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RESEARCH IN CONTEXT

Genome size and DNA base composition of geophytes: the mirror of phenology and ecology?

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• *Background and Aims* Genome size is known to affect various plant traits such as stomatal size, seed mass, and flower or shoot phenology. However, these associations are not well understood for species with very large genomes, which are laregly represented by geophytic plants. No detailed associations are known between DNA base composition and genome size or species ecology.
• *Methods* Genome sizes and GC contents were measured in 219 geophytes together with tentative morpho-anatomical and ecological traits.
• *Key Results* Increased genome size was associated with earliness of flowering and tendency to grow in humid conditions, and there was a positive correlation between an increase in stomatal size in species with extremely large genomes. Seed mass of geophytes was closely related to their ecology, but not to genomic parameters. Genomic DNA GC content showed a unimodal relationship with genome size but no relationship with species ecology.
• *Conclusions* Evolution of genome size in geophytes is closely related to their ecology and phenology and is also associated with remarkable changes in DNA base composition. Although geophytism together with producing larger cells appears to be an advantageous strategy for fast development of an organism in seasonal habitats, the drought sensitivity of large stomata may restrict the occurrence of geophytes with very large genomes to regions not subject to water stress.

Key words: Life-form, geophytes, genome size evolution, GC content, phenology, stomatal length, seed mass, ecology.

INTRODUCTION

Genome size and its consequences

Genome size (i.e. the DNA content of the unreplicated nucleus, 2C; Greilhuber *et al.*, 2005) varies considerably among eukaryotic organisms, with a minimum (1C = 2·25 Mbp) reported in *Encephalitozoon intestinalis* (Zygomycota; Vivares, 1999) and a maximum (1C = 1369 200 Mbp) disclosed in *Chaos chaos* (Amoebozoa; Friz, 1968), representing a difference of more than 600 000-fold. In tracheophytes, the difference is smaller but still remarkable: a minimum of 1C = 63·57 Mbp is present in *Genlisea margaretae* (Lentibulariaceae; Greilhuber *et al.*, 2006) and, for a long time, a known maximum of 1C = 124 597·2 Mbp has been reported for the geophyte *Fritillaria assyriaca* (Liliaceae; Bennett and Leitch, 2005). However, two larger genomes were reported in another geophytic species, *Trillium hagae* (Melanthiaceae) with 1C = 129 536·1 Mbp (Zonneveld, 2010), and *Paris japonica* (Melanthiaceae) with 1C = 148 880·9 Mbp (Pellicer *et al.*, 2010). Thus, the divergence in tracheophytes exceeds 2300-fold.

Regardless of the causes of the differences in genome size (e.g. polyploidy or the amount of repetitive and non-coding DNA; Bennetzen *et al.*, 2005), the actual amount of nuclear DNA may limit some plant traits, such as maximum height, growth rate, generation time and presence in certain types of niches (Knight *et al.*, 2005; Francis *et al.*, 2008). For species with larger genomes, the mitotic cell cycle takes additional time (Bennett, 1987; Francis *et al.*, 2008), and replicating more DNA is perhaps more energetically demanding (Cavalier-Smith, 2005). Cell division and thus the growth rates of plants with large genomes should be slower than those with smaller genomes in the same ecological conditions, as has been supported experimentally on root cells by Gruner *et al.* (2010). Moreover, mitosis and hence plant growth are inhibited by the low temperatures (López-Sáez *et al.*, 1966) that occur frequently during the spring in the temperate zone. This may imply that the very early spring ecological and temporal niche, and thus the rapid growth rate, during this period may appear to be inaccessible for herbaceous plant species with large genomes. However, among early spring-flowering species, some display rapid growth and development in spite of their unusually large genomes (Bennett and Leitch, 2005). Interestingly, most of these plants are geophytes, indicating that in addition to ecology-driven forces, the evolution of genome size is also closely related to specific life forms.

Genome size and life forms

The standardized terminology of plant life history distinguishes the following terrestrial life forms: phanerophyte, chamaephyte, hemicryptophyte, geophyte and therophyte

(Raunkiaer, 1934). According to the triangular scheme by Leitch and Bennett (2007), for every herbaceous species, there exists a minimum generation time that is determined by its genome size, and having a large genome clearly does not allow a plant species to adopt certain life strategies. A therophyte (an ephemeral or annual species) must therefore have a small genome to be able to complete its life cycle before the end of the growing season. Phanerophytes (trees and shrubs) are not temporally limited like therophytes, although some structural restriction may exist regarding cell size (correlated with genome size) (Ohri, 1998; Knight and Beaulieu, 2008). Therefore, only several woody angiosperm plants are known to posses larger genomes (cf. Bennett and Leitch, 2005). The remaining categories (monocarpic or perennial hemicryptophytes and geophytes) are also thought to show ecological constraints associated with genome size (Knight *et al.*, 2005), but they show no clear reason to be strongly limited by genome size (Leitch and Bennett, 2007). Nevertheless, extremely large genome sizes are not found randomly in all categories but clearly predominate in species with a geophytic life form (e.g. the genera *Paris*, *Trillium*, *Fritillaria*, *Erythronium* and *Leucojum*; Bennett and Leitch, 2005). Until recently, however, genome size in geophytes had not been studied systematically, so conclusions on the expected role of this life form in the evolution of plant genome gigantism could not be made. Published data on the genome size of geophytic species indicate that genome gigantism is perhaps not allowed in all phylogenetic lineages and is associated with some specific ecological conditions, such as spring growth (Grime and Mowforth, 1982). However, no detailed study of geophyte ecology in relation to genome size has been made, to date, across a wider phylogenetic spectrum.

Geophytic life form

In this study, we focused on a particular type of geophyte, namely seasonal plants with a storage organ (i.e. bulb, tuber, fusiform roots or thick rhizome) that is usually subterranean but may also be partially above ground level (e.g. *Urginea maritima*). This definition differs partly from the strict definition of geophytes by Raunkiaer (1934). In considering geophytes, we describe the seasonal type only, not those that have neither a storage organ nor seasonal behaviour (e.g. *Elytrigia repens*). Geophytism in this form should be considered an adaptation to the cyclicity of changes in environmental conditions, such as an alternation of the short light phase by a long period of shading or appearance of long periodic droughts, which are inaccessible for most species with other life strategies (De Hertogh and Le Nard, 1993; Kamenetsky *et al.*, 2005; Al-Tardeh *et al.*, 2008). Geophytes overcome unfavourable growing conditions by persisting in the form of a subterranean organ that can accumulate sufficient nutrients available for the fast development of the above-ground body during annually cycling periods of suitable conditions (Dafni *et al.*, 1981*a*, *b*). The stored reserves allow geophytes to develop successfully even at very low initial external energy supply. This trait provides them with a favourable strategy (1) in steppe vegetation in seasonally arid Mediterranean climate or (2) in temperate deciduous forests in which the light phase is restricted to cold seasons characterized by a low initial level of solar insolation – a niche generally avoided by therophytes. Unlike the therophytes, in which the plant body development depends on the speed of cell division, the fast development of a geophytic body is enabled by pumping the water to the cells, which are pre-divided underground in the storage organ during the photosynthetically inactive dormant period (Grime and Mowforth, 1982; Grime, 1983; Greilhuber, 1995; Lapointe, 2001; Werger and Huber, 2006). Because their cells are full of water, the plant body is frequently fleshy and to protect themselves from herbivore damage, they usually contain various toxic or repellent substances (e.g. the genera *Cyclamen*, *Colchicum*, *Urginea* and *Allium*; Spoerke *et al.*, 1987; Klintschar *et al.*, 1999; Al-Tardeh *et al.*, 2008).

Genome size and geophyte phenology

Geophytes are often regarded as spring-flowering plants. However, in northern temperate zones, there are geophytes that flower not only in spring (March–May, e.g. *Galanthus nivalis*, *Eranthis hyemalis*), but also in summer (June–August, e.g. *Allium flavum*, *Urginea maritima*), autumn (September–November, e.g. *Colchicum autumnale*, *Sternbergia colchiciflora*) and winter (December–February, e.g. *Helleborus niger*). With more intensive study of genome sizes, a negative correlation has been reported between genome size and the timing of shoot expansion (Grime and Mowforth, 1982; Grime *et al.*, 1985). As the timing of shoot expansion is related to the timing of flowering, a relationship may also be present between genome size and the timing of flowering. This relationship has been observed in geophytic genus *Allium* (Baranyi and Greilhuber, 1999; but see also Ohri and Pistrick, 2001), although it is not clear to what extent these observations may be generalized for geophytes.

Genome size and stomatal length

Stomata play a key role in the regulation of gas exchange and overall plant photosynthesis (balancing carbon assimilation and plant water status), and are considered to have been one of the key elements in the evolution of advanced terrestrial plants (Farquhar and Sharkey, 1982; Edwards *et al.*, 1998). The regulation by stomata of carbon dioxide uptake is related to stomatal length as large stomata are known to increase sensitivity of plants to drought (Raven, 2002; Hetherington and Woodward, 2003; Franks and Beerling, 2009).

In higher plants, stomatal guard cell length is known to be very closely correlated with genome size (Sax and Sax, 1937; Bennett, 1987; Jovtchev *et al.*, 2006; Beaulieu *et al.*, 2008; Hodgson *et al.*, 2010), implying that the evolution of genome size would necessarily also have important physiological consequences. This correlation partly results from the constraint on cell functioning given by the existence of an optimum ratio between nuclear ($\approx$ DNA content) and cytoplasm volumes, long known as the 'karyoplasmic ratio' (Hertwig, 1903; Cavalier-Smith, 2005; Jorgensen *et al.*, 2007). However, the relationship between genome size and stomatal length (cell size) has generally been studied on a limited scale in species with relatively small genomes, rarely larger than 2C = 60 000 Mbp (Beaulieu *et al.*, 2008). Thus,

the question remains whether the observed relationship is universal across the whole spectrum of genome and cell sizes and what consequences extremely large stomata may have for the ecology of species with large genomes.

Genome size and seed mass

Previous studies reported positive correlations between genome size and seed mass (e.g. Mowforth, 1985). If there is a causal relationship between cell size and genome size, some dependence of seed mass on genome size could be also expected, based on the fact that seeds are also composed of a definite number of cells (but see also Egli, 1998, 2006). However, in some cases (Bennett, 1987), a triangular type of relationship was revealed: species with small genomes can have either small or large seeds, but species with larger genomes have only larger seeds. A recent study (Beaulieu *et al.*, 2007) based on a survey of 1222 species showed a generally positive correlation between genome size and seed size. Here, we were interested to see whether a similar relationship may be found also in geophytes and whether this relationship might differ for species with extremely large genomes.

GC content variation

The genomic percentage of guanine + cytosine bases (GC content) is highly variable among prokaryotes and other unicellular organisms (Meister and Barow, 2007). In tracheophytes, GC content is generally narrower and ranges from 35 to 40 % (Barow and Meister, 2003; Meister and Barow, 2007). Only grasses (family *Poaceae*) are known, as yet, to significantly exceed this range and regularly contain species with GC contents above 40 %. Since the early studies on DNA base composition, a question has arisen regarding to what extent changing of GC content is a passive consequence of molecular mechanisms of genome size change and to what extent it may represent a selective advantage for species with certain ecology. Although the relationship between GC content and genome size has been addressed in studies of genome composition from prokaryotes to plants, knowledge of the possible ecological consequences of GC content change are completely unknown in plants.

A positive relationship between genome size and GC content has been documented in prokaryotes (Musto *et al.*, 2006) and vertebrates (Vinogradov, 1998). Some reports have shown that the relationship might also exist in plants (Bureš *et al.*, 2007; Šmarda *et al.*, 2008), where positive correlations were reported in groups of phylogenetically related taxa (e.g. species within a particular genus or family). A negative correlation is known only for teleost fishes (Vinogradov, 1998). In plants, the most detailed study across 54 species from diverse angiosperms and gymnosperm families did not show any significant trend (Barow and Meister, 2002). Until recently, the correlation of genome size and GC content has been tested only with classical statistical tests, which do not consider the phylogenetic dependence of compared species. In addition to the very poor knowledge on GC content in plants, this practice may result in biased estimates of the relationship. Therefore, a phylogenetically corrected analysis is warranted. Geophytes may be a suitable group for such an analysis, as they occur in a wide spectrum of phylogenetic lineages of tracheophytes and cover almost the entire scale of plant genome sizes, providing enough robust data for hypothesis testing. They may be also useful for the initial testing of possible consequences of GC content on plant ecology, although the restriction of the study to geophytic species necessarily limits the scale of testable conditions and associations.

MATERIAL AND METHODS

Sampling and cultivation

In total, 219 species were sampled (see Supplementary Data Table S1, available online), collected mostly from wild populations in the Czech Republic, Slovakia, Italy, France, Portugal, Romania, Bulgaria, Turkey and Israel. Some samples were obtained also from collections in the larger Czech botanical gardens: the Botanical Garden of the Faculty of Science, Masaryk University, Brno; the Botanical Garden of the Faculty of Science, Charles University, Prague; and Prague Botanic Garden, Prague. Collected plants were cultivated in pots under field conditions in the Department of Botany and Zoology, Masaryk University. When available, the fruits of cultivated plants were collected for analysis.

Flow cytometry

Fresh leaf material was taken from plants in cultivation, and the genome sizes and GC contents were estimated for each species using flow cytometry with internal standardization (ML, Partec GmbH, Münster, Germany). Intercalating propidium iodide or AT-selective DAPI dyes were used in a two-step procedure with Otto I and Otto II buffers (Otto, 1990). Simultaneous measurements with intercalating and AT-selective fluorochromes were used to estimate the AT and GC contents, according to the methodology described by Barow and Meister (2002). Detailed sample preparation and dye concentrations follow Šmarda *et al.* (2008). As standards we used primarily those recommended by Doležel and Greilhuber (2010; Table 1). In contrast to the original values

TABLE 1. *Standards used for flow cytometry measurements (when exactly known, the names of cultivars are given)*

Standard	Genome size, 2C (Mbp)	GC content (%)
Oryza sativa 'Nipponbare'	777·64	43·60
Carex acutiformis	799·93	36·46
Ipomoea quamoclit	1238·30	38·61
Solanum lycopersicum 'Stupické Polní Rané'	1696·81	38·72
Epipremnum aureum	7815·39	42·70
Pisum sativum 'Ctirad'	7841·27	41·77
Ruscus aculeatus	20 137·45	42·37
Vicia faba 'Inovec'	23 272·88	41·15
Crinum asiaticum	40 470·59	40·49
Galanthus nivalis	61 089·39	43·07
Leucojum aestivum	61 563·46	42·33

derived from a comparison with human sequence (based on human 2C = 7 pg), their genome sizes and GC contents were derived from a comparison with the fully sequenced cultivar of rice (*Oryza sativa* 'Nipponbare', 2C = 777·64 Mbp, GC = 43·6 %; International Rice Genome Sequencing Project, 2005), a gold standard with the most complete genomic sequence of any angiosperm species to date (exact genome cover 95 %). For *Solanum lycopersicum*, genomic parameters were calculated directly from the measurements with rice. For the other primary standards (Table 1), the genomic parameters were derived from the standard sample ratios with the respective dyes given by Doležel *et al.* (1992). In this way, human cells show a genome size of 6055·03 Mbp (6·19 pg), very close to the DNA content of human expected by Human Genome sequence Consortium (International Human Genome Sequencing Consortium, 2004; 6153·33 Mbp) and the estimates of human genome size obtained with alternative biochemical methods (cf. Doležel and Greilhuber, 2010). We avoided using the human genome as a gold standard because of uncertainty regarding the completeness of its repetitive portion and because of the possible effect of isochore structure and unusual GC content patterning of its genome on the binding ability of the dyes used, which might differ substantially from that in plant genomes (P. Šmarda and P. Bureš, unpubl. res.). In the case of peak overlap between standard and sample, a series of secondary standards chosen from available samples was also established (Table 1). Sample details and secondary standard measurements are given in Supplementary Data Table S1. The base contents were calculated in a spreadsheet by Šmarda *et al.* (2008), which is available at http://sci.muni.cz/botany/systemgr/download/Festuca/ATGCFlow.xls.

Phenology of flowering and growth

Data on flowering phenology were analysed in 164 species of geophytes. For 134 species (82 %) they were taken from Pignatti (1982), comprising regionally standardized data. For the remaining 30 studied species not included in the Pignatti's *Flora*, but cultivated or native to the Czech Republic (Kubát *et al.*, 2002), comparable data on flowering phenology were extrapolated from a comparison of data on species common to both regions using a logarithmic regression. The phenology of species in the Czech Republic was observed either in the Botanical Garden of the Faculty of Science, Masaryk University, or taken from Kubát *et al.* (2002). The extrapolated data were rounded to be consistent with those in the Pignatti's *Flora*. The duration of the growing season (i.e. the time between sprouting and senescence of the photosynthetic apparatus) for 93 European geophytic species was observed in the field in the surroundings of Brno and in the Botanical Garden of the Faculty of Science, Masaryk University.

Stomatal length measurement

Stomatal size is a species-specific trait with a limited variation that may be induced by environmental conditions (Lomax *et al.*, 2008) or endopolyploidy, as common in cells of other plant tissues (Melaragno *et al.*, 1993; Barow, 2006). For the majority of samples, we measured stomatal size from surface impressions by means of a microrelief method: a thin layer of transparent nail polish was applied to the leaf surface, allowed to dry, and then removed with adhesive tape and placed on a microscope slide. Slides were observed with an Olympus BX-51 microscope, under 400–1000× magnification. Digitally documented slides were analysed manually in the Olympus Cell F program. The average stomatal length for each species was based on at least 30 measurements from 1–2 leaves.

Seed mass measurement

Seed masses were determined in species where fruits were available. If possible, at least 30 dry seeds were weighed per species (Kern 770 analytical balances; Kern & Sohn GmbH, Balingen, Germany) to estimate average seed mass. Seeds from the latest harvest were preferred in the measurements. Dry single-seeded fruits (achene) were weighed whole (i.e. with the pericarp), as pericarp weight was negligible relative to seed weight.

Ecological characteristics

To characterize species ecology we used Pignatti's indicator values (Pignatti, 2005), describing the ecological requirements of species on a 12-degree ordinal scale (light, temperature, continentality, soil moisture, soil reaction and soil nitrogen). These ordinal data provide a reliable approximation of *in situ* measured ecological parameters of species habitats (Schwabe *et al.*, 2007) and are frequently used for approximation of species ecology in European ecology research. Pignatti's values were preferred over the analogous Ellenberg's values (Ellenberg *et al.*, 1991), because of their wide availability for the majority of studied species.

Statistical and phylogenetic analyses

Data were analysed by both classic and phylogenetic statistical methods using the programs Statistica 9 (Starsoft Inc., Naperville, Il, USA; Spearman's Rho correlation test, Linear regression) and Phylocom 4·1 [analysis of traits module, phylogenetically independent contrast analysis (PIC)] (Webb *et al.*, 2008). For the phylogenetic analyses of samples in Phylocom, an evolutionary tree was compiled for the test species (see Supplementary Data Table S1). The phylogeny used followed the APG 3 system (Stevens, 2001 onwards; Angiosperm Phylogeny Group, 2009), and the end-taxa were arranged according to previous studies when available (Stevens, 2001 onwards; Wang *et al.*, 2009). Clades without a clearly resolved phylogenetic history or unclear phylogenetic positions were treated as polytomies. In such cases, putative trees were drawn, based on a conventional hierarchy of taxonomic categories (e.g. species clustered in sections and sections in genera). Statistical results were rounded to three decimal places.

RESULTS AND DISCUSSION

Genome size

Genome sizes measured in 219 geophytic species in this study differed 220-fold, with a minimum 2C = 582·18 Mbp detected in *Aristolochia rotunda* (Aristolochiaceae) and maximum 2C = 128 273·07 Mbp found in *Sprekelia formosissima* (Amaryllidaceae; Table 2).

Genome sizes exceeding 2C = 40 000 Mbp were found in 33 of the 219 geophytic species analysed. They are represented by species from the orders Liliales and Asparagales. Some geophytes with large genomes were also found also in the families Ranunculaceae, Paeoniaceae, Adoxaceae and Araceae. In contrast, geophytes with small genomes were found regularly across the whole tracheophyte phylogeny.

A simple inspection of the C-value database (Bennett and Leitch, 2005) indicates that genome size in geophytes is perhaps larger than that in plant life forms, although the exact effect of geophytic life form on genome evolution remains to be tested in detail with phylogenetically independent methods. The geophytic life form is found only rarely in species with lower genome sizes and, despite the clear overrepresentation of large genomes in geophytes (406-fold; minimum 733·5 Mbp; maximum 297 761·88 Mbp; Bennett *et al.*, 1982; Pellicer *et al.*, 2010), they show a very similar range of genome size to non-geophytic plants (667-fold; Greilhuber *et al.*, 2006; Bennett and Leitch, 2005).

Genome size and stomatal length

Log-transformed genome size and stomatal length data were tightly linearly correlated (Table 2) with the parameters of the regression line very close to that observed across all angiosperms by Beaulieu *et al.* (2008) and Hodgson *et al.* (2010). This correlation was also significant when using PICs (Table 2). In addition to the former analyses with a limited number of species with extremely large genomes, our analysis suggests that this relationship may be extremely tight for plant with very large genomes that never show stomatal size less than a certain threshold (Fig. 1). Different slopes of the nregression lines may be then observed in the separate datasets with species with low to high genome sizes ($2C < 40\,000$ Mbp, $r = 0{\cdot}448$, $P < 0{\cdot}001$; Fig. 1), and extremely high genome sizes ($2C \geq 40\,000$ Mbp, $r = 0{\cdot}545$, $P = 0{\cdot}003$; Fig. 1). The steepness of the regression line in species with extremely large genomes was not simply an artefact of data division but appears to be due to the lack of small-sized stomata in plants with extremely large genomes. This trend was also documented with separate analyses of PICs for the two subsets of data, where correlation of PICs for genome size and stomatal size was much tighter at genome size >40 000 Mbp ($2C < 40\,000$ Mbp: PIC: $r = 0{\cdot}579$, $P < 0{\cdot}001$; $2C \geq 40\,000$ Mbp: PIC: $r = 0{\cdot}789$, $P < 0{\cdot}001$). To verify that the absence of smaller than expected stomatal sizes in plants with extremely large genomes (>60 000 Mbp; Fig. 1) is not an artefact of the limited life form investigated in our study, we provide measurements of stomatal sizes for known non-geophytic species with extremely large genomes (*Tradescantia virginiana*) as well as from plants with the largest known genomes (nine species of the genus *Fritillaria*). Including data from these species did not change the trend showing a strong threshold for minimum stomatal size in species with $2C > 60\,000$ Mbp (Fig. 1).

Assuming that the size of a cell may be determined jointly based on ecologically driven constraints on leaf morphology and plant physiology (Hetherington and Woodward, 2003; Hodgson *et al.*, 2010) and genome size, our data suggest that extremely large genomes do not allow stomatal size to fall below certain limits that may be necessary for correct cell functioning. At the same time, however, producing extremely large stomata could be disadvantageous given that they are only rarely observed in plants with very large genomes. With decreasing genome size, the association between genome size and minimum cell size may become less of a constraint, allowing stomatal size to be driven much more intensively by other specific eco-morphological and eco-physiological constraints. As a consequence, stomatal sizes in species with small genomes show relatively high variation and close correlation with specific eco-physiological adaptations rather than a strict correspondence with genome size (cf. Hodgson *et al.*, 2010).

Genome size and flowering phenology

A close negative relationship was observed between genome size and end of flowering (Fig. 2; Table 2). The relationship was also verified using analysis of PICs (Table 2). There was also a weak association between large genome sizes and onset of flowering, although this relationship was below accepted significance levels by means of classical statistics and also by analysing PICs (Table 2).

As mentioned in the Introduction, low temperatures inhibit mitosis and therefore growth of the entire plant. However, in temperate zones, low temperatures frequently occur in early spring for extended periods of time, so a herbaceous spring plant cannot produce a large body composed of many small cells. To overcome this problem, two basic strategies have evolved among spring herbaceous plants. The first is to be an ephemeral therophyte with a small body that may develop quickly in response to increasing temperatures. The second is to generate a larger body from fewer but larger cells (Fig. 3), as is typical of many spring (vernal) geophytes (Grime, 1983), often termed ephemeroids. The cells and whole organs of ephemeroid plants are pre-formed during the photosynthetically inactive period in the preceding season to avoid the mitotic inhibition induced by low temperatures in spring (Grime and Mowforth, 1982; Grime, 1983; Greilhuber, 1995; Lapointe, 2001; Werger and Huber, 2006). After sprouting in the spring, ephemeroids grow largely by expansion of cells rather than by cell division. As the growing season of these plants is not longer than 3 months when enough water is available, they do not invest much energy in building an organ skeleton: instead of producing thick cell walls, they maintain sufficient turgor pressure in their cells to preserve their body forms. In late spring, when water becomes scarcer, it may become difficult for plants to maintain enough turgor pressure, their leaves wither and they finish the growing season by producing fruits (Lapointe, 2001).

The fact that geophytic species with smaller genome sizes could flower at any time during the growing season (Fig. 2), whereas species with increased genome size are conspicuously

TABLE 2. *Results of statistical tests for the traits analysed*

Trait	Genome size, 2C	GC content	Stomatal length	Seed mass
Genome size, 2C		$n = 219$ $r_s = 0{\cdot}025$ $P = 0{\cdot}713$ PIC: **$r = -0{\cdot}269$** **$P = 0{\cdot}001$**	$n = 164$ **$r = 0{\cdot}591$**[a] **$P < 0{\cdot}001$**[a] PIC: **$r = 0{\cdot}600$** **$P < 0{\cdot}001$**	$n = 57$ $r_s = -0{\cdot}071$ $P = 0{\cdot}598$ PIC: $r = -0{\cdot}035$ $P = 0{\cdot}811$
GC content	$n = 219$ $r_s = 0{\cdot}025$ $P = 0{\cdot}713$ PIC: **$r = -0{\cdot}269$** **$P = 0{\cdot}001$**		$n = 164$ **$r_s = 0{\cdot}274$** **$P < 0{\cdot}001$** PIC: $r = -0{\cdot}153$ $P = 0{\cdot}087$	$n = 57$ $r_s = -0{\cdot}096$ $P = 0{\cdot}476$ PIC: $r = -0{\cdot}220$ $P = 0{\cdot}133$
Flowers from	$n = 164$ $r_s = -0{\cdot}131$ $P = 0{\cdot}094$ PIC: $r = -0{\cdot}144$ $P = 0{\cdot}119$	$n = 164$ $r_s = 0{\cdot}021$ $P = 0{\cdot}791$ PIC: $r = -0{\cdot}009$ $P = 0{\cdot}919$	$n = 113$ $r_s = -0{\cdot}053$ $P = 0{\cdot}576$ PIC: $r = 0{\cdot}015$ $P = 0{\cdot}889$	
Flowers to	$n = 164$ **$r_s = -0{\cdot}229$** **$P = 0{\cdot}003$** PIC: **$r = -0{\cdot}242$** **$P = 0{\cdot}008$**	$n = 164$ $r_s = -0{\cdot}019$ $P = 0{\cdot}811$ PIC: $r = -0{\cdot}090$ $P = 0{\cdot}334$	$n = 113$ $r_s = -0{\cdot}173$ $P = 0{\cdot}067$ PIC: $r = -0{\cdot}077$ $P = 0{\cdot}473$	
Duration of growing season	$n = 93$ $r_s = -0{\cdot}105$ $P = 0{\cdot}317$ PIC: $r = -0{\cdot}136$ $P = 0{\cdot}243$	$n = 93$ **$r_s = -0{\cdot}279$** **$P = 0{\cdot}007$** PIC: $r = -0{\cdot}009$ $P = 0{\cdot}938$	$n = 68$ **$r_s = -0{\cdot}450$** **$P < 0{\cdot}001$** PIC: **$r = -0{\cdot}313$** **$P = 0{\cdot}023$**	
EIV-light	$n = 130$ $r_s = 0{\cdot}017$ $P = 0{\cdot}849$ PIC: $r = -0{\cdot}184$ $P = 0{\cdot}071$	$n = 130$ $r_s = -0{\cdot}096$ $P = 0{\cdot}280$ PIC: $r = 0{\cdot}047$ $P = 0{\cdot}650$	$n = 87$ $r_s = 0{\cdot}076$ $P = 0{\cdot}481$ PIC: $r = -0{\cdot}077$ $P = 0{\cdot}529$	$n = 40$ **$r_s = -0{\cdot}326$** **$P = 0{\cdot}040$** PIC[b]: $r = -0{\cdot}170$ $P = 0{\cdot}338$
EIV-temperature	$n = 123$ $r_s = -0{\cdot}026$ $P = 0{\cdot}778$ PIC: $r = -0{\cdot}191$ $P = 0{\cdot}065$	$n = 123$ $r_s = -0{\cdot}026$ $P = 0{\cdot}773$ PIC: $r = 0{\cdot}028$ $P = 0{\cdot}791$	$n = 82$ $r_s = 0{\cdot}028$ $P = 0{\cdot}800$ PIC: $r = 0{\cdot}073$ $P = 0{\cdot}558$	$n = 38$ $r_s = -0{\cdot}187$ $P = 0{\cdot}260$ PIC[b]: $r = 0{\cdot}001$ $P = 0{\cdot}997$
EIV-continentality	$n = 130$ $r_s = -0{\cdot}124$ $P = 0{\cdot}159$ PIC[b]: $r = -0{\cdot}042$ $P = 0{\cdot}683$	$n = 130$ $r_s = -0{\cdot}070$ $P = 0{\cdot}428$ PIC[b]: $r = -0{\cdot}006$ $P = 0{\cdot}953$	$n = 87$ $r_s = -0{\cdot}201$ $P = 0{\cdot}063$ PIC: $r = -0{\cdot}138$ $P = 0{\cdot}257$	$n = 40$ **$r_s = 0{\cdot}376$** **$P = 0{\cdot}017$** PIC[b]: $r = 0{\cdot}137$ $P = 0{\cdot}439$
EIV-soil moisture	$n = 127$ $r_s = -0{\cdot}144$ $P = 0{\cdot}106$ PIC: $r = 0{\cdot}114$ $P = 0{\cdot}271$	$n = 127$ $r_s = -0{\cdot}152$ $P = 0{\cdot}088$ PIC: $r = -0{\cdot}104$ $P = 0{\cdot}314$	$n = 84$ $r_s = 0{\cdot}053$ $P = 0{\cdot}631$ PIC: $r = 0{\cdot}236$ $P = 0{\cdot}054$	$n = 40$ $r_s = 0{\cdot}256$ $P = 0{\cdot}112$ PIC[b]: $r = 0{\cdot}046$ $P = 0{\cdot}795$
EIV-soil pH	$n = 125$ $r_s = 0{\cdot}011$ $P = 0{\cdot}905$ PIC: $r = 0{\cdot}141$ $P = 0{\cdot}172$	$n = 125$ $r_s = -0{\cdot}102$ $P = 0{\cdot}257$ PIC: $r = 0{\cdot}064$ $P = 0{\cdot}539$	$n = 83$ $r_s = 0{\cdot}212$ $P = 0{\cdot}055$ PIC: $r = 0{\cdot}057$ $P = 0{\cdot}645$	$n = 37$ $r_s = 0{\cdot}313$ $P = 0{\cdot}059$ PIC[b]: $r = -0{\cdot}124$ $P = 0{\cdot}489$
EIV-nitrogen	$n = 126$ $r_s = 0{\cdot}005$ $P = 0{\cdot}955$ PIC: $r = 0{\cdot}172$ $P = 0{\cdot}095$	$n = 126$ **$r_s = -0{\cdot}187$** **$P = 0{\cdot}036$** PIC: $r = -0{\cdot}072$ $P = 0{\cdot}490$	$n = 84$ $r_s = 0{\cdot}026$ $P = 0{\cdot}818$ PIC: $r = 0{\cdot}245$ $P = 0{\cdot}044$	$n = 38$ $r_s = 0{\cdot}215$ $P = 0{\cdot}196$ PIC[b]: $r = 0{\cdot}172$ $P = 0{\cdot}329$

Statistically significant (at $P < 0{\cdot}05$) results are shown in bold type. r_s, Spearman's Rho correlation coefficient; [a]log-transformed values with linear regression; [b]trait had no phylogenetic signal. EIV, ecological indicator value.

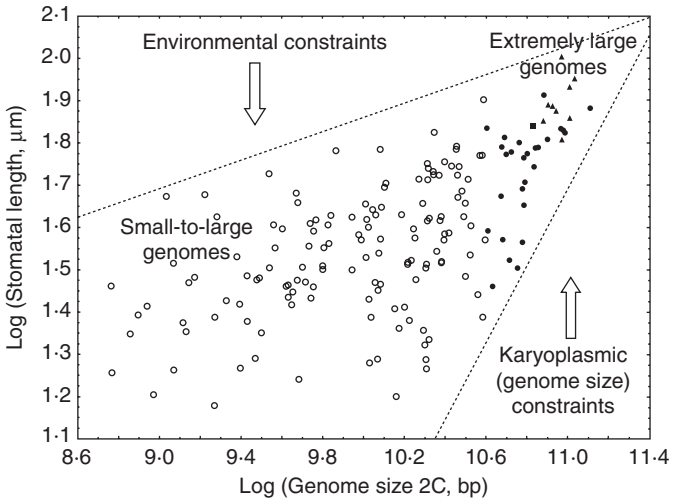

FIG. 1. The relationship between log-transformed genome size and stomatal length in geophytes. Regressions (lines not shown): overall, $y = 0{\cdot}1761x + 0{\cdot}8492$, $r = 0{\cdot}580$, $P = 0{\cdot}000$; small-to-large genomes ($2C < 40\,000$ Mbp; open symbols) $y = 0{\cdot}1434x + 0{\cdot}971$, $r = 0{\cdot}448$, $P = 0{\cdot}000$; extremely large genomes ($2C \geq 40\,000$ Mbp; closed symbols) $y = 0{\cdot}5342x - 0{\cdot}8289$, $r = 0{\cdot}545$, $P = 0{\cdot}003$. Circles, analysed geophytic species; squares, *Tradescantia virginiana*; triangles, genus *Fritillaria*.

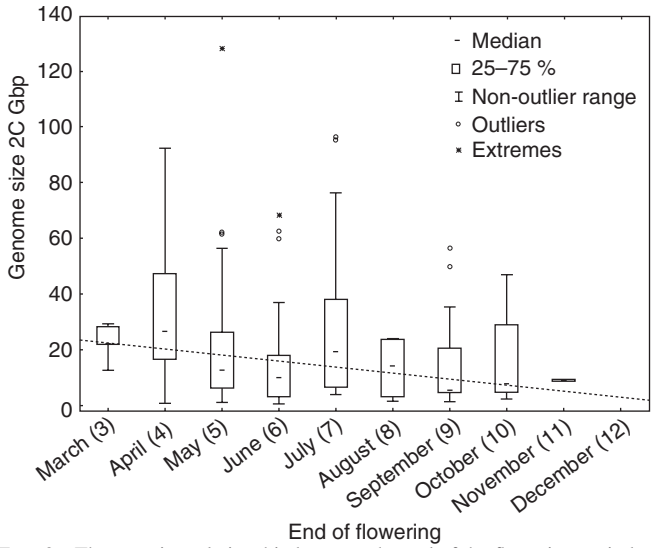

FIG. 2. The negative relationship between the end of the flowering period and genome size as resulting from Spearman rank correlation and analysis of PICs (see Table 2).

concentrated only in the spring-month categories of flowering, clearly indicates that this is not geophytism itself, which is responsible for the increase of genome size, but rather that the evolution of giant genomes in plants is associated with certain ecological conditions related to water availability. From the point of view of very large genomes, the major advantage of geophytism is the ability to replicate DNA and to pre-divide cells during the dormant period, helping to overcome the difficulties with slow DNA replication associated with large genome size. Moreover, such cells are larger because of a functional constraint on the minimum cell size at a given DNA content (Bennett, 1987; Jovtchev *et al.*, 2006). Having large cells that are only with water pumped in spring may even be advantageous and positively selected for to quickly develop a large functional body irrespective of the initial nutrition, insolation and temperature conditions. This is a preferred strategy in many temporally limited habitats that would allow principally large genomes to evolve elsewhere in environments with sufficiently long favourable conditions allowing cell division during the dormant and photosynthetically inactive period (i.e. avoiding the occurrence of large genomes in alpine geophytes, such as *Crocus* or *Veratrum*, because of long-lasting frosts). Beyond the positive selection on cell size, we cannot, however, exclude that genome gigantism in certain geophytes is the outcome of a neutral process and a passive consequence of some common molecular force (such as unconstrained retrotransposon proliferation) that is passively tolerated until reaching some metabolic limits of a plant or resource capacity of the environment. This view is in accordance with the increase in giant genomes observed with evolution of parasitism in some plants (e.g. *Viscum*), enabling them to become relatively independent of the resource limitation of the external environment.

As our data showed, the reason why large genome sizes are not more widespread in geophytes is related to water availability and perhaps to water sensitivity associated with extremely large genome size. The water status of a plant is not only related to water availability in the environment, but also critically depends on preventing its loss thorough stomata during gas exchange and carbon dioxide uptake for photosynthetic processes (Farquhar and Sharkey, 1982; Willmer and Fricker, 1996), which may be critical for the fleshy design of plant bodies in many geophytes. This process is sensitive to the regulation and fast response of stomata to changing leaf water status, which might simply be mechanistically related to stomatal size and design (Hetherington and Woodward, 2003; Franks and Farquhar, 2007; Franks and Beerling, 2009). Experiments in deciduous trees show that larger stomata are slower to close and have a potential to cause hydraulic dysfunction under drought conditions (Aasamaa *et al.*, 2001; Hetherington and Woodward, 2003). A negative role of stomatal size may be seen also in our data, where length of the growing season shortens with increasing stomatal size in 68 geophytic species (Table 2). In addition to the slower response a further disadvantage of large stomata is apparently in the less effective CO_2 uptake owing to the larger distance that a molecule has to diffuse through a stomatal pore that is larger and deeper in large sized stomata (Franks and Farquhar, 2007; Franks and Beerling, 2009). To achieve a sufficient CO_2 influx at a given total stomatal pore area, plants with larger stomata have to keep their stomata open for a longer time, which necessarily increases the sensitivity of such plants to periods of drought, forcing stomata to close. Hence, species with larger stomata may be expected generally to be less tolerant to water stress, and selection of smaller stomata improving leaf water-use efficiency is also commonly observed in response to drought treatments (Gindel, 1969; Clifford *et al.*, 1995) or treatment with abcisic acid, a plant hormone released under water stress (Franks and Farquhar, 2001). An adaptation to overcome the difficulties of CO_2 uptake and water loss may be prostrate leaf growth, which

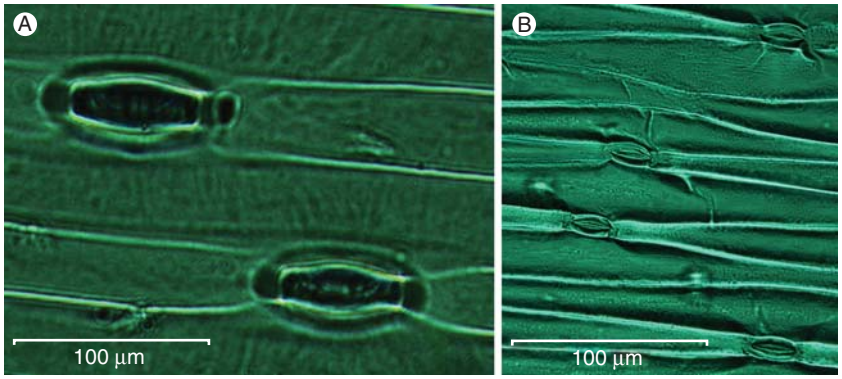

FIG. 3. The contrasting stomatal size of the spring geophyte *Gagea lutea* (A) and summer geophyte *Anthericum ramosum* (B).

developed in several lineages of South African geophytes (Esler *et al.*, 1999). This morphology supports favourable moisture conditions on the leaf underside and these plants may even utilize CO_2 produced by soil micro-organisms prospering in such conditions (Cramer *et al.*, 2007).

Because photorespiration and loss of CO_2 are increased at higher temperatures, activity of geophytic species is concentrated to cooler seasons and not to warm and moist periods of the year (Rossa and Willert, 1999). The decreased CO_2 uptake efficiency of species with large genomes and stomata suggests that they may direct their activity towards the cooler seasons and to finish their growing season before periods of high temperature. Indeed, we observed a significant negative correlation between length of the growing season and stomatal size in 68 geophytes (Table 2), indicating that despite a similar start of active growth in all geophytes enabled by minimum temperatures, these large-genomed species are forced to end growth earlier before the incidence of high temperatures.

Both slower stomata closure and decreased efficiency of CO_2 uptake generally limit the success of actively growing plants with extremely large genomes and stomata in arid or short-term volatile humid climates. Given the unexpectedly close correlation between stomatal and genome size observed in plants with very large genomes, the drought sensitivity associated with the presence of extremely large stomata may therefore be viewed as the most limiting factor in the evolution of extreme genome sizes and might mark the upper limits for maximum genome size for a plant in a given environment. This perhaps also explains why extremely large genomes are more frequently found in geophytes from habitats where a cooler climate coincides with long stable humid periods, such as occurs in temperate forests or Mediterranean mountains.

Genome size and GC content

For all 219 analysed species, GC content ranged from 35·75 % in *Allium ursinum* (Alliaceae) to 49·73 % in *Streptopus amplexifolius* (Liliaceae), 2C ranging from 582·18 to 128 273·07 Mbp (Table 2). Spearman's Rho test of the data showed no significant relationship between the two genomic measures (Table 2) but a significant negative correlation was shown to exist between genome size and GC content by using analysis of PICs (Table 2). The more detailed analysis of the data set suggests that the failure of the conventional statistics to reveal a significant correlation might result from the existing unimodal relationship between genome size and GC content with a peak at approx. 18 400 Mbp (Fig. 4). For species below this genome size threshold (126 species), a positive trend between genome size and GC content is found (Spearman's Rho: $r_s = 0{\cdot}334$, $P < 0{\cdot}001$), although this is not well supported by the analysis of PICs ($r = 0{\cdot}165$, $P = 0{\cdot}134$). In species with genomes larger than 18 400 Mbp (93 species), a negative correlation was confirmed by both tests (Spearman's Rho: $r_s = -0{\cdot}219$, $P = 0{\cdot}035$; PIC: $r = -0{\cdot}431$, $P < 0{\cdot}001$). In general, the genomic GC content showed no clear association with any of the tested ecological variables (Table 2) but trends were observable between GC and soil moisture, and GC and soil nitrogen by using non-parametric correlation (Table 2). However, these correlations were mediated only with the correlation between GC content and genome size and completely disappeared when analysed with PICs (Table 2).

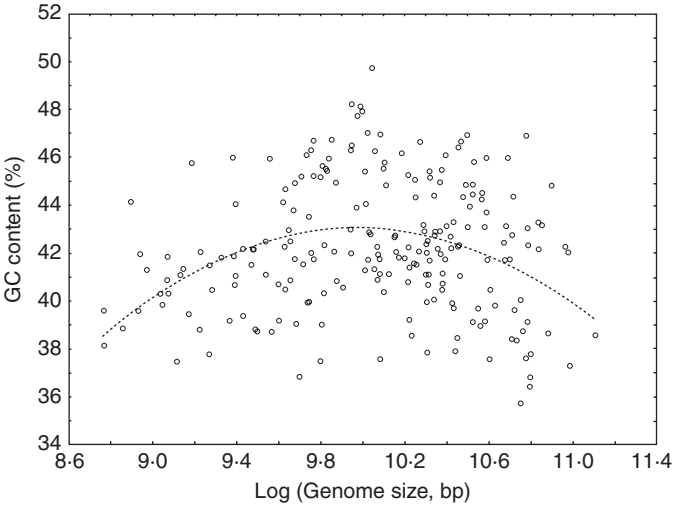

FIG. 4. The relationship between genome size and genomic GC percentage in geophytes.

Our findings contradict the reported absence of an association between genome size and GC content by Barow and Meister (2002) conducted on a limited sample of angiosperms. The positive correlation between genome size and GC content is generally not surprising, as similar trends have been reported in bacteria (Musto *et al.*, 2006), vertebrates (Romiguier *et al.*, 2010) and some small genomed genera of *Poales* (Bureš *et al.*, 2007; Šmarda *et al.*, 2008). However, the significant trend of decreasing GC with increasing genome size is unusual, so far being known only in teleost fishes (Vinogradov, 1998). This negative trend between genome size and GC content in large genomes might be explained in three ways. (1) It might result from constraints on chromatin condensation, which tends to be higher in plants with larger genomes (Vinogradov, 2005). This condensation might be facilitated in C + G-poor DNA, which is known to have higher curvature and ability to form non-linear structures compared with larger DNA regions (Vinogradov, 2003; Vinogradov and Anatskaya, 2006). (2) The synthesis of GC base pairs is economically more expensive compared with synthesis of AT base pairs (Rocha and Danchin, 2002) so that extremely large genomes may be formed from AT-rich sequences to save cell energetic resources. (3) Due to the stronger stacking interactions and triple bounding, GC base pairs are much more stable than AT base pairs (Yakovchuk *et al.*, 2006). Therefore, species growing later in the season and striving against higher temperature and UV stress might favour a GC-rich over an AT-rich structure of their genomes. The third hypothesis agrees with our observation that genomes of early-flowering geophytes are larger and generally also much more AT-rich compared with small-genomed GC-rich geophytes flowering later in the season. Nevertheless, the causality between the expected DNA stability and plant ecology, as well as the two remaining hypotheses, need to be tested in detail.

Seed mass, genome size and ecology

Seed mass was measured for 57 plant species. Comparison of their genome sizes showed no evident trend. This result is similar to the analysis by Beaulieu *et al.* (2007) showing only very weak relationships between the two parameters on a much robust dataset. Compared with stomatal size, seed mass seems to be less constrained by genome size and is very tightly driven by ecological and functional constraints. This conclusion is supported by significant correlations between seed mass and ecological indicator values for light (negative; Table 2) and continentality (positive; Table 2). In both cases, ecological indicator values for the studied species had no phylogenetic signal, and hence only results of conventional statistics are discussed. Nevertheless, the detected correlations may have an ecological explanation: germinating (non-parasitic and non-mycotrophic) plants can draw energy only from reserves stored in the seeds. This could be a favourable strategy for successful seedling development in extreme environments with limited or unpredictable supplies of energy and nutrients before the seedling becomes able to utilize energy and nutrients completely from external environment (i.e. develops enough leaf and root system). This may be the case of shady (low light input) and continental biotopes (low minimum temperatures and low precipitations), where species are known to have generally heavier seeds (Salisbury, 1974; Foster and Janson, 1985; Mazer, 1989). Analogous results were also reported by Alexander *et al.* (2009), who found a positive relationship between seed mass and altitude.

Although we did not find a significant correlation between seed mass and genome size among the species studied, a positive relationship between both these variables was documented in certain groups of related taxa (e.g. a genus or family; e.g. *Allium*, *Crepis*, *Pinus*; cf. Beaulieu *et al.*, 2007: table 1; Knight and Beaulieu, 2008: fig. 5A), where the seeds share the same general design and similar ecological conditions shape their seed size. In such a case, there exist numerous examples where seed size of closely related species differing in genome size has been used as a species discriminatory character in plant taxonomy (e.g. in polyploid relatives, such as between diploid *Stellaria pallida* and tetraploid *S. media*, or between *Ornithogalum umbellatum* and *O. divergens*; Kubát *et al.*, 2002).

CONCLUSIONS

The data presented here confirmed that the evolution of extreme genome sizes in geophytes is closely mirrored by their phenology and ecology. Although large plant genomes are present in species with a geophytic life form more frequently than in species of any other life form, this phenomenon does not indicate that the geophytic life form itself is primarily responsible for the increase in genome size. It appears that the necessary prelude to the origin of large genomes is providing enough reserves and allowing enough time for cell division during a period of unfavourable climatic conditions for plant growth. In other words, the increase of genome size and corresponding presence of large cells could be an advantageous evolutionary strategy to quickly exploit and successfully compete in seasonal environments by means of rapid development of the plant body by cell expansion. However, there seems to be a trade-off between genome size and stomatal size that increases sensitivity to droughts and limits the appearance and activity of geophytic species with extremely large genomes to temporary stable humid periods and environments. The genome size and phenology of geophytes is surprisingly also linked to remarkable changes in overall DNA base composition. Although GC content seems not to be associated directly with plant ecology, the unimodal relationship between genomic GC content and genome size indicates that some universal constraints may operate on the DNA composition of large genomes. The identification of these constraints and evaluation of their role in the evolution of genome size remains a challenge for genomic research.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Table S1: list of sampled taxa, their genome sizes, GC contents, seed masses, stomatal lengths, flowering phenology and duration of their growing season.

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