GENOME SIZE STABILITY DESPITE HIGH CHROMOSOME NUMBER VARIATION IN *CAREX* GR. *LAEVIGATA*[1]

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- *Premise of the study:* In organisms with holocentric chromosomes like *Carex* species, chromosome number evolution has been hypothesized to be a result of fission, fusion, and/or translocation events. Negative, positive, or the absence of correlations have been found between chromosome number and genome size in *Carex*.
- *Methods:* Using the inferred diploid chromosome number and 80 genome size measurements from 26 individuals and 20 populations of *Carex* gr. *laevigata*, we tested the null hypothesis of chromosome number evolution by duplication and deletion of whole chromosomes.
- *Key results:* Our results show a significant positive correlation between genome size and chromosome number, but the slope of such correlation supports the hypothesis of proliferation and removal of repetitive DNA fragments to explain genome size variation rather than duplication and deletion of whole chromosomes.
- *Conclusions:* Our results refine the theory of the holokinetic drive: this mechanism is proposed to facilitate repetitive DNA removal (or any segmental deletion) when smaller homologous chromosomes are preferentially inherited, or repetitive DNA proliferation (or any segmental duplication) when larger homologs are preferred. This study sheds light on how karyotype evolution plays an important role in the diversification of the species of the genus *Carex*.

Key words: aneuploidy; Cyperaceae; cytogenetics; genome evolution; holocentric chromosome; holokinetic drive.

Chromosome rearrangements have an important role in eukaryote evolution, as chromosome rearrangement polymorphisms are correlated with phenotypic differences being considered to confer varying levels of fitness in different habitats (Coghlan et al., 2005). Chromosome number variation in Eukaryotes result from a number of sources: the duplication or deletion of entire chromosomes, polyploidy, fission, fusion, and/or the translocation of chromosomes (Malheiros Gardé and Gardé, 1950; Greilhuber, 1995; Luceño and Guerra, 1996; Coghlan et al., 2005). In species with monocentric chromosomes, chromosome fragments without centromeres are unable to segregate normally, resulting in a loss of genetic material, and probably in the production of unviable gametes. These species also have segregation problems when chromosome fusion occurs because of the production of dicentric chromosomes that are also unable to segregate correctly. In contrast, holocentric chromosomes are characterized by having diffuse centromeres, meaning that the kinetochore activity is distributed along the whole chromosome instead of being concentrated in a single point, as in centromeric chromosomes (reviewed in Hipp et al., 2013). As a consequence, all fragments from chromosome fission or fused chromosomes may segregate normally during meiosis. This could stabilize changes in chromosome number through backcrossing or selfing, or even through crossing between different individuals that have undergone convergent rearrangements (Luceño, 1994). Holocentric chromosomes may have arisen at least 13 independent times (4 times in plants and at least 9 times in animals; Melters et al., 2012). In plants, this type of chromosome occurs predominantly in the clade Cyperaceae-Juncaceae (Greilhuber,1995; Melters et al., 2012), but also punctually in specific genera of Melanthiaceae (genus *Chionographis*), Droseraceae (genus *Drosera*) and Convolvulaceae (genus *Cuscuta*) (reviewed in Melters et al., 2012).

The genus *Carex* L. (Cyperaceae), with approximately 2000 species worldwide, is one of the most species-rich angiosperm genera (Judd et al., 2007). Moreover, *Carex* is the most diversified flowering plant genus in the temperate areas of the Northern

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Hemisphere (Escudero et al., 2012a). Somatic chromosome numbers in *Carex* range from $2n = 12$ to 124 (Hipp et al., 2009) with almost continuous variation (Roalson, 2008). Many authors have been interested in *Carex* chromosome number variation and its evolutionary consequences as a driver of diversification (Heilborn, 1924; Stebbins, 1971; Bell, 1982; Luceño and Castroviejo, 1991; Hipp, 2007; Hipp et al., 2010; Escudero et al., 2010, 2012a, b, 2013a, b). This variation in chromosome number has been suggested to result primarily from fission, fusion, and the translocation of holocentric chromosomes rather than from duplications and/or deletions of whole chromosomes (Heilborn, 1924; Wahl, 1940; Faulkner, 1972; Luceño and Castroviejo, 1991). Chromosome number variation in organisms with holocentric chromosomes can affect the recombination rates (Bell, 1982; Escudero et al., 2012b; 2013a) and the rate of gene flow among populations or closely related species (Hipp et al., 2010; Escudero et al., 2013b).

During the last years, there has been an increasing number of studies focused in genome size evolution in *Carex*. First, Nishikawa et al. (1984) found a negative correlation between chromosome number and genome size in a study including 26 *Carex* species (of approximately 2000), which suggests that DNA content is lost when chromosome number increases. By contrast, Chung et al. (2011) did not find any relationship between chromosome number and genome size in *C. scoparia*, in a study including 43 individuals from 20 populations. Later, Chung et al. (2012) in a phylogenetic approach including 87 of approximately 300 species of *Carex* subgenus *Vignea*, it was reported that the correlation between chromosome number and genome size grades from flat or weakly positive at fine phylogenetic scales, to weakly negative at deeper phylogenetic scales. In another phylogenetic approach including 96 species of *Carex*, Lipnerová et al. (2013) documented a strong negative correlation between genome size and chromosome number. More recently, in parallel with the centromere drive in monocentrics, Bureš and Zedek (2014) described the existence of a holokinetic drive mainly based on this strong negative correlation between genome size and chromosome number in organisms with holocentric chromosomes. These authors proposed that a holokinetic drive facilitates chromosomal fission and/or removal of repetitive DNA (or any segmental deletion) when smaller homologous chromosomes are preferentially inherited, or chromosomal fusion and/or repetitive DNA proliferation (or any segmental duplication) when larger homologs are favored. Repetitive DNA sequences are a major component of eukaryotic genomes and may account for up to 90% of the genome size. Therefore, knowledge of repetitive sequences assists our understanding of the organization, evolution, and behavior of eukaryotic genomes (reviewed in Mehrotra and Goyal, 2014). For example, in the grass family the D-genome of wheat has over 91% of repetitive DNA; 68% of which are transposable elements (87% of retrotransposons and 13% of DNA transposons), compared to 50% in maize (98% vs. 2%) or 14% in rice (88% vs. 12%) (Li et al., 2004).

The *Carex laevigata* group is a monophyletic clade in the phylogeny of sect. *Spirostachyae*, which comprises four species: *C. laevigata* Sm., *C. binervis* Sm., *C. camposii* Boiss. and Reut., and *C. paulo-vargasii* Luceño and J. M. Marín (Escudero et al., 2008; Escudero and Luceño, 2009). *C. laevigata* is found in Western Europe (including one population in northern Morocco) and shows chromosome numbers ranging from $2n = 69$ to $2n = 84$ (Luceño and Castroviejo, 1991; Escudero et al., 2008; Escudero et al., 2013a). *C. binervis* grows also in Western Europe and shows limited chromosome number variation ($2n =$ 72–74). *C. laevigata* and *C. binervis*, although ecologically differentiated, are sympatric in some areas of their distributions. From a phylogenetic point of view, these two species are polyphyletic within the *C.* gr. *laevigata* clade (Escudero et al., 2008; Escudero and Luceño, 2009; Escudero et al., 2013a). *Carex camposii* grows in high ranges on the southeastern Iberian Peninsula. This species shows no variation in chromosome number ($2n = 72$; Luceño and Castroviejo, 1993; Escudero et al., 2010; Escudero et al., 2013a). Finally, *C. paulo-vargasii* is also a restricted allopatric species that grows in Morocco and shows some variation in chromosome number ($2n =$ 74-75; Escudero et al., 2008, 2010, 2013a). The phylogenetic relationships among these four species are not clear and they seem to have a recent origin (Escudero et al., 2008, 2013a; Escudero and Luceño, 2009). Chromosome variation in this group has been explained by climate regime selection and neutral evolutionary processes like genetic drift (Escudero et al., 2013a).

Considering all this, the main goal of this study was to confirm, using genome size measurements, that chromosome number variation in *C.* gr. *laevigata* results from fission, fusion, and/or translocation events. The null hypothesis is that chromosome number variation is by duplication and deletion of whole chromosomes, and the alternative hypothesis is that chromosome number variation is by fission and/or fusion, and possible variation in DNA content resulting from amplification or elimination of repetitive DNA. The second goal was to evaluate the theory of the holokinetic drive in the light of the results obtained.

MATERIALS AND METHODS

***Sampling*—**This study comprises 26 individuals with known chromosome number, from 20 populations collected in the field and maintained in pots in the greenhouse. Of these, 2 individuals were from 2 populations of *C. camposii* ($2n = 72$), 2 individuals from 2 populations of *C. paulo-vargasii* ($2n = 74$), 4 individuals from 4 populations of *C. binervis* ($2n = 74$), and 18 individuals from 14 populations of *C. laevigata* (3 individuals $2n = 72$, 1 individual $2n = 78$, 1 individual $2n = 80$, 12 individuals $2n = 74$, and 2 individuals $2n = 82$) (Appendix S1; see Supplemental Data with the online version of this article). Chromosome number information was obtained from Escudero et al. (2013a).

***Genome size measurements*—**The genome size was assessed using flow cytometry following the procedure of Galbraith et al. (1983). Nuclei were isolated by chopping simultaneously with a sharp razor blade, 1 cm^2 of leaf tissue of *Carex* and 1 cm^2 of leaf tissue of *Solanum lycopersicum* 'Stupické', the internal reference standard (2C = 1.96 pg DNA, Doležel et al., 1992), in 1 mL of WPB buffer (0.2 M Tris.HCl, 4 mM $MgCl_2 0.6H_2O$, 1% Triton X-100, 2 mM EDTA $Na_2 0.2H_2O$, 86 mM NaCl, 10 mM metabissulfite, 1% PVP-10, pH adjusted to 7.5, and stored at 4°C; Loureiro et al., 2007). The suspension was filtered with a 50-µm nylon filter, and DNA was stained with 50 µg mL^{-1} of propidium iodide. Additionally, 50 µg mL^{-1} of RNAse was added to degrade double stranded RNA. The samples were analyzed in a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a 532 nm green solid-state laser, operating at 30 mW. Results were acquired using the Partec FloMax software (v. 2.5). A minimum number of 1300 nuclei per G_1 peak was analyzed (Suda et al., 2007). As a quality standard, only histograms with a coefficient of variation (CV) lower than 5% for G_1 peaks of both the sample and the standard species were accepted. Samples with CV values higher than 5% were discarded and a new sample was prepared and analyzed. Three replicates of each of the 26 individuals in two different days were performed (exceptionally, two or four replicates; Appendix S1), totaling 80 genome size measurements. The holoploid genome size in picograms (2C; sensu Greilhuber et al., 2005) was estimated for each sample by multiplying the DNA index (ratio between the mean fluorescence of the sample's and standard's G_1 nuclei) by the nuclear DNA content of the reference standard.

***Analysis of variance and linear models*—**The variance of genome size was analyzed using the following approaches: (1) among replicates within individuals vs. between individuals, (2) within vs. among species, (3) within vs. among species for $2n = 74$ (the most common diploid chromosome number in *Carex* gr. *laevigata*; Escudero et al., 2013a), and (4) within the same vs. different chromosome number.

Because of the small differences in genome size among individuals when compared with the amount of variation detected between replicates of the same individual, we decided to perform a subsampling linear model. One thousand linear models were performed with genome size as the response variable and chromosome number as the predictor variable. In each linear model, a value of the different replicates of each individual was sampled. The values of each parameter for the 1000 linear models were displayed in histograms. The observed models were compared to theoretical models of duplication and deletion of whole chromosomes. For each of the 80 genome size measurements, the mean size of a chromosome in a genome was calculated by dividing the 2C values by the diploid chromosome number. The minimum, mean, and maximum values obtained were considered as the slopes of the theoretical models of duplication and deletion of chromosomes, and compared to the observed slopes. A conservative theoretical model was also compared with the observed slopes, by considering half of the calculated minimum theoretical value. The same methodology was applied to populations instead of individuals.

All statistical analyses, including the ANOVA analyses and the subsampling linear models, were performed using R (R Development Core Team, 2014).

RESULTS

Genome size (2C) ranged from 0.854 pg in a measurement of *C. binervis* [41ME06(2), $2n = 74$, (Fig. 1), Appendix S1 (see Supplemental Data with the online version of this article)] to 0.941 pg in two measurements of *C. laevigata* from two different individuals [6701JMM(2) with $2n = 80$ and 9ME05 with $2n = 74$, Appendix S1]. Therefore, the maximum difference in genome size was only of 0.087 pg. The genome size variation within individuals from different replicates was an important component of the total variance. For example, the genome size for the individual of *C. binervis* 41ME06(2) varied from 0.854–0.873 pg (a difference of 0.019 pg), for the individual of *C. laevigata* 67JMM(2) from 0.887–0.941 pg (a difference of 0.054 pg) and for the individual of *C. laevigata* 9ME05 from 0.870–0.941 pg (a difference of 0.071 pg). This pattern of variation lead us to perform a subsampling linear model analysis rather than a standard linear model using the mean genome size for each individual.

The ANOVA displayed significant differences among individuals ($F_{25,75} = 1.871$, $P = 0.02749$), but no significant differences were found among species ($F_{3,76} = 2.295$, $P = 0.0845$) nor between individuals with $2n = 74$ chromosomes ($F_{2,46} = 2.616$, $P = 0.08391$). Finally, the ANOVA displayed significant differences among individuals with different chromosome numbers ($F_{4,75} = 2.856$, $P = 0.028557$).

Our subsampling linear model displayed a mean slope equal to 0.002263 (SD = 0.001023) and mean intercept equal to 0.715850 (SD = 0.076403). The mean P value and R^2 were 0.018729 (SD = 0.002254) and 0.124430 (SD = 0.089781), respectively (Figs. 2 and 3).

The mean ratio of genome size 2C and diploid chromosome number was 0.0118 pg per chromosome, while the minimum and maximum ratios were of 0.0107 pg and 0.0127 pg per chromosome, respectively. Also, the distribution of the slopes in the histogram did not overlap with the three theoretical slopes (minimum, mean, and maximum). Moreover, although the most conservative theoretical scenario (half of the minimum slope) overlaps with the distribution of the observed slopes, it is outside of the 95% of the distribution of the observed slopes (Fig. 3).

The analysis at population level displayed very similar results (see Appendices S2 and S3).

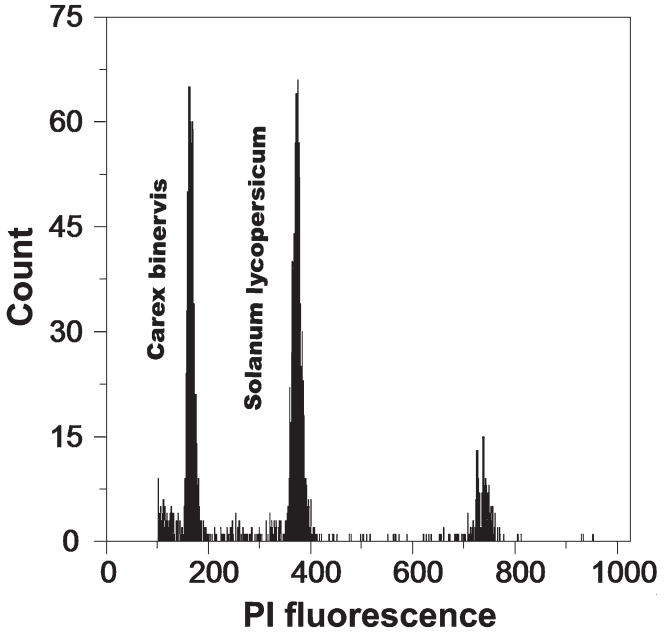

Fig. 1. Relative fluorescence histogram of propidium iodide-stained nuclei isolated from fresh leaf tissues of *Carex binervis* [individual 41ME06(2) with $2n = 74$, as an example] and of the internal reference standard, *Solanum lycopersicum* 'Stupické' (2C = 1.96 pg DNA).

DISCUSSION

Our results clearly reject the null hypothesis of duplications or losses of whole chromosomes. Thus, the alternative hypothesis of chromosome evolution by fissions, fusions, and translocations is significantly supported. The significant correlation

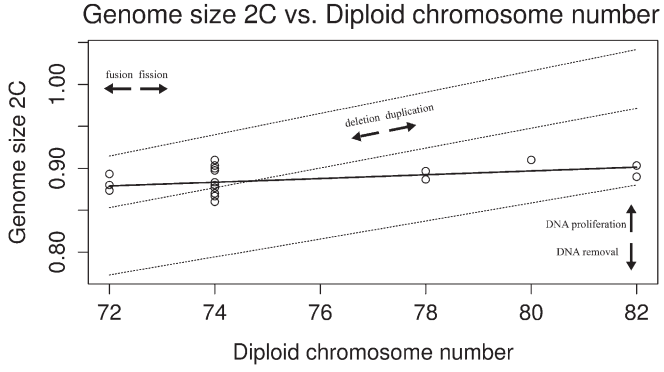

Fig. 2. Mean genome size (2C) against diploid chromosome number from the 26 individuals is plotted. The observed subsampling linear model (in blackish line) and three theoretical models (minimum, maximum, and mean; dashed lines) are also represented.

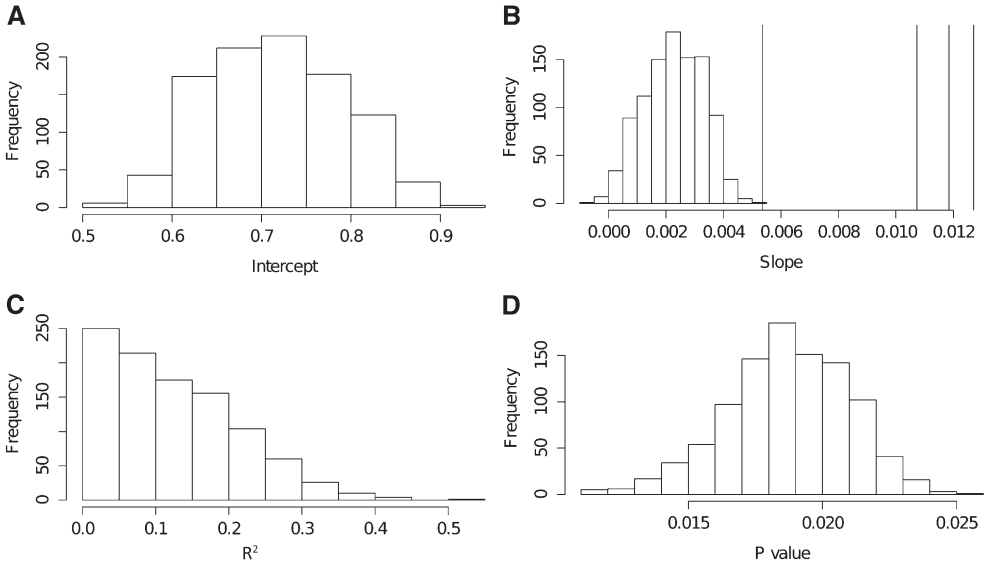

Fig. 3. Histograms of the parameters from the subsampling linear model. A, The intercept. B, The slope also including four vertical lines with four theoretical models (minimum, maximum, mean, and half of the minimum). C, The R^2. D, The P values.

between genome size and diploid chromosome number could be explained by the removal or proliferation of repetitive DNA.

Chromosome number evolution by fission, fusion, and translocation despite significant correlation between genome size and diploid chromosome number—While chromosome number variation in *Carex* has been well studied (Hipp et al., 2009), the variation in genome size has been seldom explored, with only a few studies having been performed during the last years. The first study was 30 years ago, a negative correlation was found between chromosome number and genome size in 26 *Carex* species (Nishikawa et al., 1984). This study not only rejected the hypothesis of duplication and losses of whole chromosomes, but also suggested that nuclear DNA content is lost when chromosome number increases. Similarly, a recent phylogenetic approach that included 96 species of *Carex* also documented a strong negative correlation between genome size and chromosome number (Lipnerová et al., 2013). However, other recent studies revealed different correlations from the ones reported above (Chung et al., 2011, 2012). Our results are congruent with the observations of Chung et al. (2012) as we have found a significant positive correlation between genome size and diploid chromosome number in a group of four closely related species, i.e., at a fine phylogenetic scale. This significant correlation clearly rejects the hypothesis of duplications and losses of entire chromosomes because the supported slopes are much smaller than we would expect in a scenario of quantitative aneuploidy. In fact, genome size variation could be easily explained by the removal or proliferation of repetitive DNA fragments.

In conclusion, although chromosome evolution in *Carex* has a small effect in gene subfunctionalization or neofunctionalization because of the absence of gene duplications in most of the cases (Chung et al., 2011), chromosome divergence has a demonstrated effect on the rate of hybridization and gene flow within species (Hipp et al., 2010; Escudero et al., 2013b). In addition to the effect in the rates of gene flow among populations or lineages, chromosome number variation in *Carex* affects the arrangement and constitution of linkage groups (Faulkner, 1972) and the recombination rates within species (Bell, 1982; Escudero et al., 2012b, 2013a). Finally, although homoploid hybrid speciation may have not played a major role in *Carex* diversification (Cayouette and Catling, 1992), the consequences of the high rate of rearrangements in holocentric chromosomes in the context of genome adaptation might be also important for the formation of new species (see a case of homoploid hybrid in Dragon and Barrington, 2009). Therefore, karyotype evolution through fission, fusion, and/or translocation plays an important role in species diversification within *Carex* despite the absence of duplications and losses of chromosomes.

The holokinetic drive: the selfish chromosome—The centromere drive is the theory of the selfish centromere, which exploits the asymmetric meiosis in which only one of the four meiotic products survives and the other three degenerate. The probability of being segregated depends on the size of the centromere because larger centromeres attract more microtubules (Burrack et al., 2011). A bigger centromere promotes its transmission to the surviving meiotic product at the expense of the smaller centromere in the homologous chromosome (Henikoff et al., 2001; Malik and Henikoff, 2009).

Bureš and Zedek (2014)—based on the theory of the centromere drive—proposed a parallel approach to holocentric chromosomes, the holokinetic drive. In holocentrics, the kinetochoric activity is distributed along the whole chromosome instead of concentrating in a single point, the centromere. The probability of being segregated depends on the size of the chromosome because larger chromosomes will attract more microtubules (Bureš and Zedek, 2014). In some organisms with holocentric chromosomes, like most of the species of the Cyperaceae, male meiosis can be asymmetric in addition to female meiosis (see Hipp et al., 2009).

The theory of the holokinetic drive was mainly based on the strong negative correlation between genome size and

chromosome number in organisms with holocentric chromosomes. This theory was proposed as a mechanism that facilitates chromosomal fission and/or repetitive DNA removal (or any segmental deletion) when smaller homologous chromosomes are preferentially inherited or chromosomal fusion and/or repetitive DNA proliferation (or any segmental duplication) when larger homologs are preferred. Our results revealed a significant direct correlation between genome size and chromosome number, which contribute to further refine the theory of the holokinetic drive. We believe that any significant correlation between 2*n* and 2C, negative or positive, despite the absence of quantitative aneuploidy, could be interpreted as a support for the holokinetic drive. For example, in a natural scenario that selects for higher recombination rates, chromosome fission could be positively selected (Escudero et al., 2012b, 2013a), but also the proliferation of repetitive DNA in broken chromosomes because they could be preferentially segregated later during meiosis. This would result in a positive correlation between chromosome number and genome size. In fact, in this group of species, high recombination rates are being positively selected (Escudero et al., 2013a), which could explain the significant direct correlation between chromosome number and genome size.

***Conclusions*—**Cytogenetic mutations have an important role in the evolution of eukaryotic organisms because chromosome rearrangement polymorphisms are correlated with phenotypic differences and are thought to confer varying levels of fitness in different habitats (Coghlan et al., 2005). Selection (environment selection) and neutral evolutionary processes (phylogenetic inertia, founder effect or migration patterns) have been demonstrated to explain the macro- and microevolution of holocentric chromosomes (Escudero et al., 2012b, 2013a). This study refines the theory of the holokinetic drive: this mechanism is proposed to facilitate repetitive DNA removal (or any segmental deletion) when smaller homologous chromosomes are preferentially inherited, or repetitive DNA proliferation (or any segmental duplication) when larger homologs are preferred.

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