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Patterns, causes and consequences of genome size variation in Restionaceae of the Cape flora

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Little is known about genome size (GS) variation in the hyperdiverse Cape flora of southern Africa. Towards filling this gap, we measured GS of 158 species of Cape Restionaceae and demonstrated >16-fold variation in 1C-values (0.47–7.66 pg). We also report chromosome counts of nine species and show that $2n = 32$ predominates. Patterns of GS variation were used to infer ploidy, and the variation at the holoploid level was partitioned into monoploid and ploidy variation. We show that at least 30% of the species measured contain several ploidies. The monoploid GS variation was phylogenetically constrained and 1Cx-values were significantly lower in areas near the centre of the Cape Floristic Region and in species which resprout after fire. A higher proportion of inferred polyploids were found in species with wide distribution ranges, which are smaller plants, resprout after fires and are therefore longer lived. In addition, polyploids prevail in areas at the periphery of the Cape Floristic Region, which are more likely to have been exposed to fluctuating climates in the past, and at higher elevations. This shows that monoploid and polyploid GS variation could be responding to different selection pressures.

ADDITIONAL KEYWORDS: chromosome numbers – dispersal – ecology – evolution – phylogenetic constraint – polyploid – species diversity.

INTRODUCTION

Genome size (GS) variation in angiosperms is remarkable, with a variation of 2400-fold, compared to gymnosperms with only 16-fold variation (Leitch & Leitch, 2012). The evolution of GS (*sensu* Greilhuber *et al.*, 2005) is inferred to happen at two levels. Monoploid GS (1Cx) evolution usually occurs slowly, in phylogenetic time, involving the proliferation or loss of repetitive DNA elements (Grover & Wendel, 2010). Monoploid GS change is not associated with whole genome duplication (polyploidy), although it can be associated with rounds of polyploidization followed by a slow diploidization process. Polyploidy driven changes in the GS, on the other hand, occur at or below the species level and thus not in a phylogenetic context. Polyploidy-driven GS changes are associated with whole genome duplications. In most studies, the selective regimes for monoploid- and polyploid-driven GS evolution are not separated, with a single set of predictions based on the correlations between GS, cell size and cell duplication rates. This leads to sets of predictions of correlations in phenotypic space, including plant size, stomatal size and life habits. However, it is possible that monoploid GS changes respond to different selective regimes than polyploid GS changes, and are therefore correlated with different phenotypic and environmental variables.

The Cape flora is remarkably species-rich and this richness stems from diversification in a small number of clades (Linder, 2003), thus resulting in a high degree of co-existence of closely related or congeneric species. Little is known about the degree of intraspecific ploidy polymorphism (IPP) in the Cape flora and how it compares with global figures. Ploidy-variable Cape species have occasionally been reported in monocots

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(e.g. Asparagaceae, Iridaceae and Poaceae) and eudicots (e.g. Asteraceae, Geraniaceae, Oxalidaceae and Scrophulariaceae) (Goldblatt & Johnson, 1979; Krejčíková *et al.*, 2013; Rice *et al.*, 2015), but no critical synthesis is available. Cape Poaceae seem to hold a prominent position with respect to IPP: whereas only two ploidies per species have been reported in most other groups, Cape grasses often encompass three or more ploidies and the extent of polyploidization may be relatively high (e.g. Roodt *et al.*, 2002).

Closely allied to Poaceae, Restionaceae (restios) are a major component of fire-prone vegetation in the Fynbos Biome of South Africa (Rebelo *et al.*, 2006). The Cape floristic region (CFR) is one of the two evolutionary centres of restios, harbouring nearly 60% of total diversity, *c.* 350 out of 535 recognized species (Linder, Briggs & Johnson, 1998). Despite the ecological importance of the group, data on karyological variation of Cape restios are largely lacking. Chromosome counts have been published for only three species ($2n$ = 32, 40, Krupko, 1962, 1966) and GS estimates are available for eight species from six genera, with 2C-values ranging from 1.38 pg to 5.27 pg (Hanson *et al.*, 2003; Šmarda *et al.*, 2014). Australian restios are better characterized cytologically. Briggs (2012) summarized all chromosome counts for those taxa and concluded that the overall karyological variation is quite high ($2n$ = 14, 18, 22, 24, 32, *c.* 44, 48, *c.* 104), basic chromosome numbers show some phylogenetic pattern and polyploidy and dysploidy occur in Australian restios.

The general trend emerging from comparative studies is that species with small genomes can attain a much wider array of various trait states compared to species with large genomes (the 'large genome constraint hypothesis'; Knight, Molinari & Petrov, 2005). GS is strongly correlated with cell size in some cell types (e.g. stomatal guard cells) and with the duration of mitosis and meiosis. These characteristics at cellular level constrain many functional traits related to individual growth, reproductive success and dispersal (Gregory, 2001; Greilhuber & Leitch, 2013). Of particular importance from an ecological point of view are stomatal characteristics. Stomatal size is often assumed to correlate with the rate of stomatal opening and closure (but see Elliott-Kingston *et al.*, 2016) (both scaling positively with GS) and density (scaling negatively with GS) considerably influence carbon fixation and water-use efficiency (Beaulieu *et al.*, 2008), ultimately restricting the range of suitable environmental conditions for a given plant species. In addition, nutrient-poor soils can select against large-genome species because of higher demands for phosphorus and nitrogen to synthesize DNA (Šmarda *et al.*, 2013; Guignard *et al.*, 2016). However, the ecological association between GS and ploidy is unclear. Polyploids tend to occupy ecologically somewhat different habitats than their diploid ancestors, and polyploidization (both auto- or allopolyploids) may result in a rapid niche shift (Oswald & Nuismer, 2011; Ramsey, 2011) or change the responses to selection (Etterson *et al.*, 2016).

Here we ask, first, how much GS variation is found in the Cape restios, and what are the relative contributions of polyploidy and monoploid GS evolution to the variation in holoploid GS. Second, we test whether monoploid GS changes and polyploidy are, respectively, phylogenetically constrained. Finally, we explore the hypothesis that the two processes are correlated with different environmental and biological variables of the restios and thus have different consequences for the species involved. Specifically, we test whether IPP and/or a higher monoploid GS are correlated with (1) larger range sizes, (2) dry (stressful) as compared to wet (mesic) regions, (3) well-drained habitats compared to wetlands, riverbanks and habitats with impeded drainage, (4) higher elevations, (5) resprouting fire biology, (6) myrmecochorous seed dispersal biology and (7) smaller plants.

MATERIAL AND METHODS

Phylogeny

We used the rate-corrected and age-calibrated phylogenetic hypothesis published by Bouchenak-Khelladi & Linder (in preparation), which is summarized by a set of Bayesian trees. This phylogenetic tree builds on that published by Hardy, Moline & Linder (2008) by adding missing species and additional sequence data, resulting in a combined matrix of 10 121 characters for 339 of the 350 restio species.

Chromosome numbers

To establish the relationship between chromosome number and GS, chromosome numbers were determined from actively growing root tip meristems of cultivated plants. Sixteen individuals representing nine genera of restios were analysed. Root tip meristems were collected and pre-treated with a 0.002 M solution of 8-hydroxyquinoline for 2 h at 4 °C and 2 h at room temperature. Pre-treated samples were fixed in 3:1 ethanol/acetic acid mixture at room temperature for several hours and stored at –20 °C until use. Due to the small chromosome size and bimodal karyotypes, preparations were mostly made using enzymatic digestion of cell walls with an enzyme cocktail [1% cellulase Onozuka (Serva, Heidelberg, Germany), 0.4% pectolyase and 0.4% cytohelicase (Sigma, Vienna, Austria) in citric buffer (Weiss-Schneeweiss *et al.*, 2012)] to obtain better quality data. Preparations were made in a drop of 60% acetic acid, cover slips flipped off after freezing

and material stained with 2 mg/mL DAPI dissolved in the mounting antifade medium Vectashield (Vector Laboratories, Burlingame, CA, USA). Preparations were examined with an epifluorescent microscope, Axio Imager M2 (Carl Zeiss, Vienna, Austria), and images were captured with a CCD camera using AxioVision software (Carl Zeiss). A minimum of ten complete and unambiguously countable chromosomal spreads were required for chromosome number estimation per individual.

Sampling for flow cytometry

We collected 574 samples representing 158 species of restios from the whole Cape Floristic Region between 2009 and 2013 (details about vouchers and locations are given in Table S1). Samples were transported to Prague (Czech Republic) and investigated using flow cytometry within 2 weeks of sampling (restios have hard somatic tissue and are able to survive a long time after collecting). In addition, some species were cultivated in Zurich and further 2C values obtained from Petr Šmarda (Brno, Czech Republic). Initially, sampling was designed to maximize the taxonomic coverage, so only one sample per species was collected. After discovering that at least some restios have intraspecific GS variation, we also obtained multiple, geographically spread samples per species. In all cases just one specimen was collected per population, thus assuming that most populations are monomorphic for cytotypes.

GS determination

Holoploid GS (C-value *sensu* Greilhuber *et al.*, 2005) was determined using propidium iodide flow cytometry following the simplified two-step protocol described by Esler, Greilhuber & Suda (2007). Briefly, intact stem tissue of the analysed restio plant was chopped together with an appropriate internal reference standard in 0.5 mL of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20). The sample was filtered through 42 µm nylon mesh and incubated for 10 min at room temperature. The staining solution consisted of 1 mL Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) supplemented with propidium iodide and RNase IIA (both at final concentrations of 50 µg/mL) and β-mercaptoethanol (2 µL/mL). The fluorescence intensity of isolated nuclei (5000 particles) was recorded using a Partec CyFlow SL cytometer equipped with a diode-pumped solid state laser 532 nm (Cobolt Samba, 100 mW output power). Each sample was analysed at least three times on different days and only analyses with a between-day fluctuation of <3% were considered.

We used as internal reference standards *Glycine max* (L.) Merr. 'Polanka' (2C = 2.21 pg), *Bellis perennis* L. (2C = 3.38 pg) and *Pisum sativum* L. 'Ctirad' (2C = 8.76 pg). *Bellis perennis* (Schönswetter *et al.*, 2007b) served as a primary reference standard. GSs of other reference species were calibrated against *B. perennis*, based on five measurements on different days. For each analysed plant, an internal standard was selected so that its GS was close to but not overlapping with that of the analysed sample.

Determining ploidy from GS variation

To separate the effects of genome multiplication (due to auto- or allopolyploidy) from monoploid changes in GS, we need the ploidy of the sample and its monoploid GS, 1Cx-value. Ideally, ploidy of each sample is established by counting its chromosomes and then 1Cx is calculated by dividing the 2C-value by the ploidy, as done for Orobanchaceae (Weiss-Schneeweiss, Greilhuber & Schneeweiss, 2006). Second best, if chromosome numbers are available for some samples only, these can be used to calibrate the ploidy (e.g. Frajman *et al.*, 2015). The prerequisite is that the same individuals are used for both chromosome number estimation and GS measurements. In restios we had only nine chromosome counts available for this approach, so we followed two routes to estimate the monoploid value for each species and to assign a 'DNA ploidy' (Suda *et al.*, 2006) to it. First, we plotted GS variation within each genus and species. We visually identified frequency peaks and breakpoints and sought to fit them into a series of ploidies, with the GS content going up in the monoploid steps. This assumes that GS is often closely correlated with recent ploidy, as in *Tanacetum* L. (Olanj *et al.*, 2015), equivalent with the assumption that the 1Cx is conserved between closely related species (shown by Jakob, Meister & Blattner, 2004; Leitch *et al.*, 2007). We minimized the use of the triploid level, based on the argument that triploids are rare, as has been demonstrated in detailed field surveys (Duchoslav, Šafářová & Krahulec, 2010). Ideally, each species should be analysed separately but many species are sparsely sampled and over half are represented by a single GS value and, consequently, we used groups of phylogenetically closely related pooled species samples, usually genera, to infer ploidy. We did not use the whole subfamily in one analysis, as potential evolutionary variation in 1Cx-values might obscure natural frequency levels generated by genome duplication. This method was also used by Leitch *et al.* (2008) in the study of genome downsizing in polyploids of *Nicotiana* L. and by Leitch *et al.* (2007) in the investigation of punctuated evolution in Liliaceae. Leitch *et al.* (2008) showed both downsizing and upsizing of genomes of polyploids, suggesting that an averaging approach, as followed here, might be suitable. Genome upsizing is rare in plant groups studied so far, the only other known instance is *Hordeum* L. (Jakob

et al., 2004); otherwise, all cases are of additivity or downsizing.

Secondly, once a set of ploidies was postulated for a genus or a clade, we tested assignment to these levels using *k*-means clustering, as implemented in SPSS. We analysed each genus or subgenus separately to minimize evolutionary effects and compared our assignations with the *k*-means clustering results. *K*-analysis cannot find the optimal number of groups; it simply optimizes the assignment of the samples to the groups and locates the best group media.

Each species was assigned as diploid, polyploid or unknown using the following set of explicit rules. (1) Samples inferred to be triploids were left out. This is because we have no indication of how frequent they are in the populations: if rare, they are probably the result of fusion of reduced and unreduced gametes in a diploid population. If common, they could be the result of hybridization between a diploid and a tetraploid and as such indicators of tetraploids in the species. As we could not distinguish between these two processes, we excluded triploids. (2) All species with at least one polyploid specimen (and possibly all specimens) were scored as polyploid. Polyploid is taken to mean that there may be diploids or specimens of higher ploidies in the species, and that most species assigned as polyploid contain several ploidies and thus show IPP. (3) With this approach, designating a species as diploid was based on the lack of evidence for polyploids. As this is a weak argument, a series of analyses were conducted, with species scored as diploid if all specimens were diploid, and at least two to five specimens were analysed. This tests the sensitivity of our results to 'false' diploid scoring. Due to the reasons detailed below, we eventually used only the dataset with at least two samples, in which all are diploid, as diploids. We are aware that there are numerous ways in which GS data could result in misleading ploidy estimations (Suda *et al.*, 2006) and these were taken into account.

Effect of sample size on GS and ploidy variation within species

To evaluate the confidence with which diploidy can be assigned, we tested whether our estimates of GS variation and ploidy variation within species are sensitive to sampling intensity. To this end we plotted the number of samples per species against the differences in GS size and the number of ploidies.

Potential correlates of GS variation

We inferred the distribution area of each species by modelling the potentially suitable range in Maxent (Phillips, Anderson & Schapire, 2006), using default settings. We used all available species distribution data (field notes, ecological surveys, herbarium specimens; in total, 12 299 locality records), in combination with selected climate layers, taken from Schulze *et al.* (1997). We used this approach rather than the area enclosed by the polygon of the distribution points, as many species have disjunct ranges, occurring on mountains separated by wide, inhospitable lowland areas and consequently the polygon approach would over-estimate their ranges. The range size distribution is strongly skewed and, consequently, these values were log-transformed.

Drought tolerance and climatic instability were assessed by scoring all species as present/absent to the geographical regions in the Cape used by Linder (2001). Dry regions are mostly along the arid inland margins of the region, where a small change in rainfall can result in a complete vegetation change (Esler, von Staden & Midgley, 2015). Furthermore, the eastern Cape may have been drier during the Last Glacial Maximum than the western Cape (Chase & Meadows, 2007). Consequently, the eastern regions (Eastern Cape) and the regions abutting the arid Karoo (Namaqualand, Northern Mountains and Great Swartberg) may have had more unstable climates during the Pleistocene and we therefore tested whether these have a higher proportion of polyploids than the regions thought to have had a more stable climate (e.g. the mesic coastal regions or the western end of the distribution range).

The habitat requirements of all species were taken from Linder (2001). Most species are found in well-drained habitats, where there is no evidence of impeded drainage. Here all water available to the plants comes from rainfall. Wetland habitats are recognized as habitats where rainwater from elsewhere can be concentrated, for example streambanks, impediments to free drainage (rock barriers, for example) forcing soil water to the surface, seepages or springs where ground-water comes to the surface.

The elevation for each species was estimated from all available elevation records; these include all available herbarium records and a large sample of plot data. This cannot be regarded as a random sample and, therefore, we used the mean elevation.

We used fire biology as a proxy of plant longevity, based on the argument that reseeding species are killed by fire and thus shorter lived than resprouting species, which survive fires. The interfire period in fynbos is 6–20 years (Kraaij & Van Wilgen, 2014). The typing of species as reseeders or resprouters was taken from Linder (2001). Note that only about half of the species have been designated as reseeders or resprouters.

Four dispersal modes have been postulated for the restios (Linder, 1991). The ancestral condition is ballistic dispersal, with the propagules being flung from the parent plant. In two clades the propagules are

winged, evidently adapted for wind dispersal. In one large clade, the nuts have elaiosomes and these are assumed to be myrmecochorous (Bond & Slingsby, 1983). Recently, dung beetle dispersal was described for one species (Midgley *et al.*, 2015), which was, however, not included in our sample. Plant size was taken from Linder (2001); we used the mean plant size.

Testing correlates of GS variation

To explore the phylogenetic patterns in GS and ploidy variation and the interaction between these genomic data and morphological and ecological traits, species for which no data are available were removed from the phylogenetic tree (leaving 154 species out of 339) (see Supplementary Information, Table S2). We tested for phylogenetic constraint in genome evolution using Pagel's λ (Pagel, 1994, 1997) and Blomberg's K (Blomberg, Garland & Ives, 2003) for ploidy and GS. Both methods, which rely on maximum likelihood estimates, infer whether the trait evolution is statistically different from that expected under Brownian motion (BM). Both tests were performed in *R* (R Core Team, 2012), using 'phytools' (Revell, 2012) and the maximum clade credibility (MCC) tree from the BEAST analysis.

To identify shifts in the evolution of monoploid GS among lineages, we applied an Ornstein–Uhlenbeck (OU) model for trait evolution under which several optima, rates of evolution and strength of selection can be computed (see below). We inferred the statistical patterns in both monoploid GS data and MCC tree using the reversible-jump algorithm that jointly estimates the location number and magnitude of shifts in adaptive optima implemented in the package 'bayou' (Uyeda, Eastman & Harmon, 2015). We set half-cauchy priors for α and σ^2, a normal prior for θ and a conditional Poisson for the number of shifts as priors. The analysis was run for 1 000 000 iterations. Two runs were performed to check for convergence.

We explored the correlations between, on the one hand, the two GS variables (monoploid GS as a continuous variable and ploidy as a binary categorical variable) and the correlates (continuous: elevation, range size, plant height; categorical: dispersal biology, fire, peripheral regions, wetlands) using several approaches. As the monoploid GS value was shown to be phylogenetically constrained, only phylogenetically corrected analyses were conducted.

The three-state categorical dispersal biology, which is also highly phylogenetically structured, was used to predefine three selective regimes (i.e. ballistochorous, myrmecochorous and anemochorous modes) under an OU process. Under an OU process trait values evolve towards an optimum (θ), which can be a single value for all lineages or can vary among the predefined regimes. For each model the rate of stochastic evolution (σ^2) and the strength of directed evolution towards the optima (α) can be set to be equal across all regimes (OU_M) or to differ between regimes (OU_{MA}, where only α can vary; OU_{MV}, where only σ^2 can vary; OU_{MVA}, where α and σ^2 can vary). We used the 'OUwie' package (Beaulieu *et al.*, 2012) implemented in *R*. The second-order Akaike information criterion (AICc), which takes into account sample size and increases penalty for model complexity, was used to select the optimal model. The model selection procedure was done using the MCC tree.

The latter approach could not be used for the other categorical variables, as these are phylogenetically too dispersed. Ploidy shows no phylogenetic pattern, so both phylogenetically corrected methods and non-phylogenetically corrected methods were used. All analyses (for monoploid GS and ploidy) were done on the species which could be assigned to a ploidy, by having either at least one polyploid sample or at least two inferred diploid samples.

We explored the association between continuous monoploid GS values and the continuous and categorical ecological and vegetative traits with the phylogenetic generalized least square approach (PGLS; Martins & Hansen, 1997) using the 'pgls' function (Freckleton, Harvey & Pagel, 2002) implemented in the R package 'caper' (Orme, 2012). Phylogenetically informed regressions take into account the possibility that similarity among more closely related taxa is due to a common inheritance, rather than adaptation to the explanatory variable. PGLS calculates the value of Pagel's λ (Pagel, 1994, 1997) with maximum likelihood, which allows an estimation of the degree of phylogenetic dependence of the traits. It uses the value of λ to fit a linear model of the data, taking into account phylogenetic non-independence of the traits. In all models, monoploid GS was the dependent variable and the ecological and vegetative traits were set as fixed effects. The model with the lowest AICc value was considered the best model. Model significance was assessed using a *t*-test.

To test categorical ploidies against continuous variables, we conducted phylogenetic logistic regression analyses using the 'IG10' method (Ives & Garland, 2010). This method is based on a model that allows binary traits to evolve on a phylogenetic tree by switching between values of 0 and 1. The 'IG10' method accommodates continuous and discrete independent variables and gives a correlation structure that is corrected for binary data. We performed the 'IG10' model analyses using the 'phloglm' function implemented in the 'phylolm' R package (Ho & Ané, 2014). We compared the AICc, and the model with the lowest AICc value was considered the best model. A model was considered significant when $P < 0.05$.

Correlated evolution between pairs of categorical traits was tested using BayesDiscrete as implemented in BayesTraits (Pagel, 1994; Pagel & Meade, 2006, 2011). Here, the fit of two models of evolution, one in which traits evolve independently and the other in which they evolve in a correlated fashion, is compared. The latter allows the traits to evolve such that the rate of change in one trait depends upon the state of the other (Pagel, 1994). Using maximum likelihood, we compared the results of the models with a likelihood ratio statistic, which is nominally distributed as a χ^2 with degrees of freedom equal to the differences in the number of parameters between the models.

RESULTS

GS AND DNA PLOIDY VARIATION

Holoploid GS (1C-values) ranged 16.3-fold from 0.47 to 7.66 pg, with an average of 1.39 pg, showing a strong skew towards smaller values (Fig. 1). One-third (34%) of the intraspecific GS variation was accounted for by the number of samples per species (Fig. 2), suggesting that denser sampling would most probably increase our estimate of variation in GS. The inferred DNA ploidies varied from diploid to octoploid (Fig. S1). Monoploid GS (1Cx-values) ranged from 0.47 to 2.45 pg (see Supplementary Information, Table S1). The vast majority, 122 of 158 (78%), of species had only one ploidy and only two species had diploid, tetraploid and hexaploid cytotypes. Sixteen species had more than one cytotype, if triploids were ignored. Thirty-two species had at least one sample with a higher than triploid level (Fig. 3).

PHYLOGENETIC PATTERNS

The occurrence of DNA polyploid species was not phylogenetically constrained, with Blomberg's $K = 0.10$ ($P = 0.07$) and Pagel's $\lambda = 0.45$ ($P = 0.57$) (Fig. S2). In contrast, monoploid GS showed strong phylogenetic pattern [Blomberg's $K = 0.44$ ($P = 5e\text{-}04$), Pagel's $\lambda = 0.88$ ($P = 2.84 \times 10^{-36}$)] (Table 6). The OU model was preferred over the BM model, and Bayou located six shifts in the monoploid GS values (Fig. 4).

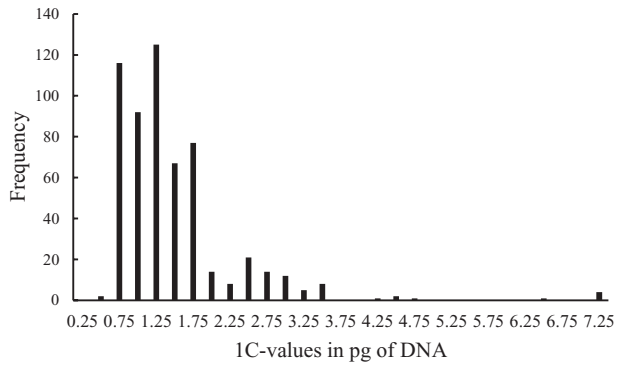

Figure 1. Histogram showing the frequency distribution of holoploid genome sizes (1C-values in pg of DNA) in African Restionaceae.

CHROMOSOME COUNTS

Chromosome numbers could be unequivocally established for nine of the ten species investigated, belonging to *Elegia* L. (four species), *Rhodocoma* Nees (one species) and *Thamnochortus* P.J.Bergius (four species) (Table 1, Fig. 5). All genera belong to Restieae; Willdenowieae remain unsampled in our study. Chromosome numbers were mostly $2n = 32$ (eight species) with exception of

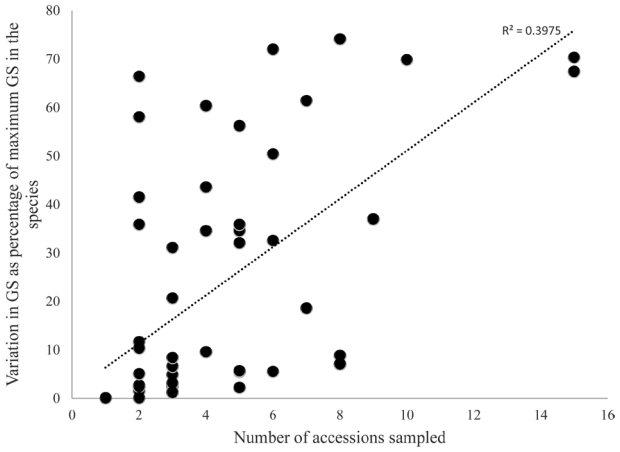

Figure 2. The correlation between the number of samples per species, and the range in GS obtained for each species ($R^2 = 0.341$, $\beta = 0.587$, $P < 0.01$) in African Restionaceae. Each point in the figure is a species. The GS range is expressed as a percentage of the maximum GS.

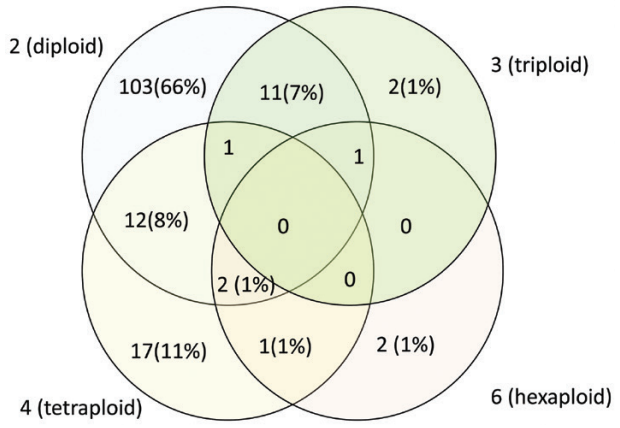

Figure 3. Distribution of the inferred DNA ploidy levels ($2x$, $3x$, $4x$ and $6x$) in African Restionaceae; each value in the Venn diagram circles is the number of species, with the percentage of all studied species in brackets.

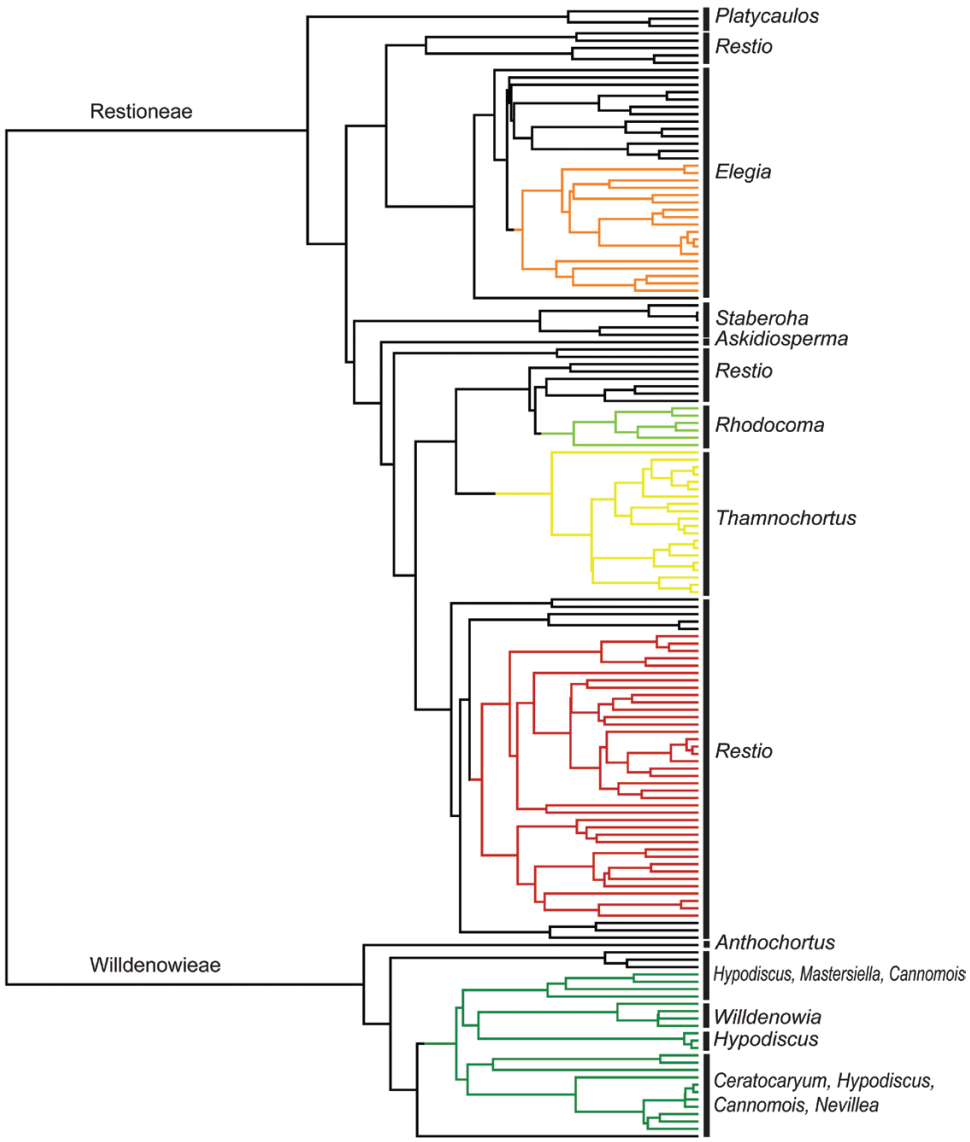

Figure 4. Mapping 1Cx-values for African Restionaceae over the maximum clade credibility phylogeny using bayou (Bayesian Ornstein–Uhlenbeck model), using six regimes of evolution of the 1Cx values found to be optimal. Several regimes are genus-specific: (1) yellow, with the smallest 1Cx-values, was found in *Thamnochortus*; (2) pale green, was marginally larger and found in *Rhodocoma*; (3) the dark red, was found in a large clade embedded in *Restio* subgenus *Restio*; (4) black, was the general background and ancestral size; (5) orange, was a part of the genus *Elegia* and (6) dark green (the largest monoploid GS) was embedded in Willdenowieae and was also the regime that was most variable (Figs S3, S4).

Elegia filacea Mast. possessing $2n = 20$. Unequivocal determination of chromosome numbers in restios is difficult due to the presence of often asymmetric and sometimes nearly bimodal karyotypes, with a few larger chromosome pairs and numerous small to very small chromosomes (Fig. 5). The chromosomes of all species were generally medium-sized to small ranging from < 1 to *c.* 5 µm. Three *Elegia* spp. with $2n = 32$ had two pairs of larger chromosomes, a few medium-sized and majority of small chromosomes (Fig. 5A, B). *Elegia filacea* possessed three pairs of larger chromosomes (Fig. 5C), but the remaining seven pairs were larger than the small chromosomes of other *Elegia* spp. with $2n = 32$. *Rhodocoma arida* H.P.Linder & Vlok, $2n = 32$, possessed two pairs of larger, two pairs of medium-sized and 12 pairs of small chromosomes (Fig. 5E). All four *Thamnochortus* spp. possessed $2n = 32$ chromosomes, again with two pairs of larger chromosomes, two to five pairs of medium-sized chromosomes and remaining chromosomes being very small (Fig. 5F, G, H, I).

Table 1. Somatic ($2n$) chromosome numbers and holoploid GS (1C) of all species of African Restionaceae for which chromosome numbers could be obtained

Species	Accession	S	ID	$2n$	Plant		Mean value for species				
					1C-value	#	1C-value	SD	CV%	Max/min	2C-values
E. cuspidata Mast.	26.11.2009		3	32	0.73	2					2.25
E. cuspidata	A 20011974	M	207	32	0.73	1	0.73	0.001	0.070	1.001	
E. equisetacea Mast.			94	32							1.55–5.13
E. filacea Mast.	4609	F	54	20	1.28	1					2.00–6.13
E. filacea		F	52	20	1.31	1					
E. filacea	04.06.2009	M	49		1.25	2	1.28	0.026	2.041	1.041	
E. tectorum (L.f.) Moline & H.P.Linder	04.06.2009	M	21	32							2.05–3.13
E. tectorum	04.06.2009		19	32							
R. arida H.P.Linder & J.H.Vlok	20050782	F	300	32	0.80	1	0.80				1.60
R. arida			48	32							
R. arida			45								
T. bachmannii Mast.	04.06.2009	M	31	32	0.61	2					
T. bachmannii	04.06.2009		30		0.59	1	0.60	0.009	1.575	1.032	
T. insignis Mast.	04.06.2009	F	68		0.62	1					1.27–1.31
T. insignis	04 Hermanus	F	76	32	0.61	1					
T. insignis	04 Hermanus	F	73		0.61	1					
T. insignis	04.06.2009	M	67		0.62	2	0.61	0.005	0.851	1.019	
T. lucens Poir.	20050779	M	14	32	0.61	1					1.13–1.20
T. lucens	20050779	F	205		0.60	1	0.61	0.007	1.169	1.017	
T. lucens			57	32							
T. lucens			60								
T. platypteris Kunth			88	32							1.30–2.71
T. platypteris	04 Nieuwoudtville		84	32							

The GS and chromosome numbers were obtained from the same plants. S = Sex; E = *Elegia*, R = *Rhodocoma*, T = *Thamnochortus*; # = Number of measurements. 2C is inferred from the whole dataset; where a range is reported this is for several values.

Correlates of GS

The phylogenetic regressions showed that only two variables were significantly correlated with monoploid GS. Species found in the presumably climatically stable, mesic, coastal regions had a significantly lower monoploid GS compared with other regions, as do species that survive fires in the seed bank, rather than by resprouting. There was no significant correlation with elevation, geographical range size, plant height or soil drainage patterns (Table 2).

Tests of contingent evolution suggested a significant association between polyploidy and higher elevations, larger geographical ranges and smaller plant size (Table 3). Furthermore, polyploids were found more commonly in peripheral areas of the CFR and are associated with fire response (Table 4). However, there is no correlation between ploidy and dispersal mode, how well-drained the habitat is and whether the species are found in the central parts of the Cape (Table 4). Higher elevations, inland distributions and well-drained habitats are correlated, all are related to a higher proportion of polyploidy and our data are not rich enough to establish whether all are individually correlated with ploidy, or whether some are auto-correlated.

Peripheral regions have proportionally more polyploids than the central areas (Fig. 6), but the difference is never significant (P = 0.2–0.3) in a region-against-region comparison. Furthermore, there is no significant variation in a combined analysis (χ^2: 8 d.f., P = 0.529). However, pooling the driest regions (Namaqualand, Northern Mountains and Swartberg) vs. the higher rainfall central and eastern regions gives a weakly significant result, at P = 0.041. There is no significant relationship with monoploid GS values. Comparison of Eastern Cape to Northern Mountains shows that there is no east/west effect in the restios, either for the proportion of polyploids or for the monoploid GS.

Although there is no significant relationship between ploidy and dispersal mode, monoploid GS varies highly significantly with dispersal mode (Fig. S5). When the

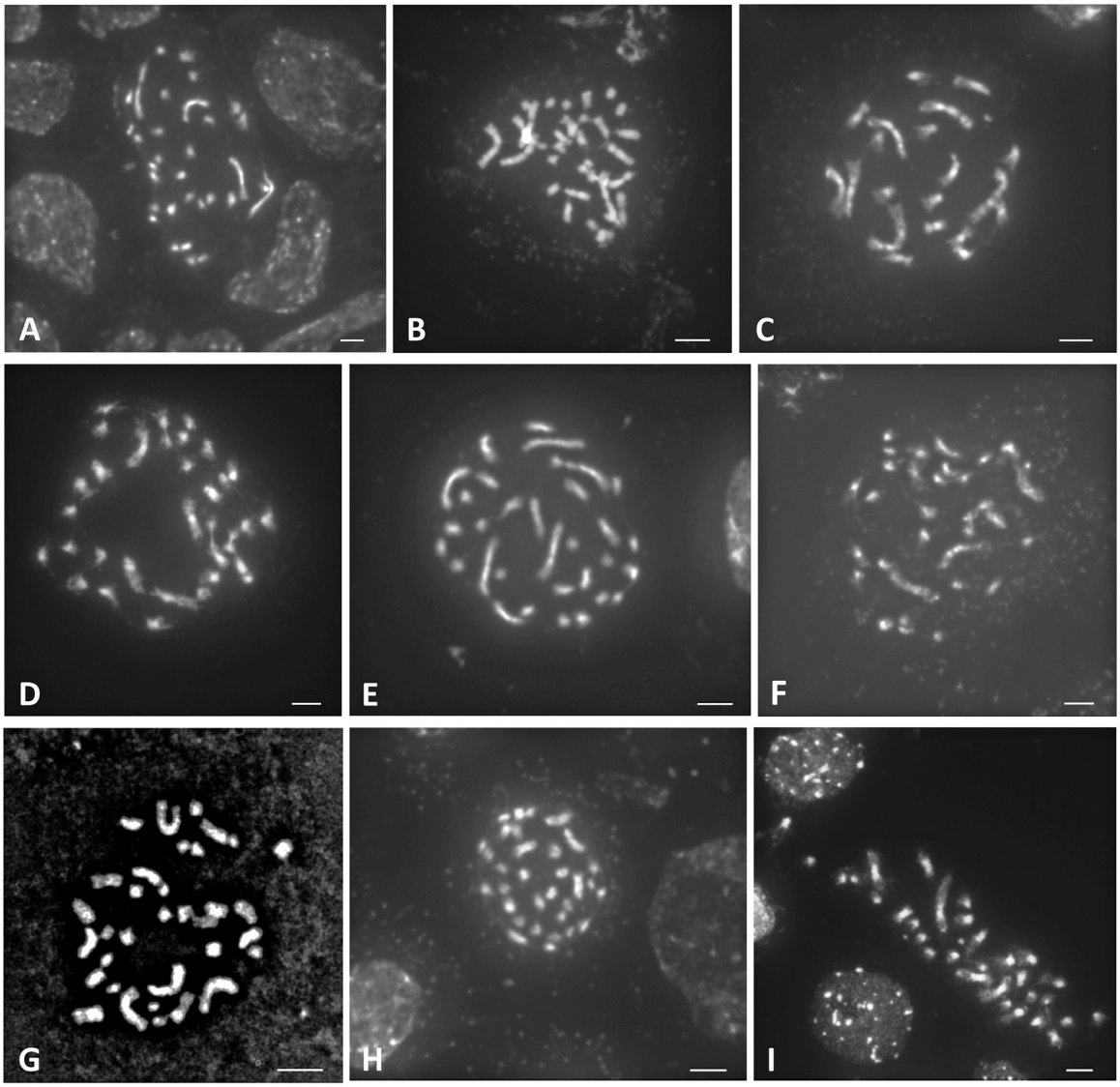

Figure 5. Mitotic chromosomes of Cape Restionaceae: (A) *Elegia cuspidata*, 2*n* = 32; (B) *Elegia equisetacea*, 2*n* = 32; (C) *Elegia filacea*, 2*n* = 20; (D) *Elegia tectorum*, 2*n* = 32; (E) *Rhodocoma arida*, 2*n* = 32; (F) *Thamnochortus bachmannii*, 2*n* = 32; (G) *Thamnochortus insignis*, 2*n* = 32; (H) *Thamnochortus lucens*, 2*n* = 32; I, *Thamnochortus platypteris*, 2*n* = 32. Scale bar, 5 μm.

three dispersal regimes are optimized using the OU model, the model that allows all three parameters to vary is found to be the best, the parameter values vary among the regimes (Table 5), showing that the highest monoploid GS is found in myrmecochorous lineages, and the lowest in anemochorous lineages. Similar results are also found without phylogenetic correction (Fig. S6).

DISCUSSION

SUMMARY

We find that there is substantial variation in GS in the African restios and that this is readily grouped into ploidy levels. We also show that polyploidy, as inferred from variation in the GS, is found in at least 34% of the species. Monoploid GS size evolution is phylogenetically constrained and fits an OU rather than a BM model. A higher proportion of polyploid species are found in arid, peripheral regions, compared to mesic, coastal regions, in species with a wider (rather than locally restricted) distribution ranges, in species with a wider elevational range (rather than restricted to lower elevations) and in more long-lived species which survive fire by resprouting (rather than in reseeders). A larger monoploid GS is associated with large-seeded myrmecochorous species and in resprouters, whereas a significantly lower monoploid GS is found in species in mesic regions (Table 6).

Table 2. Explanatory variables, for the response variable monoploid genome size (1Cx) in African Restionaceae

Variables	AICc	Parameter estimates	SE	*P*	Comments
Elevation	–34.56	1.07E-05	5.17E-05	0.846	
Log Area	–34.73	0.010	0.022	0.644	
Plant height	–38.33	0.071	0.036	0.053	
Peripheral regions	–34.52	0.001	0.041	0.970	
Central regions	**–38.48**	**–0.070**	**0.035**	**0.046**	Low 1Cx with central regions
Wetland	–35.45	–0.117	0.122	0.340	
Fire survival	**–24.37**	**–0.094**	**0.037**	**0.013**	Low 1Cx with reseeders

Each was tested individually using PGLS. Significant relationships in bold.

Table 3. Prediction of ploidy (polyploid or diploid) in African Restionaceae as a response variable to continuous variables, using IG10

Model Comparisons	AICc	ΔAICc	Parameter estimates	SE	*P*	Notes
Elevation	**169.69**	**–27.38**	**0.002**	**0.001**	**0.000**	Polyploids associated with higher elevation
Range size	**143.61**	**–1.3**	**0.57**	**0.239**	**0.017**	Polyploids with larger area
Plant height	**142.31**	**0**	**–1.148**	**0.538**	**0.033**	Polyploids with lower plant height

Significant relationships in bold.

Table 4. Correlation of ploidy (polyploid or diploid) in African Restionaceae with binary variables using the correlated evolution test

	Independent	Dependent	LRT	*P*	Notes
Peripheral regions	**–141.31**	**–135.44**	**11.732**	**0.020**	See separate notes below
Central regions	–139.68	–138.80	1.75	0.781	
Wetland	–85.45	–83.043	4.82	0.306	A non-phylogenetic test is significant, with polyploids almost absent from wetlands
Dispersal mode	–85.45	–83.03	4.846	0.31	
Fire survival	**–132.22**	**–125.041**	**14.366**	**0.006**	Resprouters with more polyploids than reseeders

Significant correlations in bold.

GS VARIATION

African restios generally have a small GS, with 67% (385 samples) with very low C-values, 31% (176 samples) with low, only 2% (12 samples) with intermediate C-values and none with high C-values, according to the size categories used by Leitch, Chase & Bennett (1998). The average 1C-value of 1.39 pg is strikingly lower than the angiosperm average of 5.8 pg (Garcia *et al.*, 2014), but similar to the 1.4 pg GS postulated to be ancestral for angiosperms (Soltis *et al.*, 2003). This is consistent with the observations by Leitch *et al.* (1998) that high GS is phylogenetically highly restricted (although one of the two clades with large GS is the monocots). These small genomes may be a common feature of the peculiar Cape flora of southern Africa (Oberlander *et al.*, 2016). The size range of *c.* 16-fold is small compared with 230-fold in Melanthiaceae (Pellicer *et al.*, 2014), which is the largest range so far reported for any angiosperm family, but larger than the six-fold range in Nymphaeaceae (Pellicer *et al.*, 2013). It is more than 14 times lower than the closely related, but much more species-rich, Poaceae (Bennett & Leitch, 2012).

MONOPLOID AND POLYPLOID GS VARIATION

Typically, polyploidy is unambiguously inferred from chromosome numbers, but chromosome numbers of only a few species could be counted due to limited

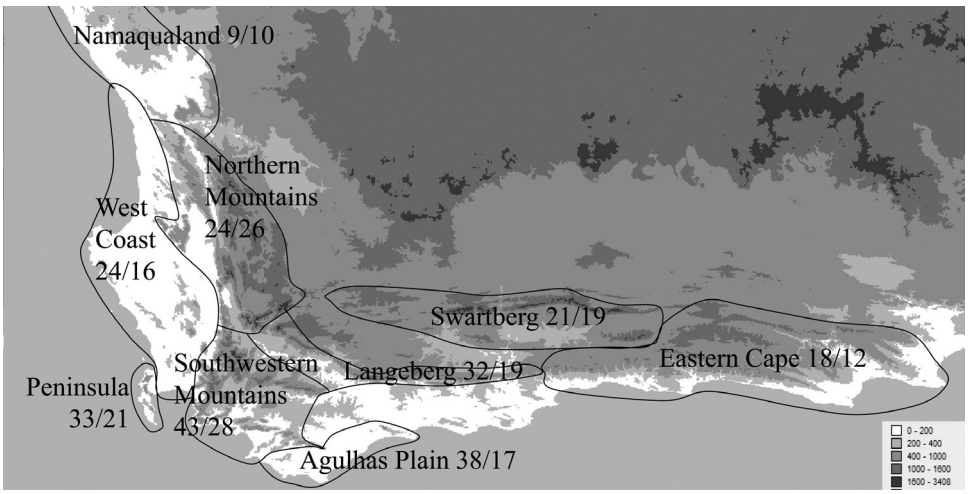

Figure 6. Regional variation in the proportion of diploid to polyploid Restionaceae in the Cape Floristic Region of South Africa. The first value in each region is the number of diploid species and the second is the number of polyploid species. The central regions (West Coast, Peninsula, South-Western Mountains, Langeberg and Agulhas Plain) have proportionally more diploids than the peripheral regions (Namaqualand, Northern Mountains, Swartberg and Eastern Cape). The key shows the elevation in metres. Fynbos vegetation dominates all regions except the West Coast and Namaqualand.

Table 5. Best OUwie model parameters for the dispersal mode in African Restionaceae, which allows all parameters to vary among the dispersal regimes

Parameter	Ballistic	Ants	Wind
α	0.0598	0.101	1.25e-01
Σ^2	0.006	0.002	3.54e-05
Optima estimated	1.038	1.425	0.820
SE	0.054	0.095	0.081

sample numbers resulting from low rate of germination, low mitotic index and the presence of asymmetric karyotypes with prevailing small to very small chromosomes. We recovered $2n = 32$ for eight of nine species analysed. This fits well with published information, where $2n = 32$ has been documented for *Hypodiscus aristatus* (Thunb.) Krauss and *Staberoha cernua* (L.f.) Dur. & Schinz (Krupko, 1962, 1966). It appears that $2n = 32$ is widespread in the African restios (*Elegia*, *Hypodiscus* Nees, *Rhodocoma*, *Staberoha* Kunth and *Thamnochortus*). This does not conform to the common monocot basic number, which is at or below $n = 10$ (Grant, 1982) and it is possible that this number resulted from previous and older polyploidy event(s) followed by chromosome rearrangements. It is thus also difficult to interpret the $2n = 20$ of *E. filacea*, especially as the number reduction appears not to be associated with a reduction in GS. Krupko (1966) recorded $2n = 40$ for *Elegia racemosa* Pers., indicating that $2n = 20$ might be diploid in this genus. This lower number might result from more frequent genome rearrangements in this lineage resulting in descending dysploidy. *Elegia filacea* is a widespread and ecologically polymorphic species and more thorough analyses of geographically distant populations might recover more chromosomal variation. The holoploid values estimated for *E. filacea* range from 2.32 to 2.94 pg/1C, whereas that of *E. racemosa* is double, from 5.03 to 6.59 pg/1C. Alternatively, $2n = 32$ might represent polyploids of taxa with $x = 10$, which subsequently experienced post-polyploidization dysploid chromosome number reduction (from $2n = 40$ to 32) and significant genome downsizing to the values reported for diploids with $2n = 20$. Although we do not have enough chromosome counts to produce robust interpretations, our results are consistent with the interpretation that within closely related species ploidy and GS co-vary, but also that, at the subfamily level, there can be substantial GS variation at the same ploidy.

Variation in GS in the restios is a result of monoploid, 1Cx size variation and of polyploidy. Whereas the former is phylogenetically conservative and can be estimated from even one sample per species, the latter is not and thus needs numerous samples per species. Consequently, we are most probably under-estimating the frequency of IPP. Ideally, we would have enough samples to detect all ploidies and so to be able to compare the frequencies of each ploidy, rather than the more categorical presence/absence of ploideis, but such an approach requires much broader sampling within and between populations of a species.

Relatively recent polyploidy, recognized by increased chromosome numbers and (near) additive or at least

Table 6. Summary of the attributes correlated with variation in GS and with inferred ploidy in African Restionaceae

Parameter	Ploidy variation	Monoploid GS
Range size	Polyploids larger***	NS
Peripheral/central	Polyploids in more arid peripheral regions**	Low monoploid GS taxa more central**
Fire biology	Resprouters with more polyploids**	Low monoploid GS in reseeders**
Dispersal mode	NS	Largest monoploid GS taxa with ant dispersal, and smallest monoploid GS in wind dispersed taxa*
Wetlands	NS	NS
Elevation	Polyploids at higher elevation***	NS
Plant height	Polyploids shorter	NS
Phylogeny	NS	Blomberg's K and Pagel's λ $P < 0.01$

*$P < 0.1$; **$P < 0.05$; ***$P < 0.01$.

significantly larger GS, might well be a phenomenon mostly observed at the tips of the phylogenetic tree, especially if autopolyploidy is the main mechanism of polyploidization. This could explain the high proportion of IPP and the absence of any clades that are exclusively polyploid. Such an interpretation fits the pattern reported for Melanthiaceae (Pellicer *et al.*, 2014) and Liliaceae (Leitch *et al.*, 2007). However, multiple rounds of older polyploidization events for which the evidence is blurred, either through diploidization or through the extinction of the polyploidy or diploid lineages, may have occurred, as in other lineages. By this model, polyploid formation is immediately followed by relatively rapid genome rearrangements (revolutionary phase) and subsequently by long periods of cytological and genetic diploidization which entails, among others, loss of coding and non-coding sequences and reduction of the chromosome number and often GS, sometimes even below the values in the lineage that gave rise to polyploids. All of these are suggested to result in both holoploid and monoploid GS reduction (Dodsworth, Chase & Leitch, 2016).

Intrinsic variables and monoploid GS evolution

Dispersal mode, fire biology and occurrence in the mesic central parts of the CFR are correlated with monoploid GS evolution and these associations could be linked to the fundamental GS-cell size correlation. Dispersal mode in the African restios is linked to seed size, with myrmecochorous species having substantially larger seeds than anemochorous and ballistochorous species. This fits the published correlation between large seed size and large GS. Kenton, Rudall & Johnson (1986) and Herben *et al.* (2012) demonstrated that species with larger seeds are associated with larger genomes and Beaulieu *et al.* (2007) demonstrated that species with small seeds generally do not have large genomes (with the notable exception of some Orchidaceae). Repeating the analysis with phylogenetic correction gave stronger results (Knight & Beaulieu, 2008). Beaulieu *et al.* (2007) showed that over the angiosperms as a whole monoploid GS holds greater explanatory power for seed mass than holoploid GS. In monoploid GS the effects of polyploidy are cancelled out and thus these results match ours. We do not have seed weight data; these may be more informative than seed categories and could have been used to test whether seed size evolution, related to monoploid GS, is punctuated. Our correlation between (long-lived) resprouters and high monoploid GS fits the pattern demonstrated by Suda *et al.* (2015) that long-lived species generally have higher ploidies and larger GS than short-lived species.

Ecological variables and polyploid GS variation

The predictions of correlations between polyploidy and particular ecological variables are largely confirmed. These include the distribution range size, fire biology, drought tolerance and tolerance of presumed unstable environments.

Polyploid species have larger distribution ranges than diploid species, consistent with the suggestion by Stebbins (1950). There could be several reasons for this. First, polyploidy may result in better establishment (te Beest *et al.*, 2012; Chen *et al.*, 2015; Suda *et al.*, 2015), so we predict larger range sizes for polyploid compared to diploid species. Second, in some IPP species investigated, the cytotypes are usually geographically, ecologically or ecogeographically separated or parapatric, with narrow overlap zones (Stebbins, 1950; Husband & Schemske, 1998) and in the overlap zones the cytotypes are often elevationally or ecologically separated

(Lumaret *et al.*, 1987; Husband *et al.*, 1998; Meirmans *et al.*, 2003; Schönswetter *et al.*, 2007a; Suda *et al.*, 2007; Duchoslav *et al.*, 2010). Consequently, it follows that IPP species should have broader ecological amplitudes than ploidy monomorphic (e.g. diploid) species. Third, it is suggested that polyploids have a wider ecological tolerance than diploids (Lumaret *et al.*, 1987, and many references therein). It probably makes a difference whether the genome duplication is a result of auto- or allopolyploidy, but we have no information on this. This interpretation is consistent with the absence of impact of the monoploid GS on the distribution range. However, it should be tested by more detailed, spatially explicit sampling in widespread species with IPP. A further potentially confounding effect might come from such widespread species harbouring several cryptic species, as might be the case in *Restio capensis* (L.) H.P.Linder & C.R.Hardy (Lexer *et al.*, 2014).

The increased proportion of polyploids in arid, peripheral regions compared to mesic, coastal regions fits three possible explanations: greater drought tolerance (Manzaneda *et al.*, 2012), greater frequency in unstable habitats (Stebbins, 1950, 1971; Ehrendorfer, 1980; Brochmann *et al.*, 2004) and greater frequency in well-drained, dry habitats (Maherali, Walden & Husband, 2009). However, non-phylogenetic analyses show that polyploids are almost absent from wetlands; this is consistent with the finding here that polyploids are more frequent in the drier regions, where wetlands are rare. The greater ability of polyploids to survive drought may not be the result of the primary consequences of polyploidy (e.g. enlarged cell sizes and associated larger stomata and wider vessels), but could be the result of faster evolution of a polyploid genome (Maherali *et al.*, 2009). Our data are not detailed enough to separate these three variables. At the broad regional level, we predict that the patterns observed for restios, with a relatively larger proportion of polyploids along the periphery of the fynbos, should also be found in all other Cape flora elements.

The positive relationship to GS and maximum elevation fits the observation that cytotypes in IPP species are often elevationally segregated (e.g. Husband *et al.*, 1998; Meirmans *et al.*, 2003; Schönswetter *et al.*, 2007a) and IPP species are thus predicted to have a wider elevational range than monoploid species. This does not fit the prediction that small C-values may be advantageous at higher elevations. Bennett (1987) assumed that plants with large genomes will probably be eliminated from high-elevation habitats due to the observed positive correlation between GS and UV damage. In addition, a shorter growing season at high elevations may support species with small genomes that have faster development (Bennett, 1972). The correlation with elevation might be via plant size, with higher elevation plants generally being smaller than lower elevation plants; this is also corroborated by a (non-significant) correlation between plant size and monoploid GS. This (weak) prediction is based on Herben *et al.* (2012) and Knight & Beaulieu (2008) and appears to be derived from a trade-off between cell size and the duration of cell divisions. These correlations have been made for dicots, but should apply even more to monocots, as tall plants have to grow quickly if they have to achieve their height in one growing season.

We show that species which resprout after fire are more likely to be polyploid. Resprouting species are usually long-lived, shorter plants (average height = 0.66 ± 0.384 m), whereas species that reseed are typically short-lived, taller plants (0.85 ± 0.587 m tall). Consequently, we do not know whether the higher ploidy is correlated with the size of the plants (as suggested for elevation), or whether it relates to the longer life cycle or whether it has something to do with the resprouting biology. Most of the life cycle comparisons have been done for annuals and perennials (e.g. Asteraceae, Enke, Fuchs & Gemeinholzer, 2011), we cannot test this in restios due to the absence of annuals in the African restios. Suda *et al.* (2015) showed that long-lived species generally have higher ploidies and larger GS than short-lived species.

Evolutionary advantage of GS variation

Variation in GS of the African restios is the result of two processes: gradual, phylogenetically constrained changes in the monoploid (1Cx) GS and rapid changes in the holoploid 2C value, presumable as a result of whole genome duplications. Changes in the monoploid (1Cx) GS and in the holoploid 2C-value affected the niche width of the species, but in different ways. Monoploid GS changes are correlated with seed size and elevation ranges, presumable ultimately as a result of changes in cell sizes. Polyploid GS changes affect a range of ecological parameters (drought tolerance, response type to fire, ability to persist in unpredictable environments and thus distribution ranges). GS variation opens new ecological possibilities for species and so might be an important contributing factor to the evolutionary radiation of the restios. Polyploidy may not only allow some niche shift, but also provide the genetic diversity on which subsequent selection can operate, thus facilitating faster evolution, leading to the prediction that evolution should be faster in peripheral areas, well-drained habitats and resprouting lineages. Such facilitation of evolution should not be the case with monoploid changes in GS. Levy & Feldman (2002, 2004) already separated these two phases: direct change as part of the dramatic reorganization with whole genome duplication and the sequent phase of evolutionary adaption. Generally, GS variation (as monoploid and

polyploid variation) has the potential to enable a lineage to fit more species into a given region, and this might be one of the processes underlying the remarkable rich Cape flora.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Figure S1. Frequency of ploidy assignations in the African restios investigated. Of the samples, 71% are diploid and 5% triploid. Thus the higher than triploid samples make up 24% of the whole dataset.
Figure S2. Mapping diploid (red) and polyploid (blue) of the phylogeny of the African restios.
Figure S3. Optimal values for each regime (1Cx values) for the African restios.
Figure S4. The stabilizing variance for each optimum – this is how strong the constraint is. Note that the *Willdenowia* clades showed the greatest variance, the others were all quite tight, most tight was *Rhodocoma* (pale green).
Figure S5. Box and whisker plots showing significant differences in the monoploid GS between the three dispersal modes (1 = ballistochorous, 2 = anemochorous, 3 = myrmecochorous) (Anova d.f. = 2, 110; $F = 48.458$, $P < 0.00$) in the African restios, but without phylogenetic correction.
Figure S6. Dispersal modes mapped on the phylogeny of the African Restionaceae. Black = ballistochorous, green = anemochorous, red = myrmecochorous.
Table S1. Vouchers, localities, genome sizes in pg, inferred genome ploidy level, and inferred monoploid GS.
Table S2. Trait data for species analysed.