* Espinosa in subgenus *Fuscospora* Hill & Read (Donoso, 2006). *Nothofagus* spp. within a clade share a similar pollen type, which facilitates the potential for interspecific crosses, and therefore pre-pollination barriers are considered weak (Veblen *et al.*, 1996). Natural hybridization among species in subgenus *Lophozonia* have been recorded between the deciduous species *N. nervosa* and *N. obliqua* (Donoso *et al.*, 1990; Marchelli & Gallo, 2001) and between *N. obliqua* and *N. glauca* (=*N. leonii* Espin.) (Donoso & Landrum, 1979). Hybrids in subgenus *Nothofagus* have also been found, e.g. between the deciduous *N. antarctica* and *N. pumilio* (Quiroga *et al.*, 2005), among the evergreen species *N. betuloides*, *N. dombeyi* and *N. nitida* (Donoso & Atienza, 1984; Premoli, 1996a, b; 1997) and between the deciduous *N. antarctica* and the evergreen *N. dombeyi* (Stecconi *et al.*, 2004).

Previous phylogeographic studies have shown that plastid sharing occurs among the five species of subgenus *Nothofagus* (Acosta & Premoli 2010; Premoli *et al.*, 2012; Acosta *et al.*, 2014), and provided evidence that past cycles of hybridization/introgression have occurred frequently in all five species with sympatric distribution and reproductive compatibility. Geological events that had catastrophic effects on the landscapes of Patagonia, such as volcanism or glaciation, may have resulted in the survival of just a few remaining individuals (i.e. mother recipient) that could receive pollen from geographically distant parental species (i.e. pollen donor) with which they hybridized (Rieseberg *et al.*, 1995; Veblen *et al.*, 1996). After a disturbance, remnant individuals in small populations may hybridize by receiving foreign pollen from related taxa in a pollen-competition scenario (Acosta & Premoli, 2010). Thus, the minority species will almost inevitably be the female parent of the hybrid. Recurrent introgressions would result in most individuals carrying exclusively maternal plastid DNAs but mostly paternal nuclear genes. This process results in plastid capture, i.e. where the cytoplasm of one species is replaced with that of another species through hybridization/introgression due to the high occurrence of interspecific gene flow in plants (Rieseberg & Soltis, 1991). Certainly, there is evidence that widespread plastid capture has taken place in species of the subgenus *Nothofagus* at different locations over > 2000 km of the southern Andes (Acosta *et al.*, 2014).

To enable extensive plastid capture in *Nothofagus* to take place, it is assumed that the hybrids must be fertile. However, *Nothofagus* spp. are ecologically distinct and clearly identifiable by diagnostic characters; this fact suggests that instead of forming hybrid swarms, the different species either diverge by ecological adaptation and/or have karyotype features that limit recombination. Hence, the question whether potential hybridization among taxa is partially due to karyotype conservation within each subgenus remains open. Hence, we constructed detailed karyotypes of the South American *Nothofagus* spp. with the aim of analysing the degree of chromosomal changes that exist within each subgenus. In addition, we aim to obtain species-specific chromosomal markers to allow us to examine hybridization and divergence in the face of potential gene flow. We hypothesize that karyotypes within each subgenus are only slightly different, so we can expect fertile interspecific hybrid formation. We also hypothesize that similar karyotype features would promote backcrosses of first generation hybrids to promote introgression, favouring adaptive variation of pure taxa.

MATERIAL AND METHODS

The provenance of the plant material studied is shown in Table 1. Distinct localities from the widespread species *N. antarctica*, *N. obliqua* and *N. pumilio* were included. Voucher specimens were identified and deposited in the herbarium of the Centro Regional Universitario Bariloche, Argentina (BCRU). Primary roots obtained by seed germination were used to study somatic chromosomes. One to four seedlings per species and three to ten somatic metaphases per individual were analysed (Table 1). Root tips were pretreated with 2 mM 8-hydroxyquinoline for 6 h at 8 °C and then fixed in 3:1 ethanol:acetic acid mixture for a minimum of 12 h. Shoot apices were macerated using an enzymatic solution of 2% cellulase (w/v) plus 1% pectinase (v/v) at 37 °C for

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Table 1. Karyotype features of *Nothofagus* species studied, all with $2n = 26$

Species and voucher specimen*	Provenance	Haploid karyotype formulae	NOR-bearing pair	HKL (µm) x (sd)	c (sd)	r (sd)	R	A_1	A_2	Stebbins CAT	Heterochromatin amount				maximum number of chromosome pairs with bands	maximum number of bands
											Total	NOR-assoc.	Peri	Para		
subgenus *Fuscospora*																
N. alessandrii W/V (2, 10)	Chile, Región del Maule, Curepto.	6m+7sm	3† (m)	24.61 (0.21)	1.89 (0.19)	1.57 (0.37)	1.34	0.33	0.10	2A	4.71	1.91		2.80	3	3
subgenus *Lophozonia*																
N. glauca W/V (2, 10)	Chile, Región del Maule, Altos de Lircay	10m+1msm+2sm	2 and 4 (m)	23.96 (2.84)	1.84 (0.41)	1.27 (0.36)	2.11	0.16	0.22	2B	3.84	3.84			2	2
N. nervosa MCA & EK 85 (1, 11)	Arg., Neuquén, San Martín de los Andes.	10m+1msm+2sm	2 and 4 (m)	16.80 (0.73)	1.29 (0.26)	1.24 (0.36)	1.81	0.14	0.20	1A	4.29	4.29			2	2
N. obliqua MCA 41 (1, 10)	Arg., Río Negro, Bariloche, cultivated.	10m+1msm+2sm	2 and 4 (m)	20.10 (2.15)	1.55 (0.33)	1.27 (0.41)	1.88	0.15	0.21	2A	5.27	4.32		0.95	2	3
MCA 42 (1, 9)	Arg., Río Negro, Bariloche, cultivated.	10m+1msm+2sm	2 and 4 (m)	20.04 (0.99)	1.54 (0.32)	1.26 (0.39)	1.94	0.15	0.21	2A	4.99	3.99		1.00	2	3
MCA & EK 86 (1, 5)	Arg., Neuquén, San Martín de los Andes.	10m+1msm+2sm	2 and 4 (m)	20.50 (1.49)	1.58 (0.34)	1.23 (0.36)	1.97	0.14	0.21	2A	4.05	3.32		0.73	2	3
N. macrocarpa W/V (2, 10)	Chile, Región de Valparaíso, Cerro La Campana	9m+1msm+3sm	2 and 4 (m)	22.80 (3.28)	1.75 (0.32)	1.35 (0.39)	1.82	0.21	0.19	2A	4.58	4.58			2	2
subgenus *Nothofagus*																
N. antarctica MCA & EK 39 (2, 5)	Arg., Río Negro, Pampa Linda.	9m+1msm+3sm	3† (m) 12 (sm)	17.85 (0.46)	1.37 (0.30)	1.33 (0.42)	1.88	0.19	0.22	2A	6.95	3.59	3.36		13	14
MCA 44 (1, 3)	Arg., Río Negro, Valle del Challhuaco.	9m+1msm+3sm	3† (m) 12 (sm)	25.40 (1.29)	1.95 (0.48)	1.35 (0.43)	2.06	0.20	0.25	2B	4.76	2.24	2.52		13	14
MCA 45 (1, 6)	Arg., Río Negro, Cerro Tronador.	9m+1msm+3sm	3† (m) 12 (sm)	23.35 (3.15)	1.80 (0.37)	1.32 (0.41)	1.93	0.19	0.21	2A	6.29	3.85	2.44		13	14
N. betuloides MCA & LG 72 (1, 9)	Arg., Santa Cruz, Parque Nacional Los Glaciares.	10m+1msm+2sm	3† (m)	21.44 (1.42)	1.65 (0.40)	1.23 (0.36)	2.16	0.14	0.24	2B	4.99	1.73	3.27		13	13
N. dombeyi MCA & EK 43 (2, 10)	Arg., Río Negro, Lago Gutiérrez.	10m+1msm+2sm	3† (m)	21.65 (1.50)	1.67 (0.39)	1.23 (0.34)	2.05	0.15	0.23	2B	4.34	1.48	2.86		13	13
N. nitida MCA & ACP 52 (2, 10)	Chile, Región de Los Lagos, Centro de esquí Antillanca	9m+2msm+2sm	3† (m)	25.28 (2.44)	1.95 (0.40)	1.27 (0.39)	2.07	0.16	0.20	2B	6.21	1.46	4.75		13	13
N. pumilio MCA & EK 40 (1, 9)	Arg., Río Negro, Cerro Otto.	10m+1msm+2sm	3† (m)	22.90 (1.89)	1.76 0.40)	1.25 (0.35)	2.02	0.16	0.23	2B	16.51	2.49	14.02		13	13
MCA & SD 46 (2, 9)	Arg., Río Negro, Valle del Challhuaco.	10m+1msm+2sm	3† (m)	24.53 (1.36)	1.89 (0.45)	1.29 (0.38)	2.12	0.18	0.24	2B	13.45	2.20	11.25		13	13

*In parentheses are the number of seedlings and somatic metaphases analysed per locality.
†Nucleolar organizing regions in long arms.
Abbreviations: Prov. province. m = metacentric; msm = meta-submetacentric; sm = submetacentric; NOR = nucleolus organizing region; HKL = haploid karyotype length in µm - mean (sd); sd = standard deviation; c = mean chromosome length; r = mean arm ratio; R = ratio between the longest and the shortest chromosome pair; A_1 = intrachromosomal asymmetry index; A_2 = interchomosomal asymmetry index; Stebbins CAT = Stebbins' classification. Heterochromatin amount expressed as percentage of HKL; NOR-assoc., NOR-associated heterochromatin; Peri, and Para pericentromeric and paracentromeric heterocromatin, respectively. Collector's names: MCA, M. C. Acosta; LG, L. Garibaldi; EK, E. Kowaljow; ACP, A. C. Premoli; SD, S. Díaz. W/V, without voucher.

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2 h and squashed in a drop of 45% acetic acid, and the coverslip was removed using liquid nitrogen. Fluorescent chromosome banding was performed using the triple staining technique with the fluorochromes chromomycin A3, dystamicin A and 4'-6-diamidino-2-phenylindole (CMA/DA/DAPI) [i.e. CDD staining] following Acosta & Moscone (2011). Enhanced or reduced fluorescence of a chromosome segment is indicated in the text by attaching + or – to the fluorochrome, respectively.

Metaphase chromosomes and interphase nuclei were observed and photographed using an Olympus BX61 microscope equipped with the appropriate filter sets (Olympus, Shinjuku-ku, Tokyo, Japan) and a JAI® CV-M4 + CL monochromatic digital camera (JAI, Barrington, NJ, USA). Digital images were imported into Photoshop 7.0 (Adobe, San Jose, CA, USA) for pseudo-coloring and final processing. For each metaphase plate, short arm length (s), long arm length (l), total chromosome length (c) and length of heterochromatic bands were measured. Chromosome terminology follows that of Levan *et al.* (1964), using the arm ratio (r = l/s) with the modifications suggested by Schlarbaum & Tsuchiya (1984): m, metacentric (r = 1.00–1.29); msm, meta-submetacentric (r = 1.30–1.69); sm, submetacentric (r = 1.70–2.99) and st, subtelocentric (r = 3.00–6.99). Satellite lengths were added to the length of the corresponding arms and lengths of the secondary constrictions [nucleolar organizer regions (NORs)] were not considered. Idiograms were constructed using the mean values for each species. In the idiograms, chromosomes were arranged first into groups according to their increasing arm ratio (from m to st) and then according to decreasing length within each group. Certain chromosomes with molecular markers (i.e. heterochromatin bands) that showed great similarity were tentatively established as homologues. The remaining chromosomes were grouped.

In addition, haploid karyotype length (HKL), average chromosome length, average arm ratio and ratio between the longest and the shortest chromosomes of the complement (R) were estimated. Karyotype asymmetry was calculated using the following parameters: the intrachromosomal asymmetry index $A_1 = 1 - [(\sum bi / Bi) / n]$ (bi = mean short arm length of each chromosome pair, Bi = mean long arm length of each chromosome pair, n = number of chromosome pairs), which indicates the length difference among the chromosome arms and the interchromosomal asymmetry index $A_2 = s/x$ (s = standard deviation, x = mean chromosome length), which indicates the size variation among chromosomes (Romero Zarco, 1986). Finally, species were also categorized following Stebbins' (1971) classification.

To visualize the patterns of chromosomal evolution in *Nothofagus*, chromosomal characters were mapped according to parsimony criteria in a pruned phylogenetic tree from Premoli *et al.* (2012) using the software Mesquite version 2.0 (Maddison and Maddison, 2007). This software coded the continuous characters; thus, subjective range construction was avoided.

RESULTS

The somatic chromosome number $2n = 2x = 26$ was found in all taxa examined. In general, karyotypes were symmetrical, considering both centromere position and chromosome size variation. All species had a majority of m chromosome pairs in their diploid complements, except for *N. alessandrii* (subgenus *Fuscospora*) with seven submetacentric chromosome pairs and consequently the highest values of r and A_1 (Table 1; Figs 4, S1).

Others asymmetrical karyotypes were found in *N. macrocarpa* of subgenus *Lophozonia* with three sm pairs, and in *N. antarctica* and *N. nitida* of subgenus *Nothofagus*, with one additional sm and one additional msm pair, respectively (Table 1). *Nothofagus alessandrii* had the lowest R and A_2 values, indicating similar chromosomal sizes (Table 1). The haploid karyotype length (HKL) for individual species ranged from 16.8 µm in *N. nervosa* to 25.4 µm in *N. antarctica* (Table 1). All species of subgenus *Lophozonia* displayed two m chromosome pairs carrying a NOR plus an attached satellite on the short arms. *Nothofagus alessandrii* and all species of subgenus *Nothofagus* showed only one m chromosome pair with a NOR on the long arm, except for *N. antarctica*, which had an additional sm chromosome bearing a NOR on the short arm (Fig. 4).

Nothofagus spp. studied here showed a comparatively low heterochromatin amount (expressed as percentage of HKL), which ranged from 3.84 in *N. glauca* to 6.95 in *N. antarctica*, except in two studied samples of *N. pumilio*, with 16.51 and 13.45 (Table 1; Fig. 4). All species examined always exhibited CMA+/DAPI- (chromomycin positive and DAPI negative) constitutive heterochromatin (Fig. 1A, B) at the satellites and a minute band proximal to the NOR, except for chromosome pair number 2 from subgenus *Lophozonia*, which bore an euchromatic satellite and the heterochromatin was distributed principally on the arm (NOR with intercalary position, Fig. 2).

Species of subgenus *Lophozonia* showed the simplest fluorescence banding pattern, with only two chromosome pairs with heterochromatin associated with NORs (Fig. 2). The individuals analysed of *N. macrocarpa* (Fig. 2B) showed one heteromorphic pair number 4, with both chromosomes bearing NORs, but one m and the other sm, whereas those of

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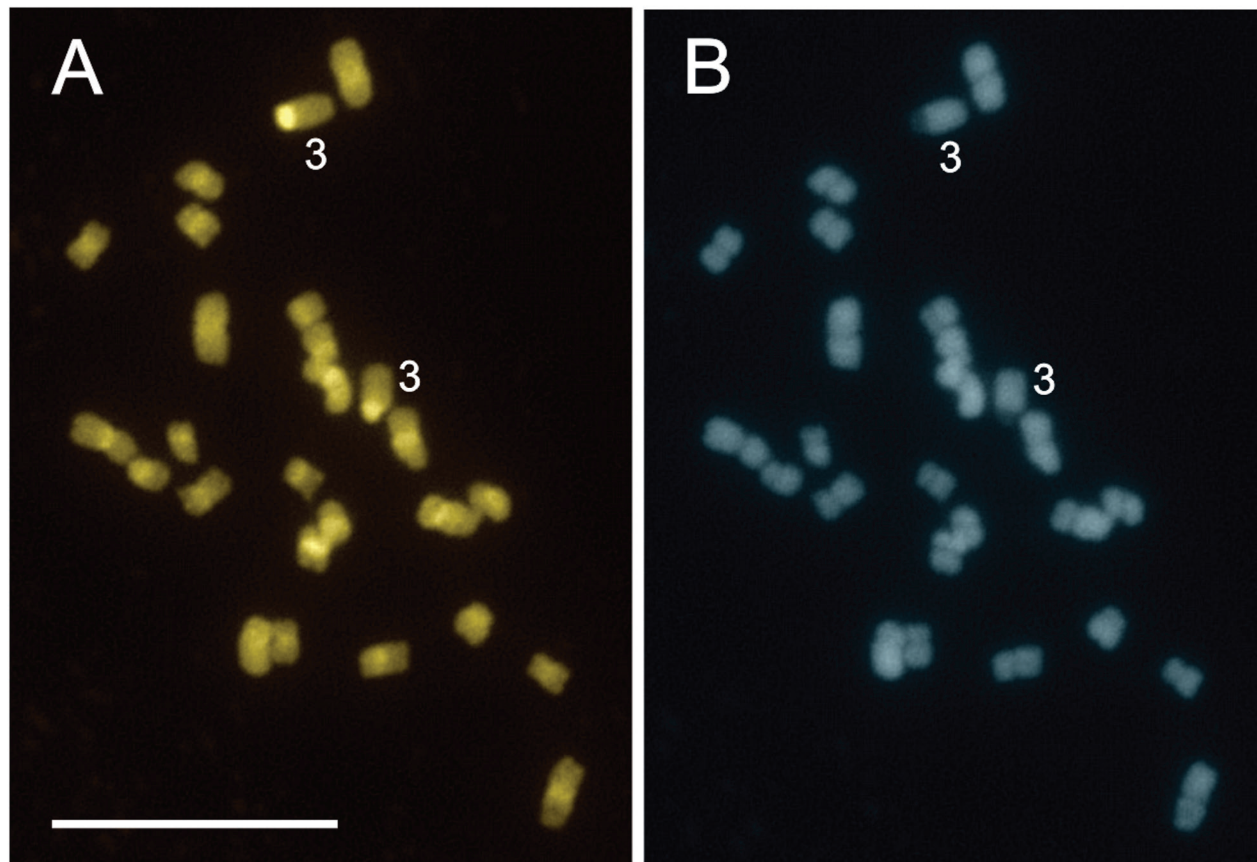

Figure 1. Somatic metaphase of *N. pumilio* ($2n = 2x = 26$) triple-stained with CMA/DA/DAPI. A, CMA fluorescence; B, DAPI fluorescence. The numbers indicated on the chromosomes are according to the idiograms of Figure 4. Scale bar = 10 µm.

N. obliqua had an additional paracentromeric band on chromosome number 4 (Fig. 2D).

The samples analysed of *N. alessandrii* (Fig. 3A) and all species of subgenus *Nothofagus* (Fig. 3B–E) had the same chromosome pair with the satellites on the long arm (number 3, Fig. 4). In addition, *N. alessandrii* samples had two chromosome pairs with paracentromeric CMA+/DAPI neutral heterochromatic bands (in chromosome pairs 8 and 9). This species showed heteromorphisms in some metaphases of the same individual with the presence or absence of minute bands; therefore, they were not considered. Centromeric CMA+/DAPI neutral heterochromatin bands were found in all the examined species of subgenus *Nothofagus* and were more conspicuous in *N. pumilio* (Fig. 1A). In all species of subgenus *Nothofagus*, the chromosomes bearing satellites lacked centromeric bands, except in pair number 12 of *N. antarctica* (Fig. 3B). Finally, the number and size of fluorochrome-stained chromocentres in interphase nuclei (Fig. 3F–I) agreed with the number and size of bands on metaphase chromosomes. The most conspicuous band was always associated with the NOR.

Mapping the chromosomal characters onto the phylogenetic tree (Figs 5, S2, S3) revealed that all analysed characters defined *Nothofagus* clades, except the c values, which did not show a clear phylogenetic pattern (Fig. S3). The lack of msm chromosomes and the presence of the highest number of sm chromosomes, highest r and lowest R values are synapomorphic for the unique South American species belonging to the *Fuscospora* clade, here represented by *N. alessandrii*. The NOR position (in chromosome number pair and arm) and heterochromatin amount associated with the NOR distinguish South American species belonging to the *Lophozonia* clade from those in the *Fuscospora* and *Nothofagus* clades. The presence of pericentromeric CMA+ heterochromatin bands is synapomorphic in subgenus *Nothofagus*, whereas paracentromeric bands were observed only in *N. alessandrii* and *N. obliqua*. Finally, the maximum number of chromosome pairs with bands and the maximum number of bands support each clade in *Nothofagus*, except in *N. obliqua* and *N. alessandrii*, which share the same number of bands; however, these are not homologous (Fig. 5).

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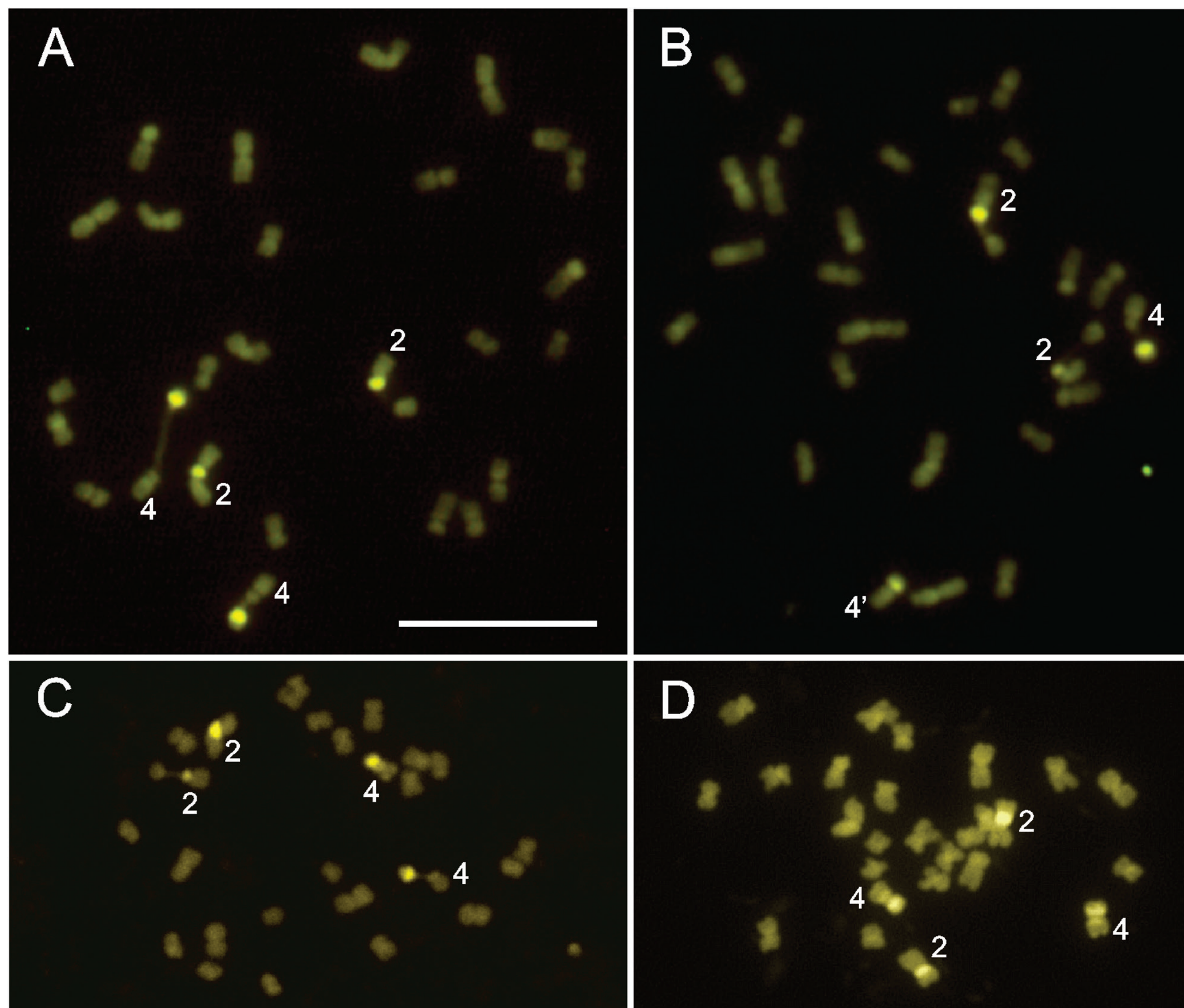

Figure 2. Somatic metaphases of the species of the *Lophozonia* clade ($2n = 2x = 26$) triple-stained with CMA/DA/DAPI (only CMA fluorescence is shown). A, *N. glauca*; B, *N. macrocarpa*; C, *N. nervosa*; D, *N. obliqua*. The numbers indicated on the chromosomes are according to the idiograms of Figure 4. Scale bar = 10 µm.

DISCUSSION

Karyotype features

Nothofagus spp. examined here are diploids with $2n = 2x = 26$, which is consistent with the chromosome number reported for all species studied in Nothofagaceae so far (Armstrong & Wylie, 1965; Wardle, 1967; Ono, 1977; Carr & McPherson, 1986; Jara-Seguel *et al.*, 2014), with the exception of *N. cunninghamii* (Hook.) Oerst. with $2n = 2x = 28$ (Wiltshire & Jackson, 2003). Chromosome numbers for *N. nervosa*, *N. antarctica*, *N. dombeyi*, *N. glauca*, *N. obliqua* and *N. pumilio* are here confirmed (Ono, 1977; Jara-Seguel *et al.*, 2014), whereas those for *N. alessandrii*, *N. betuloides*, *N. macrocarpa* and *N. nitida* are reported for the first time. This is the first detailed karyotype study examining *Nothofagus* spp. using fluorochromes.

Although the techniques used here are not comparable with those implemented by Ono (1977) and Jara-Seguel *et al.* (2014), we also found that chromosomes of *Nothofagus* are small (< 2 µm). Notably, chromosomes of *N. pumilio* are of similar size to those of the other studied *Nothofagus* spp., contrary to observations by Ono (1977). Chromosome shape coincides with that previously reported by Armstrong & Wylie (1965) and Ono (1977), who described median and submedian centromeres. Our study also highlights the asymmetry found in *N. alessandrii*, supporting the subgeneric status of *Fuscospora*; nevertheless,

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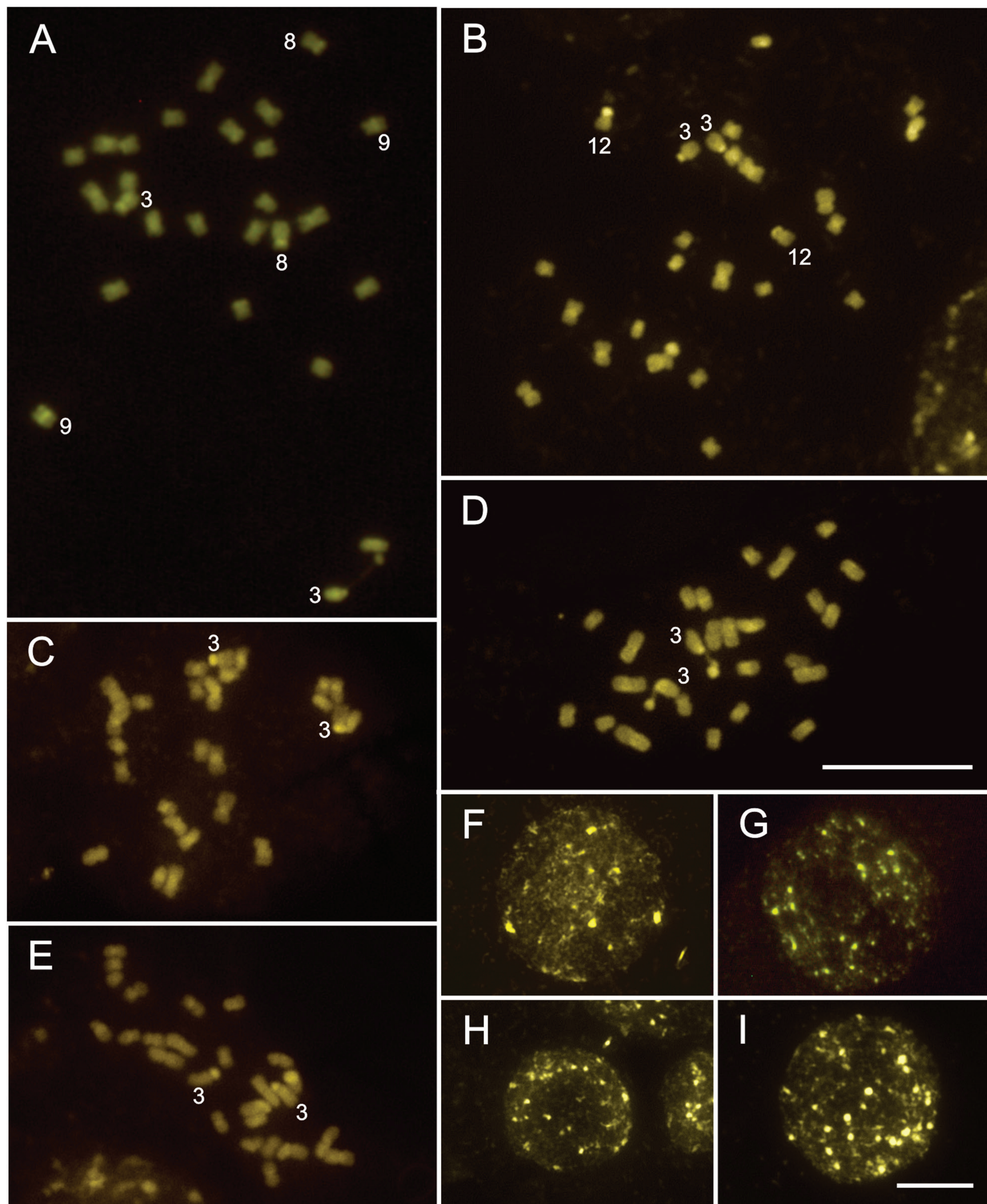

Figure 3. Somatic metaphases from *Fuscospora* (A) and *Nothofagus* clade (B–D) ($2n = 2x = 26$) and interphase nuclei from some species studied here, triple-stained with CMA/DA/DAPI (only CMA fluorescence is shown). A–E, somatic metaphases; A, *N. alessandrii*; B, *N. antarctica*; C, *N. betuloides*; D, *N. dombeyi*; E, *N. nitida*. F–I, interphase nuclei; F, *N. obliqua*; G, *N. alessandrii*; H, *N. antarctica*; I, *N. pumilio*. The numbers indicated on the chromosomes are according to the idiograms of Figure 4. Scale bar corresponds to 10 µm and is the same for all figures showing the same cell stage.

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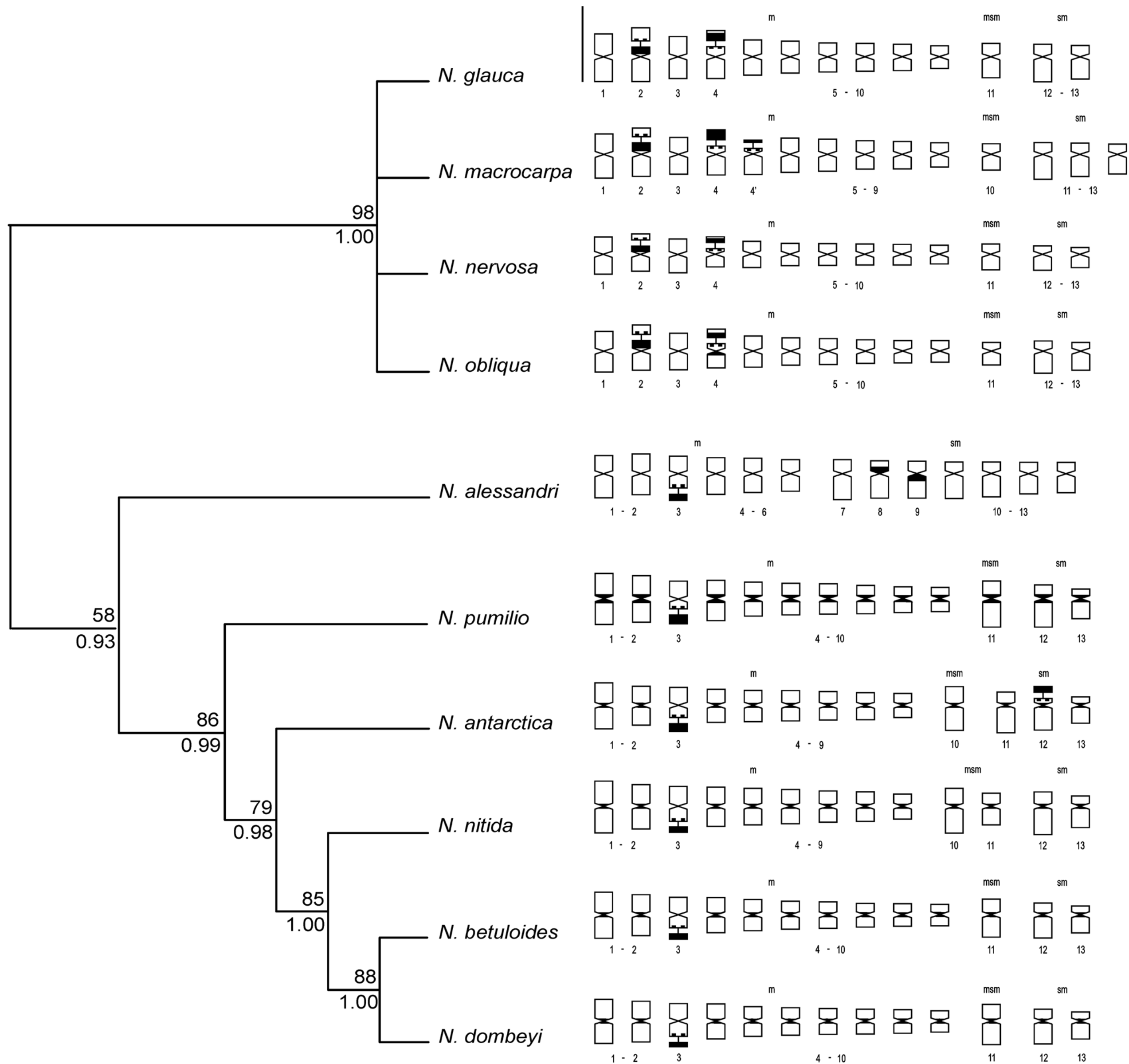

Figure 4. Phylogenetic tree and idiograms of South American *Nothofagus* species. The phylogenetic tree was taken from Premoli *et al.* (2012). Numbers above and below nodes indicate jackknife support (jk) for maximum parsimony and posterior probabilities from Bayesian inference analyses (BPP), respectively. In the idiograms, black blocks represent heterochromatic CMA+ bands. Scale bar = 4 µm.

detailed karyotypes for the other species of subgenus *Fuscospora* are needed.

Nothofagus spp. studied here have GC-rich heterochromatin, exhibiting CMA-positive and DAPI-negative bands, mainly associated with the NOR heterochromatin. In addition, *N. alessandrii* samples and the subgenus *Nothofagus* clade have additional CMA+ but DAPI neutral paracentromeric or pericentromeric bands. This characteristic and the fact that *N. alessandrii* and subgenus *Nothofagus* share the same NOR-bearing chromosome, which in turn is different from that observed in subgenus *Lophozonia*, support the sister-clade relationship between subgenera *Fuscospora* and *Nothofagus* (Premoli *et al.*, 2012). The analysis of pollen features by Fernández *et al.* (2016) yielded a similar phylogenetic signal; indeed, their analysis showed that these two subgenera also shared the fusca-type pollen but were morphologically distinct: *Fuscospora* with fusca-type (a) and *Nothofagus* with fusca-type (b).

Both chromosome pairs associated with NOR found in subgenus *Lophozonia* are similar to those

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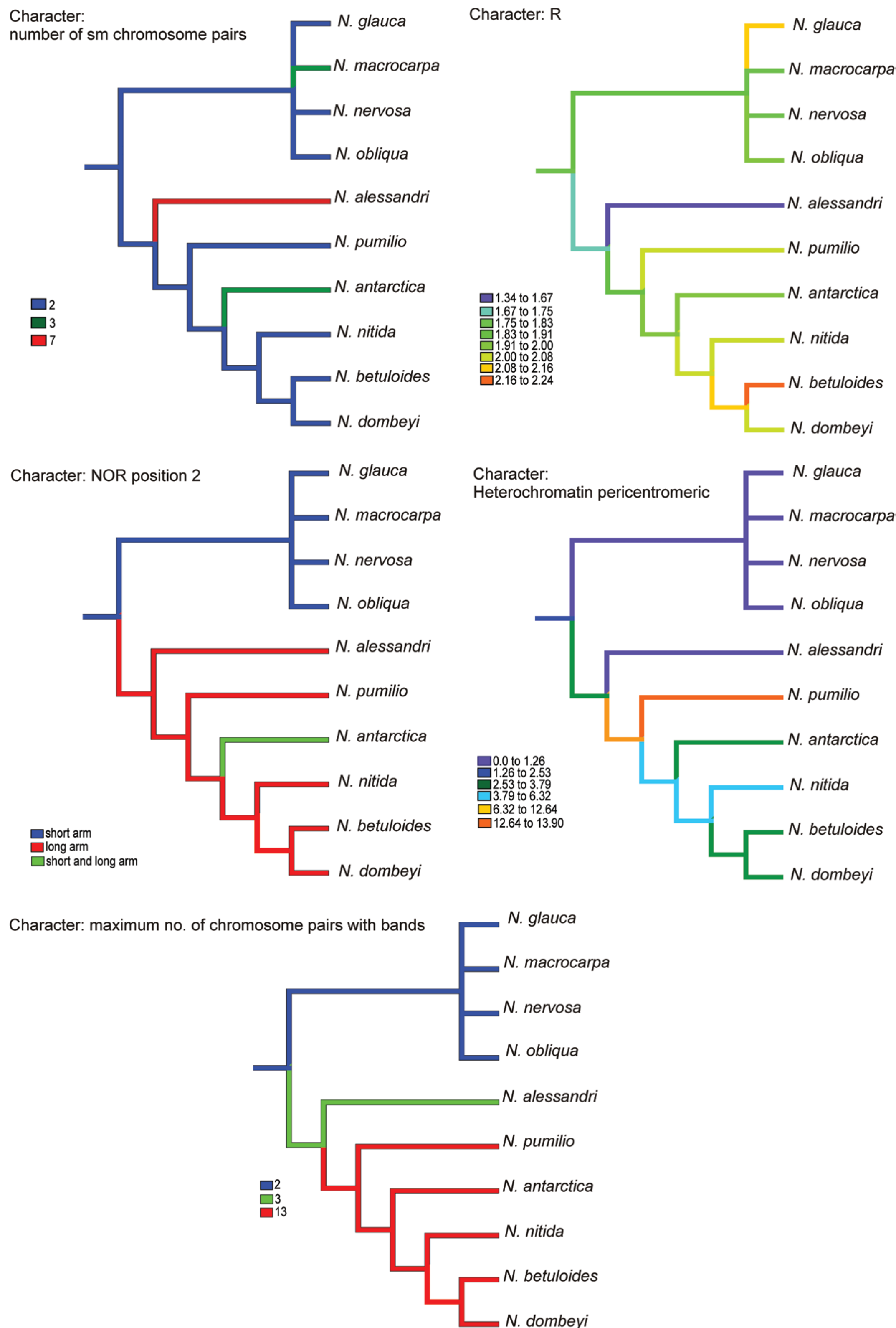

Figure 5. Mapping of chromosome characters onto the phylogenetic tree of species belonging to the South American *Nothofagus*. State reconstruction was estimated using the pruned phylogenetic tree topology from Premoli *et al.* (2012). Abbreviations: R = ratio between the longest and the shortest chromosome pair; NOR position 2: chromosome arm position of the nucleolar organizing region.

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chromosomes observed in *Quercus* L., *Castanea* Mill. and *Castanopsis* (D.Don) Spach. (Zoldos *et al.*, 1999; Chokchaichamnankit *et al.*, 2008; Ribeiro *et al.*, 2011). Thus, the *Lophozonia* clade may have retained some ancestral chromosome characters, as postulated by Jara-Seguel *et al.* (2014) due to the diploid number of $2n = 2x = 28$ (Wiltshire & Jackson, 2003) found in *N. cunninghamii* (from the *Lophozonia* clade); this chromosome number could be considered the ancestral chromosome number in *Nothofagus.* Accordingly, Armstrong & Wylie (1965) postulated that the basic chromosome number $x = 13$ could represent a reduction from $x = 14$, the predominant number in other members of the families of Fagales, including Betulaceae.

The heteromorphism observed in *N. macrocarpa* might be indicative of an ancient hybrid origin. Vergara *et al.* (2014) proposed *N. obliqua* and *N. nervosa* as progenitors; however, our data do not support this hypothesis and other progenitors should be considered. Finally, the submetacentric chromosome 12 found in *N. antarctica* bearing an additional NOR is easily identifiable and could be used in future studies of hybrids.

Genome conservation and hybridization

Based on the presence of similar karyotype formulas and distributions of heterochromatin among species of the same subgenus suggest a low rate of chromosomal change. Such genome conservation has been previously observed in other tree genera, including *Eucalyptus* L'Hér., *Fraxinus* L. and *Quercus* (Zoldos *et al.*, 1999; Siljak-Yakovlev *et al.*, 2014; Ribeiro *et al.*, 2016). For example, karyotypes of six *Eucalyptus* spp. in subgenus *Symphyomyrtus* (Schauer) Brooker, of economic and ecological importance, show a high degree of conservation, although slight differences in karyotype formulas and the distribution of AT-rich heterochromatin were found to be species-specific (Ribeiro *et al.*, 2016). Similarly, molecular-cytogenetic studies showed that heterochromatin and ribosomal DNA organization of chromosomes was conserved and are almost identical in two species of *Fraxinus* section *Fraxinus* (*F. angustifolia* Vahl and *F. excelsior* L.), which in turn were clearly differentiated from section *Ornus* (Boehm.) DC. (Siljak-Yakovlev *et al.*, 2014). Moreover, chromosome banding by means of CMA/DAPI fluorochromes and FISH patterns were identical for 11 *Quercus* spp., suggesting a close genome relationship among oaks, regardless of their geographical origin (European or American) or ecophysiology (deciduous or evergreen) (Zoldos *et al.*, 1999).

Coincidentally, in these genera extensive hybridization with resultant plastid capture was documented, with the cytoplasm of one species being replaced with that of another species. For example, McKinnon *et al.* (2001) noted that plastid sharing among Tasmanian species of *Eucalyptus* subgenus *Symphyomyrtus* 'is the rule rather than the exception' and resulted in concordant plastid DNA patterns including across major geographical disjunctions. Plastid haplotype sharing was interpreted as persistence in multiple, generally common refugia (Nevill *et al.*, 2014). Wide-ranging haplotype sharing was also documented for the phylogenetically close *Fraxinus angustifolia* and *F. excelsior*, which in turn bear morphologically similar pollen grains (Huntley & Birks, 1983). These species were considered to be the result of hybridization in common glacial refugia and/or during postglacial recolonization (Heuertz *et al.*, 2006). Similarly, extensive plastid sharing was detected in white (Petit *et al.*, 2004) and red oaks (Zhang *et al.*, 2015). Finally, widespread plastid DNA sharing was reported among species in *Nothofagus* subgenera *Nothofagus* and *Lophozonia*, and was suggested to have arisen from interspecific hybridization (Acosta & Premoli, 2010). In particular, hybridization seems to occur only between *Nothofagus* spp. with the same pollen type and therefore with weak reproductive barriers (Veblen *et al.*, 1996). Thus, the fact that species from the same clade share similar karyotypes reinforces the idea that hybrids between *Nothofagus* spp. can be fertile and that chromosome pairing in meioses could be successful. Continuous hybridization can promote the conservation of the genome organization; thus, any chromosomal change that occurs in some *Nothofagus* spp. may be selected against, therefore avoiding the persistence of such a mutation.

Nevertheless, there are some chromosome differences in *Nothofagus*. Species with the most restricted and isolated geographical range, such as *N. macrocarpa*, *N. alessandrii* and *N nitida*, have the most distinctive karyotypes. The reduced likelihood of hybridization with other species due to geographical isolation may lead to the accumulation of mutations and chromosomal changes in these taxa; this mechanism would be analogous to those that explain the evolution of sex chromosomes where they evolve from autosomes via the cessation of recombination and accumulation of mutations and chromosomal rearrangements (Abbott *et al.*, 2017). Even though hybrids have been described among the evergreen species *N. betuloides*, *N. dombeyi* and *N. nitida*, the latter is the most morphologically (Premoli, 1996a) and genetically distinct of the three (Premoli, 1996b; Premoli *et al.*, 2012). The exception to this suggestion is the distinctive karyotype observed in *N. antarctica* (e.g. very asymmetric, highest heterochromatin content) despite inhabiting the widest range of habitat types and coexisting in sympatry/parapatry with all species of subgenus *Nothofagus*. Perhaps, the extensive resprouting in this species can explain the maintenance of karyotypic differences (Premoli & Steinke, 2008). Predominantly vegetative propagation by sprouting of *N. antarctica* would mean that recombination and subsequent

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selection against chromosomal changes, which usually occur during sexual reproduction is infrequent. *Nothofaugus antarctica* occurs in different habitat types where different morphotypes and genotypes can be distinguished. Although genetically diverse, *N. antarctica* populations retain significant among-site divergence of isozymes (F_{ST} = 18%; Steinke *et al.*, 2008) probably reinforced by resprouting.

Although the deciduous *N. antarctica* and *N. pumilio* and the evergreen *N. dombeyi* can be considered 'good' (but see Mallet, 1996) ecological species, at some locations, hybrids between *N. antarctica* with either the deciduous *N. pumilio* (Quiroga *et al.*, 2005) or the evergreen *N. dombeyi* can be found (Stecconi *et al.*, 2004). Male and female floral phenologies are synchronized within a given species. However, at a given location, e.g. low elevation, the flowering phenologies of different species may overlap, i.e. between *N. antarctica* and *N. pumilio* (G. Juri, Universidad Nacional del Comahue, pers. comm.). In contrast, floral phenologies of low- and high-elevation *N. antarctica* populations are out of phase (G. Juri, Universidad Nacional del Comahue, pers. comm.), similarly to that found between low- and high-elevation *N. pumilio* populations (Premoli *et al.*, 2007; Mathiasen & Premoli, 2016). In addition, floral maturation of mid-elevation *N. dombeyi* is intermediate between *N. antarctica* or *N. pumilio*, being more decoupled with the latter (G. Juri, Universidad Nacional del Comahue, pers. comm.). Therefore, phenological overlap between *N. antarctica* with *N. pumilio* at their lower elevational extremes and with *N. dombeyi* at mid elevations may prompt greater opportunities for the potential formation of hybrids.

The high conservation of karyotypes found in woody species may account for the presence of the fertile hybrids occurring naturally between species from the same section, clade or lineage. Thus, karyotype conservation can contribute to explaining the existence of extensive plastid capture that has been observed in woody taxa. Hybridization in long-lived species, such as trees, in combination with their sprouting ability, such as that of *N. antarctica*, could be a strategy to survive and recolonize sites after natural disturbances. However, the introgression of genes from their congeners (adaptive introgression) is likely to play an important role in facilitating adaptation to climate change (Hamilton & Miller, 2016).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Idiograms of all species and accessions studied from the South American *Nothofagus* species belonging to the *Nothofagus* clade. Scale bar = 4 µm.
Figure S2. Discrete chromosomal character mapping. Character state reconstruction was estimated using the pruned phylogenetic tree topology from Premoli *et al.* (2012).
Figure S3. Continuous chromosomal characters mapping. Character state reconstruction was estimated using the pruned phylogenetic tree topology from Premoli *et al.* (2012).

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