Genome size correlates with growth form, habitat and phylogeny in the Andean genus *Lasiocephalus* (*Asteraceae*)

Velikost genomu andského rodu *Lasiocephalus* (*Asteraceae*) souvisí s životní formou, ekologií a fylogenezí

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Variation in genome size in a particular taxonomic group can reflect different evolutionary processes including polyploidy, hybridization and natural selection but also neutral evolution. Using flow cytometry, karyology, ITS sequencing and field surveys, the causes of variation in genome size in the ecologically and morphologically diverse high-Andean genus *Lasiocephalus* (*Asteraceae*, *Senecioneae*) were examined. There was a 1.64-fold variation in holoploid genome size (C-values) among 189 samples belonging to 20 taxa. The most distinct was a group of plants with large genomes corresponding to DNA triploids. Disregarding the DNA triploids, the remaining samples exhibited a pronounced (up to 1.32-fold) and rather continuous variation. Plants with the smallest genomes most likely represent intergeneric hybrids with the closely related and sympatric *Culcitium nivale,* which has a smaller genome than *Lasiocephalus.* The variation in genome size in samples of diploid *Lasiocephalus* was strongly correlated with several environmental and life history traits (altitude, habitat and growth form). However, all these factors, as well as genome size itself, were correlated with phylogeny (main split into the so-called 'forest' and 'páramo' clades), which most probably represents the true cause of the differentiation in intrageneric genome size. In contrast, relationships between genome size and phylogeny were not apparent at lower divergence levels. Instead, here we suggest that ecological conditions have played a role in driving shifts in genome size between closely related species inhabiting different environments. Collectively, this study demonstrates that various evolutionary forces and processes have shaped the variation in genome size and indicates that there is a need for multi-approach analyses when searching for the causes and consequences of changes in genome size.

K e y w o r d s: Andes, *Compositae*, flow cytometry, genome size, growth form, habitat preference, hybridization, *Lasiocephalus*, nuclear DNA amount, nucleotypic effect, phylogeny, polyploidy

Introduction

One of the current challenging questions in plant evolutionary biology is the functional significance of the approximately 2000-fold variation in genome size among angiosperms (Leitch & Bennett 2007). This tremendous variation is largely caused by different proportions of non-coding repetitive DNA, such as transposable elements, satellite DNA, introns,

and pseudogenes (Bennett & Leitch 2005). Several mechanisms, both at the chromosomal and molecular levels, are thought to be capable of generating genome size changes (Bennetzen 2005). Polyploidy, chromosome gain, presence of supernumerary or sex chromosomes, amplification of retrotransposons and genomic duplications are included among the processes that cause genome expansion. Counterbalancing mechanisms involve the loss of entire chromosomes, illegitimate recombination, unequal intrastrand homologous recombination or a higher overall rate of nucleotide deletion over insertion (reviewed in Bennett & Leitch 2005). While chromosome-level events typically cause sudden and marked changes in genome size, molecular mechanisms are more gradual with each event producing only slight modifications in genome size.

Variation in genome size has been interpreted in relation to various phenotypic and/or life-history traits. Classical observations documenting a positive relationship between genome size and cell size and duration of cell division (reviewed by Leitch & Bennett 2007) were followed by studies that revealed correlations between genome size and seed mass, leaf mass per unit area, growth rate and/or photosynthetic rate (Knight & Beaulieu 2008). In addition, genome size can also have significant consequences at the ecological level, influencing the range of environmental conditions a plant can tolerate (the so-called "large genome constraint hypothesis"; Knight et al. 2005). Over the years, relationships between genome size and temperature, water availability, latitude and altitude of the habitats have been reported (reviewed by Knight & Ackerly 2002). However, variation in genome size may not always be adaptive as documented for instance in a highly variable population of *Festuca pallens* (Šmarda et al. 2007).

Recent studies have also emphasized the importance of phylogeny in variation in genome size. For example, genome size in *Hieracium* subg. *Hieracium* was strongly correlated with the two major phylogenetic groups (Chrtek et al. 2009) and congruency between genome size and evolutionary lineages is also found in the holoparasitic *Orobanche* (Weiss-Schneeweiss et al. 2005). If phylogeny is not considered, the role of other factors (e.g. environmental conditions) in shaping genome size may be overemphasized. Therefore, when searching for the causes of variation in genome size, both adaptive and non-adaptive components need to be taken into account.

Knowledge of the patterns and dynamics of variation in genome size is largely based on the analysis of European and North American floras, and species from the world's main centres of plant diversity are neglected. One of the geographic regions that hosts an extremely-rich plant biota is the Andes of South America, which rank among the most conspicuous physiographic features on Earth (Vuilleumier & Monasterio 1986, Luteyn 1999, Young et al. 2002). Andean plant diversity largely resulted from the adaptive radiation of numerous plant groups, such as *Espeletia*, *Huperzia*, *Lupinus* and *Valeriana* (Cuatrecasas 1986, Wilkström et al. 1999, Bell & Donoghue 2005, Hughes & Eastwood 2006). Most researchers relate the adaptive radiation to the final uplift of the mountains and emergence of high-altitude non-forest habitats (páramo) about 5–3 mya and the Plio-Pleistocene climatic oscillations. Although there is an increasing number of phylogenetic studies on Andean plants (e.g. Young et al. 2002, Struwe et al. 2009) the role of genome-wide evolutionary processes (such as polyploidization and genome size change) in species radiation is largely unknown.

Lasiocephalus Willd. ex Schltdl. (*Asteraceae*, *Senecioneae*) is a neotropical high-Andean genus of ca 30 species distributed from Venezuela to Bolivia, with the highest species

richness in Ecuador (Cuatrecasas 1978). Two main growth forms are recognized in the genus: (i) the broad-leaved suffrutescent climbers of montane forests and the tree-line ecoton, and (ii) erect and ascending narrow-leaved herbaceous plants to subshrubs of the high-altitude páramo. These growth forms plausibly reflect adaptive changes that occurred during the colonization of the páramo habitats (Cuatrecasas 1978). It is likely that *Lasiocephalus* is a monophyletic group nested within *Senecio* s. str. with the high-Andean genus *Culcitium* as a sister taxon (Pelser et al. 2007). The high number of species along with the variation in growth forms and their altitudinal distribution make *Lasiocephalus* a suitable candidate for studying the evolutionary outcomes associated with adaptive radiation.

We use *Lasiocephalus* as a representative example of a group that has radiated in the Andes to address general questions concerning the extent and possible sources of the variation in DNA content. Flow cytometry, karyology and ITS sequencing were employed to address the following specific questions: (i) Does genome duplication play a role in the diversification of *Lasiocephalus*? Which cytotypes are found in this genus? Are there any mixed-ploidy populations? (ii) What is the variation in genome size (C- and Cx-values)? (iii) How does the observed pattern in DNA ploidy and variation in genome size correlate with growth forms and habitat preferences? (iv) How does the observed pattern in DNA ploidy and variation in genome size correlate with the phylogeny of the group?

Materials and methods

Plant material

Plants were collected between 2007 and 2009 in Bolivia, Ecuador and Venezuela (see Electronic Appendix 1 for locality details). In total, 189 individuals from 101 populations corresponding to 20 species of *Lasiocephalus* sensu Cuatrecasas (1978) were sampled (Table 1). Species were identified according to Cuatrecasas (1978) and by comparison with herbarium vouchers deposited at AAU, COL, G, MERF, NY, P, PRC, QCA, S and VEN. Seven distinct morphotypes of *Lasiocephalus*, which could not be assigned to any hitherto described species, were treated as separate morphospecies and referred to as *Lasiocephalus* sp. 1 to 7 (Table 1). GPS coordinates and altitude (WGS 84 system), habitat type and growth form of the plants were recorded at each locality. Young intact leaves were collected and quickly desiccated for DNA analyses using silica gel. The following plant material was sampled for flow cytometry: (i) fresh tissue (stems and leaves) of adult plants stored in plastic bags at 5-8 °C until analyses (up to 14 days), (ii) silica-dried leaf tissue of adult plants, and (iii) achenes from which seedlings were raised. In addition, three samples of closely related and often sympatric *Culcitium nivale* were also collected and analyzed for comparative purposes. Herbarium vouchers are deposited in PRC, QCA, QCNE and VEN.

Flow cytometry

DNA ploidy levels (Suda et al. 2006) were determined using flow cytometry and a Partec PA II instrument (Partec GmbH) equipped with a HBO mercury arc lamp. Genome sizes (C- and Cx-values; Greilhuber et al. 2005) were determined using CyFlow SL instrument (Partec GmbH, Münster, Germany) equipped with a green solid-state laser (Cobolt Samba, 532 nm, 100 mW). Sample preparation generally followed the simplified two-step proce-

Table 1. – Nuclear DNA contents of 13 species and seven unidentified morphospecies of *Lasiocephalus* and the closely related *Culcitium nivale*. Samples analysed = no. of populations/no. of individuals. Values are mean±SD. *Vicia faba* 'Inovec' (2C = 26.9 pg) served as an internal reference standard. In the Grouping columns, different letters indicate groups of taxa that are significantly different at $\alpha = 0.05$ using Tukey's HSD multiple comparison

Species	Genome size ('fresh' dataset)		
	Samples analysed	2C-value (pg of DNA)	Grouping
Lasiocephalus campanulatus (Sch. Bip. ex Klatt) Cuatrec.	–	–	–
Lasiocephalus cuencanus (Hieron.) Cuatrec.	–	–	–
Lasiocephalus decipiens (Benoist) Cuatrec.	2/2	16.10±0.08	ef
Lasiocephalus heterophyllus (Turcz.) Cuatrec.	–	–	–
Lasiocephalus involucratus (Kunth) Cuatrec.	5/5	15.63±0.10	def
Lasiocephalus lingulatus Schltdl. – 2x	3/3	15.07±0.15	bc
Lasiocephalus lingulatus Schltdl. – 3x	1/1	22.69	–
Lasiocephalus longipenicillatus (Sch. Bip. ex Sandw.) Cuatrec.	–	–	–
Lasiocephalus mojandensis (Hieron.) Cuatrec.	–	–	–
Lasiocephalus otophorus (Wedd.) Cuatrec.	3/3	16.10±0.47	f
Lasiocephalus ovatus Schltdl. – 2x	3/3	14.99±0.04	bc
Lasiocephalus ovatus Schltdl. – DNA 3x	1/1	22.45	–
Lasiocephalus patens (Kunth) Cuatrec.	5/6	17.27±0.15	g
Lasiocephalus pichinchensis (Cuatrec.) Cuatrec.	1/1	15.02	bcd
Lasiocephalus sodiroi (Hieron.) Cuatrec.	1/1	14.58	ab
Lasiocephalus L. sp. 1 (liana)	4/6	15.59±0.08	de
Lasiocephalus L. sp. 2 (shrub)	1/2	17.27±0.42	g
Lasiocephalus L. sp. 3 (shrubby liana)	1/1	15.70	cdef
Lasiocephalus L. sp. 4 (cf. *L. ledifolius* (Kunth) C. Jeffrey)	2/2	13.82±0.10	a
Lasiocephalus L. sp. 5 (*L. involucratus*-like)	–	–	–
Lasiocephalus L. sp. 6 (*L. sodiroi*-like) – 2x	–	–	–
Lasiocephalus L. sp. 6 (*L. sodiroi*-like) – DNA 3x	–	–	–
Lasiocephalus L. sp. 7 (*L. sodiroi*-hairy)	–	–	–
Culcitium nivale Kunth	3/3	12.35±0.10	–

dure using Otto buffers (Doležel et al. 2007). Briefly, intact leaf tissue of the *Lasiocephalus* plant to be analyzed and an internal reference standard (*Vicia faba* 'Inovec', 2C = 26.9 pg; Doležel et al. 1998) were mixed together and chopped with a sharp razor blade in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Otto 1990). The suspension was filtered through a 42-μm nylon mesh and incubated for approx. 30 min at room temperature. The staining solution consisted of 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_2O$), β-mercaptoethanol (final concentration of 2 μl/ml) and a fluorochrome. Propidium iodide (PI) and RNase IIA (both at final concentrations of 50 μg/ml) were used to determine the genome size in absolute values (pg of DNA) in fresh samples, while AT-selective DAPI (4',6-diamidino-2-phenylindole) at a final concentration of 4 μg/ml was used for estimating the DNA content in relative units in silica-dried samples. Samples were stained for 10 min at room temperature and run on the flow cytometer. Isolated nuclei were excited either with a laser (for PI staining) or a mercury arc lamp (for DAPI staining) and the fluorescence intensity of 5000 particles recorded.

test. DNA-triploids were excluded from statistical comparison. ITS clade: CN = '*C. nivale*' clade; F = 'forest' clade; P = 'páramo' clade. Growth form: BH = broad-leaved herb; L = liana; NH = narrow-leaved herb; S = shrub. Habitat type: F = Andean forest; G = grass páramo; SB = subpáramo; SP = superpáramo.

Relative DNA content ('silica' dataset)			ITS clade	Growth form	Habitat type
Samples analysed	Sample/ standard ratio	Grouping			
2/2	0.667±0.000	fghi	F	L	F
2/4	0.705±0.013	i	F	L	F
1/1	0.646	cefghi	–	L	F
2/8	0.665±0.012	gh	F	L	F
9/18	0.628±0.019	ef	F	L	SB
6/15	0.620±0.018	de	P	NH	G
2/2	0.855±0.031	–	P	NH	G
4/7	0.661±0.021	g	F	NH	SP
2/6	0.617±0.026	ce	–	BH	G
1/1	0.671	efghi	F	L	SB
7/19	0.596±0.011	bc	P	NH	SP
3/4	0.860±0.057	–	P	NH	SP
4/14	0.688±0.023	hi	F	L	F
10/21	0.612±0.016	ce	F + P	NH	G
2/3	0.599±0.003	bcd	P	NH	SP
4/5	0.622±0.011	cef	–	L	SB
–	–	–	–	S	SB
1/2	0.602±0.012	bce	–	L	SB
4/6	0.535±0.007	a	CN	NH	SP
1/6	0.626±0.010	def	F	L	SB
1/2	0.556±0.024	ab	–	NH	G
1/2	0.771±0.009	–	–	NH	G
1/4	0.545±0.007	a	–	NH	SP
1/1	0.494	–	CN	NH	SP

Two distinct datasets were produced and treated separately in subsequent statistical analyses. Firstly, relative DNA content was determined for 152 silica-dried *Lasiocephalus* and one *Culcitium nivale* sample (further referred to as the 'silica' dataset) using DAPI flow cytometry. The desiccated samples were not more than 18 months old when analyzed. DAPI was chosen because it is less sensitive to secondary metabolites and chromatin arrangement, which allowed a high-resolution analysis even of dehydrated plant tissues (Suda & Trávníček 2006). Only histograms with coefficients of variation (CVs) of the G_0/G_1 peak of the analyzed sample below 5% were considered. Secondly, absolute values of genome size of a subset of 37 fresh accessions of *Lasiocephalus* and three accessions of *Culcitium nivale* ('fresh' dataset) were determined using PI flow cytometry. More stringent criteria for the quality of the flow analysis were applied in this case: (i) peaks of both the sample and the internal standard of approximately the same height, (ii) CV of G_0/G_1 peak of the analyzed sample below 4%, (iii) three replicates of each sample on different days to minimize potential random instrumental drift, and (iv) the between-day variation in fluorescence intensity below 3%; otherwise, the most extreme

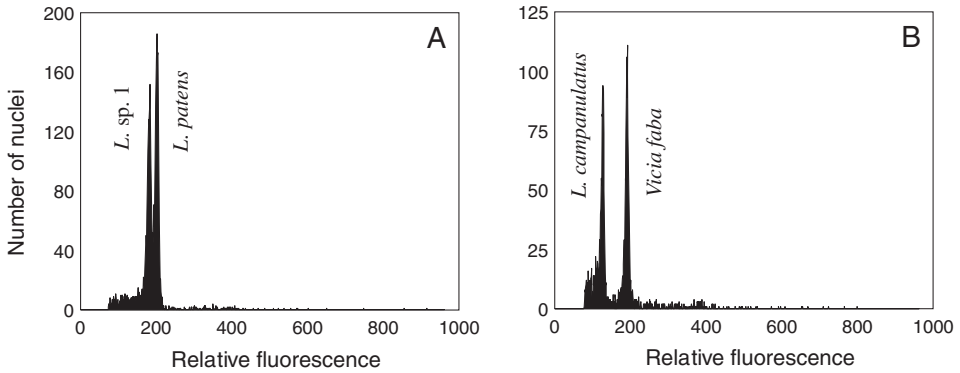


Fig. 1. – Fluorescence histograms for *Lasiocephalus* samples analyzed using flow cytometry. (A) Simultaneous analysis of propidium iodide stained nuclei isolated from fresh tissue of *L.* sp. 1 (2C = 15.59 pg, pop. no. P2) and *L. patens* (2C = 17.27 pg, pop. no. P12), which reveal interspecific differences in genome size. (B) Analysis of relative DNA content of silica gel preserved samples of *L. campanulatus* (pop. no. C1A). Isolated nuclei were stained with DAPI and fresh leaf tissue of *Vicia faba* was used as an internal reference standard. See Electronic Appendix 1 for population details.

value was discarded and the sample re-analyzed. The reliability of FCM measurements (i.e. between-plant differences) was repeatedly confirmed by simultaneous analyses of *Lasiocephalus* samples with distinct genome sizes (Fig. 1). The presence of two distinct peaks in analyses of co-processed samples is considered the most convincing evidence for genuine differences in nuclear DNA content (Greilhuber 2005).

To assess relationships between DAPI (AT-selective fluorochrome) and PI (intercalating fluorochrome without base bias) staining, a subset of thirteen fresh DNA diploid *Lasiocephalus* accessions covering the whole range of monoploid genome sizes was analyzed using both PI and DAPI flow cytometry concurrently. The analyses followed the above-defined stringent criteria with three independent replicates for each sample and each stain.

Chromosome counts

To confirm the FCM results, three individuals covering the whole genome size range at the diploid level (Table 1, Electronic Appendix 1) were subjected to conventional karyological counts using rapid squash methods. The apical root meristem of young seedlings was pre-treated with p-dichlorbenzene for 3 h, fixed in ice-cold 3 : 1 ethanol : acetic acid for 12–14 h, macerated for 1 min in 1 : 1 ethanol : hydrochloric acid, and stained with lacto-propionic-orcein (Dyer 1963).

ITS cloning and sequencing

Sequences of the nuclear ribosomal internal transcribed spacer (ITS) region were analyzed for 13 *Lasiocephalus* taxa (Table 1). Total genomic DNA was extracted from silica-dried material using the Invisorb Spin Plant Mini Kit (Invitek) according to the manufacturer's instructions. The ITS1-5.8S rDNA-ITS2 region of the nuclear ribosomal DNA was

amplified using the primers ITS5 (White et al. 1990) and ITS_Lasio (5'-AGTCGRAGCATCGTCATGAGA-3'). PCR amplifications were done in 22 µl reaction containing 0.18 mM of each dNTP (Fermentas), 0.23 mM of each primer (Sigma), 0.5 units of JumpStart REDTaq polymerase (Sigma), 1 × PCR buffer for JumpStart REDTaq (Sigma) and 5 ng of genomic DNA. An initial denaturation step at 94 °C for 1 min was followed by 35 cycles of denaturation (94 °C for 50 s), annealing (53 °C for 50 s) and extension (72 °C for 1 min) steps and a final extension at 72 °C for 10 min. Each sample was amplified in two separate reactions. Both PCR products were mixed and purified using JETQUICK PCR Product Purification Spin Kit (Genomed). The purified ITS templates were ligated to the pGEM-T Easy vector (Promega) following the manufacturer's protocol but downscaled to half volume reactions and subsequently transformed into competent cells. After an overnight culture at 37 °C on the LB ampicillin/IPTG/X-gal selective plates, colonies carrying the ITS insert were identified by colour. 10–12 colonies from each reaction were selected for colony PCR using the same conditions as in the first PCR reaction. PCR products were purified using JETQUICK PCR Product Purification Spin Kit (Genomed) and subsequently sequenced (Macrogen, lll) using the ITS5 primer.

Phylogenetic and statistical analyses

Chromatograms of the cloned sequences were inspected by eye using Finch TV sequence viewer. In addition, direct GenBank sequences of nine species identified as putative closest relatives of *Lasiocephalus* by Pelser et al. (2007) were included as an outgroup reference. Initial sequence alignment was done with ClustalW algorithm incorporated in BioEdit v5.0.6 (Hall 1999) and manually improved. The same program was used for further manual editing. Autapomorphies that were found only in one clone within the whole dataset were considered to be polymerase errors and discarded (Popp et al. 2005). Two sequences of a highly ribotype-variable species *L. pichinchensis,* which combined apomorphies of the two main clades (see Results), were identified as chimeras and therefore omitted from analyses. Sequences were submitted to GenBank (GU289931 – GU290036). Bayesian analysis (MrBayes v3.1.2; Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) was run on the complete dataset with GTR model of nucleotide substitutions, gamma model of rate heterogeneity and variable proportion of invariable sites. This model was found in hierarchical likelihood ratio tests as the model of molecular evolution best fitting the data as implemented in Modeltest v3.5 (Posada & Crandall 1998). Two parallel runs with four chains each were used, sampling every 1000th tree for 3 million generations until convergence. The first 750 trees per run were discarded as burn-in and the remaining 2251 trees per run were summarized.

Correlation between DAPI and PI measurements of the same accessions was examined in Statistica 8. Differences in nuclear DNA contents were tested using an analysis of variance (ANOVA) and further assessed by Tukey's HSD multiple comparisons incorporated in the R software 2.9.2 (R Development Core Team 2009). The differences between growth forms and preferred habitat types were assessed by ANOVA with species identity considered as a factor with random effect (nested in the particular factor) in order to avoid pseudoreplication. The relationship between DNA content and altitude was tested by linear mixed-effect model (species identity treated as a factor with random effect) incorporated in the *nlme* package in R (Lindstrom & Bates 1990). In all analyses the random effect

was modelled only for the intercept, as was suggested by the likelihood ratio test (only non-significant improvement when a more complex model taking into account also the effect of the random factor for the slope was considered).

The relationships between genome size and phylogeny were examined by testing the association of nuclear DNA content with the two main clades detected (i.e. the 'páramo' and the 'forest' clade; see Results). Due to non-homogeneity of variances (indicated by highly significant Bartlett test: $P < 0.002$ in both datasets), differences in DNA content (mean values per species) between clades were tested by the non-parametric Mann-Whitney test implemented in Statistica 8 (StatSoft 2007). Species for which there was no sequence information were omitted from this kind of analysis. Within the 'forest' clade, differences in relative genome size (the 'silica' dataset) among the three main subclades (Table 1) were tested using the Kruskal-Wallis test implemented in Statistica 8. The relationship between DNA content and altitude was assessed separately for each of the two main clades (Table 1) using the same procedure as in the whole dataset (see above).

In order to quantify the correlation between environmental factors (habitat and growth form) and phylogeny (split into two main clades), variation partitioning based on three-way ANOVA with sequential (Type I) sum of squares was done in Statistica 8. Mean DNA content values of each species were subjected to six three-way ANOVAs with different input sequence of factors and model sums of squares used as variance component measures.

Results

DAPI versus PI staining

A highly linear DAPI/PI correlation was observed across the whole range of genome sizes of the 13 selected fresh diploid *Lasiocephalus* samples (the relationship was: DAPI fluorescence = 1.126 × PI fluorescence, $R^2 = 0.99$, $P < 0.001$). This indicates a constant AT/GC content within the genus and enables a reliable conversion and interpretation of the estimated values.

Chromosome counts

Karyological analyses of three putatively diploid plants with markedly different genome sizes [*Lasiocephalus* sp. 4 (cf. *L. ledifolius*)], pop. G18, 2C = 13.82 pg; *Lasiocephalus* sp. 3, pop. no. 11567, 2C = 15.70 pg; and shrubby *Lasiocephalus* sp. 2, pop. no. 11584, 2C = 17.27 pg) confirmed the diploid number of chromosomes (2n = 2x = 40) in all samples (Fig. 2).

DNA ploidy levels

The analysis of 37 fresh and 152 silica-dried *Lasiocephalus* samples revealed a large variation in nuclear DNA content within this genus, spanning 1.64-fold range (2C = 13.82–22.69 pg) and 1.60-fold range (sample/*Vicia* ratio = 0.535–0.860), respectively (Table 1). In each dataset, two well-defined genome size groups (separated by a distinct gap) were detected. The fluorescence values of the two groups differed by ~1.5-fold, indicating that DNA diploids and DNA triploids were involved (Table 1). DNA triploids were represented by ten samples belonging to three species (*L. lingulatus*, *L. ovatus* and *L.* sp. 6)

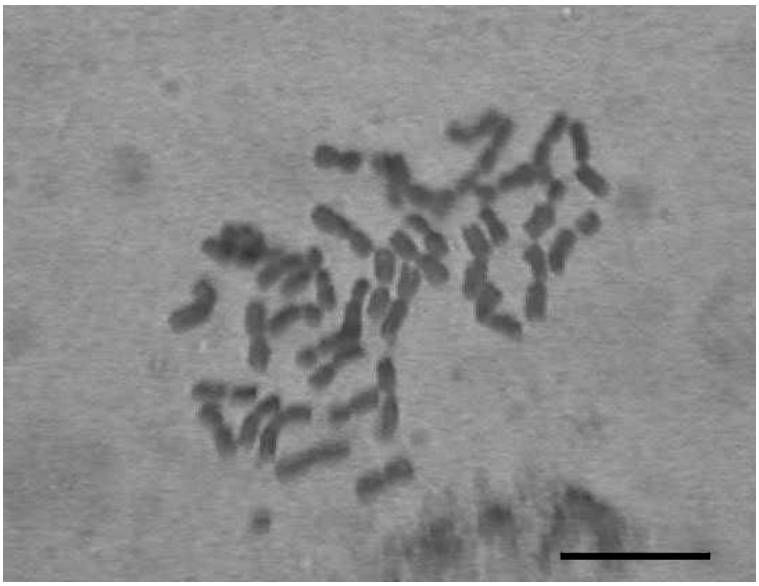


Fig. 2. – Mitotic chromosomes of *Lasiocephalus* sp. 3 (shrubby liana, pop. no. 11567), 2n = 40. Scale bar = 10 µm (photo V. Jarolímová).

and occurred in eight populations (~8% of all sampled populations). In most cases, DNA triploids grew in sympatry with more abundant diploid plants.

Genome size variation

Disregarding the DNA triploids, the remaining *Lasiocephalus* accessions still exhibit a pronounced variation in genome size. Mean 2C-values of fresh accessions ranged from 13.82 pg in *Lasiocephalus* sp. 4 (cf. *L. ledifolius*) to 17.27 pg in shrubby *Lasiocephalus* sp. 2 and *L. patens*, (1.25-fold variation). The variation in silica-dried samples was 1.32-fold, with *L.* sp. 4 (cf. *L. ledifolius*) possessing the smallest and *L. cuencanus* the largest genomes (Table 1, Fig. 3). The closely related *Culcitium nivale* possessed a smaller genome than any species of *Lasiocephalus* (mean 2C = 12.35 pg; Table 1).

In both datasets, samples with the smallest genome sizes formed a discrete group (Fig. 3). This group consisted of two taxa of putative hybrid origin – *Lasiocephalus* sp. 4 (cf. *L. ledifolius*) (a putative intergeneric hybrid between *L. ovatus* and *Culcitium nivale*) and *Lasiocephalus* sp. 7 (a putative hybrid between *L. sodiroi* and *C. nivale*). DNA content values of suspected hybrids were intermediate between values of their putative parents.

A continuous variation in nuclear DNA content was observed in the remaining samples. Nevertheless, this variation was sorted according to the growth form and preferred habitat (Fig. 3). Analysis of variance supported significant differences in genome size among the different habitats ($F_{3,23} = 7.16$, $P = 0.01$ and $F_{3,125} = 9.38$, $P = 0.001$ for 'fresh' and 'silica' datasets, respectively) and growth forms ($F_{2,23} = 6.23$, $P = 0.02$ and $F_{2,125} = 5.10$, $P = 0.02$ for 'fresh' and 'silica' datasets, respectively). Generally, the genome size of the *Lasiocephalus* species analyzed increased with decreasing altitude as documented by

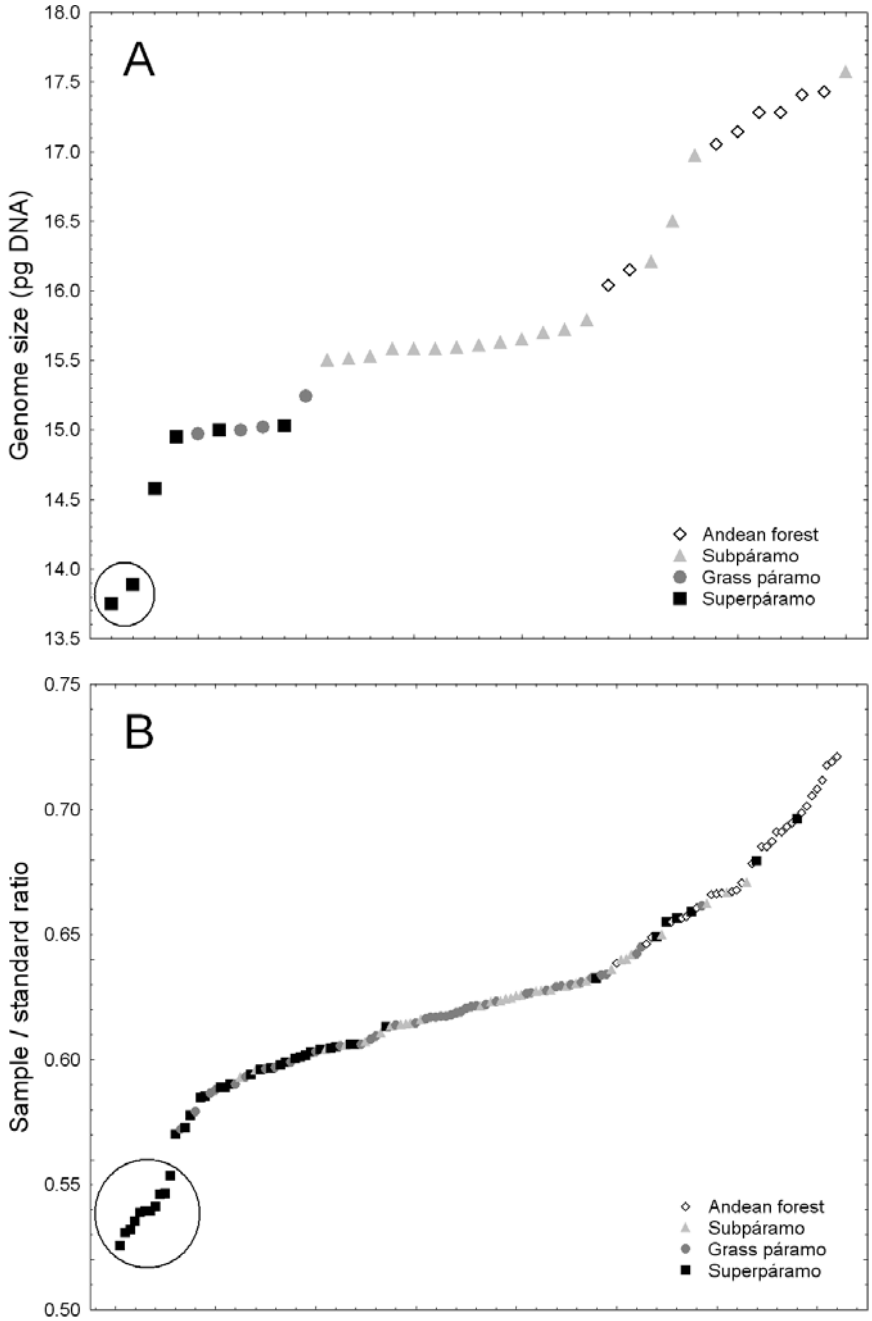


Fig. 3. – Variation in holoploid genome size of putatively diploid *Lasiocephalus*. (A) Absolute genome sizes (2C values) in pg DNA of 35 accessions of 13 *Lasiocephalus* species ('fresh' dataset, staining with propidium iodide). (B) Relative fluorescence intensity (expressed as a ratio of the sample and the internal reference standard, *Vicia faba*) in 144 accessions of 19 *Lasiocephalus* species ('silica' dataset, stained with DAPI). Different symbols refer to different habitat types (growth forms correlate with the habitat types, see Table 1). Circles depict putative hybrids between *Lasiocephalus* spp. and *Culcitium nivale*. Note the generally increasing DNA content in species inhabiting different habitats in the sequence: superpáramo < grass páramo < subpáramo < Andean forest.

the following habitat sequence: 1) superpáramo plants (mean 2C = 14.53 pg, mean sample/standard ratio = 0.591), 2) grass páramo plants (mean 2C = 15.06 pg DNA, mean sample/standard ratio = 0.616), 3) subpáramo plants (mean 2C = 15.71 pg, mean sample/standard ratio = 0.626) and 4) forest plants (mean 2C = 16.97 pg DNA, mean sample/standard ratio = 0.681). Similarly, a progressive increase in DNA content was observed among different growth forms. The narrow-leaved herbaceous plants had the lowest mean DNA values (2C = 14.74 pg, sample/standard ratio = 0.603), followed by broad-leaved herbaceous plants (sample/standard ratio = 0.617), lianas (2C = 16.15 pg, sample/standard ratio = 0.652) and a shrub (2C = 17.27 pg). The only exception was *L. longipenicillatus*, a narrow-leaved superpáramo herbaceous plant, with a distinctly higher genome size than other species of the same growth form and habitat (see Fig. 3). It should, however, be noted that the negative correlation between genome size and altitude was statistically supported only by the 'silica' dataset ($F_{1,124} = 7.611$, $P = 0.007$) but not by the less representative dataset of fresh samples ($F_{1,22} = 0.001$, $P = 0.996$).

ITS phylogeny and genome size

Bayesian phylogenetic analysis of ITS sequences run on a subset of 13 *Lasiocephalus* species revealed two well-supported lineages (Fig. 4), referred to as 'forest' and 'páramo' clades because of the markedly different habitat preferences of their members.

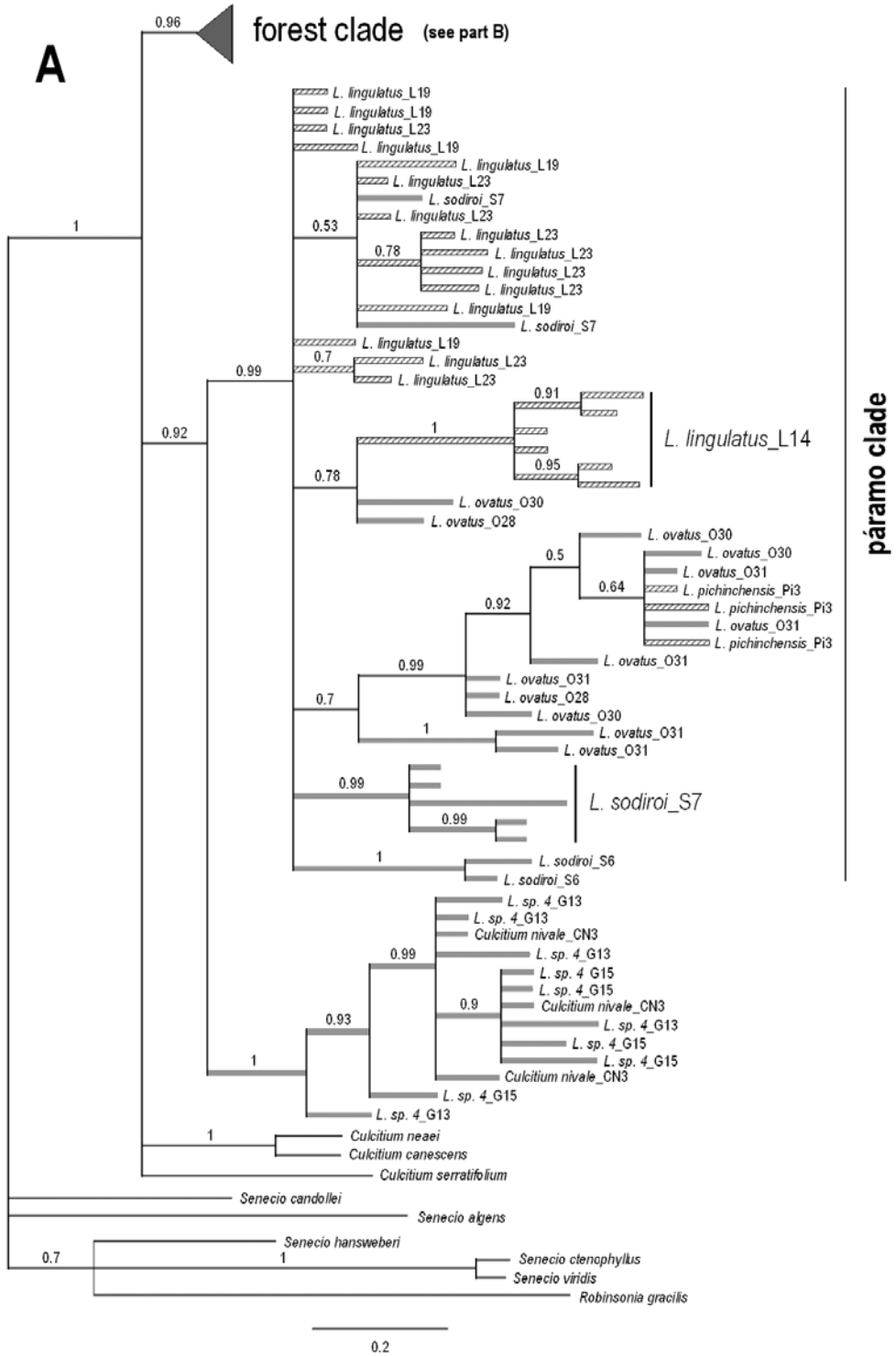
The 'forest' clade consists almost entirely of lianas inhabiting Andean forest and subpáramo habitats (see Table 1). The only exception was *L. longipenicillatus*, a superpáramo herbaceous plant from Venezuela. The 'forest' clade appears to be further subdivided into three sub-clades: (i) forest + superpáramo taxa (sub-clade 'a' at Fig. 4), (ii) entirely forest species (sub-clade 'b'), and (iii) forest + subpáramo taxa (sub-clade 'c').

The 'páramo' clade is composed of four exclusively grass páramo and superpáramo narrow-leaved herbaceous plants with unresolved relationships. The closely related *Culcitium nivale* and one putatively hybrid taxon *Lasiocephalus* sp. 4 (cf. *L. ledifolius*) with similar growth form and habitat preferences, are a sister group to the 'páramo' clade.

One species (*L. pichinchensis*) possessed ITS ribotypes characteristic of both major clades (Fig. 4); sequences were similar to *L. otophorus* and *L. ovatus* from the 'forest' and 'páramo' clades, respectively, and were isolated from a single cloned individual. For this reason, *L. pichinchensis* was omitted from subsequent tests of correlation between genome size and phylogeny. Our data do not support the monophyly of the genus *Lasiocephalus* sensu Cuatrecasas (1978) because of the polytomy at the base of the whole group, which also includes several species of the closely related genus *Culcitium* (Fig. 4).

The two main clades differ markedly in DNA ploidy and DNA content variation. DNA triploids were entirely confined to the 'páramo' clade (polyploids accounted for 17% of all samples in this clade) whereas the 'forest' clade was ploidy-uniform. In addition, diploid species from the 'páramo' clade have significantly smaller genome sizes than their 'forest' counterparts based on both the 'fresh' (Mann-Whitney $Z = 2.121$, $P = 0.034$; mean 2C = 14.97 and 16.43 pg, respectively) and 'silica' datasets ($Z = 2.717$, $P = 0.007$; mean sample/standard ratio = 0.606 and 0.658, respectively).

Relationships between DNA content and growth form, habitat preferences and altitudinal range within each of the two main clades were further analyzed in the more representative 'silica' dataset. Species occupying different habitats also differed significantly


A
forest clade (see part B)
páramo clade
L. lingulatus_L14
L. sodiroi_S7
L. lingulatus_L19
L. lingulatus_L23
L. sodiroi_S7
L. ovatus_O30
L. ovatus_O28
L. ovatus_O31
L. pichinchensis_Pi3
L. sodiroi_S6
L. sp. 4_G13
L. sp. 4_G15
Culcitium nivale_CN3
Culcitium neaei
Culcitium canescens
Culcitium serratifolium
Senecio candollei
Senecio algens
Senecio hansweberi
Senecio ctenophyllus
Senecio viridis
Robinsonia gracilis
0.2

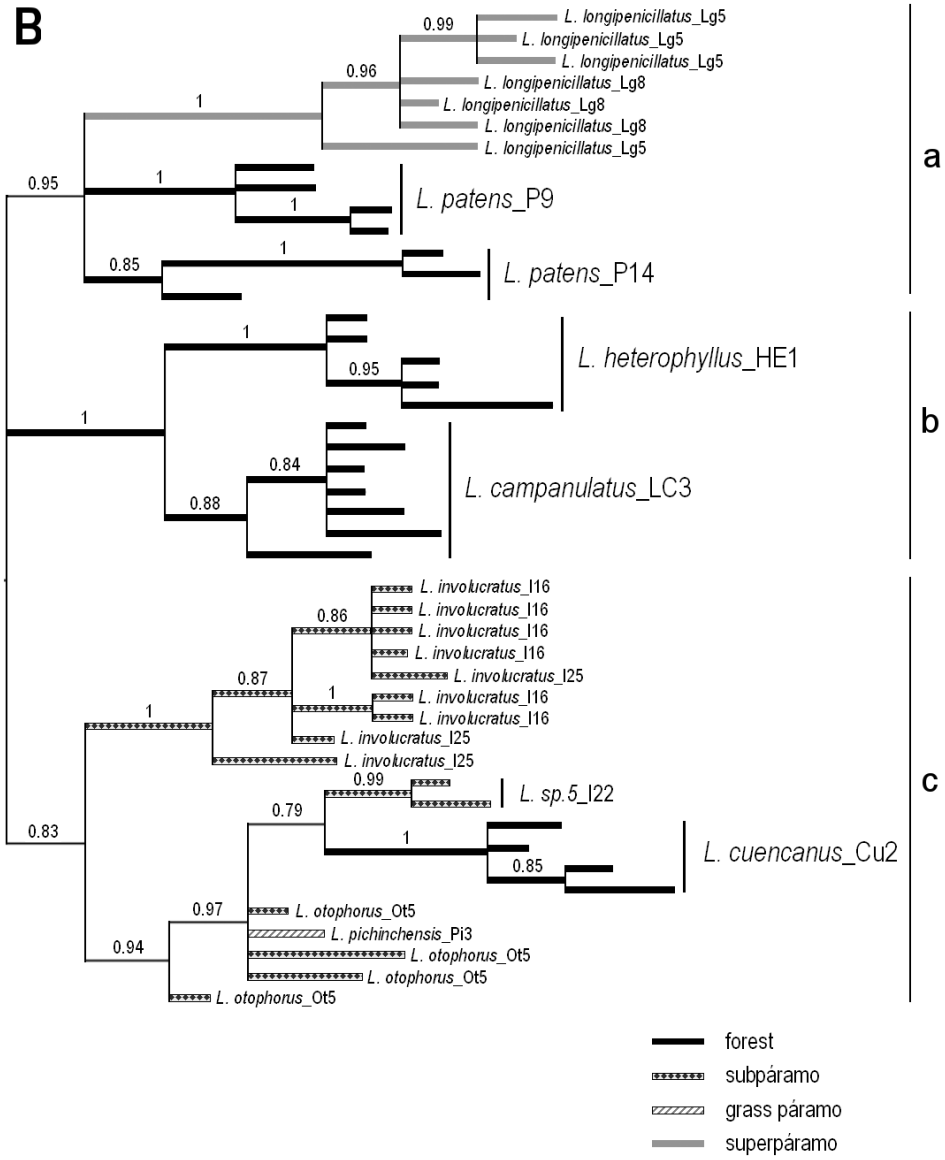


Fig. 4. – Phylogenetic analysis of ITS sequences of thirteen *Lasiocephalus* species and their closest relatives in the *Senecio* s.l. group. A Bayesian 50% probability consensus tree of 4502 trees is shown with posterior probabilities indicated above the branches. (A) Basal part of the tree and 'páramo' clade. (B) 'forest' clade further divided into three subclades (designated by a, b and c letters). Mean 2C-values for 'forest' and 'páramo' clades were 16.43 pg and 14.97 pg DNA, respectively. Different patterns of the branches indicate different habitat preferences of the respective accession/lineage.

in their DNA content values both within the 'forest' clade ($F_{2,52}$ = 12.681, P = 0.011) and within the 'páramo' clade ($F_{1,34}$ = 170.16, P = 0.048). On the other hand, C-values in the 'forest' clade were not associated with different growth forms ($F_{1,52}$ = 0.013, P = 0.915); this test was not performed for the 'páramo' clade because all species were narrow-leaved herbaceous plants. The DNA content of 'forest' clade taxa increased with decrease in altitude ($F_{1,51}$ = 4.342, P = 0.042) while no such association was found within the 'páramo' clade ($F_{1,33}$ = 0.048, P = 0.828). In addition, a clear phylogenetic structure within the 'forest' clade was subjected to further analysis. However, only non-significant differences in fluorescence values among the three subclades (Fig. 4) were detected (Kruskal-Wallis H = 0.125, P = 0.939).

Relations among the explanatory variables

Variation partitioning provided a better insight into the relations among habitat, growth form and phylogeny that were selected as possible explanatory variables for the intrageneric variation in genome size in *Lasiocephalus*. Strong correlations among the variables were obtained (Fig. 5). A large proportion of the variation was explained by a combination of all three factors (71% and 48% for 'fresh' and 'silica' datasets, respectively). Habitat seemed to be the single most important explanatory factor, although this result may be biased due to an unequal number of degrees of freedom for different variables (3 for habitat type versus 1 for ITS clade and 2 for growth form).

Discussion

This study aimed at addressing the role of different evolutionary forces in shaping genome size in a diverse plant group. We estimated relative or absolute genome size values in a representative sample set of the high-Andean genus *Lasiocephalus* (20 taxa, 189 samples) and interpreted the results in the light of the phylogenetic relationships, growth form and ecological preferences.

DNA ploidy level variation

Polyploidy is widely acknowledged as the key force in angiosperm evolution with recent or ancient genome duplication being detected in virtually all angiosperms (Soltis et al. 2009). Although no polyploids have previously been reported in *Lasiocephalus*, our FCM data provide clear evidence for intrageneric cytotype polymorphism. The most parsimonious explanation for the existence of two discrete groups of genome sizes (differing by ~1.5-fold) is the incidence of diploid and triploid plants. Because exact chromosome counts for putatively triploid individuals are not available we refer to these large-genome accessions as DNA triploids (Suda et al. 2006).

DNA triploids are most probably of autopolyploid origin as suggested by their: (i) close morphological similarity with diploid individuals of the corresponding species, and (ii) regular sympatric growth of 2x and 3x cytotypes in the same populations. Indeed, no population for which several samples were analyzed was composed entirely of DNA triploids (but triploids were detected in two populations of *L. lingulatus* for which only one individual was analyzed). Most likely, triploids originated recurrently in each population as

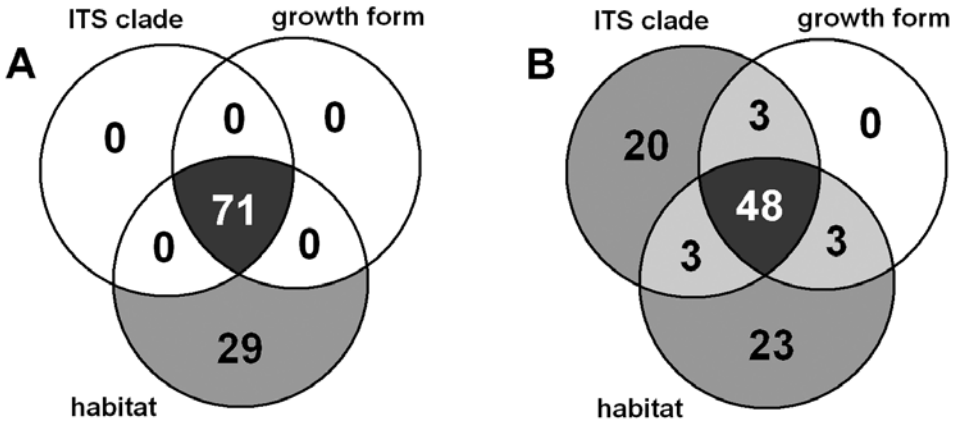


Fig. 5. – Partitioning of variance in genome size of *Lasiocephalus* samples explained by habitat, growth form and phylogeny. (A) 'fresh' dataset, (B) 'silica' dataset. The numbers represent percentages of explained variance (which was 96% and 83% of the total variance in A and B, respectively). The relative importance of a particular factor or factor combination is distinguished by different shades of grey. Note the strong correlation of all three factors. High proportion of variance explained solely by the habitat may be related to the higher number of degrees of freedom for this variable. Differences in the percentages of explained variance between both datasets reflect unequal sample sizes.

a result of fusion of reduced and unreduced gametes of diploids, such as in *Picris* (Slovák et al. 2009) and *Quercus* (Dzialuk et al. 2007).

Interestingly, all ten DNA triploids belonged to only three narrow-leaved *Lasiocephalus* taxa, namely *L. lingulatus*, *L. ovatus* and *L.* sp. 6 (resembling *L. sodiroi*). All of them grow in the high-altitude grass páramo or superpáramo and are a part of the 'páramo' clade (*Lasiocephalus* sp. 6 was not sequenced but is believed to belong to this clade because of close phenotypic similarities to another clade member, *L. sodiroi*). Genome duplication thus seems to be restricted to a small group of closely related taxa inhabiting the highest and most stressful environments. This pattern can be explained by the higher viability of polyploids in harsh conditions and/or by the higher frequency of unreduced gamete formation in a stressful and fluctuating climate (regular frost, high solar radiation, etc.; Sarmiento 1986). Relatively higher incidence of triploid plants at high altitudes is also recorded for instance in the *Cardamine pratensis* group (*C. rivularis*; Marhold 1994).

Genome size variation at diploid level

The three chromosome counts (2n = 40) determined in this study confirm the previous numbers recorded for Venezuelan *Lasiocephalus longipenicillatus* and Ecuadorian *L. involucratus*, *L. patens* and *Culcitium nivale* (Powell & Cuatrecasas 1970, Robinson et al. 1997). Despite quite a high number of somatic chromosomes, all these counts are regarded as diploid based on x = 20. This number seems to be the base chromosome number also in

some other representatives of the widely conceived genus *Senecio* (see also Suda et al. 2007), although some authors suggest x = 10 (e.g. Hodálová et al. 2007 and references therein). Because the karyologically-confirmed diploid plants are distributed across the entire range of genome sizes that remain after the exclusion of DNA triploids (Table 1), the 1.32-fold difference was interpreted as a genuine variation in monoploid genome size. It is likely that amplification or deletion of non-genic repetitive DNA (transposable elements in particular), among other mechanisms, drive this variation (Bennetzen et al. 2005, Cavalier-Smith 2005, Piegu et al. 2006).

Genome size and homoploid hybridization

Genome size is mostly stable at the species level, while it often shows considerable differences even among closely related species (Greilhuber et al. 2005, Leong-Škorničková et al. 2007, Ekrt et al. 2009, Loureiro et al. 2010). As such, genome size can be used as a supportive species-specific characteristic and is also a helpful marker for investigating interspecific hybridization. The value of genome size data lies in the fact that both heteroploid and homoploid hybrids can be identified based on intermediate DNA amounts (e.g. Šiško et al. 2003, Mahelka et al. 2005, Yahata et al. 2006, Kron et al. 2007, but see Bureš et al. 2004).

In this study, two different intergeneric hybrids between *Lasiocephalus* spp. and closely related *Culcitium nivale* were assumed on the basis of field observations and plant phenotypes. In both cases, FCM data confirmed our expectation. The average genome size of *Lasiocephalus* sp. 4 (cf. *L. ledifolius*) (2C = 13.82 pg), a putative hybrid between *L. ovatus* (2C = 14.99 pg) and *C. nivale* (2C = 12.35 pg), closely matched the theoretical DNA amount of the F1 hybrid (13.67 pg). Putative crosses showed intermediate morphological characteristics with respect to the size and shape of leaves, type of indumentum, capitula diameter, etc. Further support for their hybrid origin comes from the fact that only a small number of often non-flowering individuals scattered among abundant and spatially intermingled putative parents was observed at all the localities sampled. An analogous situation exists in a mixed population of *C. nivale* and another páramo species, *L. sodiroi*. The population of plants provisionally called *Lasiocephalus* sp. 7 (no. 11088 in Electronic Appendix 1) is comprised of morphologically transient individuals with fluorescence values (mean sample/standard ratio of 0.545) intermediate between the values of putative parents (0.494 and 0.599, respectively).

In contrast to the FCM results, the ITS sequences did not provide direct evidence supporting the hybrid origin of *Lasiocephalus* sp. 4 (cf. *L. ledifolius*). All clones isolated from two *L.* sp. 4 individuals originating from two different populations were clearly assigned to the *Culcitium nivale* lineage (most of them fell directly among the *C. nivale* accessions). The absence of *L. ovatus*-type ribotypes can be explained by at least two non-exclusive hypotheses: (i) the *L. ovatus*-belonging paralogues were not sampled due to either the limited number of clones (10 clones in total) or because of biased PCR amplification (Wagner et al. 1994, Kanagawa 2003), (ii) concerted evolution could have homogenized the paralogues in putative hybrid accessions towards a *C. nivale* copy. Rapid homogenization of rDNA in several generations is reported for instance in allopolyploid *Nicotiana* (Kovarik et al. 2004) and *Tragopogon* (Kovarik et al. 2005). Ongoing molecular work aims to shed more light on the evolutionary history of *Lasiocephalus* sp. 4 (cf. *L. ledifolius*).

Genome size and phylogeny

The recent burst of phylogeny-based studies on genome size variation revealed a strong phylogenetic component in this trait at various taxonomic levels, from seed plants as a whole (Leitch et al. 2005), through family (Leitch et al. 2007) and (sub)genus (Weiss-Schneeweiss et al. 2005, Garcia et al. 2008, Chrtek et al. 2009) up to the intraspecific level (Popp et al. 2008). Neutral evolution (i.e. random and passive changes in genome size) may often be as important as selective processes in shaping genome size (Lysak et al. 2009).

Two main clades were identified in the *Lasiocephalus* accessions analyzed (Fig. 4) – one containing lianas from the Andean forest and subpáramo habitats (plus one superpáramo species, *L. longipenicillatus*) ('forest' clade) and the other exclusively grass páramo and superpáramo narrow-leaved herbaceous plants ('páramo' clade). Interestingly, both clades differed significantly in genome size, with smaller genomes recorded in the 'páramo' clade. On the other hand, genome size in *Lasiocephalus* did not appear to be affected so much by phylogeny at the lower divergence levels as documented by the non-significant differences in genome size between the three subclades within the 'forest' clade.

Any further considerations of the ancestral state and possible direction of genome size evolution in the two main clades are, however, largely obscured by a polytomy at the base of the whole group that involves several *Culcitium* species. Moreover, *C. nivale* appears to be more closely related to the *Lasiocephalus* 'páramo' clade than to other members of its own genus (Fig. 4). Thus the data indicate that in order to re-establish the group as monophyletic *C. nivale,* at least, has to be included in *Lasiocephalus*. Nevertheless, at this stage of the investigation it is not possible to exclude that the closeness of the relationship between *Lasiocephalus* and *C. nivale* is overestimated due to the incidence of hybridization and introgression. In addition, only direct non-cloned sequences of other *Culcitium* species were obtained from the GenBank (Pelser et al. 2007). As noted by Soltis et al. (2008), the use of direct ITS sequences could mislead the phylogenetic inference especially in paralogue-rich groups. We also admit that the explanatory value of the current phylogenetic tree may be limited by the incomplete taxon sampling (only 13 out of 20 FCM-analyzed *Lasiocephalus* taxa were included). On the other hand, the sequenced dataset is sufficiently representative. It covers the whole range of estimated genome sizes and all major types of growth forms and habitats (perhaps with a single exception of the broad-leaved herbaceous *L. mojandensis*). A multi-marker molecular analysis applied to a larger dataset is currently in progress in order to draw more robust conclusions regarding the phylogenetic relationships between *Culcitium* and *Lasiocephalus*.

Genome size and environmental correlates

The influence of environmental factors on genome size evolution has been debated for years and is still a matter of controversy. Significant correlations of genome size with various ecological factors (see Leitch & Bennett 2007 for a review) differ when different model systems are compared. For example, of the correlations of genome size with altitude for 24 genera, approximately one third are positive, one third negative and one third not significant (Knight et al. 2005). Similarly, while Suda et al. (2003) report correlations between genome size and altitude, average annual temperature, rainfall and/or humidity in a number of Macaronesian genera, these relationships disappear when the sampling is more representative (Suda et al. 2005). In addition, rigorous studies on variation in

genome size at the lowest divergence levels (within species or even within population) often fail to detect any link between genome size and environmental conditions (Šmarda et al. 2007).

Significant correlations between genome size and growth form, habitat and altitude were also found in *Lasiocephalus*. On average, species from high-altitude harsh environments (superpáramo) have the smallest genomes while their counterparts from lower altitudes and more favourable conditions (Andean forest) have the largest genomes, which accords with the 'large genome constraint' theory (Knight et al. 2005). Different growth forms are associated with different altitudes: narrow-leaved herbaceous plants on the superpáramo and lianas in forests. However, it should be noted that all these variables were strongly correlated with the major phylogenetic split in *Lasiocephalus* (i.e. the division into two lineages referred to as 'páramo' and 'forest' clades; Fig. 4, see also Fig. 5). Although we do not apriori reject that environmental factors may have somehow shaped the genome size, we are convinced that phylogenetic relationships were the key determinant of the observed divergence in genome size at the highest divergence levels. This assumption is strongly supported by the fact that the only superpáramo herbaceous plant, *L. longipenicillatus* with a large genome, was included in the 'forest' clade. These findings emphasize that for the correct interpretation of the environmental correlates of genome size it is crucially important to incorporate phylogenetic information into analyses (Grotkopp et al. 2004, Jakob et al. 2004, Chase et al. 2005, Beaulieu et al. 2007, Chrtek et al. 2009).

Environmental factors seem to be more important in shaping the genome size of *Lasiocephalus* at lower divergence levels. While the three main sub-clades within the 'forest' clade do not significantly differ from each other in terms of genome size, the variation is structured according to the habitat preferences of the respective species (as also reflected by the negative correlation between genome size and altitude). Even within the sub-clades, high-altitude species have smaller DNA C-values than their low-altitude counterparts. Similar patterns are observed, for example, in the genus *Hordeum* where the environmental variable (length of vegetation period) is correlated with genome size only at the lowest taxonomic levels (Jakob et al. 2004).

Conclusions

This study is the first attempt to assess genome size variation in a radiating plant group in the tropics and interpret the results in the light of ecological conditions, life history traits and phylogeny. It indicates that the genome size in high-Andean *Lasiocephalus* has been shaped by different evolutionary processes, including polyploidy, hybridization and gradual changes at the molecular level. The variation in genome size at the higher divergence level primarily reflects phylogenetic effects, while ecological factors are more important in shaping genome size at the lower divergence levels. This highlights the need to always evaluate ecological and phenotypic correlates of genome size within a phylogenetic framework to avoid misinterpretation (e.g. overestimating the significance of environmental factors). We believe that this study is likely to stimulate further research into the promising but still largely unexplored field of tropical polyploid evolutionary biology and cytogeography.

See http://www.preslia.cz for Electronic Appendix 1.

Acknowledgements

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Souhrn

Mezidruhové rozdíly ve velikosti genomu jsou určovány souhrou různých mikroevolučních procesů, mezi něž patří např. polyploidie, hybridizace, selekční tlak, ale také neutrální evoluce. Článek se zabývá variabilitou v obsahu jaderné DNA u andského rodu *Lasiocephalus* (*Asteraceae*, *Senecioneae*) a snaží se objasnit příčiny této variability s využitím spektra metodických přístupů (průtoková cytometrie, klasická karyologie, sekvenace jaderné DNA, ekologická pozorování). V souboru 189 jedinců představujících 20 druhů byl zjištěn 1,64-násobný rozdíl mezi průměrnými C-hodnotami jednotlivých druhů. Rostliny s největšími genomy s veškerou pravděpodobností představují triploidy (první údaje v rámci celého rodu). Zbývající vzorky vykazovaly 1,32-násobnou a prakticky kontinuální variabilitu v obsahu jaderné DNA. Nejmenší genomy patrně patří mezirodovým hybridům mezi zástupci rodu *Lasiocephalus* a blízce příbuzným a sympatrickým druhem *Culcitium nivale*. Velikost genomu diploidních rostlin souvisela s různými ekologickými charakteristikami a biologickými vlastnostmi druhů (nadmořská výška, charakter stanoviště, životní forma). Nicméně všechny tyto charakteristiky (stejně jako samotná velikost genomu) výrazně korelovaly s fylogenezí skupiny. V rámci celého rodu proto považujeme fylogenetickou příslušnost za hlavní příčinu rozdílů ve velikosti genomu. Naproti tomu na nižších fylogenetických úrovních (v rámci jednotlivých evolučních linií) hrají významnou roli ekologické podmínky stanovišť, které mohou působit jako selekční faktor měnící velikost genomu. Zjištěné výsledky tedy dokládají vzájemné spolupůsobení mnoha různých evolučních činitelů při utvářejí a změnách obsahu jaderné DNA a poukazují na nutnost interdisciplinárního přístupu při studiu variability a dynamiky ve velikosti genomu.

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