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Genome size and base composition variation in natural and experimental *Narcissus* (Amaryllidaceae) hybrids

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• *Background and Aims* Although there is evidence that both allopolyploid and homoploid hybridization lead to rapid genomic changes, much less is known about hybrids from parents with different basic numbers without further chromosome doubling. Two natural hybrids, *Narcissus* × *alentejanus* ($2n = 19$) and *N.* × *perezlarae* ($2n = 29$), originated by one progenitor (*N. cavanillesii*, $2n = 28$) and two others (*N. serotinus*, $2n = 10$ and *N. miniatus*, $2n = 30$, respectively) allow us to study how DNA content and composition varies in such hybrids.
• *Methods* Flow cytometry measurements with two staining techniques, PI and DAPI, were used to estimate 2C values and base composition (AT/GC ratio) in 390 samples from 54 wild populations of the two natural hybrids and their parental species. In addition, 20 synthetic F_1 hybrid individuals were also studied for comparison.
• *Key Results* Natural hybrids presented 2C values intermediate between those found in their parental species, although intra-population variance was very high in both hybrids, particularly for PI. Genome size estimated from DAPI was higher in synthetic hybrids than in hybrids from natural populations. In addition, differences for PI 2C values were detected between synthetic reciprocal crosses, attributable to maternal effects, as well as between natural hybrids and those synthetic F_1 hybrids in which *N. cavanillesii* acted as a mother.
• *Conclusions* Our results suggest that natural hybrid populations are composed of a mixture of markedly different hybrid genotypes produced either by structural chromosome changes, consistent with classic cytogenetic studies in *Narcissus*, or by transposon-mediated events.

Key words: Amaryllidaceae, base composition, DAPI, flow cytometry, genome size, interspecific hybrids, *Narcissus*, polyploidy, propidium iodide.

INTRODUCTION

Changes in genome size as expressed by the DNA content of the non-replicated somatic genome (2C value *sensu* Greilhuber, 2005) and genome base composition following hybridization are far from completely understood in angiosperms, due to complex or as yet unknown mechanisms underlying intraspecific variation. Despite more detailed knowledge of the different molecular processes involved in chromosome evolution, such as duplications and expansions of satellite DNA, fixation of accessory chromosomes, transposable element activation or allopolyploidy (Bennetzen *et al.*, 2005; Cavalier-Smith, 2005), an insightful interpretation of genome size changes has not been satisfactorily achieved in most plant groups studied (Bennett and Leitch, 2005; Greilhuber, 2005). The evidence, now widely accepted, that reticulate evolution has played an important role in the evolution of angiosperms only adds complexity to the already complex problem of looking for patterns in DNA content change across evolution in general. Nevertheless, understanding the influence of hybridization in those genome size changes is highly relevant when realizing that hybridization is not restricted to sporadic events between species but that it is

involved in the origin of whole, even important, lineages (Wendel, 2000).

Classical studies on synthetic allopolyploids have shown that after hybridization extensive and rapid genome changes can occur (Soltis and Soltis, 1993; Wendel, 2000). These include rearrangement of chromosomes and sequences (Song *et al.*, 1995; Wendel *et al.*, 1995; Leitch and Bennett, 1997), regulation of gene expression (Scheid *et al.*, 1996; Galitski *et al.*, 1999; Matzke *et al.*, 1999; Comai, 2000), activation of transposable elements (Zhao *et al.*, 1998; Soltis and Soltis, 1999) and, most frequently, amplification, reassortment or elimination of highly repetitive sequences or low-copy genes (Feldman *et al.*, 1997; Hanson *et al.*, 1998; Liu *et al.*, 1998*a*, *b*; Salina *et al.*, 2000). Although the mechanisms responsible for some of these genomic changes are not fully understood, it is assumed that they may facilitate the viability and establishment of newly generated polyploid species (Song *et al.*, 1995; Wendel *et al.*, 1995; Feldman *et al.*, 1997; Liu *et al.*, 1998*a*, *b*; Ozkan *et al.*, 2001).

Genomic alterations also occur in homoploid hybrids (i.e. those in which there is no change in chromosome number), but they are even less understood than in allopolyploid species (Loureiro *et al.*, 2010). For instance, studies conducted on

three homoploid hybrid species of *Helianthus* originating from the same parental species have revealed significant rearrangements in just a few hybrid generations (Lai *et al.*, 2005; Karrenberg *et al.*, 2007). Although two of these three hybrid species (*H. anomalus* and *H. deserticola*) appear to have arisen several times in nature (Schwarzbach and Rieseberg, 2002), the different populations exhibit similar increases in genome size, with almost 50 % more nuclear DNA than the parental species (Baack *et al.*, 2005). Nevertheless, it is not clear whether this increase in genome size arose as a consequence of hybridization or only occurs under particular ecological conditions, such as stressful environments (Baack *et al.*, 2005).

Hybridization between species differing in basic chromosome number and ploidy levels is not unusual in plants (Krahulcova *et al.*, 1996; Bureš *et al.*, 2004) although sometimes reports of these types of hybrids are misinterpreted as cases of ascending aneuploidy (Grant, 1981). However, studies focusing on genome size changes in such hybrids are scarce or non-existent. *Narcissus* L. (Amaryllidaceae) has been the subject of several cytogenetic and flow cytometry studies supporting the view that the genus has a highly dynamic cytological organization (Fernandes, 1951, 1967, 1968, 1975; Zonneveld, 2008). Here, we seek to understand the effects of natural interspecific hybridization in the genome size of two closely related hybrids: *N. × alentejanus* Fernández Casas and *N. × perezlarae* Font Quer (Amaryllidaceae). These natural hybrids provide a suitable system to study genome evolution because both the phylogenetic relationships between the progenitor species and their hybrid origin are well documented (Marques *et al.*, 2010). The two morphologically similar hybrids share one parental species (*N. cavanillesii* A. Barra and G. López $2n = 4x = 28$; subgenus *Narcissus*) but differ in the other. *Narcissus serotinus* L. ($2n = 2x = 10$; subgenus *Hermione*) is the progenitor of *N. × alentejanus* while *N. miniatus* Donnison-Morgan, Koopowitz and Zonneveld ($2n = 6x = 30$; subgenus *Hermione*) is the progenitor of *N. × perezlarae* (Marques *et al.*, 2010). On the other hand, the latter two progenitors are closely related as *N. miniatus* is an allohexaploid that originated between *N. elegans* (Haworth) Spach ($2n = 4x = 20$; subgenus *Hermione*) and *N. serotinus* L. (Díaz-Lifante *et al.*, 2009; Marques *et al.*, 2010) (Fig. 1). The two hybrids exhibit odd chromosome numbers that are intermediate between those of their parental species ($2n = 19$, *N. × alentejanus*; $2n = 29$, *N. × perezlarae*). In addition to being well documented on molecular grounds, the case studies allow a comparison of genome size in different natural hybrid populations. These hybrid populations arose independently and thus might exhibit different DNA content, as reported in some studies involving distinct ecological conditions (Bureš *et al.*, 2004). Secondly, the inclusion of F_1 synthetic hybrids in the study aims to complement the dynamic picture of DNA content changes in the early stages of hybridization events by comparison with those in established natural hybrids. We cannot, however, identify the generation of those natural hybrids because in previous studies we obtained partially contradictory evidence. On the one hand, chromosome numbers recorded in hybrids were intermediate between the parents (Marques *et al.*, 2010). On the other, a thorough sampling of nuclear ribosomal cloned sequences in hybrids revealed that in
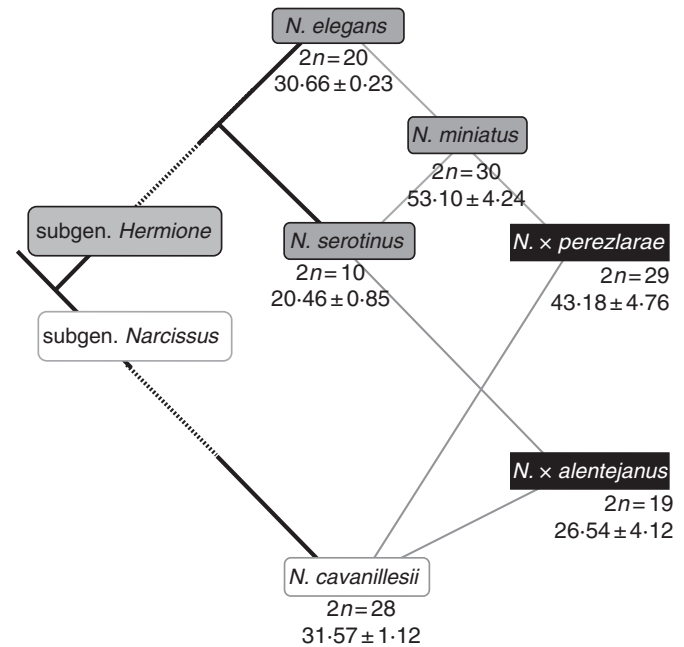

FIG. 1. Scheme of the phylogenetic relationships among the studied species, based on DNA sequences from two mitochondrial regions (*atpA* and *cob*), two plastid regions (*ndhF* and *matK*) and nrITS; chromosome numbers are also reported (based on Marques *et al.*, 2010). Mean PI 2C values gathered in this study are also indicated.

a number of individuals there was only a single internally transcribed spacer (ITS) sequence type of one of the parents, usually the mother, which is not expected in F_1s (Marques *et al.*, 2010).

In this study, we used flow cytometry based on two different fluorochrome staining techniques to assess the quality and quantity of genomic changes in a group of naturally hybridizing species of *Narcissus* differing in basic chromosome number and ploidy level. While DNA-intercalating dyes such as PI (propidium iodide) bind to all bases homogeneously, DAPI (4′,6-diamidino-2-phenylindole) binds preferentially to AT-rich regions so that results are influenced by the differences in base composition of each species in comparison with the reference standard (Meister and Barow, 2007). Thus, comparing the results obtained with the two dyes allows some estimation of whether hybridization leads to bias in genome base composition (Šmarda and Bureš, 2006).

Specifically, this work tries to answer the following questions in the two hybrids *Narcissus × alentejanus* and *N. × perezlarae*: (1) Are the values of genome size of natural hybrids within the expected range given the genome size and ploidy level of the parental species? (2) Do synthetic hybrids resulting from reciprocal crosses exhibit differences in genome size? (3) Is genome size similar in synthetic F_1 hybrids and hybrid plants from natural populations? (4) Does hybridization affect genome base composition in addition to genome size?

MATERIAL AND METHODS

Study system

A total of 390 samples from 59 wild populations were collected in the field: 14 populations of *Narcissus cavanillesii*,

15 populations of *N. serotinus*, 16 populations of *N. miniatus*, four populations of *N.* × *alentejanus*, four populations of *N.* × *perezlarae* and six populations of *N. elegans* (Supplementary Data Table S1, available online). Populations selected span the geographical range in which the two species hybridize in the southern part of the Iberian Peninsula although samples outside this area were also collected for comparison. *Narcissus elegans*, the other progenitor of the allopolyploid *N. miniatus*, was included in this study for comparison purposes. Nuclear DNA content was estimated from 4–8 individuals per population (Table S1). In addition to natural populations, we analysed 20 F_1 synthetic hybrids obtained from controlled reciprocal pollinations between *N. cavanillesii* and *N. serotinus* (CS and SC) and between *N. cavanillesii* and *N. miniatus* (CM and MC). The first letter indicates the ovule-donor in the crossing experiment (C, *N. cavanillesii*; S, *N. serotinus*; M, *N. miniatus*). Experimental pollinations were performed in 2002 for analyses of crossability in both directions (Marques and Draper, 2004). All 410 individuals (390 natural plus 20 F_1 synthetic hybrids) were grown in the greenhouse at the Real Jardín Botánico Madrid, Spain (40 °24′45″N, 3 °41′2″E; 667 m a.s.l). A previous study documented chromosome numbers in all the populations (Marques *et al.*, 2010; Table S1).

Flow cytometry

Measurements were made on a Cell Lab Quanta Beckman Coulter flow cytometer (Beckman Coulter Inc., Brea, CA, USA) equipped with a 100-W Mercury arc lamp with excitation line optimized at 366 nm for UV measurements with DAPI, and a 2–22-mW, 488-nm laser diode using the 570-nm long-pass filter for PI measurements. Two types of fluorochromes were used for assays: intercalating PI for absolute nuclear DNA content estimations and AT-selective DAPI for estimation of the AT portion of nuclear DNA content. PI has been previously used to investigate systematic relationships in *Narcissus* (Zonneveld, 2008). For sample preparation, a piece of fresh young tissue collected from leaves was chopped using a razor blade together with an internal standard in a Petri dish containing 600 µL Galbraith's buffer (Galbraith *et al.*, 1983) supplemented with 100 µg ml^{-1} RNase A (Boehringer, Meylan, France). *Pisum sativum* 'Express Long' (2C = 8·37 pg; GC = 40·5 %; AT = 59·5 %) was used as internal standard (seeds provided by the Institut des Sciences du Végétal, Gif-sur-Yvette, France). The suspension was filtered through a 33-µm SEFAR PA 1000 140/355-35W nylon filter (SEFAR, Barcelona, Spain) and subsequently supplemented with either 60 µg mL^{-1} PI (Sigma-Aldrich, Alcobendas, Madrid, Spain) or 2·0 µg mL^{-1} DAPI (Partec, Münster, Germany) depending of the staining technique. The two measurements were made on the same day using a portion of the leaf obtained from the same individual. Prior to the first measurement, the cytometer was checked for linearity using standard fluorescent beads (Coulter Electronics). Amplification settings were kept constant throughout the experiment. All measurements were taken twice on different days to avoid instrument drift. In each sample, 10 000 particles were counted. The 2C-value was calculated by multiplying the known DNA content of the standard by the quotient of the 2C peak positions of the target species and the standard in the histogram of fluorescence intensities, under the assumption that there is a linear correlation between the fluorescent signals from stained nuclei of the unknown specimen and the internal standard. The AT frequency was calculated using the equation:

$$f\,(\mathrm{At_{species}}) = (R - \mathrm{DAPI_{species}}/R - \mathrm{PI_{species}}) \times f\,(\mathrm{AT_{reference}})$$

where R-$\mathrm{DAPI_{species}}$ is the ratio between mean fluorescence peaks for a sample and reference stained by DAPI, R-$\mathrm{PI_{species}}$ is the ratio between mean fluorescence peaks for a sample and reference stained by PI and $f(\mathrm{AT_{reference}})$ is the AT frequency of the reference sample (Barow and Meister, 2002).

As generally accepted, genome size is the 2C-value divided by ploidy level (Bennett, 1998), so that the genome size corresponds to the 1C-value in diploids but can vary in polyploids due to the 'Cx-paradox' (Greilhuber, 2005). However, in our study we have used 2C-values instead of 1C, to allow comparisons between hybrids and between them and their progenitors. The cytogenetic complexity of hybrids between parents differing in basic chromosome number and ploidy level prevents a reliable calculation of 1C values in the hybrids.

Sampling design and statistical analyses

The mean and s.d. of each sample was calculated from the means of the two measurements performed. Only histograms with symmetrical peaks and with a coefficient of variation (CV) of the sample G1 peak below 2 % were considered. We also performed some simultaneous measurements of hybrid samples and their respective parental species. The normality of the distribution of genome size of all samples was tested using the Kolmogorov–Smirnov test. Differences of genome size (PI and DAPI) among species, populations and individuals were evaluated using analysis of variance (ANOVA). In those cases in which ANOVA revealed significant differences, the Tukey HSD post-hoc test was performed. In addition, a *t*-test was used to compare genome size values between the two natural hybrids and between the reciprocal synthetic F_1 hybrids. To explore if the values of genome size obtained for *N.* × *alentejanus* and *N.* × *perezlarae* differed from those expected assuming an intermediate value between that of their parents, we used a chi-square test. To assess differences in base composition (DAPI) with respect to whole genome size (PI) we computed the DAPI/PI ratio for each individual, population and species.

RESULTS

Accuracy of measurements

CV of G0/G1 peaks for PI staining of the standard *Pisum sativum* 'Express Long' and for the measured samples were always below 3·01 % in 410 estimates (mean of standard CV = 1·99 ± 0·32 %, mean of samples CV = 2·10 ± 0·45 %). Histograms yielded by DAPI staining did not exceed 3·51 %, either for the standard or for the sample (mean of standard CV = 2·99 ± 0·33 %, mean of samples CV = 3·12 ± 0·15 %).

TABLE 1. *2C-values (pg) assessed with PI and DAPI from the studied species of* Narcissus

Species	$2n$	N	N_{pop}	PI				DAPI				%AT
				Min.	Max.	Mean	s.d.	Min.	Max.	Mean	s.d.	
N. cavanillesii	28	88	14	30·52	33·77	31·57	1·12	12·30	14·44	13·21	1·33	24·95
N. serotinus	10	94	15	19·52	21·30	20·46	0·85	13·67	19·78	17·64	2·89	51·37
N. miniatus	30	111	16	48·24	56·18	53·10	4·24	25·16	38·20	35·02	3·96	39·28
N. elegans	20	37	6	30·62	30·78	30·66	0·23	26·05	29·00	27·89	3·22	54·14
N. × *alentejanus*	19	28	4	22·92	29·84	26·54	4·12	14·03	16·78	15·52	4·22	36·15
N. × *perezlarae*	29	32	4	39·61	46·14	43·18	4·76	26·49	29·47	28·11	4·17	39·39

$2n$ = Mitotic chromosome number reported for each species (Marques *et al.*, 2010). N = Number of individuals measured per species. N_{pop} = Number of populations measured per species. Min. = Minimum 2C-value recorded considering all populations. Max. = Maximum 2C-value recorded considering all populations.

Genome size in natural hybrids and parental species

The mean 2C-value assessed with PI increased in the following order: *Narcissus serotinus* < *N.* × *alentejanus* < *N. elegans* < *N. cavanillesii* < *N.* × *perezlarae* < *N. miniatus*, which is congruent with the number of chromosomes reported for each taxon (Table 1). Nevertheless, 2C-values assessed with DAPI increased in a different order: *N. cavanillesii* < *N.* × *alentejanus* < *N. serotinus* < *N. elegans* < *N.* × *perezlarae* < *N. miniatus*. These values showed a significant interspecific variation (ANOVA test, PI: $F_{5,51} = 28·876$, $P = 0·0001$; DAPI: $F_{5,51} = 39·444$, $P = 0·0001$).

The 2C-values of the two natural hybrids always fell between those of the parental species when estimated both with PI (*N.* × *alentejanus* vs. parental species: $F_{2,12} = 61·900$, $P = 0·0001$; *N.* × *perezlarae* vs. parental species: $F_{2,12} = 92·895$, $P = 0·0001$) and with DAPI (*N.* × *alentejanus* vs. parental species: $F_{2,14} = 83·950$, $P = 0·0001$; *N.* × *perezlarae* vs. parental species: $F_{2,15} = 79·589$, $P = 0·0001$). Simultaneous measurements of hybrid samples with their parental species always resulted in separate peaks, the intermediate peak corresponding to the natural hybrid plants (Fig. 2). However, 2C-values of the natural hybrids did not always conform to expectation [(DNA content of parent$_1$ + DNA content of parent$_2$)/2] but depended on the fluorochrome technique. The 2C-values obtained with DAPI were always higher than expected whereas the 2C-values measured with PI did not differ from those expected (Table 2).

Inter- and intra-population variation in natural hybrids

For *N.* × *alentejanus*, the 2C-values measured with PI varied between 22·92 pg in population AJU and 29·84 pg in population UPO, with an intra-population variance of 21·32 pg. Estimations with DAPI ranged between 14·03 pg in population FTA and 16·78 pg in population AJU, with an intra-population variance of 10·15 pg (Fig. 3). This high intra-population variance was demonstrated as a clear double peak in the simultaneous measurement of several samples (Fig. 4). Likewise, in *N.* × *perezlarae*, genome size measured with PI varied between 39·61 pg in population GTG and 46·14 pg in population MDS, with an intra-population variance of 27·32 pg. The 2C-values measured with DAPI varied between 26·49 pg in population DEN and 29·47 pg in population MDS, with a variance of 27·41 pg (Fig. 3).
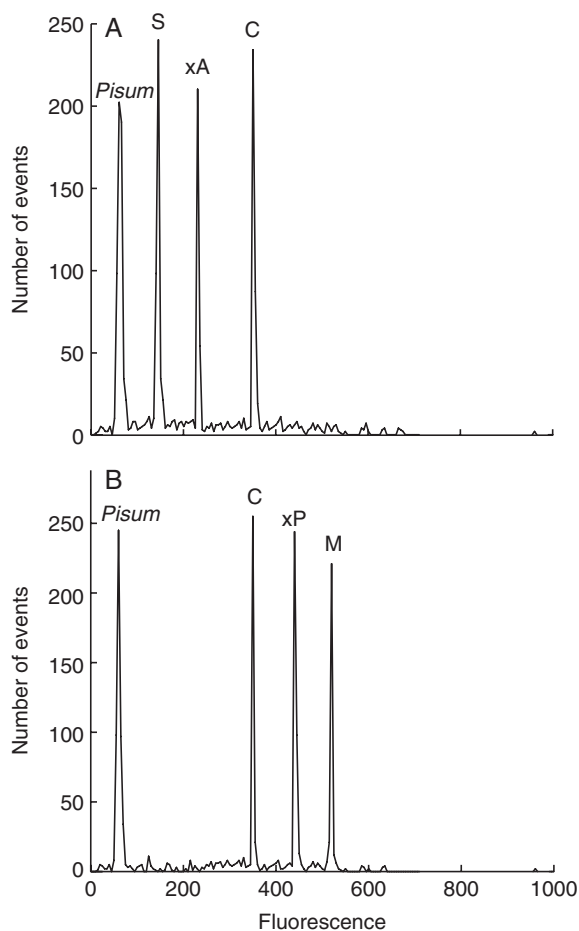

FIG. 2. Fluorescence histogram of propidium iodide-stained (PI) nuclei of parental species and their hybrids analysed in the same sample. (A) *Narcissus* × *alentejanus* and parental species. (B) *N.* × *perezlarae* and parental species. Abbreviations: C, *N. cavanillesii*; S, *N. serotinus*; M, *N. miniatus*; ×A, *N.* × *alentejanus*; ×P, *N.* × *perezlarae*.

No statistically significant differences in 2C-values were found across populations of *N.* × *alentejanus* (ANOVA, PI: $F_{3,5} = 5·071$, $P = 0·054$; DAPI: $F_{3,6} = 1·466$, $P = 0·241$) or across populations of *N.* × *perezlarae* (PI: $F_{3,5} = 4·583$, $P = 0·052$; DAPI: $F_{3,6} = 1·695$, $P = 0·561$; Fig. 3).

Genome size of synthetic hybrids

Comparing the four classes of experimental crosses, hybrids generated between *N. cavanillesii* and *N. miniatus* always had higher 2C-values than hybrids between *N. cavanillesii* and *N. serotinus* (Table 3), which is expected based on the genome size of *N. miniatus* as compared with *N. serotinus*. Comparing the reciprocal hybrids, 2C-values depended on the fluorochrome used. When assessed with PI, CS hybrids yielded higher 2C-values than SC hybrids while MC hybrids yielded higher 2C-values than CM hybrids (Table 3). In contrast, when genome was estimated with DAPI, no significant differences were found between the reciprocal synthetic hybrids CS and SC and between CM and MC (Table 3).

Comparisons between synthetic and natural hybrids

Estimated with PI, synthetic CS hybrids yielded higher 2C-values than the natural hybrid *N.* × *alentejanus* ($t = 23.418$, d.f. $= 32$, $P = 0.0001$) while synthetic CM hybrids had lower 2C-values than the natural hybrid *N.* × *perezlarae* ($t = 22.789$, d.f. $= 36$, $P = 0.015$; Fig. 5). The remaining reciprocal hybrids showed no differences from their corresponding natural hybrids (SC vs. *N.* × *alentejanus*: $t = 1.054$, d.f. $= 32$, $P = 0.306$; MC vs. *N.* × *perezlarae*: $t = 1.086$, d.f. $= 36$, $P = 0.067$; Fig. 5). When stained with DAPI, synthetic F_1 hybrids always yielded higher values than their natural hybrid counterparts (*N.* × *alentejanus* vs. synthetic hybrids: $F_{2,21} = 21.312$, $P = 0.0001$; *N.* × *perezlarae* vs. synthetic hybrids: $F_{2,37} = 21.379$, $P = 0.0001$).

TABLE 2. *Comparison of expected and obtained genome size values estimated with PI and DAPI for the two natural hybrids* Narcissus × alentejanus *and* N. × perezlarae

Natural hybrids	PI			DAPI		
	χ^2	d.f.	P	χ^2	d.f.	P
N. × *alentejanus*	36·026	27	0·0054	38·897	27	**0·05***
N. × *perezlarae*	27·200	31	0·2810	38·200	31	**0·01****

Comparisons were performed with a χ^2-test. Significant results are indicated in bold. *$P < 0.05$, **$P < 0.01$.

DISCUSSION

The genome size values here obtained are largely congruent with those previously reported in a study of the genus *Narcissus* as a whole using PI staining (Zonneveld, 2008). However, our extensive sampling provides a more accurate picture of the intraspecific variation of genome size in *Narcissus* (Table 1). Specifically, our results attest to quantitative and qualitative genome changes found in synthetic F_1 interspecific hybrids, corresponding to the unstable phase that is characterized by structural changes and heterochromatic transposon amplification (McClintock, 1984).
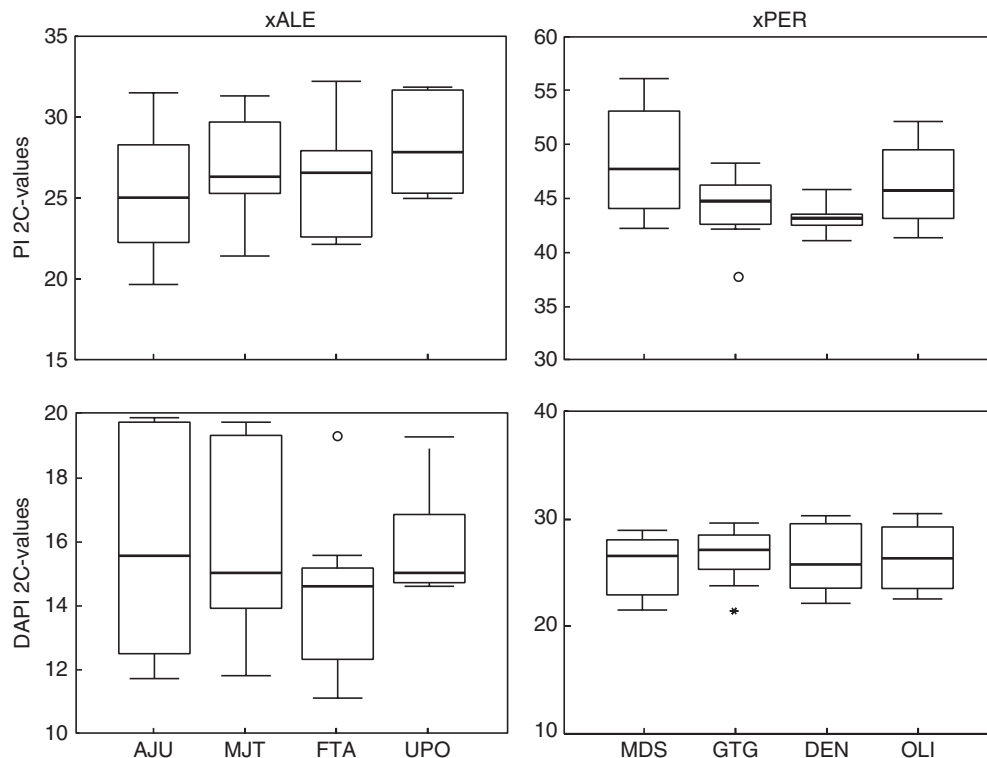

FIG. 3. Intra-population variation of 2C values obtained for the natural hybrids *N.* × *alentejanus* (xALE) and *N.* × *perezlarae* (xPER). Top: genome size measured with PI. Below: genome size measured with DAPI. Values are expressed in pg. The horizontal line across the box represents the median, and the box represents the interquartile range that contains 50 % of the values. The whiskers are the lines that extend from the box to the highest and lowest values excluding outliers. Asterisks represent extreme values (cases with values more than 3 box lengths from the upper or the lower edge of the box) and open circles represent outliers (cases with values between 1·5 and 3 box lengths from the upper or the lower edge of the box).
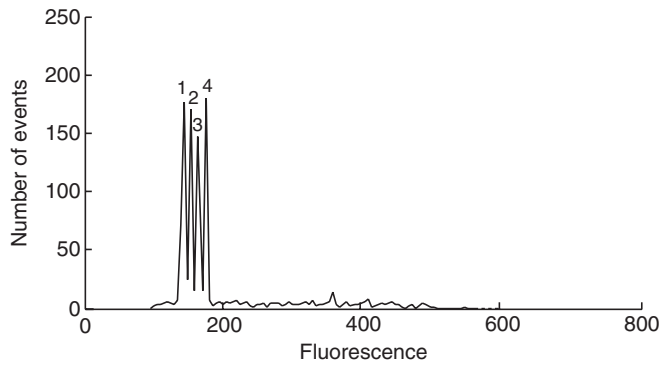
F IG . 4. Differences in genome size among *Narcissus × perezlarae* samples from four different populations. The nuclei of all individuals were stained with propidium iodide and analysed simultaneously. The peak ratios were 1 : 1·068 : 1·127 : 1·164. The mean 2C-values were 39·61 pg (population 1: GTG), 42·32 pg (population 2: DEN), 44·68 pg (population 3: OLI) and 46·14 pg (population 4: MDS).

T ABLE 3. *Results of* t-*test analyses assessing genome size values (PI and DAPI) obtained for the synthetic hybrids*

Synthetic hybrids	PI			DAPI		
	d.f.	*t*	*P*	d.f	*t*	*P*
CS and SC vs. CM and MC	19	11·230	**0·0001*****	19	18·082	**0·0001*****
CS vs. SC	9	13·077	**0·012****	9	11·928	0·847
CM vs. MC	9	18·637	**0·0001*****	9	18·637	0·610

CS, *N. cavanillesii × N. serotinus*; SC, *N. serotinus × N. cavanillesii*; CM, *N. cavanillesii × N. miniatus*; MC, *N. miniatus × N. cavanillesii*. The first species of each pair is the ovule donor while the second species corresponds to the pollen donor. Significant differences are indicated in bold type. **P < 0·01, ***P < 0·001.

A general observation is that the range of genome size values is usually lower for those species not considered of hybrid origin such as *N. cavanillesii*, *N. serotinus* and *N. elegans* as compared with those of hybrid origin such as *N. × alentejanus*, *N. × perezlarae* and the allopolyploid *N. miniatus* where s.d. values fall above 4 (Table 1). This observation is also true for 2C-values obtained with DAPI and therefore calls for an explanation as intraspecific variation in genome size is considered to be rather scarce (Šmarda and Bureš, 2006; Suda *et al.*, 2007; but see Slovák *et al.*, 2008).

A first relevant aspect of our results is the differences found in the 2C-values assessed with PI between the reciprocal F_1 hybrids (Fig. 5, Table 3). A maternal effect for genome size in hybrids has been previously reported in synthetic homoploid hybrids of *Helianthus* (Baack *et al.*, 2005), and in synthetic *Brassica* polyploids (Song *et al.*, 1995), among other groups. To explain such effects, there is increasing evidence, observed in polyploids, that the maternal cytoplasm produces some selective pressure via seed inviability on the nuclear genome, which discards non-harmonious combinations and thus contributes to stabilize the newly synthesized hybrid (Soltis and Soltis, 1993; Caceres *et al.*, 1998). One possible mechanism is that in many angiosperms the viability of embryos depends on the balance of maternal and paternal
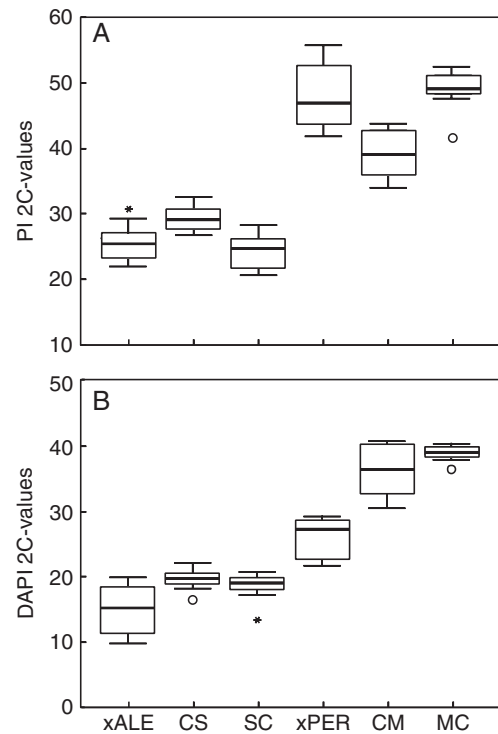

F IG . 5. 2C-values in natural hybrids, *N. × alentejanus* (xALE) and *N. × perezlarae* (xPER) and the equivalent artificial hybrids, CS (*N. cavanillesii × N. serotinus*), SC (*N. serotinus × N. cavanillesii*), CM (*N. cavanillesii × N. miniatus*) and MC (*N. miniatus × N. cavanillesii*). (A) Genome size obtained with PI. (B) Genome size obtained with DAPI. Values are expressed in pg. For details of the box-and-whisker plots see Fig. 3.

genomes in the endosperm. This balance should normally be 2 : 1 because of fusion of the polar nuclei with one sperm nucleus to form the central cell on the maternal side (Martienssen, 2010). Our two hybrids deviate from this ratio because of the divergent parental genomes involved, so we could expect a strong differential maternal effect.

A second relevant aspect of our study is the genome size differences, estimated with PI, found between natural hybrids and some of the synthetic hybrids. Although no significant differences were found between natural hybrids and some of the corresponding synthetic crosses (SC, MC), *N. × alentejanus* had lower values than synthetic CS hybrids while *N. × perezlarae* had higher values than synthetic CM hybrids (Fig. 5). One possible explanation for this pattern is that individuals studied from the natural hybrid populations represent late hybrid generations, which would have undergone substantial genome size changes as compared with F_1s or are simply a mixture of hybrid genotypes, not necessarily from late hybrid generations. We cannot answer this question conclusively because in previous studies partially contradictory evidence was obtained as to the type of generation the natural hybrids belong to (see Introduction). Regardless, the fact that significant PI 2C-value differences are found in comparisons involving synthetic hybrids in which *N. cavanillesii* acts as ovule donor (CS, CM) suggests that those differences are representative of natural conditions because *N. cavanillesii* has been found to be the mother species in most natural hybrids

(Marques *et al.*, 2010). Therefore, our results suggest two different trends in the natural hybrids: to increase genome size in *N. × perezlarae* and to decrease it in *N. × alentejanus* after the first stages of hybridization.

Another conclusion is that because 2C-values obtained with DAPI were significantly larger in the synthetic F_1 hybrids than in the respective natural hybrids (Fig. 4), an increase in GC-rich regions should occur in nature following hybridization. Although there are uncertainties as to the fine interpretation of DAPI values due to nucleotide requirements for the binding of this dye (Barow and Meister, 2002), our results are quite consistent in this respect.

In summary, our results from natural and synthetic hybrids indicate genome size variation and changes of different sorts. These include differences between reciprocal crosses for 2C values estimated with PI as well as differences between natural hybrids and some of the synthetic hybrids, an overall higher variation of genome size estimates in hybrids (both estimated with PI and DAPI), and higher 2C-values obtained with DAPI in synthetic F_1 hybrids compared with their natural counterparts. With the exception of the first item already discussed (maternal effects), the others require specific and perhaps also some general underlying mechanisms.

The tendency towards genome size reduction, documented especially in allopolyploids (Rieseberg *et al.*, 1995; Pikaard and Chen, 1998; Bureš *et al.*, 2004; Garnatje *et al.*, 2006), has been explained by rapid elimination of sets of non-coding sequences (Ozkan *et al.*, 2001), and which can even be strongly biased towards one of the progenitor genomes (Levy and Feldman, 2004; Skalická *et al.*, 2005). Although rarer, there are also reports of the opposite trend, of increasing genome size in hybrids (Baack *et al.*, 2005).

In *Narcissus*, we suggest that chromosome rearrangements and recombination are involved in the genome size changes detected here since they can explain both the variation in genome size and the rate of the changes produced as fast as (but not only) in the F_1 generation. In previous chromosome studies of the two hybrids, we observed a high frequency of aberrant meioses with chromosome bridges and lagging chromosomes, sometimes even preventing chromosome counts (Marques *et al.*, 2010). Such aberrant meioses, due to lack of homology between chromosomes, may result in the asymmetric loss and gain of chromosome regions (Song *et al.*, 1995). This is consistent with the extensive cytogenetic pioneering studies of Fernandes (1943, 1951, 1967, 1968, 1975) in *Narcissus*. He pointed out the importance of chromosome rearrangements in the evolution of *Narcissus*, specifically in the origin of the two base numbers $x = 5$ (subgenus *Hermione*) and $x = 7$ (subgenus *Narcissus*) and in the diversification of sections such as *Jonquillae* or *Apodanthae*. Recently, karyological polymorphisms that have been detected in *N. serotinus* and *N. miniatus* are clearly attributed to chromosome rearrangements caused by hybridization (Díaz Lifante *et al.*, 2009).

Not much can be concluded regarding the functional and evolutionary significance of changes and variation in genome size in hybrids. In a previous study, we found that the natural hybrids studied here exhibit high fitness for vegetative components and are even able to set some seeds albeit at low frequency (Marques *et al.*, 2011). Taking into account the higher range of genome size values in hybrids as well as the frequency of structural changes in *Narcissus* detected from cytogenetic approaches, it seems that a strong selection operating over a wide variety of genomes has resulted in a number of evolutionary viable genotypes in the two hybrids of *Narcissus* studied here.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford journals.org and consist of Table S1: 2C-values assessed with PI and DAPI from the species of *Narcissus* studied.

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