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The Evolution of Genome Size Variation in Drumstick Onions (*Allium* subgenus *Melanocrommyum*)

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Abstract—*Allium* subgenus *Melanocrommyum* is a species-rich group of perennial onions with uniform karyology, ecology, and breeding system. Thus, the cytological, ecological, and physiological factors often correlated with genome size should have negligible effect on genome size variation in *Melanocrommyum*. We measured DNA content in subgenus *Melanocrommyum* using flow cytometry based on propidium iodide staining and analyzed the evolution of genome size in a phylogenetic context. The observed 2C genome size variation in 160 accessions of 70 species of the subgenus was high, varying from 26.26–78.73 pg. The significant phylogenetic signal in genome size data suggests that distribution of genome size is in accordance with phylogenetic clades identified by the analysis of nuclear ITS sequences. Estimation of ancestral genome sizes using generalized least squares revealed lineages with increasing as well as decreasing DNA content. We found within-species genome size variation to be mostly below 2.5%. In species where intraspecific genome size differences were in a range of 6–9%, we suggest the existence of cryptic species, as previously inferred by molecular markers. Thus, genome size variation reflects incipient speciation or diversification in *Allium* subgenus *Melanocrommyum*. About two-fold differences in DNA content in several *Melanocrommyum* species indicate the occurrence of diploid and tetraploid cytotypes in these taxa, which for some species has been confirmed by chromosome counts.

Keywords—*Allium* subgenus *Melanocrommyum*, C-value, cryptic species, evolution, flow cytometry, genome size, phylogeny, polyploidy.

Angiosperms vary tremendously in nuclear DNA content and much of this genome size variation is due to non-genic, repetitive DNA. It is well documented that in addition to polyploidization (Leitch and Bennett 1997; Soltis and Soltis 2000), transposon amplification is a major cause of genome expansion (Kalendar et al. 2000; Hawkins et al. 2008; Grover and Wendel 2010). The mechanisms responsible for genome size decrease include unequal crossing over, illegitimate recombination, a higher overall rate of deletions than insertions, and selection against transposable elements (Morgan 2001; Devos et al. 2002; Petrov 2002; Wendel et al. 2002; Ma et al. 2004; Bennetzen et al. 2005; Grover et al. 2007). The biological and evolutionary significance of wide variation in DNA content is a longstanding question in evolutionary biology. Most theories about genome size evolution emphasize the effects of DNA mass and volume on phenotype. Such effects include an increase in nucleus and cell size, prolonged duration of both mitosis and meiosis, and possible consequences of these changes such as slower cell growth and increased minimum generation time (Bennett 1972, 1987; Grime 1986; Wyman et al. 1997). While the above-mentioned theories focus on the adaptive importance of genome size variation, others point to the role of stochastic processes in genome size evolution (Lynch and Conery 2003). Oliver and colleagues (2007) propose a proportional model of evolution, implying that larger genomes evolve faster without imposing selection pressure. Petrov (2001) argues that while the mutations affecting the phenotype are subject to natural selection, the mutations with negligible selective effects are influenced mainly by genetic drift. Therefore, in the latter case the fixation of genome size will be a result of neutral stochastic events (Jakob et al. 2004).

Recent accumulation of C-value data (e.g. Plant DNA C-values database; http://data.kew.org/cvalues/homepage.html) and the availability of phylogenies for many plant groups present an opportunity to study genome size evolution in angiosperms within a phylogenetic context. Few studies have followed this approach at the family or genus level (Bennetzen and Kellogg 1997; Cox et al. 1998; Jakob et al. 2004; Johnston et al. 2005; Leitch et al. 2007), while others have conducted broader analyses across all angiosperms (Leitch et al. 1998; Soltis et al. 2003) or the entire embryophyta (Leitch et al. 2005). However, rigorous analyses of genome size variation among closely related species in a phylogenetic framework are still missing for most plant groups. Several studies found a correlation of genome size with life history, breeding system, and ecology within several taxonomic groups (Albach and Greilhuber 2004; Weiss-Schneeweiss et al. 2006) and even within single species. Within-species genome size variation has been found in samples from geographically separated populations (Jakob et al. 2004; Schmuths et al. 2004; Šmarda and Bureš 2006), while in other cases variation in DNA content was correlated with ecological differences (Kalendar et al. 2000; Knight and Ackerly 2002; Jakob et al. 2004) or with differences in plant phenotype (Knight et al. 2005; Murray 2005; Beaulieu et al. 2007; Achigan-Dako et al. 2008). However, neither spatial pattern nor correlation of genome size with vegetation types or microclimatic conditions was found in a highly variable population of *Festuca pallens* Host (Šmarda et al. 2008).

In this study we analyze genome size evolution using phylogenetic and statistical approaches in a species-rich group of drum-stick onions (*Allium* L. subg. *Melanocrommyum* (Webb et Berthel.) Rouy). Subgenus *Melanocrommyum* is one of the largest in the genus *Allium* comprising approximately 160 perennial species with an extremely short developmental period (ephemeral growth form; Hanelt et al. 1992) and outcrossing mating system (R. M. Fritsch, pers. comm.). *Melanocrommyum* taxa are almost exclusively adapted to arid conditions, and possess uniform karyotypes (Fritsch and Astanova 1998). The members of the subgenus are diploid (base chromosome number $x = 8$) with a few exceptions. Occasionally, tetraploid individuals have been reported in species otherwise known to be diploid but they were mostly neglected due to their rare occurrence (*A. atropurpureum* Waldst. et Kit., Ohri et al. 1998; *A. nigrum* L., Shopova 1972; *A. akaka* Gmelin ex Roem. et Schult., Vakhtina 1969). The observed karyological and ecological

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uniformity (i.e. absence of adaptive factors that often are correlated with genome size) makes the group particularly interesting to study genome size variation and evolution. Thus, any variation within the subgenus is expected to be phylogenetically constrained and influenced by the number of speciation events and neutral population-level phenomena (e.g. genetic drift and population size). Studies concerning genome size variation across the genus *Allium* using Feulgen cytophotometry (Ohri et al. 1998; Ohri and Pistrick 2001) reported variation in genome size in *Melanocrommyum* even though only 15 species of the subgenus were studied. Interestingly, the authors found no general correlation between genome size and life strategy differences among species of the genus *Allium* (Ohri and Pistrick 2001). In the present investigation genome size variation among 70 taxa of subgenus *Melanocrommyum* was determined using flow cytometry, and analyzed in a phylogenetic framework using the generalized least squares (GLS) method (Pagel 1997, 1999). Here, we (1) examine the range of genome size variation in *Melanocrommyum*, (2) study the correlation between genome size variation and phylogenetic relationships, (3) determine if there is evidence for alterations in genome sizes (both increase and decrease) in phylogenetic groups, (4) provide evidence that diploid and tetraploid cytotypes occur within single species of subgenus *Melanocrommyum*, and (5) suggest that within-species genome size differences confirm the existence of cryptic species inferred by molecular markers and emphasize the taxonomic importance of genome size variation.

Materials and Methods

Plant Material—Fresh leaf material of 160 accessions (70 taxa) representing all current taxonomic sections and phylogenetic groups of subgenus *Melanocrommyum* was obtained in early spring from the *Allium* Reference Collection of the IPK Gatersleben (Germany) (Table 1). For the majority of taxa two or more accessions were analyzed. Voucher specimens were deposited in the herbarium of the IPK Gatersleben (GAT).

Nuclear Genome Size Estimation—For flow cytometric measurements of the genome size, samples of fresh young leaves were chopped with a sharp razor blade together with *Vicia faba* L. cultivar 'Tinova' (2C = 26.21 pg) (Genebank Gatersleben accession number: FAB602) as an internal reference standard (Fig. 1) in a Petri dish containing 1 mL nuclei isolation buffer (Galbraith et al. 1983) supplemented with DNase-free RNase (50 μg/mL) and propidium iodide (50 μg/mL). The mixture was filtered using tubes with cell-strainer caps (35 μm; BD Biosciences, New Jersey). For species with genomes larger than 60 pg/2C, *Allium cepa* L. cultivar 'Vsetatska' (2C = 33.69 pg, Genebank Gatersleben accession number: ALL47), and with genomes smaller than 30 pg/2C, *Secale cereale* L. subsp. *cereale* (2C = 16.01 pg, Genebank Gatersleben accession number: R737) were used as internal reference standards. The relative fluorescence intensities of stained nuclei were measured using either a FACStar^{PLUS} (BD Biosciences) flow sorter equipped with an argon ion laser INNOVA 90C (Coherent GmbH, Dieburg, Germany) or a FACSAria flow sorter (BD Biosciences). Usually, 10,000 nuclei per sample were analyzed. Accessions with the most contrasting values were measured several times. The absolute DNA amounts of samples were calculated based on the values of the G1 peak means.

Chromosome Counts—Chromosome numbers were determined from root tip meristems for 34 accessions of 14 species (Table 1). The growing root tips were pre-treated in ice-cold (0°C) tap water for 24 hr. Root tips were then fixed in acetic acid:ethanol mixture (1:3) for 24 hr. After washing in distilled water, and hydrolyzing in 0.2N HCl for 20 minutes at room temperature, roots were stained with a saturated acetocarmine solution (carmine boiled in 45% acetic acid and filtered to remove undissolved crystals) and squashed in 45% acetic acid.

Phylogenetic Analyses—Phylogenetic relationships of *Melanocrommyum* species were based on a study by Gurushidze et al. (2008), which used sequences of the nrDNA ITS to infer phylogenetic relationships. The analyses presented here were based on a simplified Bayesian tree (Fig. 2) in which only a single individual per species was retained, and which depicts the estimated ancestral genome sizes as well as the 2C DNA values of the measured taxa. In cases when the individuals of one species showed topological differences in the phylogenetic tree, the individuals with different topologies were kept.

C-value Statistics and Evolution of Genome Size—Nuclear DNA-values (2C) for each species were calculated as mean values from at least three accessions. The statistical analyses were done both with and without known tetraploid accessions to see if well-known genome reorganization after polyploidization (Wendel 2000; Leitch et al. 2008) would significantly change the results. We used Kolmogorov-Smirnov test for every phylogenetic clade to check if the genome sizes were normally distributed. The entire data set was normally distributed, but the data did not show normal distributions within each clade. Therefore, Kruskal-Wallis test was used to determine whether DNA-value differences among phylogenetic clades were significant, defining clades resulting from the ITS phylogenetic analysis as grouping variable. Kruskal-Wallis is a nonparametric test that checks the null hypothesis that the different samples in the comparison were drawn from the same distribution or from distributions with the same median (Hill and Lewicki 2006). Both above mentioned statistical tests were conducted with SPSS 12.0 (SPSS Inc., Chicago, Illinois). However, statistical analysis of genome size differences is problematic because conventional statistical methods consider species as independent samples, although they are evolutionarily related. A more suitable approach for analyzing data that may not be statistically independent is generalized least squares (Pagel 1997, 1999). The method is implemented in the computer program BayesContinuous (within the package Bayestraits, www.evolution.rdg.ac.uk) and can be used to test for trait evolution, correlated trait evolution, and to perform regressions for continuously varying traits (Pagel and Meade 2007). Useful feature of BayesContinuous are the three scaling parameters kappa (κ), delta (δ), and lambda (λ), that can be estimated for a given data set and phylogeny. These scaling parameters allow tests of the mode, tempo, and phylogenetic associations of trait evolution (Pagel 2004). The kappa (κ) parameter differentially scales individual phylogenetic branch lengths and can be used to test for a punctuated versus gradual mode of trait evolution. If $\kappa = 0$, trait evolution is independent of the length of the branch and is consistent with a punctuated mode of evolution, while $\kappa > 1$ indicates that longer branches contribute more to trait evolution (gradual mode). Parameter delta (δ) scales the overall path in the tree (distance from the root to the species, as well as the shared path lengths) and can be used to test for adaptive radiation. If $\delta > 1$, then longer path lengths have contributed disproportionately to trait evolution, which is interpreted as accelerated evolution over time (species-specific adaptation), whereas an estimate of $\delta < 1$ is the signature of an adaptive radiation. The parameter λ is particularly important, as it tests for phylogenetic signal in the comparative data, i.e. assesses the contribution of phylogeny to the covariance among species on a given trait. A value of $\lambda > 0$ indicates strong phylogenetic signal, whereas $\lambda = 0$ suggests a star phylogeny (values of taxa are independent). The parameters κ, δ, and λ were estimated using the 'random-walk' model of evolution (model A). Determining the most appropriate model as well as evaluation of the above mentioned parameters were conducted using likelihood ratio statistics (Huelsenbeck and Rannala 1997).

Ancestral genome sizes within *Melanocrommyum* were assessed using the generalized least squares (GLS) method implemented in the computer program Compare version 4.6b, http://compare.bio.indiana.edu/ (Martins 2004). GLS tests the evolution of continuous characters along the phylogenetic tree from extant taxa and predicts each ancestral state as the weighted average of other taxa in the phylogeny (Martins and Hansen 1997). As both programs require a completely resolved binary tree, polytomies were resolved introducing short branches according to the authors' recommendation. The predicted genome sizes with standard errors (SE) for major lineages were plotted on the phylogenetic tree (Fig. 2).

Results

Genome Size Variation in Subgenus Melanocrommyum—The 2C DNA values of all measured species of subgenus *Melanocrommyum* varied from 26.26 pg in diploid *A. fetisowii* Regel to 78.73 pg in tetraploid *A. cyrilli* Tenore (Table 1). Several examples of the histograms of relative fluorescence

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TABLE 1. Accessions, chromosome numbers (when available), and genome sizes of the *Melanocrommyum* taxa used in this study. N refers to the number of analyzed individuals from each species; SD refers to standard deviation calculated from measurements of all individuals of each species. Chromosome counts are either from the present study, or taken from the literature (Ohri et al. 1998; Friesen et al. 2006). [1]Measurements were based on one or two individuals (thus SD-s are not available); [2]two distinct 2C values available within one taxon (for details see text); [3]chromosome counts from present study. Herbarium specimens for each taxon are deposited at GAT (see Gurushidze et al. 2008).

Species and subspecies	Accession No.	Chromosome number ($2n$)	2C DNA content (pg), species' average ± SD	N
Allium aflatunense B. *Fedtsch*.	5562, 5694, 5632	16	43.71 ± 0.33	7
Allium akaka S. G. Gmelin ex Roem. et Schult.	6397	32[3]	74.71[2] ± 0.49	3
Allium akaka S. G. Gmelin ex Roem. et Schult.	6398, 5980	16[3]	39.39[2] ± 0.10	4
Allium alexeianum Regel	6291	16[3]	34.18[2] ± 0.29	3
Allium alexeianum Regel	6303		31.63[2] ± 0.13	3
Allium altissimum Regel	2976, 6357	16	43.89 ± 0.12	4
Allium aroides Popov & Vved.	3703, 6189	16	38.09 ± 0.02	4
Allium atropurpureum Waldst. et Kit.	1017, 2194	16; 32	52.24 ± 0.05	4
Allium backhousianum Regel	616, 2680	16	44.30 ± 0.15	6
Allium bakhtiaricum Regel	3924, 6623		41.28 ± 0.58	4
Allium breviscapum Stapf	6478, 6480	16	45.95 ± 1.13	4
Allium cardiostemon Fischer et Meyer	3947	16	41.14[1,2]	2
Allium cardiostemon Fischer et Meyer	6081		38.83[2] ± 0.41	3
Allium caspium (Pall.) M. Bieb. subsp. *baissunense* (Lipsky) Khassanov et R. M. Fritsch	5873	16	34.92 ± 0.35	4
Allium caspium (Pall.) M. Bieb. subsp. *caspium*	5927	16	31.34 ± 0.37	3
Allium chelotum Wendelbo	6236	20	39.74 ± 0.24	3
Allium chitralicum Wang et Tang	6153	32	68.69[1]	1
Allium chychkanense R. M. Fritsch	5057, 5060		33.98 ± 0.96	7
Allium costatovaginatum Kamelin et Levichev ex Krassovskaja et Levichev	5783, 6364		34.46 ± 0.06	6
Allium cristophii Trautv.	5920	16	45.96 ± 0.25	3
Allium cupuliferum Regel	6350, 6354	16	40.83 ± 0.26	6
Allium cyrilli Tenore	1150, 1550, 5296, 5349	32[3]	78.73 ± 1.79	12
Allium darwasicum Regel	6134, 6138	16[3]	33.90[2] ± 0.05	4
Allium darwasicum Regel	6431, 6553		37.60[2] ± 0.09	6
Allium decipiens Fischer ex Schult. et Schult. f.	2709, 5837	16; 20	42.22 ± 0.12	5
Allium derderianum Regel	6390, 6394	16	43.42 ± 0.61	5
Allium dodecadontum Vved.	5695	16	37.41 ± 0.31	3
Allium elburzense Wendelbo	6519, 6658		51.26 ± 1.54	3
Allium ellisii J. D. Hooker	6249, 6254, 6255		44.73 ± 1.2	6
Allium fetisowii Regel	2975	16	26.26[2] ± 0.12	3
Allium fetisowii Regel	3693, 5052, 5688		28.37[2] ± 0.34	6
Allium giganteum Regel	6122, 6148, 6258	16	42.81 ± 0.86	6
Allium gypsaceum Popov et Vved.	5669	16	33.82[1]	2
Allium hamedanense R. M. Fritsch	6487		46.28 ± 0.57	4
Allium hollandicum R. M. Fritsch	1631, 2615, 2800	16	43.94 ± 0.61	10
Allium intradarvazicum R. M. Fritsch	6452, 6454	16[3]	37.92 ± 0.23	4
Allium isakulii R. M. Fritsch et F. O. Khass. subsp. *balkhanicum* R. M. Fritsch et Khassanov	5932		40.82 ± 0.64	3
Allium isakulii R. M. Fritsch et F.O. Khass. subsp. *subkopetdagense* R. M. Fritsch et Khassanov	5259, 5919	16	41.39 ± 0.32	3
Allium jesdianum Boiss. et Buhse subsp. *angustitepalum* (Wendelbo) Khassanov et R. M. Fritsch	1083, 3671	16[3]	38.57 ± 0.10	6
Allium jesdianum Boiss. et Buhse subsp. *jesdianum*	3951, 6261	16[3]	38.27 ± 0.24	4
Allium karataviense Regel	5040, 5793, 6133, 6366	18	42.66 ± 0.41	9
Allium koelzii (Wendelbo) K.Persson et Wendelbo	6501, 6509, 6491		54.91 ± 0.85	8
Allium komarowii Lipsky	3144, 6282, 6296	16[3]	40.66 ± 0.68	6
Allium kuhsorkhense R. M. Fritsch et Joharchi	6262		46.45[1]	2
Allium lipskyanum Vved.	1384, 3118, 5076	16[3]	33.62 ± 0.48	9
Allium macleanii J. G. Baker	2218, 2415, 6150, 6445	16	42.63 ± 0.83	12
Allium majus Vved.	5073		36.56 ± 0.65	3
Allium materculae Bordz. subsp. *graveolens* R. M. Fritsch ined.	6612		45.24 ± 0.58	3
Allium materculae Bordz. subsp. *materculae*	3948, 6402, 6406	16	42.74 ± 0.21	10
Allium motor Kamelin	3355, 6208	16	36.46 ± 0.06	4
Allium nevskianum Vved. ex Wendelbo	5078, 5451	16[3]	36.60 ± 0.18	7
Allium nigrum L.	515, 2616, 5321	16	54.81 ± 2.06	7
Allium orientale Boiss.	5352, 5365, 5371	16	43.68 ± 0.82	5
Allium protensum Wendelbo	2162	16	59.81[2] ± 2.06	3
Allium protensum Wendelbo	3672		32.49[1,2]	1
Allium pseudobodeanum R. M. Fritsch et Matin	3932, 3936, 6388		51.68 ± 1.41	7
Allium regelii Trautv.	5917	16	42.83 ± 0.86	5
Allium robustum Kar. et Kir.	6565	16	32.14 ± 0.30	3
Allium rosenbachianum Regel subsp. *kwakense* R. M. Fritsch	6159, 6461	16	47.58 ± 0.52	6
Allium rosenbachianum Regel subsp. *rosenbachianum*	6120, 6132	16	41.28 ± 0.48	3
Allium rosenorum R. M. Fritsch	1869, 2938, 6158	16[3]	40.83[2] ± 0.57	11
Allium rosenorum R. M. Fritsch	6290, 6298, 5081		38.68[2] ± 0.38	7

(Continued)

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Table 1. Continued

Species and subspecies	Accession No.	Chromosome number ($2n$)	2C DNA content (pg), species' average ± SD	N
Allium saposhnikovii E. Nikitina	6276	16	60.15[1]	2
Allium saralicum R. M. Fritsch	6506		42.80 ± 0.24	6
Allium sarawschanicum Regel	1323, 2946	16	34.20[2] ± 0.29	6
Allium sarawschanicum Regel	6248		36.21[2] ± 0.38	3
Allium schachimardanicum Vved.	5066		33.94 ± 0.05	4
Allium severtzovioides R. M. Fritsch	5043, 5879		32.09 ± 0.72	6
Allium sewerzowii Regel	5047	16	56.45 ± 1.25	4
Allium shelkovnikovii Grossh.	6404		41.72[2] ± 0.69	5
Allium shelkovnikovii Grossh.	6405		39.43[2] ± 0.55	5
Allium stipitatum Regel	3246, 3958, 3962, 3967, 5263	16[3]	43.93 ± 0.90	12
Allium suworowii Regel	5791, 6141	16	37.62 ± 0.27	6
Allium taeniopetalum Popov & Vved subsp. *mogoltavicum* (Vved.) R. M. Fritsch et Khassanov	5910, 6372	16	30.62 ± 0.02	4
Allium taeniopetalum Popov & Vved subsp. *taeniopetalum*	5249, 6358, 6531	16	31.27 ± 0.50	7
Allium tashkenticum Khassanov et R. M. Fritsch	6171	16	30.44 ± 0.36	6
Allium trautvetterianum Regel	6130	16	41.32 ± 0.51	3
Allium tulipifolium Ledeb.	6599	16	32.16 ± 0.28	6
Allium ubipetrense R. M. Fritsch	6475, 6507		43.54 ± 0.33	6
Allium verticillatum Regel	6162, 6525	16	33.49 ± 0.62	6
Allium vvedenskyanum Pavlov	3723	16	33.44 ± 0.52	3
Allium winklerianum Regel	6434, 6564	16[3]	32.87 ± 1.10	8

intensity are shown in Fig. 1. As chromosome numbers were not available for approximately one-third of the measured taxa, we report here only the 2C values and not the monoploid genome size (Cx), as the latter corresponds to the DNA content of the chromosome base number (Greilhuber et al. 2005).

Usually, genome size measurements from different accessions of the same species were similar, as the coefficient of variation was below 2.5%. However, we found conspicuous within-species genome size variability, which could be classified as (i) relatively small (6–9% of the measured genome size) and (ii) large (two-fold) differences in DNA content. The relatively small within-species genome size variation was detected in the following species: 6% of the whole genome size in *A. shelkovnikovii* Grossh. and *A. cardiostemon* Fischer et Meyer, 7.4% in *A. rosenorum* R. M. Fritsch, 7.8% in *A. fetisowii* Regel, 8% in *A. darwasicum* Regel, and 8.8% in *A. alexeianum* Regel. To test if the observed differences indeed reflected intraspecific genome size variations rather than technical artifacts, we co-chopped the leaves of accessions 6298 (38.28 pg/2C) and 1869 (41.11 pg/2C) of *A. rosenorum* and measured the fluorescent intensity of the nuclei mixture of these two accessions. A similar procedure was undertaken for accessions 6553 (37.25 pg/2C) and 6134 (33.94 pg/2C) of *A. darwasicum*. This experiment resulted in two clearly distinguishable individual peaks (Fig. 3), indicating that the genome sizes of the two corresponding accessions of the same species are unambiguously different from each other. Roughly two-fold differences in genome size were observed in two species: 74.71 pg and 39.39 pg DNA in *A. akaka*, and 59.81 pg and 32.49 pg DNA in *A. protensum* Wendelbo. To date, *A. akaka* has mainly been reported to be diploid ($2n = 16$; Pedersen and Wendelbo 1966; Pogosian 1983; Özhatay 1986), while one study noted tetraploid and even hexaploid individuals (Vakhtina 1969). We counted chromosomes of the *A. akaka* accession with doubled 2C DNA content and confirmed the tetraploid status of this accession ($2n = 4x = 32$; Table 1). Although chromosome counts for *A. protensum* were available only for one accession ($2n = 16$, Pedersen and Wendelbo 1966), the two-fold difference in DNA amount also suggests the occurrence of both diploid and tetraploid cytotypes in this species.

In *A. saposhnikovii* E. Nikitina (60.15 pg) and *A. sewerzowii* Regel (56.45 pg) the measured genome sizes were relatively large and corresponded to a doubled amount compared to closely related taxa (*A. tashkenticum* Khassanov et R. M. Fritsch 0.44 pg and *A. severtzovioides* R. M. Fritsch 32.09 pg). However, to date, both species (*A. saposhnikovii* and *A. sewerzowii*) have been reported to be diploid (Pogosian and Seisums 1992; Fritsch and Astanova 1998).

***Genome Size Variation Among Phylogenetic Clades*—**The phylogenetic tree based on ITS sequences of 110 species of subgenus *Melanocrommyum* consists of a "basal" grade (eight species) and a core clade consisting of the remaining species. In total, 12 well-supported groups (A-L) were circumscribed according to the ITS phylogeny (Fig. 2) (Gurushidze et al. 2008). The variation of genome size among the defined phylogenetic clades is represented in a box plot (Fig. 4). Table 2 shows the mean genome sizes, number of the species, maximum and minimum genome size values, and standard deviations for each phylogenetic group. The genome sizes in the "basal" grade were generally lower than in the core clade (33.65 ± 5.72 pg DNA and 42.01 ± 8.73 pg DNA, respectively). The basal-most group A possessed the lowest (29.70 ± 4.11 pg) and group C, belonging to the core clade, the highest (50.21 ± 5.71 pg) average DNA values (Fig. 4). The latter group unites morphologically similar circum-Mediterranean taxa, all belonging to section *Melanocrommyum,* and also contains the tetraploid species *A. cyrilli.*

The Kruskal-Wallis tests showed that genome size differences among the phylogenetic clades were significant. The ranking of the groups differed from one clade to another (Kruskal-Wallis $\chi^2 = 40.19$, df = 11, $p < 0.001$).

***Genome Size Evolution and Ancestral Genome Size Estimation*—**The presence of a phylogenetic signal was suggested by the generalized least squares (GLS), which predicted the maximum likelihood value of λ to be significantly

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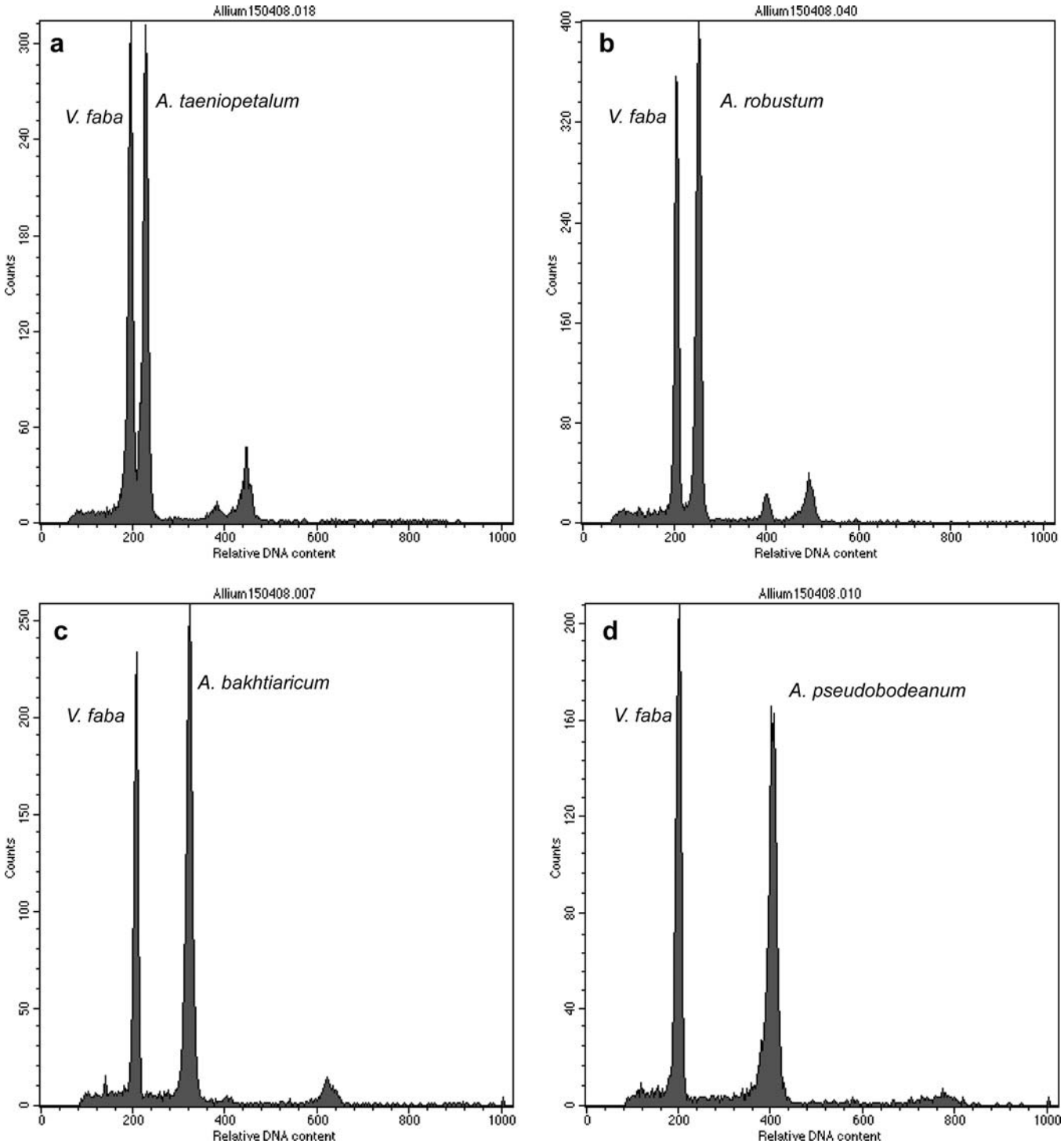


FIG. 1. Histograms of flow-cytometric genome size measurements in taxa of *Allium* subgenus *Melanocrommyum*. Examples of relative fluorescence intensity of propidium-iodide stained nuclei of the species *A. taeniopetalum* Popov & Vved., *A. robustum* Kar. et Kir., *A. bakhtiaricum* Regel, *A. pseudobodeanum* R. M. Fritsch et Matin measured with the internal standard *Vicia faba*.

greater than 0. The maximum likelihood estimates of the parameters λ, κ, δ, and respective interpretations are given in Table 3. The results of GLS (values of κ and δ) supported a punctuated mode of genome size evolution in *Melanocrommyum*, and suggests that most of the genome size variation evolved later rather than earlier in the phylogeny of the subgenus (species-specific adaptations).

Ancestral genome size estimation showed that genome size both increased and decreased during evolution along the lineages leading to extant species. In the "basal" grade most of the taxa show relatively low DNA contents, and GLS predicted for the root of the subgenus a 2C DNA content of 35.68 ± 6.21 pg, which is smaller than genome sizes of most contemporary taxa. However, it is obvious that both gain and

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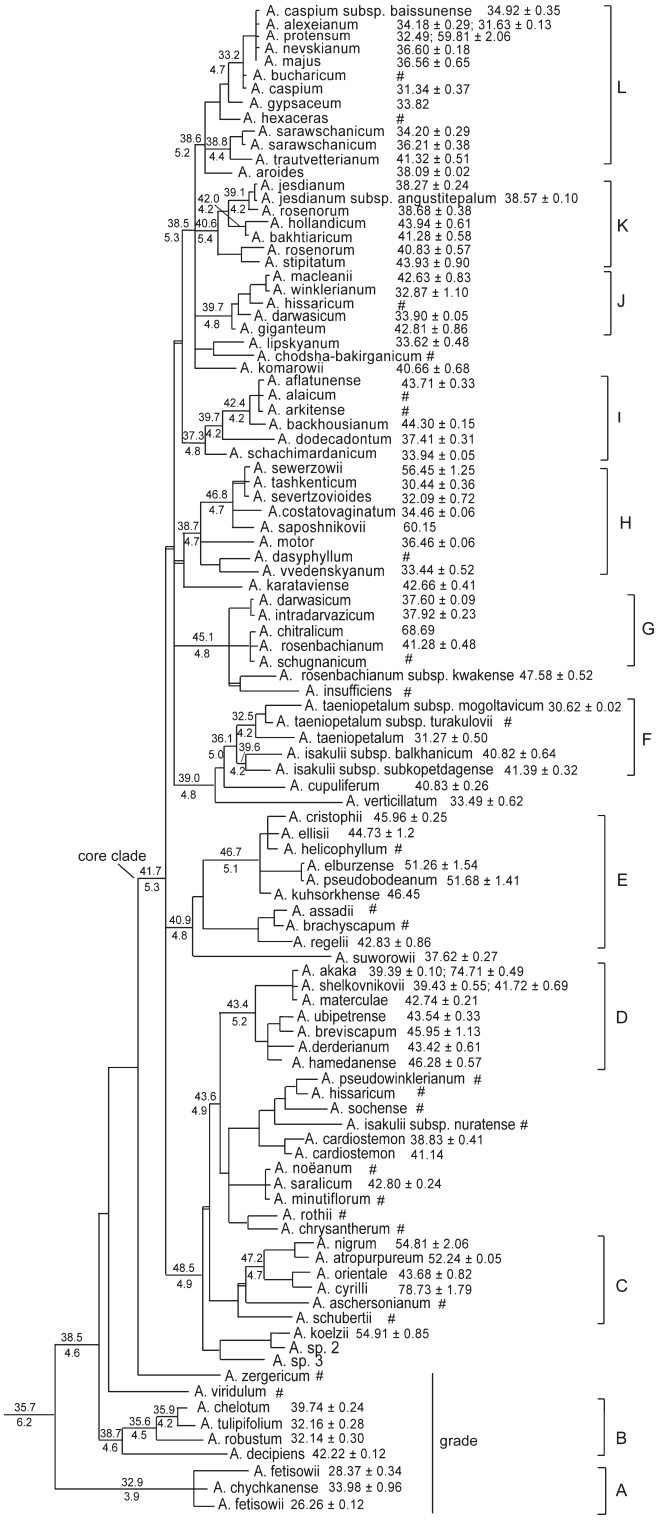


FIG. 2. Phylogenetic tree resulting from a Bayesian analysis of ITS sequences of subgenus *Melanocrommyum* (from Gurushidze et al. 2008). Gray double-lines indicate branches not present in the strict consensus (MP) tree. The numbers next to the species names depict 2C DNA values and standard deviations of respective species (wherever available), and the numbers along the branches show estimated ancestral genome sizes with standard errors (SE). Species for which genome sizes are not available, are depicted by #, the well-supported groups are indicated by uppercase letters on the right side (clades A – L).

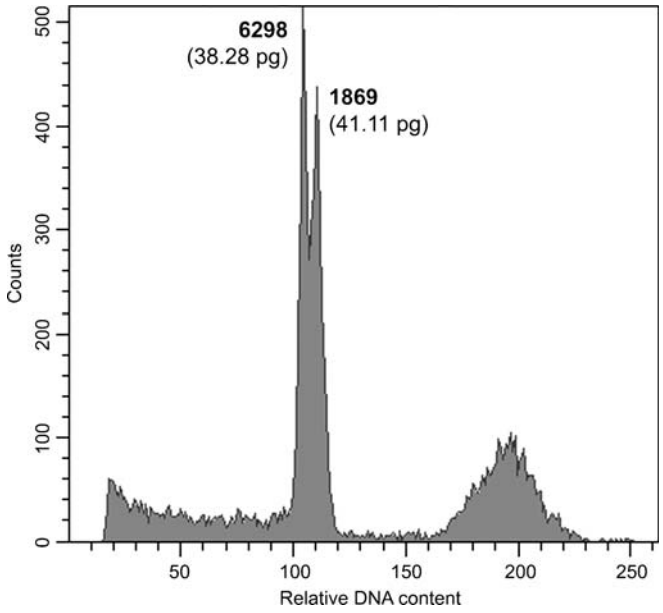


FIG. 3. A histogram of relative fluorescent intensity of co-measured *A. rosenorum* individuals with deviating genome sizes. The histogram shows two clearly distinguishable individual peaks (38.28 pg and 41.11 pg), indicating real genome size differences between individuals.

loss could have occurred in closely related lineages. For example, in clades F, I, and K genome size increased in one sister lineage, while it decreased in another (Fig. 2).

DISCUSSION

Genome Size Variation and Ploidy Level—The high genome size values (2C DNA content ranging from 26.26–78.73 pg) in

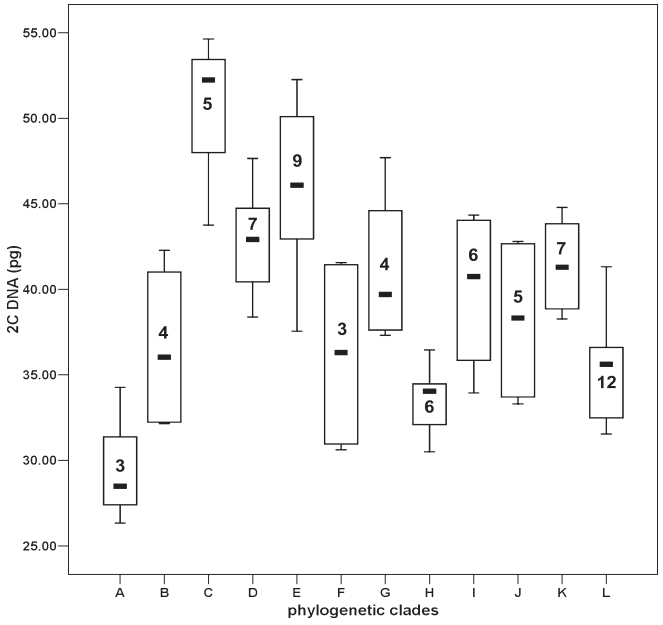


FIG. 4. Box plot displaying the distribution of 2C DNA values (Y – axis) across the defined phylogenetic clades (X – axis), resulting from the ITS analyses (phylogenetic relationships among clades A – L are depicted on Fig. 2). A solid box represents the median, whiskers – standard deviation, and Arabic numerals the number of the species in the respective clade.

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TABLE 2. Genome size amount (pg) in phylogenetic clades.

Clade	Species No.	Mean	Minimum	Maximum	SD
A	3	29.70	26.26	33.98	4.11
B	4	36.62	32.14	42.22	5.17
C	5	50.21	43.68	54.81	5.71
D	7	42.76	39.39	46.28	2.93
E	9	46.87	42.83	51.68	4.13
F	3	36.20	30.62	41.39	6.06
G	4	41.11	37.60	47.58	4.77
H	6	33.52	30.44	36.46	2.29
I	6	39.95	33.94	44.30	4.98
J	5	38.19	32.87	42.81	5.18
K	7	41.38	38.27	43.94	2.58
L	12	35.43	31.34	41.32	3.16

Melanocrommyum are in agreement with previous reports (Ohri et al. 1998; Ohri and Pistrick 2001). The observation that plants with large genomes have short periods of growth followed by long periods of dormancy (e.g. geophytes in the orders Asparagales and Liliales; Chase et al. 2005) holds also in *Melanocrommyum*. Although the underlying mechanisms are unknown, the subgenus *Melanocrommyum* is another example of plants having an extremely short developmental period and high amounts of DNA. Genome size differences across species are more than two-fold. Although it is clear, that all known tetraploids possess doubled DNA amount compared to confirmed diploid species (Table 1), insufficient information about the ploidy of many taxa with high amounts of DNA does not allow for conclusions as to whether the genome sizes among species are always correlated to ploidy level. For example, the high 2C DNA values in *A. saposhnikovii* (60.15 pg) and *A. sewerzowii* (56.45 pg) could indicate that tetraploid cytotypes occur in these species, taking into account that the closely related species have only half of those genome sizes (30–34 pg). However, the tetraploidy of these individuals first needs to be confirmed by chromosome counts. In contrast, within *A. protensum* where DNA content differs by two-fold between different accessions, the occurrence of diploid and tetraploid cytotypes is highly likely, and in *A. akaka* the detected doubled DNA amount corresponds to a confirmed tetraploid cytotype.

Taxonomic Relevance of Genome Size Variation—Pronounced within-species differences in genome size are often reported (e.g. Price et al. 1981; Graham et al. 1994; Bennett and Leitch 1995) and correlated with differences in the habitat (e.g. Kalendar et al. 2000; Jakob et al. 2004) or in plant phenotype (Knight et al. 2005; Murray 2005; Beaulieu et al. 2007). However, as noted by Obermayer and Greilhuber (2005), intraspecific variation in genome size must be taken with utmost caution, as some earlier studies probably reported differences caused by technical artifacts. Nevertheless, intraspecific genome size variations were also found to indicate microevolutionary differentiation and could be taxonomically significant (Murray 2005; Achigan-Dako et al. 2008). This was demonstrated in *Juncus biglumis* L. (Schönswetter et al. 2007), where genome size differences and molecular markers revealed congruent groups.

Although the aim of this paper was not to analyze intraspecific genome size variation in *Melanocrommyum*, in some taxa within-species differences were observed. In *A. rosenorum* and *A. darwasicum*, morphologically cryptic lineages were phylogenetically clearly separated by molecular data (ITS sequences and fingerprint markers; Gurushidze et al. 2008), and they also differed in genome sizes. Two species showing intraspecific genome size variation (*A. cardiostemon* and *A. fetisowii*) are not polyphyletic according to ITS sequences. However, the genetic differentiation at the ITS locus of these species is high (0.5% and 2.9% genetic distance, respectively). Thus, genetic divergence coupled with genome size differences within *A. rosenorum* and *A. darwasicum* indicates separately evolving lineages within morphologically indistinguishable (cryptic) species, while in *A. cardiostemon* and *A. fetisowii* genome size differences could suggest the process of ongoing microevolutionary differentiation. These examples underline the potential of genome size variation for species delimitation. There are several other examples (e.g. *Hordeum spontaneum* K. Koch, Kalendar et al. 2000; *Dactylis glomerata* L., Reeves et al. 1998; *Juncus biglumis* L., Schönswetter et al. 2007) where intraspecific genome size variation might be indicative of incipient speciation, and thus have taxonomic relevance.

Another explanation for the congruent differences of genome size and ITS sequences within the above-mentioned species could be ancient interspecific hybridization. Thorough population genetic analyses with sampling multiple individuals and analyzing additional unlinked loci would be required to distinguish between ongoing microevolutionary diversification and ancient hybridization in these cases.

In *A. shelkovnikovii* (6% difference in 2C DNA content) and *A. alexeianum* (8.8%), ITS data provided no indication for intraspecific differentiation. Nevertheless, the range of variation of genome size between different accessions seems high compared to the "normal" range of variation (up to 3% of measured genome size for most taxa). Although it is possible that the genetic differentiation within these species is too young for the resolution of the molecular markers used (Gurushidze et al. 2008, 2010), the hypothesis that differences in genome size in *A. shelkovnikovii* and *A. alexeianum* are the result of ancient introgression, is also plausible. Although we obtained no indication for the occurrence of B chromosomes in the karyotyped species, Fritsch and Astanova (1998) reported the presence of B chromosomes in one species of *Melanocrommyum*, thus the hypothesis that differences in genome size in *A. shelkovnikovii* and *A. alexeianum* are due to B chromosomes cannot be ruled out.

Genome Size Changes in Relation to Phylogeny—Both analyses, the generalized least squares and Kruskal-Wallis test, support the assumption that distribution of genome size is not random but rather dependent on phylogenetic

TABLE 3. Maximum likelihood parameter estimates for λ, δ, and κ calculated using the generalized least squares (Pagel 1997, 1999).

Parameter	Value	Interpretation
λ (test for phylogenetic signal)	0.60 (significantly > 0)	Significant phylogenetic signal
δ (test for tempo of evolution)	3.00 (significantly > 1)	Temporally later change (Species-specific adaptation)
κ (test for mode of evolution)	0.00	Punctuated evolution

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relationships. The outcomes of the statistical analyses are comparable for both data sets, including and excluding polyploids. The test for phylogenetic signal was positive using GLS, suggesting that genome size variation among species is adequately predicted by our phylogenetic tree. Based on comparison of the genome size between the "basal" grade and core clade, and estimated ancestral and contemporary genome size, the data suggest that the ancestral genome size in subgenus *Melanocrommyum* was relatively small. However, genome size changes do not always show a trend of increasing size, as both DNA gain and loss were detected even within closely related lineages. This is in agreement with other evolutionary reconstructions showing that both increases and decreases have occurred in different taxonomic groups (e.g. Wendel et al. 2002; Jakob et al. 2004).

According to GLS analyses, the evolution of genome size in subgenus *Melanocrommyum* follows the punctuated rather than gradual mode. In other taxa, the hypothesis of punctuated genome size evolution received support in *Orobanche* L. (Weiss-Schneeweiss et al. 2006) and Liliaceae (Leitch et al. 2007), whereas a gradual mode of evolution was inferred in *Hieracium* L. (Chrtek et al. 2009). Intraspecific stability of genome size (Greilhuber 1998; Gregory 2004) and reports of rapid genome reorganization after speciation (Rieseberg et al. 1995; Song et al. 1995; Özkan et al. 2001) promoted the idea that a punctuated model with most changes occurring immediately after speciation is more realistic rather than a gradual change in genome size (Albach and Greilhuber 2004; for review see Gregory 2004). However, to prove this hypothesis (punctuated mode more plausible than gradual), statistical analyses regarding the mode of the genome size evolution in a phylogenetic framework are needed in many other eukaryotic groups.

Considering the ecological uniformity of the entire subgenus, a pattern of non-adaptive evolution ($\delta > 1$) was rather expected. However, high variability of genome size in *Melanocrommyum* prompts to invoke non-adaptive theories of genome size evolution. Oliver et al. (2007) have proposed a simple proportional model of genome size evolution without invoking selection pressure. The model implies that the rate of genome size evolution is simply proportional to genome size. The authors showed that the fastest rates of evolution occur in the largest genomes, and this trend is evident across 20 major eukaryote taxonomic groups. The fixation of genome size changes (particularly the ones caused by neutral or effectively neutral length mutations) might also be neutral, primarily determined by stochastic events such as drift, especially in small populations or after founder events. Unfortunately, population genetic studies in non-model groups of organisms like *Allium* are largely missing, which makes this challenging hypothesis currently untestable.

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