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RESEARCH PAPER

Using chromosomal data in the phylogenetic and molecular dating framework: karyotype evolution and diversification in *Nierembergia* (Solanaceae) influenced by historical changes in sea level

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Keywords

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ABSTRACT

Karyotype data within a phylogenetic framework and molecular dating were used to examine chromosome evolution in *Nierembergia* and to infer how geological or climatic processes have influenced in the diversification of this solanaceous genus native to South America and Mexico. Despite the numerous studies comparing karyotype features across species, including the use of molecular phylogenies, to date relatively few studies have used formal comparative methods to elucidate chromosomal evolution, especially to reconstruct the whole ancestral karyotypes. Here, we mapped on the *Nierembergia* phylogeny one complete set of chromosomal data obtained by conventional staining, AgNOR-, C- and fluorescent chromosome banding, and fluorescent *in situ* hybridisation. In addition, we used a Bayesian molecular relaxed clock to estimate divergence times between species. *Nierembergia* showed two major divergent clades: a mountainous species group with symmetrical karyotypes, large chromosomes, only one nucleolar organising region (NOR) and without centromeric heterochromatin, and a lowland species group with asymmetrical karyotypes, small chromosomes, two chromosomes pairs with NORs and centromeric heterochromatin bands. Molecular dating on the DNA phylogeny revealed that both groups diverged during Late Miocene, when Atlantic marine ingressions, called the 'Paranense Sea', probably forced the ancestors of these species to find refuge in unflooded areas for about 2 Myr. This split agrees with an increased asymmetry and heterochromatin amount, and decrease in karyotype length and chromosome size. Thus, when the two *Nierembergia* ancestral lineages were isolated, major divergences occurred in chromosomal evolution, and then each lineage underwent speciation separately, with relatively minor changes in chromosomal characteristics.

INTRODUCTION

Karyotype analyses have been an important tool to aid in the study of plant systematics and to reveal patterns of chromosome evolution. Despite their relatively infrequent use in phylogeny, chromosomes undoubtedly provide powerful information for inferring phylogenetic relationships, since they are hereditary elements of the whole nuclear genome and discrete hereditary units of mutation. Moreover, karyotype features do not depend on gene expression, environmental conditions, age, developmental phase or other confounding factors. Thus, karyotype analyses combine the advantages of molecular and morphological characters (O'Brien *et al.* 1999; Dobigny *et al.* 2004; Guerra 2012). Cytogenetic studies are often limited to the description of karyotypes and ancestral karyotypes or patterns of chromosome evolution are frequently described without any statistical support and sometimes with poor understanding of the underlying mechanisms (Guerra 2012). The evolutionary history of a karyotype is often difficult to determine, especially for deep events. Over time, the accumulation of chromosome rearrangements will obscure the exact identity, number and order of events that have occurred along a lineage leading to extant karyotypes (Schubert & Lysak 2011). Moreover, some laws or preconceived assumptions need additional evidence, such as median centromere, symmetry, higher basic number and larger chromosomes, which are considered a normal ancestral condition. Different organisms may evolve their chromosome complements in different ways, hindering extrapolations from one group to another (Jones 1970). Therefore, the use of karyological data in combination with evolutionary inferences within a phylogenetic context should be encouraged (Guerra 2012). Advances in cytogenetics have occurred together with development in molecular biology, flow cytometry, bioinformatics and phylogenetics, especially in vertebrates (Robinson & Yang 2012 and references therein), whereas plant cytogenetics lags somewhat behind in this respect. The development of new methods that facilitate the combination of cytogenetic and phylogenetic analyses is expected to allow 'the cytogenetic renaissance' (Pires & Hertweck 2008).

Karyotype changes are considered to significantly contribute to speciation and macroevolution. The fixation of chromosomal arrangements in different lineages can generate reproductive barriers and speciation, especially by reducing gene flow through the suppression of recombination. In flowering plants, allo- and auto-polyploidy are intensely studied mechanisms, and together with gene duplication (caused by mobile genetic elements or duplication of genes or chromosomal segments) are factors responsible for adaptive divergence and reproductive isolation. In contrast, a generally accepted explanation of how chromosome structure changes caused by rearrangements, *i.e.* translocation and inversion, has still not become established, and whether they contribute to speciation is still unknown (Livingstone & Rieseberg 2003). Most chromosomal speciation models assume that some sort of geographic isolation is necessary to accumulate independent mutations (Rieseberg & Willis 2007). A final consideration is the speed with which new chromosomal rearrangements arise and become established in populations. Most models rely on spontaneous mutation that drives chromosomal evolution but, in many taxa, this process is slow relative to the development of ecological isolating barriers or the accumulation of genic sterility factors (Rieseberg 2001). Only isolated polyploid species may arise in one or two generations.

Finally, advances in cytogenetic tools allow the construction of increasingly detailed karyotypes, especially among species with similar chromosome size and morphology (Acosta *et al.* 2012). Fluorochrome banding or *in situ* hybridisation may substantially improve the cytogenetic analysis and infer homeologies and differences between species (Robledo & Seijo 2010; Scaldaferro *et al.* 2013). In addition, cytogenetics can implement methods of phylogenetic analyses, allowing statistically informed inferences. For example, treating the chromosome data as morphological data, cytogeneticists might construct their own phylogenies. In addition, the development of comparative phylogenetic methods could provide a powerful tool to infer patterns of chromosomal evolution and reconstruct ancestral karyotypes by mapping the chromosome data in the phylogenies (Watanabe *et al.* 1999). Despite the numerous studies comparing karyotype features across species, including the use of molecular phylogenies, the application of formal phylogenetic approaches to the study of chromosomal evolution has only just begun. To date, relatively few studies have used formal comparative methods to elucidate chromosomal evolution (*i.e.* Watanabe *et al.* 1999; Vaio *et al.* 2013; Enke *et al.* 2015), and in general, chromosome data from only one source (especially basic chromosome number) have been mapped. However, comparative methods permit the use of discontinuous (*e.g.* chromosome number, heterochromatin types, number of heterochromatic bands and rDNA loci) and continuous chromosomal data (*e.g.* haploid karyotype length, asymmetry indices, heterochromatin amount).

Here, using detailed *Nierembergia* karyotypes within a phylogenetic and molecular dating framework, we reveal patterns of chromosomal evolution and diversification in *Nierembergia,* a New World genus of Solanaceae that includes 21 species indigenous to South America and one species native to Mexico. The genus is diverse in growth forms, ranging from rhizomatous, tuber-bearing or prostrate herbs to small shrubs, and is unusual because the flowers do not produce nectar; instead, they bear elaiophores inside the corolla that produce fatty oils as a reward to oil-collecting bees (Cocucci 1991; Cocucci & Hunziker 1995). This genus, specialised in relation to pollination ecology, probably arose and evolved in relation to the exomalopsine oil bees (Cocucci 1991); however, no probable cause for diversification has yet been postulated. The genus is cytogenetically interesting because species have two base chromosome numbers ($2n = 16$ and 18) and some populations consist of polyploids or individuals bearing B-chromosomes (Acosta *et al.* 2006; Acosta & Moscone 2011). Moreover, a previous study (Tate *et al.* 2009) showed that *Nierembergia* species group in two mayor clades that differ in haploid karyotype length, asymmetry indices, morphology and geographic distribution. Then chromosome data were obtained with conventional staining techniques; therefore, the application of modern cytogenetic tools, together with comparative methods and molecular dating should lead us to a more reliable hypothesis formulation about the causes of the observed chromosomal variation. We expect that major chromosomal changes had occurred earlier, during the split of the main groups, establishing a barrier to gene flow and avoiding hybridisation.

We characterised cytologically 21 samples belonging to 17 species and six varieties of *Nierembergia*, *Bouchetia*, *Leptoglossis* and *Petunia* using AgNOR-, C- and fluorescent chromosome banding and fluorescent *in situ* hybridisation. We mapped on the phylogeny, the chromosomal data obtained here, including data from conventional staining (Tate *et al.* 2009) with the aims of testing chromosomal evolution and reconstructing the entire ancestral karyotypes using statistical comparative methods. In addition, we used a Bayesian molecular relaxed clock to estimate the date of main karyotype changes and of species divergence in order to infer the possible role of chromosomal changes in diversification of *Nierembergia*.

MATERIAL AND METHODS

Plant material

Karyotypic data were taken from verified herbarium specimens of 13 species and six varieties from *Nierembergia*. The outgroup consisted of one species of *Bouchetia* and *Leptoglossis* and two species of *Petunia*. The source of the studied samples is shown in Table S1. Voucher specimens were deposited in the herbarium of the Botanical Museum of Cordoba, Argentina (CORD). For molecular dating, sequences from *Nierembergia* and other Solanaceae species and *Ipomoea batatas* (L.) Lam. were obtained from GenBank.

Cytogenetic procedures

Somatic chromosomes were observed in squashed root meristems obtained from seed germination. The root apices were fixed in a 1:3 acetic acid:ethanol mixture for 12 h after a pretreatment in 2 mM 8-hydroxyquinoline at 8 °C for 8 h. Root tips were squashed and pectinase-cellulase macerated according to the method of Schwarzacher *et al.* (1980) at 37 °C for 60 min. After removal of the coverslip with CO_2, slides were air-dried, aged for 1 to 2 days at room temperature, and then maintained at −20 °C until use. Triple staining with chromomycin A3, distamycin A and 4-6-diamidino-2-phenylindole (CMA/DA/DAPI; *i.e.* CDD staining) was performed as described by Schweizer (1980). Enhanced, neutral or reduced

fluorescence of a chromosome segment is indicated in the text by attaching +, o or −, respectively, to the fluorochrome or fluorochrome combination. C-banding procedure was performed according to Schwarzacher *et al.* (1980) with the modification suggested by Bennett *et al.* (1995), *i.e.* staining with DAPI instead of Giemsa. AgNOR banding was based on the Ag-I procedure of Bloom & Goodpasture (1976), with the modification of Kodama *et al.* (1980), *i.e.* using nylon cloth (0.242-mm mesh size) instead of coverslips. Fluorescent *in situ* hybridisation (FISH) was carried out following the protocol of Moscone *et al.* (1996). The 5S rDNA and 18S-25S (45S) rDNA repeated sequences were localised using the following DNA probes: pXV1, a 349-bp fragment of the 5S rRNA gene repeated unit from *Beta vulgaris*, including the adjacent spacer (Schmidt *et al.* 1994); and R2, a 6.5-kb fragment of the 18S-5.8S-25S rDNA repeat unit from *Arabidopsis thaliana*, including the internal transcribed spacers ITS1 and ITS2, and a short 5′ segment of the intergenic region (IGR; Wanzenböck *et al.* 1997). The first probe was labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany), and the second probe was labelled with biotin-11-dUTP (Sigma, St. Louis, MO, USA), both by nick translation. Somatic chromosomes and interphase nuclei were observed and photographed on a Leica DMLB epifluorescence microscope (Leica, Heerbrugg, Switzerland) equipped with a computer-assisted Leica DC 250 digital camera system. For epifluorescence microscopy, images were captured in black and white using appropriate filter sets in each case. Digital images were pseudo-coloured (and combined in the case of FISH images) using the IM 1000 Leica software and imported into Photoshop, version 7.0 (Adobe, San Jose, CA, USA) for final processing. Chromosome features and indices were calculated as in Tate *et al.* (2009). In addition, the position of intercalary bands was calculated by the di index ($di = d \times 100/a$ where d = distance of band centre from the centromere, a = length of corresponding chromosome arm), according to Greilhuber & Speta (1976). Finally, correlation analyses between heterochromatin amount, intrachromosomal asymmetry index (A1) and karyotype length were performed in INFOSTAT version 2012 (Di Rienzo *et al.* 2012) using the non-parametric Spearman test (Sokal & Rohlf 1995).

Phylogenetic analysis and molecular dating

The phylogeny of *Nierembergia* was constructed using the rpl16 and ITS sequences from Tate *et al.* (2009) obtained from GenBank. Sequences were aligned using MEGA 3.1 (Kumar *et al.* 2004) with manual adjustments as needed. Bayesian analysis was conducted using MRBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001) with a model of sequence evolution generated by MRMODELTEST version 2.2 (Nylander 2004) which implements the hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AIC). The evolution model that best fitted the datasets was GTR + I + G (Rodríguez *et al.* 1990). The analysis consisted of two independent runs of 2×10^6 generations with four chains each (three heated and one cold) and trees were saved every 100 generations in each run. Approximately 10% of the trees (corresponding to the burn-in period) were discarded and a 50% majority rule consensus tree was constructed from the remaining trees. Calibration of Solanaceae molecular clock is difficult because of the family's poor fossil records (Collinson *et al.* 1993). To estimate the genetic divergences in *Nierembergia* we used two different and independent approaches. First, we obtained sequences of *ndh*F region from GenBank of different species belonging to Solanaceae suprageneric groups, according to Olmstead *et al.* (2008) and *Ipomeae batatas* (Convolvulaceae) in order to calibrate a Solanaceae molecular clock. The split of the Solanaceae stem from the outgroup *Ipomoea batatas* was considered the root of the tree. The calibration point of this node was set to 52 Myr, according to the first appearance of the Convolvulaceae from Collinson *et al.* (1993). Additional time constraints were set for the split between Solanaceae genera: at 44.9 Myr according to fossil age of *Cantisolanum daturoides*, the most recent common ancestor between *Schizanthus* and *Nolana*, and 14 Myr for the appearance of *Solanum* and *Physalis* (Collinson *et al.* 1993; Franco & Brea 2008). Divergence times were estimated using BEAST version 1.6.2 (Drummond & Rambaut 2007). The input file was prepared in BEAUti version 1.6.2 (provided in the BEAST package). The substitution model was GTR with a Gamma site heterogeneity model with four categories following the Mr. Modeltest result; the clock was set as an uncorrelated log-normal relaxed model; and the Yule process was selected as a prior for the distribution of divergence dates. The Monte Carlo Markov Chain was set to run for 10^7 generations, sampling every 100 cycles. Then, the chronogram resulting (Fig. S1) was used to indirectly obtain the ages of the split of *Petunia axillaris* as root height (30 Myr), and *Leptoglossis linifolia* (19 Myr) and *Bouchetia erecta* (14 Myr) as the split of these genera. These ages were employed as secondary calibration point in the molecular dating of *Nierembergia* phylogeny. The second method employed was the use of the average substitution rate for angiosperm plastid DNA of 0.007 substitutions/site/million year (Hewitt 2001) and different ITS substitution rates for Boraginaceae and Rubiaceae from the survey of Kay *et al.* (2006) to transform rpl 16 and ITS distances in *Nierembergia* into absolute time, respectively.

Chromosome evolution and character mapping

To visualise the patterns of chromosomal evolution and reconstruct the ancestral karyotype in *Nierembergia*, chromosomal characters were mapped according to parsimony, maximum likelihood criteria, and Bayesian inference in a pruned phylogenetic tree from Fig. S2 using the software Mesquite version 2.0 (Maddison & Maddison 2007) and BayesTraits version 2.0 (Pagel *et al.* 2004). The MCMC multistate and continuous random walk modules were implemented to analyse discrete and continuous states, respectively. Characters and coding are listed in Table 1. Discrete characters were coded as indicated in Table 1, without the use of ranges. Continuous characters were coded with Mesquite software, thus avoiding subjective range construction. The ancestral reconstruction of each character was used to reconstruct the whole ancestral karyotypes in Fig. 1. The numbers of msm, sm and st chromosomes and of 45S and 5S chromosomes are only illustrative because the homeologies are uncertain. Finally, to map the geographic distribution in the pruned *Nierembergia* phylogenetic tree (Fig. 2), the states were as follows: 0, semi-arid lowlands; 1, mountain area (highland); 2, Brazilian shield (lowland); 3, 'Paranense sea' (lowland).

Table 1. Chromosomal characters and coding used in character mapping.

								C-DAPI+						no. cr 45S					
species	2n	no. NOR	pos NOR	no. msm cr	no. sm cr	no. st cr	bhci CMA+	term	interc	centr	no. cr bhc	no. 5S cr	pos 5S	term 1	term 2	c	r	R	Hc
Ban	0	1	0	0	1	1	0	1	1	1	3	8	1	6	1	2.54	1.44	1.37	27.79
Lli	3	0	0	0	5	0	1	1	1	0	4	7	1	3	0	2.33	1.68	1.35	9.78
ari	0	1	0	3	0	0	0	0	0	1	3	0	0	2	2	2.39	1.29	1.51	20.68
bro	0	0	0	2	0	0	0	0	0	0	0	1	0	5	0	2.76	1.18	1.40	1.58
cal	0	0	1	2	0	0	1	0	0	0	1	5	0	3	0	2.59	1.22	1.33	3.38
eri	1	0	0	1	0	1	0	0	0	0	1	6	0	4	5	2.61	1.40	2.00	3.11
gra	0	1	0	1	1	0	0	0	1	1	3	5	1	3	5	2.21	1.21	1.39	21.21
linli	0	0	0	2	0	0	1	0	0	0	1	1	0	3	0	2.84	1.17	1.39	1.98
lingl	0	1	0	1	0	0	1	0	0	0	2	1	0	3	5	2.86	1.11	1.38	3.41
linpa	0	0	0	1	0	0	1	0	0	0	1	1	0	3	0	2.85	1.12	1.49	2.77
linpi	0	0	0	2	0	0	1	0	0	0	1	1	0	3	0	2.73	1.14	1.46	3.03
pulpu	0	0	0	1	0	0	0	0	0	0	0	3	0	7	0	2.87	1.12	1.34	1.92
pulma	0	0	0	0	0	0	0	0	0	0	0	4	0	8	0	2.81	1.10	1.37	1.43
rep	0	1	0	1	1	0	0	0	0	1	3	2	0	2	4	2.17	1.21	1.40	23.70
rig	0	0	0	1	2	0	1	0	0	1	3	2	0	1	0	2.22	1.41	1.45	30.29
riv	0	1	0	1	2	0	1	0	0	1	3	2	0	3	3	2.22	1.34	1.58	20.62
sco	1	1	0	1	1	1	0	0	0	0	2	5	0	3	4	2.33	1.47	1.74	3.62
tuc	0	0	0	1	0	0	0	0	0	0	0	1	0	7	0	2.75	1.17	1.38	1.77
vei	0	1	0	2	1	0	0	0	0	1	3	0	0	2	2	2.25	1.28	1.43	24.75
Pax	2	0	0	1	3	0	0	0	1	0	1	8	0	9	6	2.87	1.51	1.35	2.54
Ppa	1	1	0	1	4	0	0	0	1	1	2	8	0	9	7	3.01	1.48	1.23	4.32

All values correspond to haploid complement except for 2n. Coding: 2n, 0 = 16, 1 = 18, 2 = 14, 3 = 20; number of nucleolus organising region (no. NOR), 0 = 1, 1 = 2; position of active NOR (pos NOR) and position of 5S loci (pos 5S), 0 = short chromosome arm, 1 = long chromosome arm; number of msm, sm and st chromosomes (no. msm cr, no. sm cr, no. st cr), 0 = 0, 1 = 1, 2 = 2, 3 = 3, 4 = 4, 5 = 7; presence/absence of C-DAPI+/CMA+DAPI− intercalary heterochromatin bands (bhci CMA+), presence/absence of terminal (term), intercalary (interc) and centromeric (centr) C-DAPI+/CMAoDAPIo (bhc C-DAPI+) heterochromatin bands, 0 = absence, 1 = presence; maximum no. of pairs with bands (no. bhc cr), 0 = 1, 1 = 2, 2 = 3, 3 = 8, 4 = 10; chromosome identification number with 5S loci (no. cr 5S), 0 = 1 (m), 1 = 4 (m), 2 = 5 (m), 3 = 6 (m), 4 = 7 (m), 5 = 7 (msm), 6 = 8 (msm), 7 = 6 (sm), 8 = 7 (sm); chromosome identification number with the first or unique terminal 45S loci (45S term 1), 1 = 1 (m), 2 = 2 (m), 3 = 3 (m), 4 = 4 (m), 5 = 5 (m), 6 = 6 (m), 7 = 7 (m), 8 = 8 (m), 9 = 5–8 (sm); chromosome identification number with the second terminal 45S loci (45S term 2), 0 = absence, 1 = 8 (st), 2 = 7 (msm), 3 = 8 (sm), 4 = 4 (m), 5 = 5 (m), 6 = 6 (sm), 7 = 9 (sm); mean chromosome length (c), mean arm ratio (r), ratio between the longest and the shortest chromosome pair (R) and heterochromatin amount expressed as percentage of haploid karyotype length. Species codes as in Table S1.

RESULTS

Karyotype analyses

Species of *Nierembergia* can be grouped according to their karyotypic features. Group **A** consists of *N. browallioides* (Fig. S3A–C), *N. calycina* (Fig. 3A–C), *N. ericoides* (Fig. S4A–C), *N. linariifolia* (Figs 4J–L and S3J–R), *N. pulchella* (Figs 4M–O and S3D–F), *N. scoparia* (Fig. 4G–I) and *N. tucumanensis* (Fig. S3G–I), with comparatively high haploid karyotype length (HKL), and the most symmetrical karyotypes considering both centromere position and chromosome size variation (for more detailed classical karyotype characteristics refer to Tate *et al.* 2009). All species have 2n = 2x = 16 chromosomes, except for *N. ericoides* and *N. scoparia* with 2n = 2x = 18. The karyotypes of these taxa consist only or mainly of m pairs and one or two msm pairs (Table S2, see idiograms of Fig. 1) and species with 18 chromosomes are characterised by an additional and small st pair, being only half as long as the largest pair of the complement. They have only one chromosome pair with nucleolar organising regions (NORs) and two maximum number of nucleoli in interphase nuclei (except *N. linariifolia* var. *glabriuscula* with two and four, respectively; Fig. S5G and H; and *N. scoparia* with sometimes one additional NOR in only one of the st chromosomes; Fig. 5O and P),which were impregnated with silver in AgNOR banding (Figs 5I, J, Q–T and S5A–F, I–L). AgNORs are located on the short arm, except in *N. calycina* (Fig. 5I). In addition, species of group A show the simplest fluorescence banding pattern (Fig. 1). They have comparatively low CMA+/DAPI− (chromomycin positive and DAPI negative) heterochromatin amount (expressed as percentage of HKL), ranging from 1.43 in *N. pulchella* var. *macrocalyx* exhibited in only one satellite pair, through 3.38 in *N. calycina* with one NOR and additional intercalary band, to 3.41 in *N. linariifolia* var. *glabriuscula*, with intercalary band (as other *N. linariifolia* varieties) and two NORs (Table S3). The terminal band observed in pair 9 (st) in *N. scoparia* is in heterozygosis, since it is carried in one of the homologues. The fluorescent heterochromatic bands CMA+/DAPI− indicate a GC-rich heterochromatin constitution. Finally, the number of 45S rDNA loci ranges from one to two pairs (coincident with CMA+ bands), whereas there is invariably only one pair of 5S sites located in the short arm (Table S3).

Group **B** contains *N. aristata* (Fig. S4D–F), *N. graveolens* (Fig. 4A–C), *N. repens* (Fig. S4G–I), *N. rigida* (Fig. S4J–L),

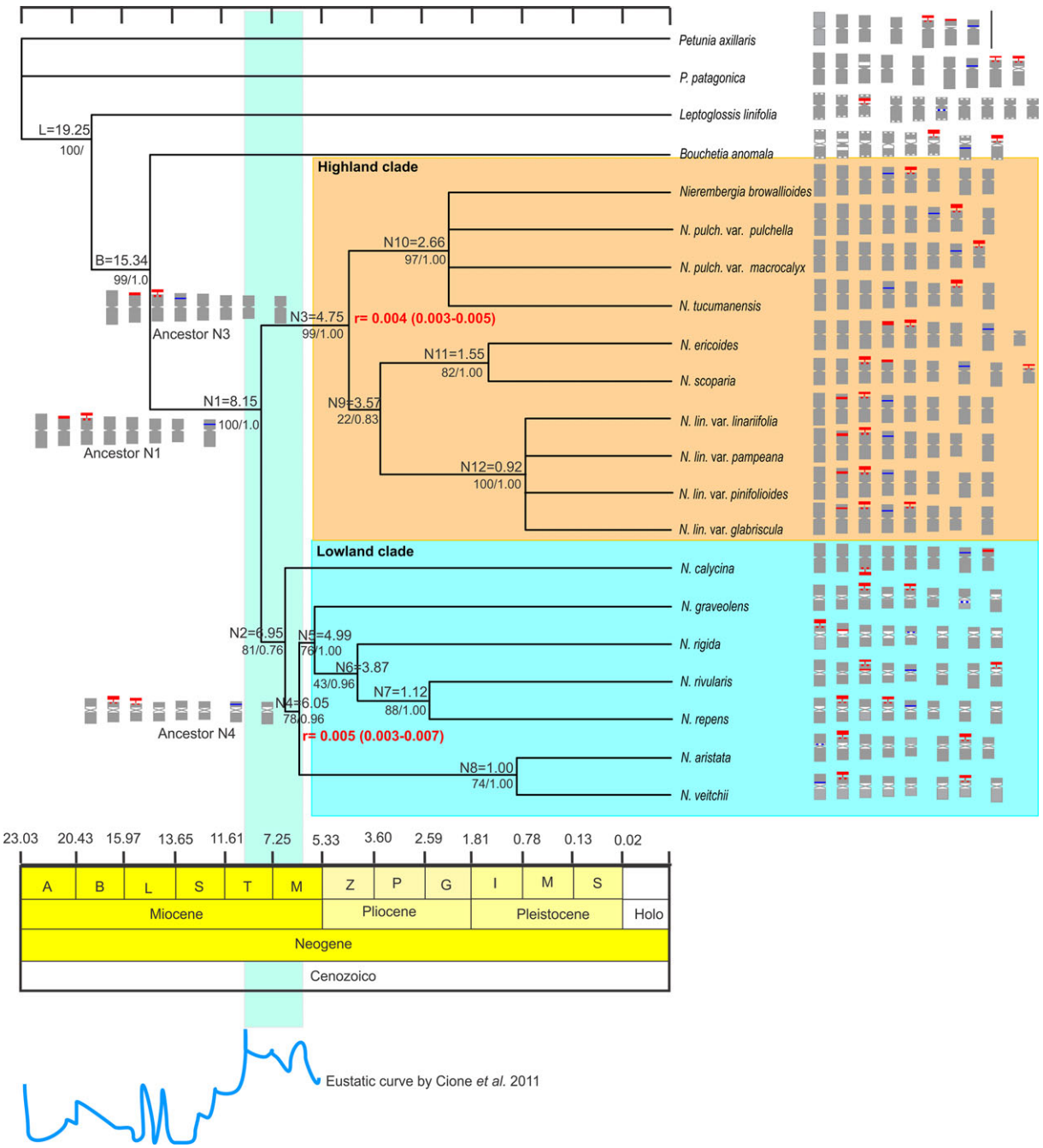

Fig. 1. Chronogram showing relationships between the studied species of *Nierembergia*, ages of divergences and idiograms using rpl 16 and ITS sequence data. Numbers above nodes indicate the ages from the molecular dating analysis (obtained by averaging indirect fossil calibration values from Table S4). Numbers below nodes indicate jackknifing support (jk) for maximum parsimony and posterior probabilities from Bayesian inference analyses (BPP), respectively. Average substitution rate and ranges are indicated in red for each lineage (r). In each idiogram, single chromosomes representing the pairs are ordered sequentially. Ancestral karyotypes are shown in some nodes following the character mapping. Type chromosome bands are coded as follows: white = C-DAPI bands, red = CMA+/DAPI− bands and 45S loci, blue = 5S loci. Different colours indicate the phylogenetic groups discussed in the text (light orange: group A, light blue: group B). Scale bar corresponds to 4 μm.

N. rivularis (Fig. S4M–O) and *N. veitchii* (Fig. 4D–F), with 2n = 2x = 16 and the most asymmetrical karyotypes with one or two sm in addition to one to three msm pairs and lower values of HKL than group A (Table S2, see idiograms of Fig. 1). They have a maximum of two pairs of chromosomes carrying NORs (except *N. rigida* with one; Fig. S5Q and R) stained with AgNORs. The maximum number of silver-stained nucleoli is always four (Figs 5L, N and S5H, P, U). The AgNORs are always located on the short arm. They show CMA+/DAPI− bands on two chromosome pairs with NORs, except in *N. rigida* with only one NOR but an additional intercalary band (Table S3, idiograms of Fig. 1). Remarkably, this group

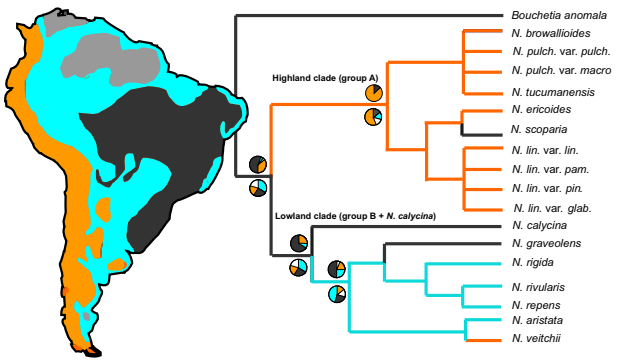

Fig. 2. Ancestral range reconstruction for *Nierembergia*. Map of South America showing the biogeographic subdivisions used to encode the distributions of individual species: (orange) mountainous areas, (dark grey) Brazilian shield; (light blue) Paranense sea; (white) semi-arid lowland (modified from Donato *et al.* 2003). The reconstruction models used were parsimony, maximum likelihood and Bayesian inference. Pie graphs at nodes show ancestral state probabilities based on either maximum likelihood or Bayesian inference, above and below each node, respectively. Ancestral state reconstruction was estimated using the pruned phylogenetic tree topology from Fig. S2.

shows C-DAPI+ centromeric heterochromatin in all chromosomes (C-DAPI+/CMAoDAPIo; Figs 4B, E and S4E, H, K, N). Thus, total heterochromatin amount ranges (expressed as percentage of HKL) between 20.62 in *N. rivularis* and 30.29 in *N. rigida* (Table 1 and S3). *Nierembergia graveolens* (Fig. 4B and idiogram of Fig. 1) is unique in having paracentromeric bands in 3, 4, 6 and 7 chromosome pairs in addition to pericentromeric bands. Moreover, this species has an intercalary band in pair 7, which corresponds to rDNA 5S loci (see FISH results). The number of 45S rDNA loci is two pairs (coincident with CMA+ bands). Finally, there is only one pair of 5S sites always in the short chromosome arm except in *N. graveolens*, where the 5S is located in the long chromosome arm (Table S3, idiograms of Fig. 1).

Bouchetia anomala (2n = 2x = 16) has two CMA+ DAPI− bands, which correspond to the AgNORs in addition to terminal, intercalary, para- and pericentromeric C-DAPI+/CMAoDAPIo bands (Figs 3D–F and 5A, B, idiograms of Fig. 1). *Leptoglossis linifolia* (2n = 2x = 20) shows one intercalary band, which corresponds to the AgNORs site and terminals and one intercalary C-DAPI+/CMAoDAPIo band, which corresponds to the 5S site (6 sm pair) and is located in only one homologous chromosome (Figs 3G–I and 5C, D, idiogram of Fig. 1). Finally, *P. axillaris* (2n = 2x = 14) and *P. patagonica* (2n = 2x = 18) show one and two chromosome pairs with CMA+/DAPI− NOR associated bands; and one and two C-DAPI+/CMAoDAPIo bands, respectively (Figs 3J–O and 5E–H, idiograms of Fig. 1). *Bouchetia anomala* and *L. linifolia* have the 5S loci located in the long chromosome arm (idiograms of Fig. 1).

The AgNOR banding in *Nierembergia* shows that the number of active NORs in metaphase and the number of nucleoli in the interphase nucleus differs within each individual. In addition, the relative size of the nucleoli in the interphase nucleus and metaphase NORs is not constant, although variations in both structures show no correlation. The satellites are not differentially stained with silver staining (Fig. 5Q). AgNORs are generally located at a subterminal position and have attached satellites of variable size (micro- or macrosatellites), but sometimes in terminal position (Fig. 5K and O). *Leptoglossis linifolia* has a linear satellite and *Nierembergia rigida* has a satellite in tandem (Figs 5C and S5Q). Finally, the number and size of fluorochrome-stained chromocenters in interphase nuclei correspond with the number and size of bands on metaphase chromosomes. The most conspicuous band is always associated with the NOR. The NOR-associated heterochromatin comprises the distal satellite and, usually, a minute band proximal to the NOR, except in *L. linifolia*, in which only a minute band proximal to satellite is heterochromatic (Fig. 3G).

While the FISH patterns of the 45S ribosomal RNA gene family differ among taxa regarding number, position and size of loci, the 5S position is more constant (see character mapping below). *Bouchetia anomala* (Fig. 3F), *L. linifolia* (Fig. 3I), *P. axillaris* (Fig. 3L) and *P. patagonica* (Fig. 3O) show the 5S loci in sm chromosomes, whereas in *Nierembergia* species it is located in m chromosomes (see idiogram of Fig. 1). Exceptionally, *N. ericoides* (Fig. S4C), *N. scoparia* (Fig. 4I), *N. calycina* (Fig. 3C) and *N. graveolens* (Fig. 4C) have 5S loci in msm chromosomes. In particular, the 5s loci in *N. aristata* (Fig. S4F) and *N. veitchii* (Fig. 4F) are on the largest m chromosome (pair 1), whereas the other *Nierembergia* species have this marker in smaller chromosomes (no. 4 or lower). The loci 5S never are in synteny with 45S (idiograms of Fig. 1).

Haploid karyotype length (HKL) has a significant and negative correlation with intrachromosomal asymmetry index (A_1) ($r = -0.56$, $P = 0.02$) and heterochromatin amount ($r = -0.72$, $P < 0.01$). In addition, heterochromatin amount is positively correlated with A_1 ($r = 0.63$, $P = 0.01$; Fig. 6).

Phylogenetic analysis, karyotype ancestral reconstruction and molecular dating

The phylogenetic tree (Figs S2 and 1) from chloroplast DNA and ITS sequences within *Nierembergia* samples shows two major divergent clades: a group A (N3: jk (jackknifing support) = 99%, BPP (Bayesian posterior probability) = 1.00) and a group B (N2: jk = 81%, BPP = 0.76). The group A has symmetrical karyotypes, large chromosomes, only one NOR and does not have centromeric heterochromatin; the group B has asymmetrical karyotypes, small chromosomes, two chromosomes pairs with nucleolar organising regions and centromeric heterochromatin bands (Fig. 1). *N. calycina*, which belongs to the group A (according chromosomal data), is geographically and phylogenetically related to group B.

The mapping of the chromosomal characters onto the phylogeny (Figs S6 and S7) revealed that the maximum pair numbers with bands, the presence of centromeric C-DAPI+/CMAoDAPIo heterochromatin bands, position of 5S loci, heterochromatin amount, mean arm ratio and mean chromosome length were synapomorphic for *Nierembergia*. Diploid chromosome complement of 18 and a small st chromosome pair are synapomorphic to *N. ericoides* and *N. scoparia*. NOR number and 45S position did not show a clear pattern, representing independent losses or gains in *Nierembergia* species. Particularly, the number of NORs supports clade B with convergences invoked for *N. linariifolia* var. *glabriuscula* and *N. scoparia* from clade A, respectively, and one reversion in *N. rigida*.

Estimating an ancestral karyotype suggested that 2n = 16 [ML (maximum likelihood) = 100%, BPP = 0.58; Figs 1 and

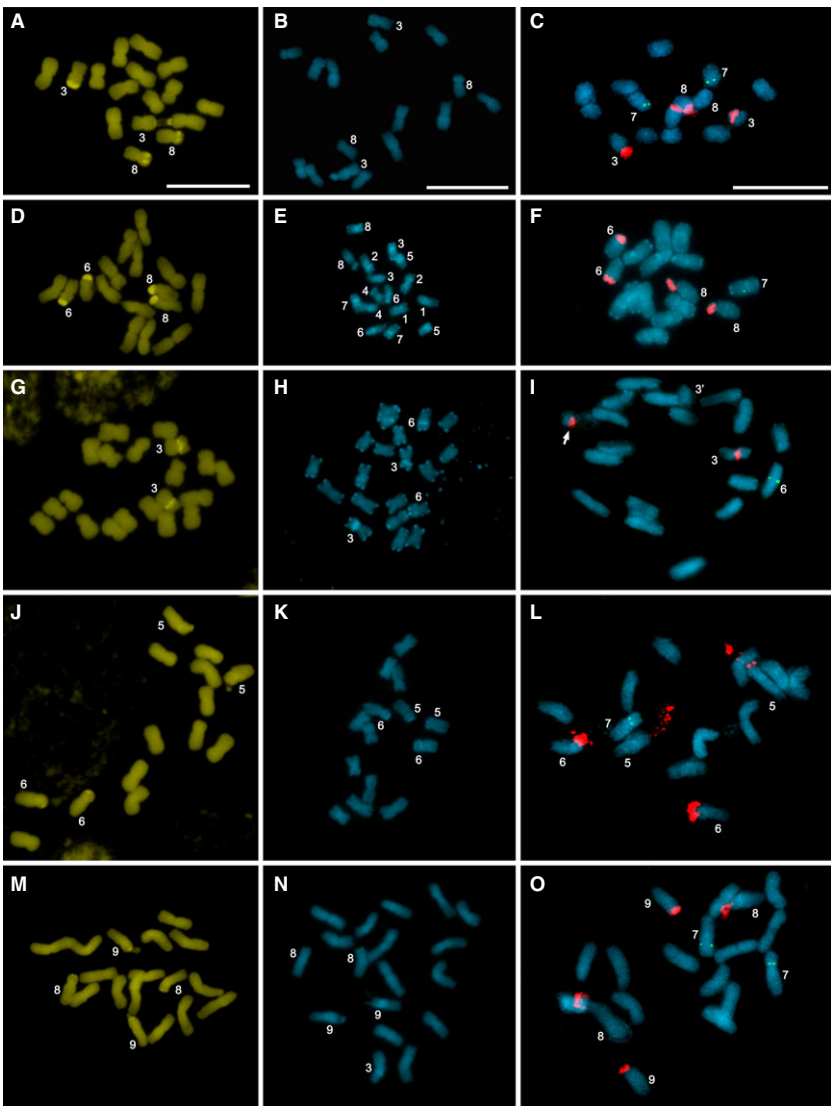

Fig. 3. Somatic metaphases of the studied genera. A, D, G, J, M: double-stained with CMA/DAPI (only CMA fluorescence is shown); B, E, H, K, N: DAPI C-banding; C, F, I, L, O: Double fluorescent *in situ* hybridisation using probes for the 45S and 5S rRNA genes. Red signals indicate hybridisation with the biotin-labelled 45S probe, which was detected with TRITC-conjugated antibodies; green signals indicate hybridisation with the digoxigenin-labelled 5S probe, which was detected with FITC-conjugated antibodies. A–C: *Nierembergia calycina* (2n = 16); D–F: *Bouchetia anomala* (2n = 16), G–I: *Leptoglossis linifolia* (2n = 20), J–L: *Petunia axillaris* (2n = 14), M–N: *Petunia patagonica* (2n = 18). Numbers on chromosomes are according to idiograms of Fig. 1. Scale bar corresponds to 10 μm and is the same for all figures showing the same technique.

S6], only one chromosome pair with NOR in a short arm (ML = 0.61, 100, BPP = 0.42, 0.72, respectively), one asymmetric chromosome pair (msm) (ML = 0.40, BPP = 0.35) and a small heterochromatin amount (only CMA+/DAPI− bands: 11.03%; confidence interval = 10.74–11.31; Fig. S7) are the most likely character states for the common ancestor of *Nierembergia* (see Ancestor N1 of Fig. 1). In addition, these characters are reconstructed for the ancestor of the lineage A (N3), whereas the pericentromeric C bands (ML = 0.90, BPP = 0.97; Fig. S7) and the second NOR (ML = 0.64, BPP = 0.90) and chromosomes smaller (2.37, 97% confidence interval = 2.36–2.38) and more asymmetric (1.27, 97% confidence interval = 1.27–1.28; Fig. S7) are reconstructed for the ancestor (N4) of the lineage B. Finally, it is remarkable that the values estimated for the continuous character using the Bayesian approach fall within the range estimated by parsimony analysis (Fig. S7). The mapping of the geographic distribution shows that the groups A and B correspond to highland (mountain) (ML = 0.87, BPP = 0.59) and lowland clades (Brazilian shield and Paranense sea; ML = 0.30, BPP = 0.45), respectively (Fig. 2).

The Bayesian molecular relaxed clock shows that divergence between the highland and lowland clades occurred approximately 8 million years ago (Ma) (obtained by averaging indirect fossil calibrations, N1; Table S4). Pericentromeric C bands and two NORs have appeared at *ca.* 6.05 (N4) Ma, when chromosomes were smaller and more asymmetric. Finally, the most recent divergences were dated at around 1 Ma (Fig. 1).

DISCUSSION

Classical and molecular cytogenetic practices, such as conventional staining, fluorescent, C- and AgNOR banding method combined with FISH technique using ribosomal RNA gene probes (45S and 5S rDNA) provided useful markers for chromosome identification and valuable data to enhance knowledge about genome organisation and evolution in *Nierembergia*. In addition, this chromosomal information in a phylogenetic and molecular dating framework allows the construction of hypotheses about speciation processes and chromosomal evolution in the genus. Karyotype features presented here show that *Nierembergia* is more related to *Bouchetia* (with the

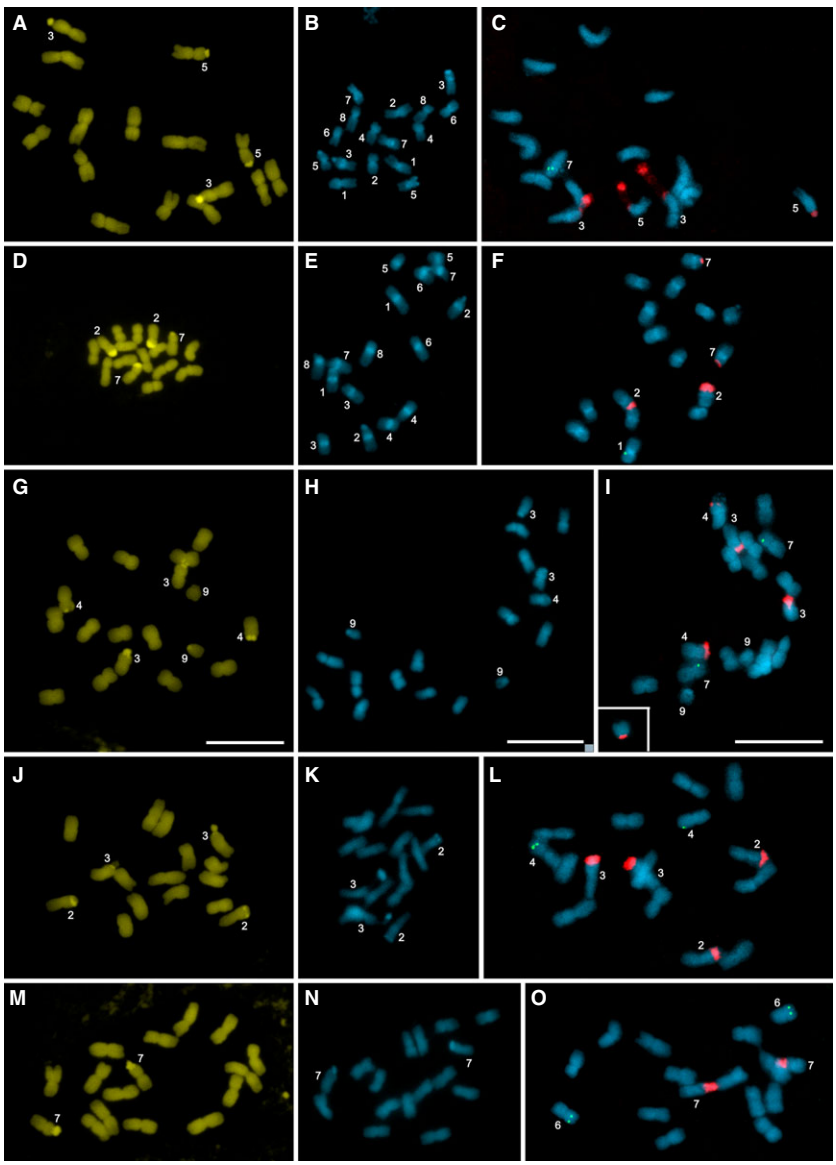

Fig. 4. Somatic metaphases of some *Nierembergia* species studied. A, D, G, J, M: double-stained with CMA/DAPI (only CMA fluorescence is shown); B, E, H, K, N: DAPI C-banding; C, F, I, L, O: Double fluorescent *in situ* hybridisation using probes for the 45S and 5S rRNA genes. Red and green signals indicate hybridisation with the biotin-labelled 45S probe and digoxigenin-labelled 5S probe, respectively. A–C: *N. graveolens* (2n = 16); D–F: *N. veitchii* (2n = 16), G–I: *N. scoparia* (2n = 18), in box, one st (9) chromosome with 45S site belonging to another metaphase is shown. J–L: *N. linariifolia* var. *pinifolioides* (2n = 16), M–O: *N. pulchella* var. *pulchella* (2n = 16). Numbers on chromosomes are according to idiograms of Fig. 1. Scale bar corresponds to 10 μm and is the same for all figures showing the same technique.

same base chromosome number, x = 8), than *Leptoglossis* (x = 10) and *Petunia* (x = 7). Even though *N. ericoides* and *N. scoparia* have the same base chromosome number as *P. patagonica*, it seems to be apomorphic in the genus. In Acosta *et al.* (2006), we suggest that *P. patagonica* could be included in the genus *Calibrachoa*, although a recent molecular work (Reck-Kortmann *et al.* 2015) suggests that *P. patagonica* is close to *Fabiana* (both with x = 9; Moscone 1989; Acosta *et al.* 2006).

Karyotype changes and chromosomal evolution

Earlier comparative karyotype studies of related species have described patterns and directions of chromosomal evolution within a group lacking a rigorous phylogenetic framework. More recently, this problem has been addressed and there are now an increasing number of studies that have analysed karyology based on phylogenetic information (Peruzzi *et al.* 2009). However, few of them have used statistical methods to formalise the comparative cytogenetic studies and to infer ancestral karyotypes (Watanabe *et al.* 1999; Escudero *et al.* 2012; Vaio *et al.* 2013). Here, we reconstructed ancestral karyotypes in *Nierembergia* using chromosome character mapping. The study shows that clades supported by sequence data are also recovered as distinct groups based on cytogenetic and morphological characteristics and geographic distribution. Thus, the clade (A) contains mostly taxa of woody plants with pollen grains shed singly, crescent-shaped stigmas and anthocyanins in the corolla, symmetrical karyotypes, large chromosomes, only one NOR and no centromeric heterochromatin; these taxa (hereafter referred to as highland clade) generally grow in mountainous regions. The clade B includes herbs, with aggregated pollen (except the shrubs *N. graveolens* and *N. repens*, which shed pollen singly) and capitate or broad but not crescent-shaped stigmas, asymmetrical karyotypes, small chromosomes, two chromosomes pairs with NOR and centromeric heterochromatin bands; these species grow in lowland areas (lowland clade). Chromosome characters belonging to the

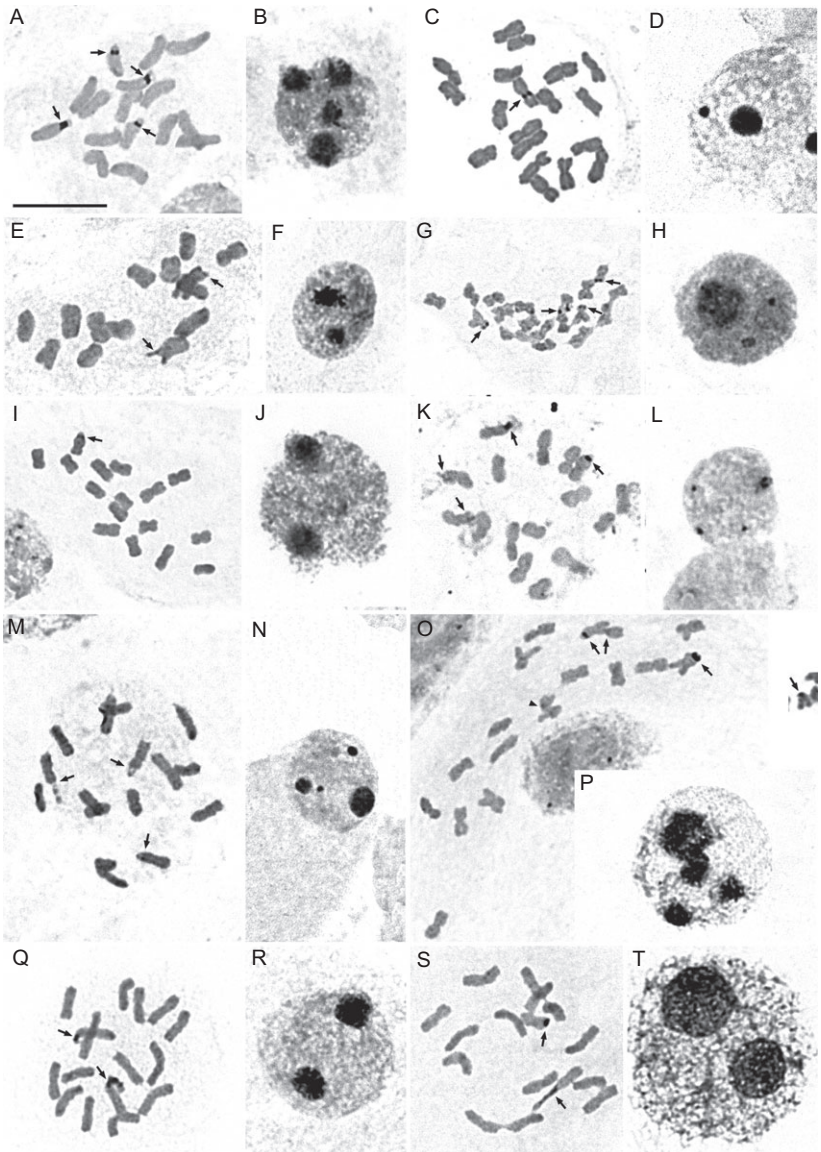

Fig. 5. Somatic metaphases and interphase nuclei after AgNOR banding. A, B: *Bouchetia anomala*; C, D: *Leptoglossis linifolia*; E, F: *Petunia axillaris*; G, H: *Petunia patagonica*; I, J: *Nierembergia calycina*; K, L: *N. graveolens*; M, N: *N. veitchii*; O, P: *N. scoparia*; Q, R: *N. linarifolia* var. *pinifolioides*; S, T: *N. pulchella* var. *pulchella*. Arrows indicate AgNORs. Scale bar corresponds to 10 μm.

highland clade are considered an ancestral condition in *Nierembergia*. The lowland clade, in agreement with its species' life cycle (herbaceous), has (slightly) the highest rate of mutation and the largest number of species (12 from 19; Figs 1 and S2).

The new cytogenetic methodologies applied here have revealed novel insights into the karyological characteristics and karyotype evolution of *Nierembergia*. According to the different banding methods used in the present work, two types of constitutive heterochromatin were observed in *Nierembergia*: chromomycin-positive and DAPI− negative (CMA+/DAPI−) and C-DAPI+ heterochromatin. The studied species have comparatively low CMA+/DAPI− heterochromatin amount exhibited at the satellites (terminal) and some intercalary bands. Heterochromatin type GC-rich loci are coincident with 45S ones and therefore NOR-associated, as observed in most Solanaceae species studied so far (Acosta *et al.* 2012; Miguel *et al.* 2012; Scaldaferro *et al.* 2013; Chiarini *et al.* 2014). It is known that in most plant species, ribosomal DNA is rather GC-rich (Roa & Guerra 2012). Remarkably, lowland *Nierembergia* species (except *N. calycina*) show C-DAPI+ bands in all centromeres. This kind of heterochromatin has been found in other Solanaceae, such as *Cestrum* (Fregonezi *et al.* 2006; Fernandes *et al.* 2009), but never exhibiting the phylogenetically consistent pattern observed here. This constitutive heterochromatin appeared only after performing C-banding or FISH. While C-banding requires a physicochemical pretreatment, base-specific banding of fluorochrome banding depends on the affinity of heterochromatin for a specific fluorochrome (Guerra 2000; Barros e Silva & Guerra 2010). The C-DAPI banding revealed 5S loci in *N. graveolens* and *L. linifolia*. In *Capsicum*, 5S loci were also revealed but as CMA+ bands (Scaldaferro *et al.* 2006, 2013), meaning that the heterocromatin nature of 5S loci differs in Solanaceae. Finally, 45S loci were more variable in number and position between species, whereas 5S loci were more stable and the 5S position had a clear phylogenetic pattern, such as in *N. aristata* and *N. veitchii*, and *N. repens*, *N. rigida* and *N. rivularis*.

The constitutive heterochromatin amount was negatively correlated with karyotype length, which is unexpected because heterochromatin amount may be regarded as an additional component of the genome. Thus, in highland *Nierembergia*,

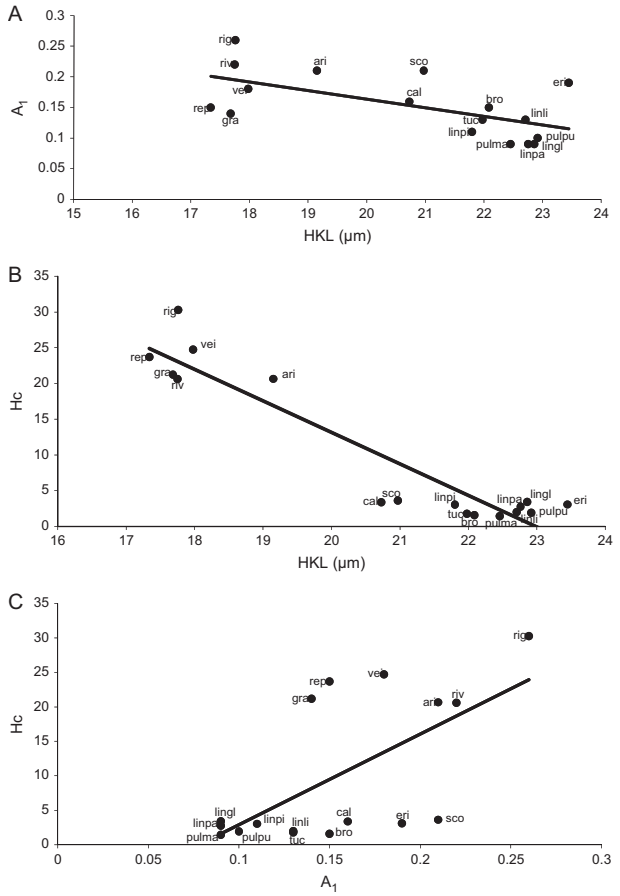

Fig. 6. Correlation analysis between different karyological data in *Nierembergia*. A: Intrachromosomal asymmetry index (A_1) *versus* haploid karyotype length (HKL); r = −0.56, $P = 0.02$. B: Heterochromatin amount (expressed as percentage of HKL) *versus* HKL; r = −0.72, $P < 0.01$. C: Heterochromatin amount *versus* A_1; r = 0.63, $P = 0.01$. Species codes as in Table S1.

chromosomal size must have increased by repeated sequences spreading throughout the complement (dispersed repeated DNA, *i.e.* transposable elements) rather tandemly repeated sequences, mainly asymmetrical in each arm, increased the chromosome symmetry. In contrast, in the lowland clade, chromosomal size seems to have been reduced due to the asymmetric packing of heterochromatin in the chromosome arms. A similar lack of positive correlation between genome size and heterochromatin amount has also been observed in *Cyphomandra* clade and *Solanum* (Pringle & Murray 1993; Acosta *et al.* 2012), but this correlation was present in *Capsicum* L. (Moscone *et al.* 2003; Scaldaferro *et al.* 2013). In addition, genome size and asymmetry indices were negatively correlated, indicating that the genome size increase observed in the highland clade is generally accompanied by decreasing intrachromosomal asymmetry. Such pattern suggests that the additional DNA is not being added uniformly across the karyotype, indicating that an increase in genome size is accompanied by decreasing size differences between chromosome arms of the karyotype (*i.e.* a more symmetrical karyotype). This correlation was also found for some other plants, such as *Fritilaria*, *Tricyrtis*, Lilioideae and Tulipeae (Peruzzi *et al.* 2009). The emergence of C-DAPI heterochomatin and consequent decrease in chromosome size occurred only after 2 Myr of isolation of the *Nierembergia* lineages, whereas other structural changes such as translocations and inversions occurred gradually over 8 Myr. Thus, if chromosome changes were involved in *Nierembergia* diversification, the appearance of C-DAPI heterochromatin probably with a previous geographic isolation (see below) would favour the suppression of recombination and gene flow between the two lineages.

Molecular dating and times of speciation

We used two independent sources of calibration, the fossil record and the previously calculated rate of mutation (for nuclear ITS and chloroplast DNA; Hewitt 2001; Kay *et al.* 2006). We decided to discuss our results with the average ages obtained with fossil calibration. Even though the fossil record used is ancient, we consider that it is more appropriate than the use of mutation rate derived from other studies, because in the latter case it is assumed that the mutation rate is the same in all lineages. The calibration rate calculated for each dataset using a known date of divergence is more successfully used to estimate the rate for the whole phylogeny, because rates of change can vary between taxa and between genes (Bromham & Penny 2003).

Dates obtained here are comparable with those reported by Särkinen *et al.* (2013) with respect to the ages obtained in the stem node of Solanaceae (*ca.* 49 *versus* 51 Ma in the present work; see Fig. S1) and for the split between *Leptoglossis*, *Bouchetia* and *Nierembergia* (*ca.* 17 and 13 Ma *versus* 19 and 15–14 Ma, see additional file 2 from Särkinen *et al.* 2013 and Figs S1 and 1 in this work, respectively). However, *Nierembergia* tree and ages of genus diversification disagree completely with their work, even though we used the same ITS sequences (obtained from Tate *et al.* 2009). In that work, they discussed only the relationships with major Solanaceae clades and encourage readers to refer back to available clade-specific studies for detailed species-level phylogenies. Notably, a recent work about oil bees dated the crown of *Nierembergia* at 8 Ma (Martins *et al.* 2015), which is in agreement with our molecular dating analysis.

Historical climate changes have had a major effect on the distribution and evolution of plant species. Distinct points of views concerning the origin of diversification of current species are still under debate. The controversy is if relatively recent Pleistocene climatic changes have driven speciation, or current species diversity is more ancient (Pennington *et al.* 2004). Until the 19th century, species diversity in biomes of South America, such as Amazonia, was attributed to Pleistocene refuges. However, more recently, an older origin for the Amazonia diversity has been proposed (Hoorn *et al.* 2010). Moreover, deep intraspecific divergences have been proposed for some South American lineages such as *Nothofagus* (Premoli *et al.* 2012; Acosta *et al.* 2014), which pre-date Pleistocene epochs. Although links between the Andean orogeny and neotropical diversification have long been suggested, only recently have researchers started to explore dated phylogenetic trees, in combination with more realistic, complex geological scenarios (Hoorn *et al.* 2010).

Molecular dating on the DNA phylogeny revealed that *Nierembergia* diverged in two clades probably after the mid-Miocene (8 Ma), during Atlantic marine ingressions called the 'Paranense Sea' (Aceñolaza 2000; Marengo 2000; Hernández *et al.* 2005; Acosta *et al.* 2013). This marine transgression filled the Pampean and Mesopotamic region spanning from 'Sierras

Pampeanas' to 'Chaco Paraguayo' and South Bolivia from the Middle Miocene to the Late Miocene–Pliocene (15 to 5.33–3.6 Ma; Aceñolaza 2000; Cione *et al.* 2011). Some authors have proposed that a sea strait crossed South America, even reaching the Caribbean Sea (Aceñolaza 2000). This event probably forced ancestors of terrestrial organisms to find refuge on emerging and more stable highlands of the Brazilian shield. For *Nierembergia*, the ancestor of the lowland clade probably grew on the Brazilian shield, which is currently inhabited by two species exhibiting several ancestral characteristics (Fig. 2): *N. calycina* (with intermediate characters between clades) and *N. graveolens* (with chromosomal characters similar to *B. anomala*). In addition, *N. calycina*, *N. graveolens* and *B. anomala* share the 5S chromosomal position. These results are consistent with the notion that geologically older and more stable areas of Amazonia (such as the Brazilian shields and mountains) functioned as 'species-pumps', whereas geologically more dynamic areas (such as the western Amazonian lowlands) mostly 'captured' part of the diversity generated nearby, indicating a more recent history of the biota in this regions (Aleixo & Rosetti 2007). The floristic distinctiveness of this region was recognised in an early work from Rambo (1954), who hypothesised that elevated metamorphic formations, such us mountainous regions from Rio Grande do Sul, Uruguay, Córdoba and Buenos Aires Hills in Argentina, may have been isolated by marine ingressions in the past (Speranza *et al.* 2007).

During the Pliocene and Pleistocene, most of the living *Nierembergia* species diversified. The Andean Orogeny and Sierras Pampeanas uplift are associated with deformation phases that occurred from the Miocene to the Pleistocene (20–2 Ma; Ramos *et al.* 2002); this event created new habitats for species colonisation and diversification. In addition, once the Paranense Sea receded, probably as a result of the uplift of the mountains, the extended flooded area originated plains, spanning from northern Patagonia to northern Argentina and along the eastern slopes of the rising Andes of northwest Argentina, northern Bolivia, southern Peru and Venezuela. This period is known as 'the Age of the Southern Plains' (ASP; Ortiz-Jaureguizar & Cladera 2006). During the ASP, the climate was supposedly cooler, and had a stronger seasonality than in the Mid-Miocene. The rising of the eastern Sierras Pampeanas led to the rain-shadow effect that transformed the occidental regions into the current semi-deserts (see Ortiz-Jaureguizar & Cladera 2006 and references therein). Finally, the Great Patagonian Glaciations produced an extreme aridisation in zones far away from glaciers about 2–1 Ma (Ab'Sáber 1977; Rabassa & Coronato 2009). Thus, the subsequent *Nierembergia* speciation could be the result of the uplift of mountainous regions and the isolation of ancestral lineages in warmer and wetter refuges and adaptations to arid habitats.

CONCLUSION

The geographic isolation of the ancestor of *Nierembergia* species as a consequence of the marine ingression called the Paranense Sea could have produced the split of lineages within the genus and the presence of new chromosomal characteristics that have favoured the suppression of gene flow and promoted diversification. Then, each linage might have undergone speciation separately in response to the Andean Orogeny and Sierras Pampeanas uplift, and the increase of aridity and cold with relatively minor changes in chromosomal characteristics. Finally, the renaissance of cytogenetics depends on the implementation of modern techniques, incorporating statistical analysis methods to test hypotheses (*e.g.* from the phylogeny), and the use of cytogenetics only as a tool to answer evolutionary and ecological questions, among others.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Chronogram showing relationships between species of Solanaceae and the ages of divergences obtained using sequences of ndhF.

Figure S2. Chronogram showing relationships between species of *Nierembergia* and ages of divergences obtained using rpl 16 and ITS sequence data.

Figure S3. Somatic metaphases of some *Nierembergia* species studied (2n = 16).

Figure S4. Somatic metaphases of some *Nierembergia* species studied.

Figure S5. Somatic metaphases and interphase nuclei after AgNOR banding.

Figure S6. Ancestral state reconstruction of chromosome data for the studied *Nierembergia* species.

Figure S7. Ancestral state reconstruction of chromosome data for the studied *Nierembergia* species.

Table S1. List of species studied, code, provenance and voucher specimen.

Table S2. Karyotype features of *Bouchetia, Leptoglossis, Nierembergia* and *Petunia*, obtained with conventional staining (Tate *et al.* 2009) and AgNOR banding.

Table S3. Karyotype features of *Bouchetia, Leptoglossis, Nierembergia* and *Petunia* using fluorescent chromosome banding, C-DAPI banding and fluorescent *in situ* hybridisation (FISH).

Table S4. Bayesian relaxed molecular clock age estimates (Ma) for *Nierembergia* phylogeny according to different calibration scenarios using BEAST.

REFERENCES

Ab'Sáber A.N. (1977) Espacos ocupados pela expansao dos climas secos na America do Sul, por ocasiao dos períodos glaciais quaternários. *Paleoclimas*, **3**, 1–19.

Aceñolaza F.G. (2000) La Formación Paraná (Mioceno medio): estratigrafia, distribución regional y unidades equivalentes. In: Aceñolaza F.G., Herbst R. (Eds), *El Neógeno de Argentina, Serie Correlación Geológica*. INSUGEO, Tucumán, Argentina, Vol. **14**, pp 9–27.

Acosta M.C., Moscone E.A. (2011) B chromosomes in *Nierembergia aristata* (Solanaceae): nucleolar activity and competition with the A chromosomes. *Cytogenetic and Genome Research*, **132**, 105–112.

Acosta M.C., Ordoñez A.D.V., Cocucci A.A., Moscone E.A. (2006) Chromosome reports in South American Nicotianeae (Solanaceae) with particular reference to *Nierembergia*. *Annals of Missouri Botanical Garden*, **93**, 634–646.

Acosta M.C., Guerra M., Moscone E.A. (2012) Karyological relationships among some South American species of *Solanum* (Solanaceae) based on fluorochrome banding and nuclear DNA amount. *Plant Systematics and Evolution*, **298**, 1547–1556.

Acosta M.C., Moscone E.A., Cocucci A.A. (2013) *Patterns of chromosomal evolution in Solanaceae: a phylogenetic approach*. IV Simposio Latinoamericano de Citogenética y Evolución, Guarujá, Sao Pablo, Brazil.

Acosta M.C., Mathiasen P., Premoli A.C. (2014) Retracing the evolutionary history of *Nothofagus* in its geo-climatic context: new developments in the emerging field of phylogeology. *Geobiology*, **12**, 497–510.

Aleixo A., Rosetti D.D.F. (2007) Avian gene trees, landscape evolution, and geology: towards a modern synthesis of Amazonian historical biogeography? *Journal of Ornithologhy*, **148**, S443–S453.

Barros e Silva A.E., Guerra M. (2010) The meaning of DAPI bands observed after C-banding and FISH procedures. *Biotechnic and Histochemistry*, **85**, 115–125.

Bennett S.T., Leitch I.J., Bennet M.D. (1995) Chromosome identification and mapping in the grass *Zingeria biebersteiniana* (2n = 4) using fluorochromes. *Chromosome Research*, **3**, 101–108.

Bloom S.E., Goodpasture C. (1976) An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. *Human Genetics*, **34**, 199–206.

Bromham L., Penny D. (2003) The modern molecular clock. *Nature Reviews Genetics*, **4**, 216–224.

Chiarini F.E., Santiñaque F.F., Urdampilleta J.D., Las Peñas M.L. (2014) Genome size and karyotype diversity in *Solanum* sect. *Acanthophora* (Solanaceae). *Plant Systematics and Evolution*, **300**, 113–125.

Cione A.L., Cozzuol M.A., Dozo M.T., Acosta Hospitaleche C. (2011) Marine vertebrate assemblages in the southwest Atlantic during the Miocene. *Biological Journal of the Linnean Society*, **103**, 423–440.

Cocucci A.A. (1991) Pollination biology of *Nierembergia* (Solanaceae). *Plant Systematics and Evolution*, **174**, 17–35.

Cocucci A.A., Hunziker A.T. (1995) Nierembergia. In: Hunziker A.T. (Ed), *Flora Fanerogámica Argentina. 256 Solanaceae, parte 1. Subtr. VIIb. Nierembergiinae. Fasc*. Vol. **15**, pp 3–14.

Collinson M.E., Boulter M.C., Holmes P.R. (1993) Magnoliophyta ("Angiospermae"). In: Benton M. J. (Ed), *The fossil record 2*. Chapman & Hall, London, UK, pp 809–841.

Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., Tablada M., Robledo C.W. (2012) InfoStat versión 2012, Grupo InfoStat, FCA. Universidad Nacional de Córdoba, Argentina. Available from http://www.infostat.com.ar

Dobigny G., Ducroz J.-F., Robinson T.J., Volobouev V. (2004) Cytogenetics and cladistics. *Systematic Biology*, **53**, 470–484.

Donato M., Posadas P., Miranda-Esquivel D.R., Ortiz Jaureguizar E., Cladera G. (2003) Historical biogeography of the Andean region: evidence from Listroderina (Coleoptera: Curculionidae: Rhytirrhinini) in the context of the South American geobiotic scenario. *Biological Journal of the Linnean Society*, **80**, 339–352.

Drummond A.J., Rambaut A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.

Enke N., Kunze R., Pustahija F., Glöckner G., Zimmermann J., Oberländer J., Kamari G., Siljak-Yakovlev S. (2015) Genome size shifts: karyotype evolution in *Crepis* section *Neglectoides* (Asteraceae). *Plant Biology*, **17**, 775–786.

Escudero M., Hipp A.L., Waterway M.J., Valente L.M. (2012) Diversification rates and chromosome evolution in the most diverse angiosperm genus of the temperate zone (Carex, Cyperaceae). *Molecular Phylogenetics and Evolution*, **63**, 650–655.

Fernandes T., Rego L.N.A.A., Nardy M., Yuyama P.M., Vanzela A.L.L. (2009) Karyotype differentiation of four *Cestrum* species (Solanaceae) revealed by fluorescent chromosome banding and FISH. *Genetics and Molecular Biology*, **32**, 320–327.

Franco M.J., Brea M. (2008) Leños fósiles de la Formación Paraná (Mioceno Medio), Toma Vieja, Paraná, Entre Ríos, Argentina: registro de bosques estacionales mixtos. *Ameghiniana*, **45**, 699–717.

Fregonezi J.N., Fernandes T., Torezan J.M.D., Vieira A.O.S., Vanzela A.L.L. (2006) Karyotype differentiation of four *Cestrum* species (Solanaceae) based on the physical mapping of repetitive DNA. *Genetics and Molecular Biology*, **29**, 97–104.

Greilhuber J., Speta F. (1976) C-banded karyotypes in the *Scilla hohenackeri* group, *S. persica*, and *Puschkinia* (Liliaceae). *Plant Systematics and Evolution*, **126**, 149–188.

Guerra M. (2000) Patterns of heterochromatin distribution in plant chromosomes. *Genetics and Molecular Biology*, **23**, 1029–1041.

Guerra M. (2012) Cytotaxonomy: the end of childhood. *Plant Biosystems*, **146**, 703–710.

Hernández R.M., Jordan T.E., Dalenz Farjat A., Echavarría L., Idleman B.D., Reynolds J.H. (2005) Age, distribution, tectonics, and eustatic controls of the Paranense and Caribbean marine transgressions in southern Bolivia and Argentina. *Journal of South American Earth Science*, **19**, 495–512.

Hewitt G.M. (2001) Speciation, hybrid zones and phylogeography – or seeing genes in space and time. *Molecular Ecology*, **10**, 537–549.

Hoorn C., Wesselingh F.P., ter Steege H., Bermudez M.A., Mora A., Sevink J., Sanmartín I., Sanchez-Meseguer A., Anderson C.L., Figueiredo J.P., Jaramillo C., Riff D., Negri F.R., Hooghiemstra H., Lundberg V., Stadler T., Särkinen T., Antonelli A. (2010) Amazonia through time: Andean uplift, climate change, landscape evolution, and biodiversity. *Science*, **330**, 927–931.

Huelsenbeck J.P., Ronquist F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754–755.

Jones K. (1970) Chromosome changes in plant evolution. *Taxon*, **19**, 172–179.

Kay K.M., Whittall J.B., Hodges S.A. (2006) A survey of nuclear ribosomal internal transcribed spacer substitution rates across angiosperms: an approximate molecular clock with life history effects. *BMC Evolutionary Biology*, **6**, 36.

Kodama Y., Yoshida M.C., Sasaki M. (1980) An improved silver staining technique for nucleolus organizer regions by using nylon cloth. *Japanese Journal of Human Genetics*, **25**, 229–233.

Kumar S., Tamura K., Nei M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150–163.

Livingstone K., Rieseberg L. (2003) Chromosomal evolution and speciation: a recombination-based approach. *New Phytologist*, **161**, 107–112.

Maddison W.P., Maddison D.R. (2007) Mesquite: a modular system for evolutionary analysis, version 2.0. Available from http://mesquiteproject.org.

Marengo H. (2000) Rasgos micropaleontológicos de los depósitos de la transgresión Entrerriense-Paranense en la Cuenca Chaco-paranense y Noroeste Argentino, República Argentina. In: Aceñolaza F.G., Herbst R. (Eds) *El Neógeno de Argentina, Serie Correlación Geológica*. INSUGEO, Tucumán, Argentina, Vol. **14**, pp 29–45.

Martins A.C., Melo G.A., Renner S.S. (2015) Gain and loss of specialization in two oil-bee lineages, *Centris* and *Epicharis* (Apidae). *Evolution*, **69**, 1835–1844.

Miguel V., Acosta M.C., Moscone E.A. (2012) Karyotype analysis in two species of *Solanum* (Solanaceae) sect. *Cyphomandropsis* based on chromosome banding. *New Zealand Journal of Botany*, **50**, 217–225.

Moscone E.A. (1989) Karyotype analyses in three Patagonian and S. Andean endemic genera of Nicotianeae (Solanaceae). *Plant Systematics and Evolution*, **166**, 31–39.

Moscone E.A., Matzke M.A., Matzke A.J.M. (1996) The use of combined FISH/GISH in conjunction with DAPI counterstaining to identify chromosomes containing transgene inserts in amphidiploid tobacco. *Chromosoma*, **105**, 231–236.

Moscone E.A., Baranyi M., Ebert I., Greilhuber J., Ehrendorfer F., Hunziker A.T. (2003) Analysis of nuclear DNA content in *Capsicum* (Solanaceae) by flow cytometry and Feulgen densitometry. *Annals of Botany*, **92**, 21–29.

Nylander J.A. (2004) *MrModelTest*. Program Distributed by the Author. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

O'Brien S.J., Menotti-Raymond M., Murphy W.J., Nash W.G., Wienberg J., Stanyon R., Copeland N.G., Jenkins N.A., Womack J.E., Marshall Graves J.A. (1999) The promise of comparative genomics in mammals. *Science*, **286**, 458–481.

Olmstead R.G., Bohs L., Migid H.A., Santiago-Valentin E., Garcia V.F., Collier S.M. (2008) A molecular phylogeny of the Solanaceae. *Taxon*, **57**, 1159–1181.

Ortiz-Jaureguizar E., Cladera G.A. (2006) Paleoenvironmental evolution of southern South America during the Cenozoic. *Journal of Arid Environments*, **66**, 498–532.

Pagel M., Meade A., Barker D. (2004) Bayesian estimation of ancestral character states on phylogenies. *Systematic Biology*, **53**, 673–684.

Pennington R.T., Lavin M., Prado D.E., Pendry C.A., Pell S.K., Butterworth C.A. (2004) Historical climate change and speciation: neotropical seasonally dry forest plants show patterns of both Tertiary and Quaternary diversification. *Philosophical Transactions of the Royal Society B*, **359**, 515–537.

Peruzzi L., Leitch I.J., Caparelli K.F. (2009) Chromosome diversity and evolution in Liliaceae. *Annals of Botany*, **103**, 459–475.

Pires J.C., Hertweck K.L. (2008) A renaissance of cytogenetics: studies in polyploidy and chromosomal evolution. *Annals of Missouri Botanical Garden*, **95**, 275–281.

Premoli A.C., Mathiasen P., Acosta M.C., Ramos V.A. (2012) Phylogeographically concordant chloroplast DNA divergence in sympatric *Nothofagus s.s.* How deep can it be? *New Phytologist*, **193**, 261–275.

Pringle G.J., Murray B.G. (1993) Karyotypes and C-banding patterns in species of *Cyphomandra* Mart. ex Sendtner (Solanaceae). *Botanical Journal of the Linnean Society*, **111**, 331–342.

Rabassa J., Coronato A. (2009) Glaciations in Patagonia and Tierra del Fuego during the Ensenadan Stage/Age (Early Pleistocene–earliest Middle Pleistocene). *Quaternary International*, **210**, 18–36.

Rambo B. (1954) Análise histórica da flora de Pôrto Alegre. *Sellowia*, **6**, 9–112.

Ramos V.A., Cristallini E.O., Pérez D.J. (2002) The Pampean flat-slab of the Central Andes. *Journal of South American Earth Sciences*, **15**, 59–78.

Reck-Kortmann M., Silva-Arias G.A., Stehmann J.R., Greppi J.A., Freitas L.B. (2015) Phylogenetic relationships of *Petunia patagonica* (Solanaceae) revealed by molecular and biogeographical evidence. *Phytotaxa*, **222**, 17–32.

Rieseberg L.H. (2001) Chromosomal rearrangements and speciation. *Trends in Ecology & Evolution*, **16**, 351–358.

Rieseberg L.H., Willis J.L. (2007) Plant speciation. *Science*, **317**, 910–914.

Roa F., Guerra M. (2012) Distribution of 45S rDNA sites in chromosomes of plants: structural and evolutionary implications. *BMC Evolutionary Biology*, **12**, 225.

Robinson T.J., Yang F. (2012) Molecular cytogenetics: karyotype evolution, phylogenomics and future prospects. *Heredity*, **108**, 1–3.

Robledo G., Seijo G. (2010) Species relationships among the wild B genome of *Arachis* species (section *Arachis*) based on FISH mapping of rDNA loci and heterochromatin detection: a new proposal for genome arrangement. *Theoretical and Applied Genetics*, **121**, 1033–1046.

Rodríguez F., Oliver J.L., Marín A., Medina J.R. (1990) The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology*, **142**, 485–501.

Särkinen T., Bohs L., Olmstead R.G., Knapp S. (2013) A phylogenetic framework for evolutionary study of the nightshades (Solanaceae): a dated 1000-tip tree. *BMC Evolutionary Biology*, **13**, 214.

Scaldaferro M.A., Seijo J.G., Acosta M.C., Barboza G.E., Ducasse D.A., Moscone E.A. (2006) Genomic characterization of the germplasm in peppers (*Capsicum* – Solanaceae) by fluorescent *in situ* hybridization. *Plant Science*, **43**, 291–297.

Scaldaferro M.A., Grabiele M., Moscone E.A. (2013) Heterochromatin type, amount and distribution in wild species of chili peppers (*Capsicum*, Solanaceae). *Genetic Resources and Crop Evolution*, **60**, 693–709.

Schmidt T., Schwarzacher T., Heslop-Harrison J.S. (1994) Physical mapping of rRNA genes by fluorescent *in-situ* hybridization and structural analysis of 5S rRNA genes and intergenic spacer sequences in sugar beet (*Beta vulgaris*). *Theoretical and Applied Genetics*, **88**, 629–636.

Schubert I., Lysak M.A. (2011) Interpretation of karyotype evolution should consider chromosome structural constraints. *Trends in Genetics*, **27**, 207–216.

Schwarzacher T., Ambros P., Schweizer D. (1980) Application of Giemsa banding to orchid karyotype analysis. *Plant Systematics and Evolution*, **134**, 293–297.

Schweizer D. (1980) Simultaneous fluorescent staining of R bands and specific heterochromatic regions (DA-DAPI bands) in human chromosomes. *Cytogenetics and Cell Genetics*, **27**, 190–193.

Sokal R.R., Rohlf F.J. (1995) *Biometry, the principles and practice of statistics in biological research*, 3rd edition. W.H. Freeman, New York, NY, USA.

Speranza P.R., Seijo J.G., Grela I.A., Solís Neffa V.G. (2007) Chloroplast DNA variation in the *Turnera sidoides* L. complex (Turneraceae): biogeographical implications. *Journal of Biogeography*, **34**, 427–436.

Tate J.A., Acosta M.C., McDill J., Moscone E.A., Simpson B.B., Cocucci A.A. (2009) Phylogeny and character evolution in *Nierembergia* (Solanaceae): molecular, morphological, and cytogenetic evidence. *Systematic Botany*, **34**, 198–206.

Vaio M., Gardner A., Emshwiller E., Guerra M. (2013) Molecular phylogeny and chromosome evolution among the creeping herbaceous *Oxalis* species of sections *Corniculatae* and *Ripariae* (Oxalidaceae). *Molecular Phylogenetics and Evolution*, **68**, 199–211.

Wanzenböck E.-M., Schöfer C., Schweizer D., Bachmair A. (1997) Ribosomal transcription units integrated via T-DNA transformation associate with the nucleolus and do not require upstream repeat sequences for activity in *Arabidopsis thaliana*. *The Plant Journal*, **11**, 1007–1016.

Watanabe K., Yahara T., Denda T., Kosuge K. (1999) Chromosomal evolution in the genus *Brachyscome* (Asteraceae, Astereae): statistical tests regarding correlation between changes in karyotype and habit using phylogenetic information. *Journal of Plant Research*, **112**, 145–161.